THE UNIVERSITY OF YAOUNDE I
****
FACULTY OF SCIENCE
****
POST GRADUATE SCHOOL FOR LIFE SCIENCES, HEALTH AND ENVIRONMENT
****
DOCTORAL RESEARCH UNIT FOR LIFE SCIENCES
****

UNIVERSITE DE YAOUNDE I
****
FACULTE DES SCIENCES
****
CENTRE DE RECHERCHE ET DE FORMATION DOCTORALE EN SCIENCES DE LA VIE, SANTE ET ENVIRONNEMENT
****
UNITE DE RECHERCHE ET DE FORMATION DOCTORALE EN SCIENCES DE LA VIE
****

DEPARTMENT OF ANIMAL BIOLOGY AND PHYSIOLOGY
DEPARTEMENT DE BIOLOGIE ET PHYSIOLOGIE ANIMALES
LABORATORY OF ANIMAL PHYSIOLOGY AND THERAPEUTIC RESEARCH
LABORATOIRE DE PHYSIOLOGIE ANIMALE ET RECHERCHE THERAPEUTIQUE

Effects of potassium sorbate (food preservative) and tartrazine (food dye) on the female reproductive system

Ph.D. DISSERTATION

Submitted in partial fulfilment of the requirements for the degree of **Doctorat/Ph.D** in **Biology of Animal Organism**

Option: **Animal Physiology and Therapeutic Research**

By

NDJENGUE MINDANG Elisabeth Louise

***Master ès-sciences in* Biology of Animal Organism**

Option: **Animal Physiology**

Registration number: **12Q0944**

Public defense held on May, 27 2025 in front of a board members of the jury made up of:

President:	**M. DIMO Théophile, Professor,**	**University of Yaoundé I**
Supervisor:	**M. NJAMEN Dieudonne, Professor**	**University of Yaoundé I**
Examiners:	**Mrs. JATSA BOUKENG Hermine, Associate Professor,**	**University of Yaoundé I**
	M. DJIOGUE Sefirin, Associate Professor,	**University of Yaoundé I**
	Mrs. MVONDO Marie Alfrede, Associate Professor,	**University of Ebolowa.**

Year 2025

REPUBLIQUE DU CAMEROUN

Paix-Travail-Patrie

****

UNIVERSITÉ DE YAOUNDÉ I

FACULTÉ DES SCIENCES

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REPUBLIC OF CAMEROUN

Peace-Work-Fatherland

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THE UNIVERSITY OF YAOUNDE I

FACULTY OF SCIENCE

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DEPARTMENT OF ANIMAL BIOLOGY AND PHYSIOLOGY

DEPARTEMENT DE BIOLOGIE ET PHYSIOLOGIE ANIMALES

ATTESTATION DE CORRECTION

Nous soussignés, membres du jury de la soutenance de thèse de Doctorat/PhD en **Biologie des Organismes Animaux**, Option : **Physiologie Animale et Recherche Thérapeutique**, de Madame **NDJENGUE MINDANG Elisabeth Louise**, matricule **12Q0944**, soutenance autorisée par la correspondance N° 25-1832/UY1/VR-EPDTIC/DAAC/DA-AAC/CDR/SR/SR-A/mna de Monsieur le recteur de l'Université de Yaoundé I en date du 30 avril 2025 sur le sujet intitulé : « Effects of potassium sorbate (food preservative) and tartrazine (food dye) on the female reproductive system», attestons que les corrections exigées à la candidate lors de cette évaluation, qui a eu lieu le mardi 27 mai 2025 dans la salle S01/S02 de la Faculté des Sciences, ont réellement été effectuées.

En foi de quoi, la présente attestation lui est délivrée pour servir et valoir ce que de droit.

Fait à Yaoundé, le 17 JUIL 2025

L'Examinateur

Le Président du jury

Le chef de Département

Pr. Sevilor KEKEUNOU
Faculté des Sciences
Université de Yaoundé I

THE UNIVERSITY OF YAOUNDE I **Faculty of Science** Division of Programming and Follow-up of Academic Affaires		UNIVERSITÉ DE YAOUNDÉ I **Faculté des Sciences** Division de la Programmation et du Suivi des Activités Académiques
LIST OF PERMANENT TEACHING STAFF		**LISTE DES ENSEIGNANTS PERMANENTS**

ACADEMIC YEAR 2024/2025
(By Department and by Grade)
UPDATE: 16 January 2025

ADMINISTRATION

DEAN: OWONO OWONO Luc Calvin, *Professor*
VICE-DEAN / DPSAA: NDJIGUI Paul-Désiré, *Professor*
VICE-DEAN / DSSE: NYEGUE Maximilienne Ascension, *Professor*
VICE-DEAN/ DRC: NOUNDJEU Pierre, *Associate Professor*
Head of Administrative and Financial Division: NDOYE FOE Florentine Marie Chantal, *Associate Professor*
Head of Academic Affairs, Education and Research Division: AJEAGAH Gideon AGHAINDUM, *Professor*

1- DEPARTMENT OF BIOCHEMISTRY (BC) (44)

N°	SURNAMES AND FIRST NAMES	GRADE	OBSERVATIONS
1.	BIGOGA DAIGA Jude	Professor	On duty
2.	FEKAM BOYOM Fabrice	Professor	On duty
3.	KANSCI Germain	Professor	On duty
4.	MBACHAM FON Wilfred	Professor	On duty
5.	MOUNDIPA FEWOU Paul	Professor	*Head of Department*
6.	NGUEFACK Julienne	Professor	On duty
7.	NJAYOU Frédéric Nico	Professor	On duty
8.	OBEN Julius ENYONG	Professor	On duty
9.	ACHU Merci BIH	Associate Professor	On duty
10.	BEBEE Fadimatou	Associate Professor	On duty
11.	BEBOY EDJENGUELE Sara Nathalie	Associate Professor	On duty
12.	FONKOUA Martin	Associate Professor	On duty
13.	AKINDEH MBUH NJI	Associate Professor	On duty
14.	ATOGHO Barbara MMA	Associate Professor	On duty
15.	AZANTSA KINGUE GABIN BORIS	Associate Professor	On duty
16.	BELINGA née NDOYE FOE F. M. C.	Associate Professor	*Head DFA / FS*
17.	DAKOLE DABOY Charles	Associate Professor	On duty
18.	DONGMO LEKAGNE Joseph Blaise	Associate Professor	On duty
19.	DJUIDJE NGOUNOUE Marceline	Associate Professor	On duty
20.	DJUIKWO NKONGA Ruth Viviane	Associate Professor	On duty
21.	EFFA ONOMO Pierre	Associate Professor	*VD/FS/Univ Ebwa*
22.	EWANE Cécile Annie	Associate Professor	On duty
23.	KENGNE NOUEMSI Anne Pascale	Associate Professor	On duty

24.	KOTUE TAPTUE Charles	Associate Professor	On duty
25.	LUNGA Paul KEILAH	Associate Professor	On duty
26.	MANANGA Marlyse Joséphine	Associate Professor	On duty
27.	MBONG ANGIE M. Mary Anne	Associate Professor	On duty
28.	MOFOR née TEUGWA Clotilde	Associate Professor	*Dean FS / Uds*
29.	NANA Louise épouse WAKAM	Associate Professor	On duty
30.	NGONDI Judith Laure	Associate Professor	On duty
31.	Palmer MASUMBE NETONGO	Associate Professor	On duty
32.	PECHANGOU NSANGOU Sylvain	Associate Professor	On duty
33.	TCHANA KOUATCHOUA Angèle	Associate Professor	On duty

34.	BAKWO BASSOGOG Christian Bernard	Senior Lecturer	On duty
35.	ELLA Fils Armand	Senior Lecturer	On duty
36.	EYENGA Eliane Flore	Senior Lecturer	On duty
37.	FOUPOUAPOUOGNIGNI Yacouba	Senior Lecturer	On duty
38.	KOUOH ELOMBO Ferdinand	Senior Lecturer	On duty
39.	MADIESSE KEMGNE Eugenie Aimée	Senior Lecturer	On duty
40.	MANJIA NJIKAM Jacqueline	Senior Lecturer	On duty
41.	MBOUCHE FANMOE Marceline J.	Senior Lecturer	On duty
42.	OWONA AYISSI Vincent Brice	Senior Lecturer	On duty
43.	WILFRED ANGIE ABIA	Senior Lecturer	On duty
44.	WOGUIA Alice Louise	Senior Lecturer	On duty

2- DEPARTMENT OF ANIMAL BIOLOGY AND PHYSIOLOGY (A.B.P) (50)

1.	AJEAGAH Gideon AGHAINDUM	Professor	*DAARS/FS*
2.	DJIETO LORDON Champlain	Professor	On duty
3.	DZEUFIET DJOMENI Paul Désiré	Professor	On duty
4.	ESSOMBA née NTSAMA MBALA	Professor	*CD and Vice-Dean/FMSB/UYI*
5.	KEKEUNOU Sévilor	Professor	*Head of Department*
6.	MEGNEKOU Rosette	Professor	On duty
7.	NJAMEN Dieudonné	Professor	On duty
8.	NOLA Moïse	Professor	On duty
9.	TAN Paul VERNYUY	Professor	On duty
10.	TCHUEM TCHUENTE Louis Albert	Professor	*Service Inspector/ Program leader MINSANTE*
11.	ZEBAZE TOGOUET Serge Hubert	Professor	On duty

12.	ALENE Désirée Chantal	Associate Professor	*Vice-Dean/ Uty Ebwa*
13.	ATSAMO Albert Donatien	Associate Professor	On duty
14.	BILANDA Danielle Claude	Associate Professor	On duty
15.	DJIOGUE Séfirin	Associate Professor	On duty
16.	GOUNOUE KAMKUMO Raceline épse FOTSING	Associate Professor	On duty
17.	JATSA BOUKENG Hermine épse MEGAPTCHE	Associate Professor	On duty

18.	KANDEDA KAVAYE Antoine	Associate Professor	On duty
19.	LEKEUFACK FOLEFACK Guy B.	Associate Professor	On duty
20.	MAHOB Raymond Joseph	Associate Professor	On duty
21.	MBENOUN MASSE Paul Serge	Associate Professor	On duty
22.	MOUNGANG Luciane Marlyse	Associate Professor	On duty
23.	NOAH EWOTI Olive Vivien	Associate Professor	On duty
24.	MONY Ruth épse NTONE	Associate Professor	On duty
25.	MVEYO NDANKEU Yves Patrick	Associate Professor	On duty
26.	NGUEGUIM TSOFACK Florence	Associate Professor	On duty
27.	NGUEMBOCK	Associate Professor	On duty
28.	TADU Zephyrin	Associate Professor	On duty
29.	TAMSA ARFAO Antoine	Associate Professor	On duty
30.	TOMBI Jeannette	Associate Professor	On duty
31.	YEDE	Associate Professor	On duty

32.	AMBADA NDZENGUE GEORGIA ELNA	Senior Lecturer	On duty
33.	BASSOCK BAYIHA Etienne Didier	Senior Lecturer	On duty
34.	ETEME ENAMA Serge	Senior Lecturer	On duty
35.	FEUGANG YOUMSSI François	Senior Lecturer	On duty
36.	FOKAM Alvine Christelle Epse KENGNE	Senior Lecturer	On duty
37.	FOSSI TANKOUA Olivia Epse DJEUTCHOUANG SAYANG	Senior Lecturer	On duty (transfer Uty of Dla)
38.	GONWOUO NONO Legrand	Senior Lecturer	On duty
39.	KOGA MANG DOBARA	Senior Lecturer	On duty
40.	LEME BANOCK Lucie	Senior Lecturer	On duty
41.	MAPON NSANGOU Indou	Senior Lecturer	On duty
42.	METCHI DONFACK MIREILLE FLAURE EPSE GHOUMO	Senior Lecturer	On duty
43.	NDENGUE Jean De Matha	Senior Lecturer	On duty
44.	NGOUATEU KENFACK Omer Bébé	Senior Lecturer	On duty
45.	NJUA Clarisse YAFI	Senior Lecturer	*Head Div. Uty Bamenda*
46.	NWANE Philippe Bienvenu	Senior Lecturer	On duty
47.	YOUNOUSSA LAME	Senior Lecturer	On duty
48.	ZEMO GAMO Franklin	Senior Lecturer	On duty

49.	KODJOM WANCHE Jacguy Joyce	Assistant	On duty

3- DEPARTMENT OF PLANT BIOLOGY AND PHYSIOLOGY (P.B.P) (37)

1.	AMBANG Zachée	Professor	*Head of Department*
2.	BIYE Elvire Hortense	Professor	On duty
3.	DJOCGOUE Pierre François	Professor	On duty
4.	MBOLO Marie	Professor	On duty
5.	NDONGO BEKOLO	Professor	On duty
6.	ZAPFACK Louis	Professor	On duty

7.	ANGONI Hyacinthe	Associate Professor	On duty

8.	DJEUANI Astride Carole	Associate Professor	On duty
9.	MAHBOU SOMO TOUKAM. Gabriel	Associate Professor	On duty
10.	MALA Armand William	Associate Professor	On duty
11.	NGALLE Hermine BILLE	Associate Professor	On duty
12.	NGONKEU MAGAPTCHE Eddy L.	Associate Professor	*CT / MINRESI*
13.	TONFACK Libert Brice	Associate Professor	On duty
14.	TSOATA Esaïe	Associate Professor	On duty
15.	ONANA JEAN MICHEL	Associate Professor	On duty

16.	DIDA LONTSI Sylvere Landry	Senior Lecturer	On duty
17.	GONMADGE CHRISTELLE	Senior Lecturer	On duty
18.	MAFFO MAFFO Nicole Liliane	Senior Lecturer	On duty
19.	MANGA NDJAGA JUDE	Senior Lecturer	On duty
20.	NNANGA MEBENGA Ruth Laure	Senior Lecturer	On duty
21.	NOUKEU KOUAKAM Armelle	Senior Lecturer	On duty
22.	NSOM ZAMBO EPSE PIAL ANNIE CLAUDE	Senior Lecturer	*On loan/UNESCO MALI*
23.	GODSWILL NTSOMBOH NTSEFONG	Senior Lecturer	On duty
24.	KABELONG BANAHO Louis-Paul-Roger	Senior Lecturer	On duty
25.	KONO Léon Dieudonné	Senior Lecturer	On duty
26.	LIBALAH Moses BAKONCK	Senior Lecturer	On duty
27.	LIKENG-LI-NGUE Benoit C	Senior Lecturer	On duty
28.	TAEDOUNG Evariste Hermann	Senior Lecturer	On duty
29.	TEMEGNE NONO Carine	Senior Lecturer	On duty
30.	BOLIE Hubert	Assistant	On duty
31.	MACHE NKOUANDEU Pasma	Assistant	On duty
32.	MAFFO FOKOU Adèle	Assistant	On duty
33	METSEBING Blondo-Pascal	Assistant	On duty
34.	NTONMEN YPNKEU Ammandine	Assistant	On duty
35.	ONANA EBODE Clotaire	Assistant	On duty

4- -DEPARTMENT OF INORGANIC CHEMISTRY (I.C) (28)

1.	GHOGOMU Paul MINGO	Professor	*Minister In charge of missions at the P.R*
2.	NANSEU NJIKI Charles Péguy	Professor	On duty
3.	NDIFON Peter TEKE	Professor	*CT MINRESI*
4.	NGOMO Horace MANGA	Professor	*Vice Chancelor/UB*
5.	NJIOMOU C. épse DJANGANG	Professor	On duty
6.	NJOYA Dayirou	Professor	On duty

7.	ACAYANKA Elie	Associate Professor	On duty
8.	EMADAK Alphonse	Associate Professor	On duty
9.	KAMGANG YOUBI Georges	Associate Professor	On duty
10	KEMMEGNE MBOUGUEM Jean C.	Associate Professor	On duty
11	KENNE DEDZO GUSTAVE	Associate Professor	On duty
12	MBEY Jean Aime	Associate Professor	On duty

13	NDI NSAMI Julius	Associate Professor	*Head of Department*
14	NEBAH Née NDOSIRI Bridget NDOYE	Associate Professor	*Senator/SENAT*
15	NYAMEN Linda Dyorisse	Associate Professor	On duty
16	PABOUDAM GBAMBIE AWAWOU	Associate Professor	On duty
17	TCHAKOUTE KOUAMO Hervé	Associate Professor	On duty
18	BELIBI BELIBI Placide Désiré	Associate Professor	*Head of Service/ HTTC Bertoua*
19	CHEUMANI YONA Arnaud M.	Associate Professor	On duty
20	KOUOTOU DAOUDA	Associate Professor	On duty

21	MAKON Thomas Beauregard	Senior Lecturer	On duty
22	NCHIMI NONO KATIA	Senior Lecturer	On duty
23	NJANKWA NJABONG N. Eric	Senior Lecturer	On duty
24	PATOUOSSA ISSOFA	Senior Lecturer	On duty
25	SIEWE Jean Mermoz	Senior Lecturer	On duty
26	BOYOM TATCHEMO Franck W.	Assistant	On duty
27	DANTIO NGUELA Christian Brice	Assistant	On duty
28	LEKENE NGOUATEU Reine	Assistant	On duty

5- DEPARTMENT OF ORGANIC CHEMISTRY (O.C) (33)			
1.	Alex de Théodore ATCHADE	Professor	*DEPE / Univ Bertoua*
2.	DONGO Etienne	Professor	*Vice-Dean/FES /UYI*
3.	NGOUELA Silvère Augustin	Professor	*Head of Department UDS*
4.	PEGNYEMB Dieudonné Emmanuel	Professor	*Rector UBertoua/ / Head of Department*
5.	MBAZOA née DJAMA Céline	Professor	On duty
6.	MKOUNGA Pierre	Professor	On duty

7.	AMBASSA Pantaléon	Associate Professor	On duty
8.	EYONG Kenneth OBEN	Associate Professor	On duty
9.	FOTSO WABO Ghislain	Associate Professor	On duty
10.	KAMTO Eutrophe Le Doux	Associate Professor	On duty
11.	KENMOGNE Marguerite	Associate Professor	On duty
12.	MVOT AKAK CARINE	Associate Professor	On duty
13.	NGOMO Orléans	Associate Professor	On duty
14.	NGO MBING Joséphine	Associate Professor	*Chief Unit MINRESI*
15.	NGONO BIKOBO Dominique Serge	Associate Professor	*C.E.A. / MINESUP*
16.	NOTE LOUGBOT Olivier Placide	Associate Professor	*Dir HTTC/Uty Bertoua*
17.	NOUNGOUE TCHAMO Diderot	Associate Professor	On duty
18.	TABOPDA KUATE Turibio	Associate Professor	On duty
19.	TAGATSING FOTSING Maurice	Associate Professor	On duty
20.	OUAHOUO WACHE Blandine M.	Associate Professor	On duty
21.	ZONDEGOUMBA Ernestine	Associate Professor	On duty

22.	MELONG Radius	Senior Lecturer	On duty
23.	MESSI Angélique Nicolas	Senior Lecturer	On duty
24.	MUNVERA MFIFEN Aristide	Senior Lecturer	On duty

25.	NGNINTEDO Dominique	Senior Lecturer	On duty
26.	NONO NONO Éric Carly	Senior Lecturer	On duty
27.	OUETE NANTCHOUANG Judith Laure	Senior Lecturer	On duty
28.	SIELINOU TEDJON Valérie	Senior Lecturer	On duty
29.	TCHAMGOUE Joseph	Senior Lecturer	On duty
30.	TSAFFACK Maurice	Senior Lecturer	On duty
31.	TSAMO TONTSA Armelle	Senior Lecturer	On duty
32.	TSEMEUGNE Joseph	Senior Lecturer	On duty

33	NDOGO ETEME Olivier	Assistant	On duty
34	NGUEMDJO CHIMEZE Valery Wilfried	Assistant	On duty

6- DEPARTMENT OF RENEWABLE ENERGY (RE) (1)			
1.	BODO Bertrand	Professor	*Head of Department*

7- DEPARTMENT OF COMPUTER SCIENCES (C.S) (22)			
1.	ATSA ETOUNDI Roger	Professor	*Chief Division MINESUP*
2.	FOUDA NDJODO Marcel Laurent	Professor	*Academic General Inspector / MINESUP*
3.	NDOUNDAM Réné	Professor	On duty

4.	ABESSOLO ALO'O Gislain	Associate Professor	*Chief of Unit Head MINFOPRA*
5.	MELATAGIA YONTA Paulin	Associate Professor	On duty
6.	TSOPZE Norbert	Associate Professor	On duty
7.	AMINOU HALIDOU	Senior Lecturer	*Head of Department*
8.	DJAM Xaviera YOUH - KIMBI	Senior Lecturer	On duty
9.	DOMGA KOMGUEM Rodrigue	Senior Lecturer	On duty
10	EBELE Serge Alain	Senior Lecturer	On duty
11	EKODECK Stéphane Gaël Raymond	Senior Lecturer	On duty
12	HAMZA Adamou	Senior Lecturer	On duty
13	JIOMEKONG AZANZI Fidel	Senior Lecturer	On duty
14	KOUOKAM KOUOKAM E. A.	Senior Lecturer	On duty
15	MESSI NGUELE Thomas	Senior Lecturer	HOD/info engeneering/ UEbwa
16	MONTHE DJIADEU Valery M.	Senior Lecturer	On duty
17	NZEKON NZEKO'O ARMEL JACQUES	Senior Lecturer	On duty
18	OLLE OLLE Daniel Claude Georges Delort	Senior Lecturer	*Assistant Director ENSET Ebolowa*
19	TAPAMO Hyppolite	Senior Lecturer	On duty

20	BAYEM Jacques Narcisse	Assistant	On duty
21	MAKEMBE. S. Oswald	Assistant	*Director CUTI*

22	MAXWELL NDONGNKON MANGA	Assistant	On duty
23	NDOM Francis Rolin	Assistant	On duty
24	NGUIMEYA TSOFACK Baudoin	Assistant	On duty
25	NKONDOCK. MI. BAHANACK.N.	Assistant	On duty

8- DEPARTEMENT OF MATHEMATICS (MA) (34)

1.	AYISSI Raoult Domingo	Professor	*Head of Department/ D ENSPY*

2.	KIANPI Maurice	Associate Professor	On duty
3.	MBANG Joseph	Associate Professor	On duty
4.	MBEHOU Mohamed	Associate Professor	*Head of Division/ENSPY*
5.	MBELE BIDIMA Martin Ledoux	Associate Professor	On duty
6.	NOUNDJEU Pierre	Associate Professor	*VDRC/FS/UYI*
7.	TAKAM SOH Patrice	Associate Professor	On duty
8.	TCHAPNDA NJABO Sophonie B.	Associate Professor	*Director/AIMS Rwanda*
9.	TCHOUNDJA Edgar Landry	Associate Professor	On duty

10	AGHOUKENG JIOFACK Jean Gérard	Senior Lecturer	*Chief Unit MINEPAT*
11	BOGSO ANTOINE Marie	Senior Lecturer	On duty
12	BITYE MVONDO Esther	Senior Lecturer	On duty
13	CHENDJOU Gilbert	Senior Lecturer	On duty
14	DJIADEU NGAHA Michel	Senior Lecturer	On duty
15	DOUANLA YONTA Herman	Senior Lecturer	On duty
16	KIKI Maxime Armand	Senior Lecturer	On duty
17	KOKOMO AYISSI Eric Brice	Senior Lecturer	On duty (Transfer of the University of Douala)
18	LOUMNGAM KAMGA Victor	Senior Lecturer	On duty
19	MBAKOP Guy Merlin	Senior Lecturer	On duty
20	MBATAKOU Salomon Joseph	Senior Lecturer	On duty
21	MENGUE MENGUE David Joël	Senior Lecturer	*HOD / HTTC, Uty Ebwa*
22	MBIAKOP Hilaire George	Senior Lecturer	On duty
23	NGUEFACK Bernard	Senior Lecturer	On duty
24	NIMPA PEFOUKEU Romain	Senior Lecturer	On duty
25	OGADOA AMASSAYOGA	Senior Lecturer	On duty
26	POLA DOUNDOU Emmanuel	Senior Lecturer	On duty
27	TENKEU JEUFACK Yannick Léa	Senior Lecturer	On duty
28	TCHEUTIA Daniel Duviol	Senior Lecturer	On duty
29	TETSADJIO TCHILEPECK M. Eric.	Senior Lecturer	On duty

30	EBODE ATANGANA Pie Désiré	Assistant	On duty
31	FOKAM Jean Marcel	Assistant	On duty
32	GUIDZAVAI KOUCHERE Albert	Assistant	On duty
33	MAMA ASSANDJE Prosper	Assistant	On duty
34	MANN MANYOMBE Martin Luther	Assistant	On duty
35	MEFENZA NOUNTU Thiery	Assistant	On duty

36	NYOUMBI DLEUNA Christelle	Assistant	On duty

9- DEPARTMENT OF MICROBIOLOGY (MB) (24)

1.	ESSIA NGANG Jean Justin	Professor	*Head of Department*
2.	KOUITCHEU MABEKU Epse KOUAM Laure Brigitte	Professor	On duty
3.	MUNE MUNE Martin Alain	Professor	On duty
4.	NYEGUE Maximilienne Ascension	Professor	*VICE-DEAN / DSSE*
5.	RIWOM Sara Honorine	Professor	On duty
6.	SADO KAMDEM Sylvain Leroy	Professor	On duty

7.	ASSAM ASSAM Jean Paul	Associate Professor	*Dean/ FASA/UDs*
8.	BOUGNOM Blaise Pascal	Associate Professor	On duty
9.	NJIKI BIKOÏ Jacky	Associate Professor	On duty
10	TCHIKOUA Roger	Associate Professor	*Chief Serv. Of education*
11	EHETH Jean Samuel	Senior Lecturer	On duty
12	ESSONO Damien Marie	Senior Lecturer	On duty
13	EZO'O MENGO Fabrice Télésfor	Senior Lecturer	On duty
14	LAMYE Glory MOH	Senior Lecturer	On duty
15	MEYIN A EBONG Solange	Senior Lecturer	On duty
16	MONI NDEDI Esther Del Florence	Senior Lecturer	*Chief Serv/DAAC/UY1*
17	NKOUDOU ZE Nardis	Senior Lecturer	On duty
18	NKOUE TONG Abraham	Senior Lecturer	On duty
19	NGOUENAM Romial Joël	Senior Lecturer	On duty
20	NJAPNDOUNKE Bilkissou	Senior Lecturer	On duty
21	TAMATCHO KWEYANG Blandine Pulchérie	Senior Lecturer	On duty
22	SAKE NGANE Carole Stéphanie	Senior Lecturer	On duty
23	TOBOLBAÏ Richard	Senior Lecturer	On duty

24	ZO'O EZO'O Fabrice Télesfor	Assistant	On duty
25	MAYI Marie Paule Audrey	Assistant	On duty

10. -DEPARTMENT OF PHYSICS (PH) (45)

1.	BEN- BOLIE Germain Hubert	Professor	On duty
2.	BIYA MOTTO Frédéric	Professor	*GD/HYDRO Mekin*
3.	DJUIDJE KENMOE épouse ALOYEM	Professor	On duty
4.	EKOBENA FOUDA Henri Paul	Professor	*Vice-Rector Uty Ngaoundéré*
5.	ESSIMBI ZOBO Bernard	Professor	On duty
6.	EYEBE FOUDA Jean sire	Professor	On duty
7.	FEWO Serge Ibraïd	Professor	On duty
8.	HONA Jacques	Professor	On duty

9.	NANA ENGO Serge Guy	Professor	On duty
10.	NANA NBENDJO Blaise	Professor	On duty
11.	NDJAKA Jean Marie Bienvenu	Professor	*Head of Department*
12.	NJANDJOCK NOUCK Philippe	Professor	On duty
13.	SAIDOU	Professor	*Head of Center/IRGM/MINRESI*
14.	SIEWE SIEWE Martin	Professor	On duty
15.	SIMO Elie	Professor	On duty
16.	TABOD Charles TABOD	Professor	*Dean FS/ Univ Bda*
17.	TCHAWOUA Clément	Professor	On duty
18.	WOAFO Paul	Professor	On duty
19.	ZEKENG Serge Sylvain	Professor	On duty
20.	VONDOU Derbetini Appolinaire	Professor	On duty
21.	ENYEGUE A NYAM épse BELINGA	Associate Professor	*Head of Continuing and Distance Training Division/ ENSPY*
22.	FOUEJIO David	Associate Professor	*Chief unit/ MINADER*
23.	MBINACK Clément	Associate Professor	On duty
24.	MBONO SAMBA Yves Christian U.	Associate Professor	On duty
25.	MELI'I Joelle Larissa	Associate Professor	On duty
26.	MVOGO ALAIN	Associate Professor	On duty
27.	NDOP Joseph	Associate Professor	On duty
28.	WAKATA née BEYA Annie Sylvie	Associate Professor	*General secretary /UYII*
29.	WOULACHE Rosalie Laure	Associate Professor	*Absent from her duty*
30.	ABDOURAHIMI	Senior Lecturer	On duty
31.	AYISSI EYEBE Guy François Valérie	Senior Lecturer	On duty
32.	CHAMANI Roméo	Senior Lecturer	On duty
33.	DJIOTANG TCHOTCHOU Lucie Angennes	Senior Lecturer	On duty
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28.	NGO'O ZE ARNAUD	Senior Lecturer	On duty
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30.	TEHNA Nathanaël	Senior Lecturer	On duty
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32.	FEUMBA Roger	Senior Lecturer	On duty
33.	MBANGA NYOBE Jules	Senior Lecturer	On duty

Number of permanent teachers of the University of Yaounde I

NUMBER OF TEACHERS					
DEPARTMENT	**Professors**	**Associate Professor**	**Senior Lecturer**	**Assistants**	**Total**
BCH	08 (01)	24 (14)	11 (02)	00 (01)	**44 (21)**
BPA	08 (01)	18 (07)	16 (06)	01 (01)	**49 (15)**
BPV	12(02)	10 (02)	14 (07)	06 (00)	**32 (14)**
CI	08 (02)	14 (03)	05 (01)	03 (00)	**27 (05)**
CO	06 (01)	14 (05)	12 (05)	02 (00)	**33 (09)**
RE	06 (01)	/	/	/	**01 (0)**
IN	03 (00)	03 (00)	13 (01)	06 (00)	**22 (01)**
MAT	01 (00)	08 (00)	20 (02)	07 (01)	**34 (02)**
MIB	05 (01)	05 (03)	13 (05)	01 (02)	**24 (11)**
PHY	17 (01)	11 (04)	15 (01)	04 (00)	**41(08)**
ES	10 (00)	14 (03)	09 (03)	00 (00)	**34 (07)**
Total	**67 (11)**	**122 (41)**	**128 (32)**	**25 (09)**	**342 (88)**

A total of **360 (94)** including:

- Professors **67 (11)**
- Associate Professor **123 (41)**
- Senior Lecturer **122 (32)**
- Assistants **30 (09)**

( ) = Number of women **94**

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18.	KOFANE TIMOLEON CREPIN							PR
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20.	BITOM DIEUDONNE	547050X	23/11/1958	Ambam	M			PR

DEDICATION

To people hoping for children. *This work is for you, from me, your loving researcher. It is the fruit of so many years of effort and arduous sacrifices. Find in it one of the causes of the current decline fertility.*

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SOMMARY

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LIST OF ABBREVIATIONS AND SYMBOLS

ADI	Authorized Daily Intake
ARH	Arcuate nucleus of the hypothalamus
BPA	Bisphenol A
CBG	Corticosteroid Binding Globulin
CNS	Central nervous System
DNA	Deoxyribonucleic Acid
EDCs	Endocrine-Disrupting Chemicals
EFSA	European Food Safety Authority
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Estrogen Receptor
ERE	Estrogen Response Element
FSH	Follicle-Stimulating Hormone
GnRH	Gonadotropin-Releasing Hormone
GnRs	Gonadotrophins receptors
HDL	High Density Lipoprotein
HPG	Hypothalamic–Pituitary–Gonadal
HPO	Hypothalamic-Pituitary-Ovarian
HRE	Hormone Response Element
HRP	Horseradish Peroxidase
HSP 90 and 70	Heat shock proteins 90 and 70
LBD	Ligand binding domain
LDL	Low density lipoprotein
LH	Luteinizing Hormone
MPN	Medial Preoptic Nucleus
OS	Oxidative Stress
PCOS	polycystic Ovary Syndrome
PND	Postnatal Day
PRs	Progesterone Receptors
PS	Potassium sorbate
ROS	Reactive Oxygen Species
SHBG	Sex Hormone Binding Globulin
T	Tartrazine
TMB	Tetramethylbenzidine
TRE	Trans-regulatory elements
VMH	Ventromedial nucleus of the hypothalamus

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ABSTRACT

Over the past century, a growing number of couples find it difficult to procreate without medical assistance. World Health Organization (WHO), suggest that around one in every six people of reproductive age worldwide experience infertility in their lifetime, and it has an impact on their families and communities. Worldwide, an increase in the prevalence of this reproductive disorder, observed in both humans and wildlife has been more and more linked to the lifestyle factors such as exposure to endocrine-disrupting chemicals (EDCs). Several food additives present in our food may enhance the risk of infertility in both sexes. Therefore, it is crucial to understand the impact of human exposure to these substances in order to protect the health of the population and the perpetuation of species. On this basis, a pre-pubertal exposure to potassium sorbate (12.5, 45, and 78 mg/kg BW doses) and tartrazine (7.5, 27, and 47 mg/kg BW doses) and an-extended three generations reproductive toxicity were evaluated in immature female Wistar rats. The impact of their daily oral administration from postnatal day (PND) 21 till the parturition was evaluated. The work aimed to study their effects on the activation of hypothalamic-hypophysis-ovarian axis, sexual behavior, fertility of parents (F0) and offsprings of the first, second and third generations. Results from the evaluation of their effects on sexual maturation showed that potassium sorbate and tartrazine had no significant effect on the body weight gain of the parents (F0). However, compared to the control group, animals receiving potassium sorbate (78 mg/kg BW), showed significantly decrease in percentage of animal with vaginal opening ($p < 0.001$) on postnatal day 46, the number total follicles ($p < 0.001$), primary follicles ($p < 0.01$), relative weight of ovaries ($p < 0.01$) and an increase in the number of atresia follicles ($p < 0.001$). Concerning tartrazine (47 mg/kg BW), a significant high percentage of early vaginal opening was observed from day 46 of age ($p < 0.05$). Likewise, the results showed that tartrazine at the dose of 47 mg/kg BW significantly increased the number of total ($p < 0.001$), primary ($p < 0.01$), secondary ($p < 0.001$), and antral ($p < 0.01$) follicles, estrogen and LH serum concentration ($p < 0.001$) and uterine epithelial thickness at 27 ($p < 0.05$) and 47 ($p < 0.01$) mg/kg/BW. On sexual behavior, the present study showed that the administration of potassium sorbate (12.5, 45, and 78 mg/kg BW) or tartrazine (7.5, 27, and 47 mg/kg BW) to sexually immature rats F0 was associated with the decrease ($p < 0.05$) of estrous cycle ratio (tartrazine at the dose of 7.5 mg/kg BW ($p < 0.05$)), the number of 4 to 5 days cycles (tartrazine at the doses of 7.5 and 47 mg/kg BW, $p < 0.05$) and an increase ($p < 0.01$) of the number of irregular cycles (potassium sorbate at the dose 78 mg/kg BW, $p < 0.01$). Following to this, the analysis of the videos recorded during the sexual behavior test showed that the daily oral

administration of a single dose potassium sorbate or tartrazine to F0 was responsible for the increase the compartment change frequency (T 27, $p < 0.01$ and T 47, $p < 0.001$), hopping latency (PS 45, $p < 0.05$), number of rejections (PS 12.5, $p < 0.001$) and lordosis latency (PS 78, $p < 0.001$ and T 27, $p < 0.05$) and a decrease in the darting latency (T 7.5, $p < 0.01$), hopping latency (T at all the tested doses), and lordosis frequency (PS 12.5 and 45, $p < 0.01$ and T 27, $p < 0.001$). The evaluation of their effects on fertility revealed that exposure to potassium sorbate or tartrazine from PND 21 to the parturition is responsible for the decrease of fertility index, gestational rate, implantation index and the increase of the resorption index and post-implantation loss rate as compared to the control group. The administration of potassium sorbate or tartrazine to pregnant females was responsible for the decrease of the offspring's weight gaining (PS 12.5, F3; PS 45, F2; PS 78, F1 and F3; T 7.5, F1; F2; and F3; T 27, F3; T 47, F1 and F3), sex ratio (potassium sorbate and tartrazine at all the tested doses), mean age of vaginal opening (T 47, F1), percentage of rats with vaginal opening and relative weight of uterus (PS 12.5, F1) and an increase of the mean age of animals with vaginal opening (PS 12.5, F1 and F2). Statistical differences regarding the number of fetuses and fetal and placental weight were also significant. Tartrazine at the doses of 7.5 and 47 mg/kg BW was responsible for the decrease of fertility index and gestational rate at the F1. As compared to the control group, potassium sorbate significantly increased the uterine epithelial thickness in all the generations (PS 12.5 F1, $p < 0.001$; PS 12.5 F2, $p < 0.01$; PS 45 F2, $p < 0.05$; PS 12.5 F3, $p < 0.001$; PS 45 F3, $p < 0.001$). Concerning biochemical parameters evaluated in the third generation, potassium sorbate decreased the ovarian total protein (PS 78, $p < 0.001$) and progesterone serum concentration (PS 45 and 78). On folliculogenesis, the results obtained showed that potassium sorbate decreased the number of primary follicles (PS 78, $p < 0.001$) and increased the number of atresia follicle (PS 78) at the third generation. To conclude, these results could validate at least in part the global observations on increasingly declining fertility linked to the exposure to food additives and provide a substantial scientific prove confirming potassium sorbate and tartrazine as endocrine disruptors.

Key words: food additive; tartrazine; potassium sorbate; rat; infertility; folliculogenesis; endocrine disruptor

RESUME

Au cours du siècle dernier, un nombre croissant de couples éprouve des difficultés à procréer sans assistance médicale. Selon les estimations de l'Organisation Mondiale de la Santé (OMS), environ une personne sur six en âge de procréer dans le monde est confrontée à l'infertilité au cours de sa vie, ce qui impacte sa famille et sa communauté. Dans le monde, l'augmentation de la prévalence de ce trouble de la reproduction, observée tant chez les humains que chez les animaux, est de plus en plus liée au mode de vie, tel que l'exposition aux perturbateurs endocriniens (PEs). Plusieurs additifs alimentaires présents dans notre alimentation peuvent augmenter le risque d'infertilité autant bien chez l'homme que chez la femme. Il est donc crucial de comprendre leur impact afin de protéger la santé de la population et la fonction de reproduction. Sur cette base, les effets d'une exposition des rates immatures de souche Wistar au sorbate de potassium (doses de 12,5, 45 et 78 mg/kg PC) et à la tartrazine (doses de 7,5, 27 et 47 mg/kg PC) ont été évalués. Les effets d'une administration orale quotidienne du sorbate de potassium et de la tartrazine chez les rates de 21 jours d'âge jusqu'à la mise bas ont fait l'objet de cette étude. Il était question pour nous d'étudier leurs effets sur l'activation de l'axe hypothalo-hypophyso-ovarien, le comportement sexuel, la fertilité des parents et des descendants de la première, deuxième et troisième génération. Comparé au groupe témoin, aucune variation significative n'a été observée en ce qui concerne le poids corporel des parents (F0) traités au sorbate de potassium et à la tartrazine. Les résultats ont montré que, les rats recevant le sorbate de potassium (78 mg/kg PC) présentaient une diminution significative ($p < 0,001$) du pourcentage d'animaux avec ouverture vaginale au jour 46 postnatal, ainsi qu'une diminution du nombre de follicules primaires ($p < 0,01$), du poids relatif des ovaires et une augmentation du nombre de follicules en atrésie ($p < 0,001$). La tartrazine quant à elle, a entrainé à la dose de 47 mg/kg PC, une augmentation significative du pourcentage de rates avec ouverture vaginale au jour 46 postnatal ($p < 0,05$), une augmentation significative du nombre de follicules totaux ($p < 0,01$), primaires ($p < 0,01$), secondaires ($p < 0,001$) et antraux ($p < 0,01$) chez les animaux F0. Apres 40 jours de traitement des parents F0, la tartrazine a également entrainé à la dose de 47 mg/kg, une augmentation significative du taux sérique d'œstrogène et de LH, ainsi qu'une augmentation de la taille de l'épithélium utérin aux doses de 27 et 47 mg/kg PC. Sur le comportement sexuel, la présente étude montre que l'administration d'une dose unique de sorbate de potassium (12,5, 45 et 78 mg/kg PC) et de la tartrazine (7,5, 27 mg/kg PC et de 47 mg/kg PC) aux rates F0 a entrainé une diminution ($p < 0.05$) du ratio du cycle œstral (tartrazine à la dose de 7,5 mg/kg PC), du nombre des cycles de 4

à 5 jours (tartrazine aux doses de 7,5 et 47 mg/kg PC) et une augmentation ($p < 0,01$) du nombre des cycles irréguliers (sorbate de potassium à la dose de 78 mg/kg PC). L'analyse des vidéos enregistrées lors du test de comportement sexuel a montré que l'administration du sorbate de potassium et de la tartrazine à la F0 a induit une augmentation de la fréquence de changement de compartiment (T 27, $p < 0,01$; et T 47, $p < 0,001$), de la latence de « hopping » (SP 45, $p < 0,05$), du nombre de rejets (SP 12,5, $p < 0,001$) et de la latence de lordose (SP 78, $p < 0,001$ et T 27, $p < 0,05$) et d'une diminution de la latence de « darting » (T 7,5, $p < 0.01$), de « hopping » (tartrazine à toutes les doses testées), et de la fréquence de lordose (SP 12,5 et 45, $p < 0,01$). L'évaluation des effets sur la fertilité a révélé que l'exposition au sorbate de potassium ou à la tartrazine du jour 21 postnatal à la parturition a entrainé une diminution de l'index de fertilité, du taux de gestation, de l'index d'implantation et de l'augmentation de l'index de résorption et des pertes avant implantation, ceci comparé au groupe témoin. Les résultats sur la toxicité reproductive montrent que l'administration du sorbate de potassium ou de la tartrazine aux femelles gestantes a provoqué une diminution du gain pondéral (SP 12,5, F3 ; SP 45, F2 ; SP 78, F1 et F3 ; T 7,5, F1 ; F2 ; et F3 ; T 27, F3 ; T 47, F1 et F3), du sex ratio (sorbate de potassium et tartrazine à toutes les doses), de l'âge moyen de l'ouverture vaginale (T 47 F1), du pourcentage de rates avec ouverture vaginale et du poids relatif de l'utérus avec (SP 12.5 F1), et une diminution de l'âge moyen de l'ouverture vaginale (SP 12.5 F1 et F2). La tartrazine aux doses de 7.5 et 47 mg/kg PC a entrainé une diminution de l'index de fertilité et du taux de gestation en F1. Comparé au groupe témoin, le sorbate de potassium a significativement augmenté la taille de l'épithélium utérin (SP 12.5 F1, $p < 0.001$; SP 12.5 F2, $p < 0.01$; SP 45 F2, $p < 0.05$; SP 12.5 F3, $p < 0.001$; SP 45 F3, $p < 0.001$). En ce qui concerne l'évaluation des paramètres biochimiques chez les animaux de la troisième génération, le sorbate de potassium a entrainé une diminution significative de la concentration en protéines totales ovariennes (SP 78, $p < 0.001$) et de la concentration sérique en progestérone (PS 45 et 78. Sur la folliculogenèse, les résultats montrent que le sorbate de potassium a également entrainé une diminution significative du nombre de follicules primaires (PS 78, $p < 0.001$) et a augmentation significative du nombre de follicules en atrésie (PS 78). En conclusion, ces résultats pourraient valider en partie les observations mondiales sur la baisse de la fertilité liee a l'exposition aux additifs alimentaires et apportent une preuve scientifique étayant les effets perturbateurs du système endocrinien du sorbate de potassium et de la tartrazine.

Mots clés : Additifs alimentaires ; Tartrazine ; Sorbate de potassium ; Rat ; Infertilité ; Folliculogenèse ; Perturbateurs endocriniens

INTRODUCTION

Infertility is defined as the incapacity of a couple to conceive and achieve pregnancy after 12 months of consistent unprotected sexual intercourse with no contraceptive measures taken (Gnoth *et al*., 2005; Abebe *et al*., 2020; Chorosho *et al*., 2023). This disease of the reproductive system (Nagórska *et al*., 2019) can be categorized into two sub-categories: primary and secondary infertility. The former is the inability to conceive and have a successful live birth when you have never had a child, while the latter occurs when individuals have at least one previous biological child (Romeiro *et al*., 2017). Infertility represents one of the most important complications in gynecology (Moridi *et al.,* 2019) with a pooled prevalence of 30-40% in Sub-Saharan Africa (Inhorn and Patrizio, 2015) and 20-30% in Cameroon (Charlotte *et al.,* 2021); environments where a woman's capacity for childbearing determines whether a marriage will be successful or not (Bokaie *et al.,* 2015). In addition, many studies have shown that females related causes of couple's infertility represents for about 54.01% against 22.26% in men (Abebe *et al*., 2020). Being infertile can affect all aspects of lives of infertile women, which can cause serious psychological trauma and social humiliation. In some cases, infertility can lead to social disgrace and exclusion, verbal and physical abuse, marriage violence, and breakup. Especially for women, infertility decreases their quality of life and exposes them to multiple sexual partners, sexually transmitted diseases, and increased sexual dysfunction (Monga *et al*., 2004; Abebe *et al.,* 2020; Silva *et al*., 2023). In addition, dealing with infertility results in a loss of privacy surrounding the couple's sexual life and frequently leads to having sex for reproduction rather than pleasure (Onat and Kizilkaya, 2012; Esselstrom, 2014).

It is assumed that physical, stress-related, and lifestyle factors influence fertility. These include environmental factors such as fetal nutrition, childhood eating habits, physical activity, and exposure to electromagnetic fields and/or endocrine-disrupting chemicals (EDC) (Belcher *et al*., 2019; Stagi *et al*., 2020). It has been shown that urbanization and the professionalization of couples, as well as continued consumption of industrially processed foods damage the endocrine system. This growth in the consumption of processed foods is the source of a lively debate about the release of toxins into the environment (Marques-Pinto and Carvalho, 2013; Kabir *et al.,* 2015; Silva *et al*., 2023**)**. In recent decades, the global food industry makes use of increasing amounts of natural and synthetic food additives which are suspected to be harmful and potentially toxic to health (Scippo and Maghuin-Rogister, 2007; Zingue *et al*., 2021). An increase in the prevalence of reproductive disorders in both humans and wildlife has been linked to accidental exposure to endocrine-disrupting chemicals (EDCs) and their increase production (Ricard, 2011; Frade Costa *et al*., 2014; Tassinari *et al*., 2021; Delbes *et al*., 2022). Many concerns such as early puberty, declining fertility, and cancers, are emerging about the long-term effects on human health following chronic exposure to these substances (Frade Costa *et al*., 2014; Tassinari *et al*., 2021;

Zingue *et al*., 2021**)**. Endocrine disruptors are identified as one of the causes of genital tract malformations, metabolic disorders, neurological problems, learning disabilities, decreased immune defences, allergies, polycystic ovarian syndromes, precocious/delay puberty, cancers, decreased fertility that can lead to the extinction of entire population (Samuel *et al.,* 2014; Belcher *et al*., 2019). According to the literature, the timing of EDC exposure represents a key to determine their outcomes. Their effects on health vary according to the age (early childhood, puberty) and physiological state (pregnant women) of the exposed individuals (Gore *et al*., 2015). It has also been shown that exposure to endocrine-disrupting chemicals (EDCs) during gestation influences the development of a generation of offspring; this may be responsible for certain diseases and dysfunctions in adulthood (Gore *et al*., 2014). The concern is bigger for children considered more vulnerable (Ricard, 2011) as the intensive use of food additives exposes them more (Ricard, 2011; Frade Costa *et al*., 2014; Tassinari *et al*., 2021). Therefore, it is crucial to understand the impacts of human exposure to EDCs in order to protect the health of the population and to preserve species. Unfortunately, few studies on their effects on women's reproductive function are available. In addition, compared to boys, alterations in the female tract are likely to remain invisible until they reach sexual maturity (puberty). Faced with the hypothesis that food additives contribute to the current decline fertility, it becomes urgent not only to identify and quantify each substance used as food additives in our diet but also to evaluate their potential endocrine-disrupting activity (Gore *et al*., 2014). In addition, the access to secure, and healthy food is crucial for the maintenance of health and wellbeing. Among these potential EDC, our interest was based on potassium sorbate and tartrazine. In line with this, the present study was designed and carried out to evaluate the potential endocrine disruptor activity of potassium sorbate and tartrazine on the reproductive system in immature female Wistar rats.

According to the literature, potassium sorbate is one of the food preservatives and personal care products, suspected to have side effects on the female reproduction system (EFSA *et al*., 2019; Hasson, 2020). It has been demonstrated that it is responsible for the increase of atresia follicles in rat ovaries, inflammation of oviducts, and degeneration of endometrium in the uterus of female rats (Hasson, 2020). Potassium sorbate is a water-soluble salt of sorbic acid isolated from the berries of the mountain ash tree in 1859. According to the literature, potassium sorbate is responsible for oxidative stress (Sugihara *et al*., 1998), mutagenicity, and DNA (Deoxyribonucleic acid)-damaging activity (Kitano *et al*., 2002), genotoxicity and cytotoxicity. To assess the damage caused by potassium sorbate in humans, Mamur and colleagues, (2010) evaluated its genotoxic potential in cultured and isolated human lymphocytes. From the findings, potassium sorbate was seen to be genotoxic to the human peripheral blood lymphocytes (Mamur *et al*., 2010). The aforementioned effects can negatively affect immunity and lead to various chronic diseases such

as cancers, diabetes mellitus and others. In 2019, following a re-evaluation study of sorbic acid (E 200) and its potassium salt potassium sorbate (E 202) as food additives, the European Food Safety Authority (EFSA) alerted to the revision of the authorized daily intake (ADI) (25 mg/kg BW).

Tartrazine is one of the most used colorants by children and adolescents and this makes them the most exposed population (Dixit *et al*., 2010). Known as E102, FD and C Yellow 5, C.I 19140, acid Yellow 23, Food yellow 4 or trisodium 1-(4-sulfonatophenyl)-4- sulfonatophenylazo)-5pyrazolonz-3-carboxylate, tartrazine is a synthetic lemon yellow azo dye made by coal tar (Wopara *et al*., 2021; Shakoor *et al*., 2022) with the chemical formula: 4-5-Dihydro-5-oxo-1-(4-sulfophenyl)4- ((4-sulfophenyl) aso) 1H-pyrazole-3 carboxylic acid (Khera and Munro, 1979**)**. Tartrazine is an orange water-soluble powder very widespread (in drinks, cookies, confectionery, preserves, yogurts, cosmetics, drugs, among others.) used as a dye. It has been shown in previous *in vitro* studies to be responsible for allergies, tumor diseases, mutagenic and genotoxic effects, and neuro-behavioural disorders (hyperactivity and sleep disturbance in children) (Rowe and Rowe, 1994; Dawodu and Akpomie, 2016; Khayyat *et al*., 2017; Zingue *et al*., 2021). Prolonged usage of tartrazine increases the number of gastric mucosa lymphocytes and eosinophils (Moutinho *et al*., 2007). Several studies showed that tartrazine also has adverse effects on male reproduction especially on sperm parameters (negative impact on sperm maturation process and decrease in sperm density, mobility, and viability) (Boussada *et al*., 2017; Wopara *et al*., 2021). These effects are accompanied by a significant decrease in serum testosterone concentration (Boussada *et al*., 2017). The combined treatment of tartrazine and erythrosine mixture in adult male rats impairs testicular architecture and function and is accompanied by an increase in a serum hormone (Luteinizing Hormone (LH), Follicle Stimulating Hormone (FSH) and testosterone (Mehedi *et al*., 2009). In female rats, the frequent intake or increase of tartrazine affects thyroid and reproductive hormones (LH, FSH, estrogen, progesterone) and mineral content in tissues; increases the chances of free radical production, leading to the development of oxidative stress in the body (Shakoor *et al*., 2022). In an *in vito* study, tartrazine has also been classified as a xenoestrogen (Axon *et al*., 2012; Nasri and Pohjanvirta, 2021) that can bind to estrogen receptor α (ERα) in the Michigan Cancer Foundation-7 (MCF-7) cell line and induce a proliferative effect in breast cancer cells and increase the expression of an estrogen receptor gene (Datta and Lundin-Schiller, 2008). Despite the multiple effects of tartrazine, especially on reproductive hormones (Mehedi *et al*., 2009; Axon *et al*., 2012; Boussada *et al*., 2017; Nasri and Pohjanvirta, 2021; Shakoor *et al*., 2022) which are responsible for sex maturation, such as folliculogenesis, ovulation, reproductive behaviors and successful of pregnancy, there is still a lack of available information

about its harmful effects on female reproductive function. This work aimed at assessing if potassium sorbate and tartrazine disrupt the endocrine system and lead to decrease fertility.

RESEARCH HYPOTHESIS

Potassium sorbate and tartrazine disrupt the endocrine system leading to the alterations of sexual maturation, sexual behavior and fertility.

GENERAL OBJECTIVE

This study was undertaken to evaluate the effects of potassium sorbate and tartrazine on the female reproductive system in a model of immature female Wistar rats.

SPECIFIC OBJECTIVES

Specifically, our goals aimed to evaluate:

- The effects of potassium sorbate and tartrazine on the activation of the hypothalamic-hypophysis-ovarian axis, sexual behavior and the fertility of parents (F0).
- The effects of potassium sorbate and tartrazine on hypothalamic-hypophysis-ovarian axis, sexual behavior and fertility of F1, F2 and F3.

CHAPTER I

LITERATURE REVIEW

I.1. Female reproductive system

I.1.1. Sexual maturation in females

According to the literature, the timing of pubertal onset depends on genetic and environmental factors. The genes involved in this process are the ones that control GnRH hypothalamic secretion, pituitary development and its functioning, hormone synthesis and bioactivity, energy homeostasis and growth, and potential peripheral feedback from sex steroids (Perry *et al*., 2014). Puberty is a major developmental event at the end of the juvenile stage, with marked physical and psychological changes, which prepare for adulthood (Eckert-Lind *et al*., 2020; Stagi *et al*., 2020). It can be seen as a complex sequence of biological events marked by the reactivation of the hypothalamic-pituitary-gonadal axis after a period of quiescence during childhood; followed by an important increase in sex hormone secretion by the gonads, which leads to a gradual maturation of sexual characteristics which culminate into the attainment of full adult reproductive capacity (Teilmann *et al*., 2003; Bellis *et al*., 2006; Eckert-Lind *et al*., 2020). The onset of secondary sexual characteristics, and the pubertal growth spurt, are markers of this developmental process that lead to sexual and reproductive maturity, the development of mental processes and adult identity (Brito *et al*., 2016). Puberty is accompanied by bodily changes, encourages curiosity, promotes interest in sexual activity, increases aggression in adolescents, and can intensify risky behaviors (Bellis *et al*., 2006). Before the puberty, the ovaries continuously grow and secrete in small number of oestrogens, which inhibits the release of gonadotropin-releasing hormone (GnRH) from the hypothalamus. At puberty, when leptin levels are appropriate, the hypothalamus becomes less sensitive to oestrogen and begins to release GnRH in a cyclical mode. GnRH stimulates the release of FSH and LH from the adenohypophysis which induce ovarian folliculogenesis, steroidogenesis, ovulation and formation of corpus luteum. In return, these two hormones act on the ovaries to allow the secretion of ovarian hormones, especially oestrogens. Oestradiol produced by the ovary is responsible for the development of secondary sexual characteristics (growth and development of the breasts and reproductive organs, redistribution of fat mass (hips, breasts) and bone maturation). All these events, responsible for maturation of reproductive neuroendocrine function are responsible for to the onset of ovulatory menstrual cycles (Marieb and Hoehn, 2010). Any dysregulation of these events can lead to the early or delay sexual maturation (Dallos-Lara and Mendoza-Rojas, 2020). Thus, delay and early sexual activity have many consequences such as the current decline fertility (Scippo and Maghuin-Rogister, 2007; Ricard, 2011; Gore *et al*., 2014; López-Rodríguez *et al*., 2019; Faghani *et al*., 2022).

I.1.1.1. Ovarian cycle

I.1.1.1.1. Follicular phase

The follicular phasis consists of initiation, basal follicular growth, selection and pre-ovulatory maturation with the acquisition of dominance (Yamoto *et al*., 1992). The ovary represents a key female reproductive organ that is essential for synthesis and secretion of hormones that are necessary for reproductive function. It is a complex organ that undergoes continual structural and functional changes throughout a female's reproductive lifespan (Monget *et al*., 2021; Fletcher *et al*., 2022). The ovary's initial course is set during embryonic development when primordial germ cells migrate to the developing ovary and form germ cell nests (Rimon-Dahari *et al*., 2016). During embryonic development, the germ cell nests are disintegrate and combine with somatic cells to form a limited pool of primordial follicles, starting then the process of folliculogenesis. Primordial follicles develop and mature into primary follicles, then preantral follicles, and finally antral follicles during folliculogenesis (Figure 1). Only antral follicles have the capacity for ovulation and the massive production of sex steroid hormones (Zhou *et al*., 2019; Fletcher *et al*., 2022). During this phasis, there is a slow increase in the number of LH receptors on the cells of the internal theca of the ovarian follicles, while the number of FSH receptors on the cells of the granulosa does not vary (Yamoto *et al*., 1992). It is thought to be dependent on factors secreted by the oocyte and/or granulosa cells such as EGF (Epidermal growth factor), TGFβ ("transforming growth factor") or GDF-9 ("growth differentiation factor-9") (Maruo *et al*., 1993; Dong *et al*., 1996). In the follicular phasis, the biosynthesis of 17 β-estradiol (E2) is predominant. In the cells of the internal theca, under the effect of LH, cholesterol is transformed into androgens. In the granulosa cells, under the influence of FSH, androgens are converted into estradiol in a process called aromatization. Estrogen production increases as follicles grow and as their size (in this case the dominant follicle, the follicle that survived the wobble in FSH release initiated by activin and inhibin, both produced by the granule cells during follicular growth) reaches a certain threshold, the level of estrogen produced briefly retro activates the hypothalamus and adenohypophysis causing an sudden release of LH to a certain extent and FSH, around the middle of cycle (Marieb and Hoehn, 2010).

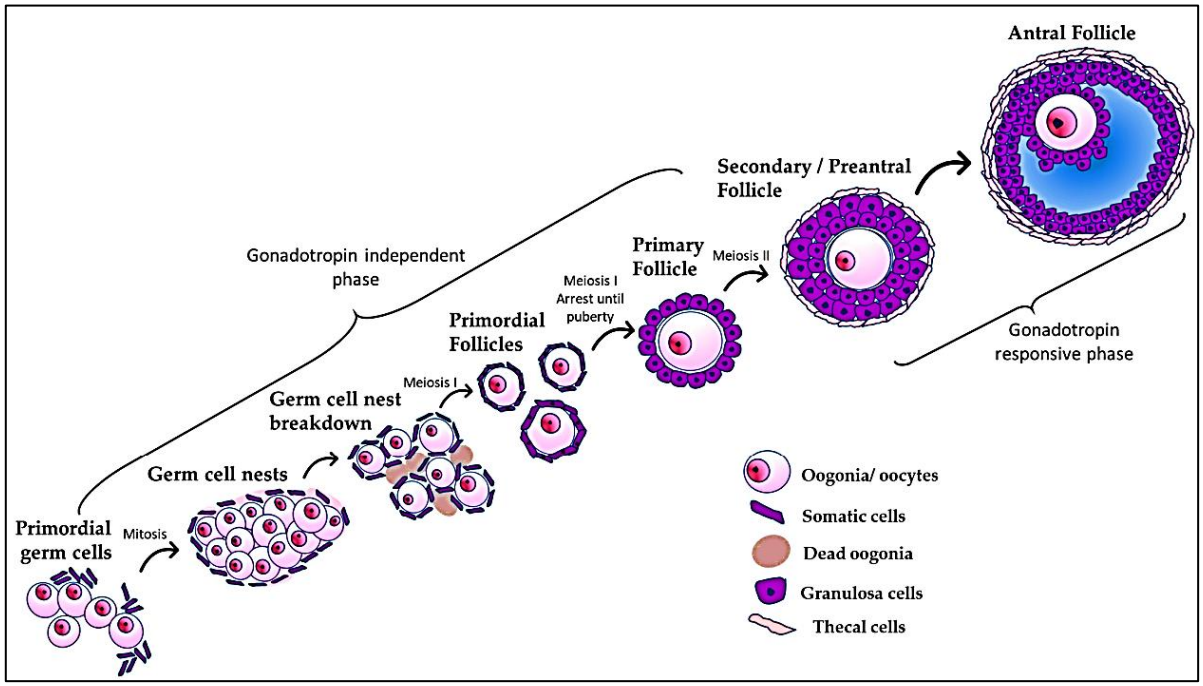


Figure 1: Different stages of the process of folliculogenesis (Fletcher *et al*., 2022).

I.1.1.1.2. Ovulatory phase

Before puberty, the oocytes in primordial follicles are stopped in the first division of meiosis (prophase I). During puberty, the releasing of GnRH by hypothalamus leads to the secretion of gonadotropic hormones known as follicle-stimulating hormone (FSH) and luteinizing hormone LH). These hormones induce the follicular growth and cause the primordial oocytes of the dominant follicle to complete meiosis I, forming secondary oocytes (Marieb and Hoehn, 2010; Fletcher *et al*., 2022). LH also increases vascular permeability, stimulates the release of prostaglandins E and F (PgE and PgF) and triggers an inflammatory response that promotes the release of enzymes called metalloproteins, which help weaken the ovarian wall. Then the blood stops flowing to the protruding area of the follicle. Within minutes, this region thins, bulges, and then ruptures, producing ovulation (expulsion of the mature oocyte) (Marieb and Hoehn, 2010).

I.1.1.1.3. Luteal phase

Following ovulation, the ruptured follicle collapses and the antrum fills with coagulated blood and eventually resorbs. The granular cells increase in volume and, together with the cells of the internal theca, form a new endocrine gland (corpus luteum). All these events are under the control of LH, which then stimulates the production of progesterone and a small amount of estrogen by this new gland. The increase in blood levels of progesterone and estrogen exerts a feedback inhibition on the hypothalamic release of GnRH and consequently on the release of LH and FSH from the adenohypophysis. The decrease in gonadotropins prevents the

development of new follicles and the influx of additional LH that could cause the release of more oocytes. If there is no signal of successful fertilization and implantation, the corpus luteum begins to degenerate by apoptosis and the process of follicular maturation restarts. All that will remain is a scar called "corpus albicans". When the oocyte is fertilized and pregnancy occurs, the corpus luteum persists until the placenta is ready to produce hormones in its place, which is about three months (Marieb and Hoehn, 2010; Fletcher *et al*., 2022). The ovary gradually loses its follicular supply due to the limited number of primordial follicles that are present at the time birth, either through ovulation or atresia and this leads to the onset of menopause or reproductive senescence (Fletcher *et al*., 2022; Ding *et al*., 2022).

I.1.1.2. Uterin cycle

The uterine or menstrual cycle is the series of cyclical changes undergone by the endometrium each month in response to variations in blood levels of ovarian hormones. These endometrial changes are coordinated with the phases of the ovarian cycle, which are governed by gonadotropins released by the adenohypophysis (Chaitra *et al*., 2020). Mentrual cycle stages are as follows (Marieb and Hoehn, 2010):

I.1.1.2.1. Menstrual phasis

Going from day-1 to 5; during which, the thick hormone-dependent functional layer of the endometrium detaches from the uterine wall, a process that causes bleeding. The blood and tissue flow into the vagina and constitutes the menstrual flow or menses. On day 5, the ovarian follicles begin to secrete more estrogen.

I.1.1.2.2. Proliferative (pre-ovulatory) phasis

It lasts from day 6 to 14. Here, the endometrium is reconstituted under the influence of increased estrogen. Its basal layer generates a new functional layer. As this new layer thickens, its glands enlarge and its spiral arteries become more numerous. The endometrium becomes velvety, thick and well-vascularized. During this phase, estrogen also causes the synthesis of progesterone receptors in the endometrial cells, which prepares them to interact with the progesterone secreted by the corpus luteum. Cervical mucus is normally thick and sticky but estrogen makes it clear and crystalline. It then forms channels that facilitate the passage of sperm to the uterus. Ovulation, which occurs in less than 5 minutes, takes place in the ovary at the end of the proliferative phase (day 14), in response to the abrupt release of LH from the adenohypophysis.

I.1.1.2.3. Secretory phasis (postovulatory)

It goes from day 15 to 28. its duration is more constant (14 days). The increase in progesterone levels produced by the corpus luteum acts on the endometrium, which has been

sensitized by estrogen. The functional layer transforms into a secretory mucosa and secretes nourishing glycogen into the uterine cavity which is supposed to support the embryo in case of fertilization until it is implanted in the highly vascularized mucosa. Under the effect of progesterone, the adenohypophysis release of LH is inhibited and the cervical mucus becomes viscous again, forming a mucous plug that prevents the entry of spermatozoa or some pathogens into the uterus. If fertilization does not occur, the corpus luteum begins to degenerate towards the end of the secretory phase, when the blood level of LH decreases. The decrease in progesterone levels deprives the endometrium of its hormonal support, and the spiral arteries become tortuous and spasmodic. The lysosomes of the endometrial cells deprived of oxygen and nutrients die, making menstruation possible on day 28. The spiral arteries contract one last time, then suddenly relax, generously irrigating the endometrium. Blood gushes into the weakened capillary beds, fragmenting them and causing the functional layer to peel off; this day consists in the first day of a new cycle.

I.1.2. Estrous cycle in rats

In the rat the reproductive cycle called estrus cycle, important marker of sexual maturity or gonadotropin responsiveness, lasts 4-5 days and is under the control of central nervous system (CNS). It consists of 4 phases called proestrus, estrus, metestrus and diestrus (Marcondes *et al*. 2002; Adeniyi and Agoreyo, 2019; Chaitra *et al*., 2020; Jorge *et al*., 2021) characterized by different cell types desquamated from the vaginal epithelium, the presence or absence of leukocytes and mucus in vaginal smears (Hubscher *et al*., 2005; Freeman, 1994; Paccola *et al*., 2013). Like menstrual cycle, the pre-ovulatory and post-ovulatory phases take place during the estrous cycle. They are related to proestrus, metestrus and diestrus respectively (Marcondes *et al*., 2002; Adeniyi and Agoreyo, 2019). Ovulation (sexual receptivity or heat) occurs during the night of the estrus phase after the luteinizing hormone (LH) surge (Johnson, 2007; Paccola *et al*., 2013). Many parameters such as estrous frequency and regularity, estrous cycle length, estrous phase interval and diestrus index have been study for long but do not help to have detailed information about durations of folliculogenesis, luteogenesis and luteolysis. Thus, recent study suggested that estrous cycle ratio can be a predictor of luteogenesis and luteolysis intervals (Adeniyi and Agoreyo, 2019; Agoreyo and Adeniyi, 2018; Chaitra *et al*., 2020). It appears that any alteration in this parameter may be a consequence of the interference of EDCs in the hypothalamic-pituitary-ovarian axis (Gore *et al*., 2018; Jorge *et al*., 2021).

I.1.3. Sexual behavior

Normal sexual function represents an important aspect of wellbeing and quality of life of all living animals, from insects to mammals, including human (Yakubu and Olytoye, 2016; Melis *et al*., 2022). Generally, the acquisition of maturity is accompanied by bodily changes,

increase of curiosity and interest in sexual activity (Bellis *et al*., 2006) and this represents excellent predictors of fertility (Pfaus *et al*., 2001; Olivier *et al*., 2016). For all mammals, sexual activity consists in the insertion of the male's penis into the female's vagina to allow sperm transport indispensable for fertilization and for vaginocervical stimulation to facilitate pregnancy (Pfaus *et al*., 2001). It is well nkwon that female rats copulate readily during the post-ovulation heat phase. During this period, many of the female's behaviors reflect her desire to the male, by soliciting its attention and displaying sexual behavior in its presence (Yakubu and Olutoye, 2016). If females are not receptive, males are not motivated to achieve an ejaculation with them (Jenkins and Becker, 2005). According to Micevych and Meisel (2017), the hypothalamus is most often linked with innate behaviors such as sexual behavior (Micevych and Meisel, 2017). In the rat, as in most mammals, the medial preoptic nucleus (MPN) and the ventromedial nucleus (VMH) of the hypothalamus have been identified as the neuroanatomical sites at which sexual behavior is promoted by the oestrogen. Its pharmacology is dependent on the reproductive cycle and consists of attractivity, proceptivity, and receptivity (Olivier *et al*., 2011).

I.1.3.1. Attractivity

Attractivity reflects behavior, smell and sounds by the female that attract the male attention and most often leads to proceptive behavior of the female, including solicitation, hopping and darting (Olivier *et al*., 2011; Karabaşoğlu and Erbaş, 2021). It is known that all female sexual behaviors, including being attractive for males and being attracted to males (Le Moëne and Ågmo, 2018) as well as the display of paracopulatory behaviors and lordosis (Ogawa *et al*., 1998), are dependent on the estrogen (Ogawa *et al*., 1999; Walf *et al*., 2008a, 2008b; Antal *et al*., 2012). Although, the exact mechanism by which estrogen work is still being elucidated. Whatever, literature showed that the estrogen modulates several neurotransmitters involved in the control of motivational behavior, mood and cognition such as dopamine (Amin *et al*., 2005, Micevych and Meisel, 2017; Melis *et al*., 2022). This neurotransmitter is associated with the increasing sexual motivation and desire and induces its effects through G-protein receptors, coupling mechanism that is essential for the activation of cellular responses (Karabaşoğlu and Erbaş, 2021).

I.1.3.2. Proceptivity

During sexual interaction, the female displays a series of stereotyped motor activities including ear wiggling, running, and darting. The behavior patterns have been termed "proceptive" or "precopulatory". It is defined them as 'appetitive activities shown by females in response to stimuli received from males. A female approaching a male or staying in close

proximity to a male increases the possibility of sexual interactions. During this step, the female engages in a behavior known as darting (the female initiates a forward flight, followed by an abrupt stop with orientation of her hindquarters towards the male); hopping (the female makes a short jump with frozen legs), and solicitations (the female makes an orientation with her head towards the male, followed by a flight towards her initial compartment) (Karabaşoğlu and Erbaş, 2021).

I.1.3.3. Receptivity

The level and frequency of lordosis can be used as an index to measure the status of female sexual receptivity. During copulation, female rats display lordosis. It represents a very stereotyped posture in response to a mounting male, a distinct spinal reflex posture with flexion of the back, extension of the neck, and elevation of the hindquarters and rump. This posture usually lasts 0.5 – 1.5 second (s) (Olivier *et al*., 2011; Melis *et al*., 2022). The tactile stimulation stimulates cutaneous receptors in the flank, rump, tail base and perineum, which feed their information to the brain where primarily areas in the hypothalamus (notably the VMH) are crucial in the control of lordosis. To be inducing, this required the activation of oestrogen receptor. Moreover, a latent period is needed for receptivity development. A female displaying this behavior in response to male mounting is frequently described as sexually receptive (Olivier *et al*., 2011; Melis *et al*., 2022). The main stimulus leading to the presentation of lordosis is tactile stimulation of the back and flanks provided by the mounting male. Additionally, lordosis activation can be regulated by various neurotransmitter systems across the brains, which are process depending on the differential regulation of the signaling pathways mediated by the various neurotransmitters such as serotonin. Since sexual behavior in rats is organized by brain areas, it has been demonstrated that a decrease of serotonin leads to affecting female sexual behaviors directly such as lordosis (Snoeren *et al.,* 2010; Karabaşoğlu and Erbaş, 2021). Estrogen and serotonin are known to play an important role in the sexual activity in the female brain (Karabaşoğlu and Erbaş, 2021). The stimulation or inhibitions of sexual activity by serotonin depend on which receptor subtypes are activated. The preovulatory surge in luteinizing hormone (LH), caused by positive feedback exerted by estrogen, is thought to be mediated by serotonin (Morello and Taleisnik, 1985; Amin *et al*., 2005). In the presence of estrogen, serotonin elicits LH secretion and ovulation, whereas it can have the opposite effect in estrogen's absence (Vitale and Chiocchio, 1993; Amin *et al*., 2005). It has been demonstrated a hypothalamic circuit regulates consummatory aspects of reproductive behavior such as lordosis behavior, a measure of sexual receptivity that involves estradiol membrane-initiated

signaling in the arcuate nucleus (ARH), activating β-endorphin projections to the MPN, which in turn modulate VMH activity the common output from the hypothalamus. Sexual experience sensitizes the response of nucleus accumbens neurons to dopamine signaling through the induction of a long lasting early immediate gene. While estrogen alone increases spines in the ARH, sexual experience increases dendritic spine density in the nucleus accumbens. These two circuits appear to converge onto the medial preoptic area where there is a reciprocal influence of motivational circuits on consummatory behavior and vice versa (Micevych and Meisel, 2017). Taking into account the importance of the reproductive development in acquisition of fertility, it is important to investigate on the contaminants capable of affecting the hormonal balance of an organism, since they can compromise the reproductive function (Marques-Pinto and Carvalho, 2013; Hannon and Flaws, 2015; Fletcher *et al*., 2022; Silva *et al*., 2023).

I.2. Female infertility

Defined as the incapacity of a couple to conceive and achieve pregnancy after 12 months of consistent unprotected sexual intercourse with no contraceptive measures taken (Gnoth *et al*., 2005; Abebe *et al*., 2020; Chorosho *et al*., 2023), infertility is the disease of the reproductive system (Nagórska *et al*., 2019). It represents one of the most important complications in gynecology (Moridi *et al*., 2019) and can be categorized into two sub-categories: primary and secondary infertility. The former is the inability to conceive and have a successful live birth when you have never had a child, while the latter occurs when individuals have already had at least one previous biological child (Romeiro *et al*., 2017; Abebe *et al*., 2020; Charlotte *et al*., 2021). This inability to conceive affects 30-40% of couples in Sub-Saharan Africa (Inhorn and Patrizio, 2015) and 20-30% in Cameroon (Charlotte *et al*., 2021). In these regions, the sexual lives of couples are significantly impacted by infertility, particularly for infertile women (Bokaie *et al*., 2015), for whom a capacity for childbearing determines whether a marriage will be successful or not (Larsen, 2000, Telefo *et al*., 2012; Bokaie *et al*., 2015). The most identified causes of infertility include a history of tubal pregnancy (tubal factors), abnormal menstruation, uterine fibroids, intrauterine adhesion, uterine polyps, endometriosis, pelvic inflammatory disease, sexual transmitted diseases, ovarian disorders (Abebe *et al*., 2020; Chorosho *et al*., 2023). It is also observed that women over 35 years old experience problems conceiving. Metabolic disorders such as polycystic ovary syndrome (PCOS), obesity and diabetes mellitus also enhance the epidemiology of infertility in affected couples (Chorosho *et al.,* 2023). Furthermore, abortion, smoking, consumption of alcohol and exposure to environmental toxic like pesticides, and food additives (tartrazine, Bisphenol A, phthalates) may enhance the risk of infertility in both sexes (Hashem *et al*., 2019; Fletcher *et al*., 2022; Chorosho *et al.,* 2023).

I.2.1. Causes of female infertility

Many factors such as a sufficient number of ovarian follicles containing good quality oocytes, adequate hormonal secretions, and permeable tubes to allow migration of the oocyte and sperm, or the presence of uterus can influence female fertility (Figure 2) (Abebe *et al*., 2020; Silva *et al*., 2023; Chorosho *et al*., 2023).

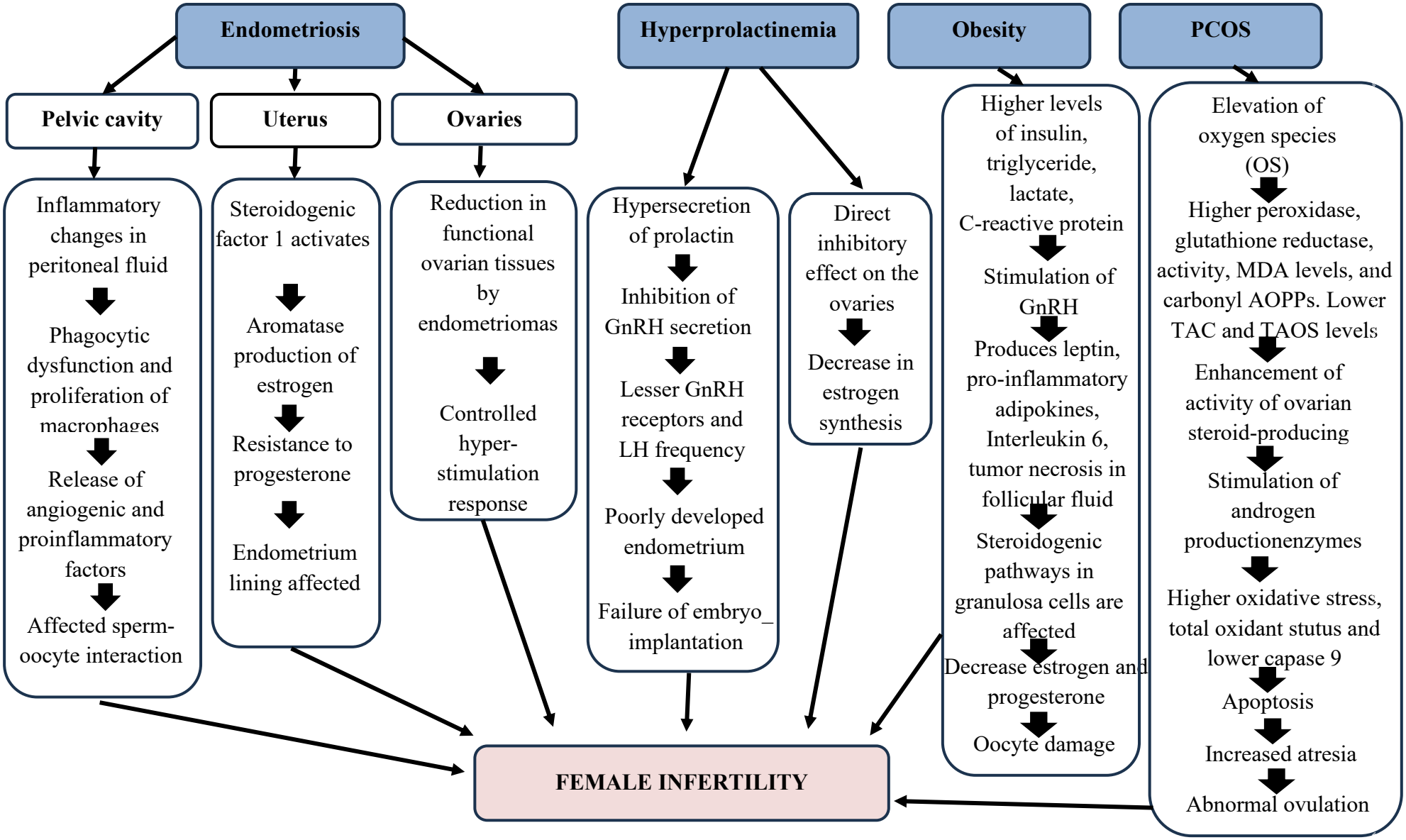


Figure 2: Factors leading to female infertility (modified from Chorosho *et al*., 2023).

I.2.1.1. Age related problems

According to the literature, the success of reproduction depends on several physiological interactions in which the ovary and the ovarian follicular reserve play a fundamental role. Nowadays, the delay in pregnancy and the postponed marriage until after 30 years of age represent a sociological change compared with the previous century. It represents an important factor for the decreased fertility rate due to its relationship with the damaged follicles and a reduction in the reserve pool of follicles (Cruz *et al*., 2017; Moridi *et al*., 2019; Chorosho *et al*., 2023). Getting pregnant after the age of 30 has been associated with a higher risk of miscarriage, hypertension and diabetes mellitus, increased risk of genetic malformation of the fetus due to a greater probability of damaged follicles/oocytes (Cruz *et al*., 2017).

I.2.1.2. Ovulatory dysfunction

The health of the ovaries is one of the important parameters of the female reproduction. The ovaries undergo ongoing structural changes that are essential for the maturation of ovarian follicles and the synthesis of sex steroids, the ones regulate reproduction and endocrine function (Fletcher *et al*., 2022). Therefore, any disruptions in the ovulatory surge of luteinizing hormone (LH) or of the ability of the maturing follicle to respond to the gonadotropic signal hormonal communication between FSH and LH, estrogen and progesterone, and their receptors may result in anovulation or amenorrhea, leading to fertility problems (Awounfack *et al*., 2018; Fletcher *et al*., 2022; Faghani *et al*., 2022).

I.2.1.3. Tubal diseases

One of the commonest causes of female infertility is tubal factors, representing for about 39.17% of all infertility problems (Abebe *et al*., 2020). For a normal function of the reproductive system, women need to have functioning fallopian tubes; the ones are involved in the early development of the embryo and in the transport of the embryo into the uterine cavity. It is known that fertilization takes place in the outer extremity or the ampullary section of Fallopian tubes. Their total or partial occlusions are mainly due to pelvic inflammatory disease (PID) and sexually transmitted illnesses (Ericksen and Brunette, 1996; Tsevat *et al*., 2017; Abebe *et al*., 2020). Therefore, this anatomical or functional alteration is associated with ectopic pregnancies responsible for infertility (Jose-Miller *et al*., 2007; Abebe *et al*., 2020; Silva *et al*., 2023).

I.2.1.4. Endometriosis

Endometriosis represents one of the major causes of infertility. It is defined as a condition where the tissue that composes the uterine lining grows in the Fallopian tubes and or

around the ovaries (Eskenazi and Warner, 1997; Steinkeler *et al*., 2009). Because the fragmented tissue still acts the same as the one found in the uterus and responds to hormones changes, the tissue can break down and bleeds, causing pain before and after the period. All these events lead to heavy, painful and long menstrual periods as well as painful intercourse. It can sometimes induce alterations in the expression of follicular genes, ovarian follicle stock, and disorders of embryo implantation (Jose-Miller *et al*., 2007; Steinkeler *et al*., 2009).

I.2.1.5. Ovarian disorder

Ovulation depends on the complex balance of pituitary-gonadal hormones and the success of their interaction. During the puberty, Gonadotropin Hormone-Releasing Hormone (GnRH) released in a pulsatile pattern of rhythmic secretory bursts whose amplitude and frequency varies according to cycle stage. The pituitary cells called gonadotropins, responding to GnRH stimulation, synthesize and release Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH), which induce ovarian folliculogenesis, steroidogenesis, ovulation and formation of corpus luteum. The intricate coordination of pituitary-ovarian hormones and the effectiveness of their interaction are necessary for ovulation (Marieb and Hoehn, 2010; Chaitra *et al*., 2020; Fletcher *et al*., 2022). Any disturbance of this balance could have an impact on fertility. The causes of ovarian disorders include failure to produce mature follicles, polycystic ovary syndrome, dysfunction of the hypothalamus or pituitary gland, and premature ovarian failure. In that case, ovulation is rare and the chances of fertilization become non-existent (Figure 3).

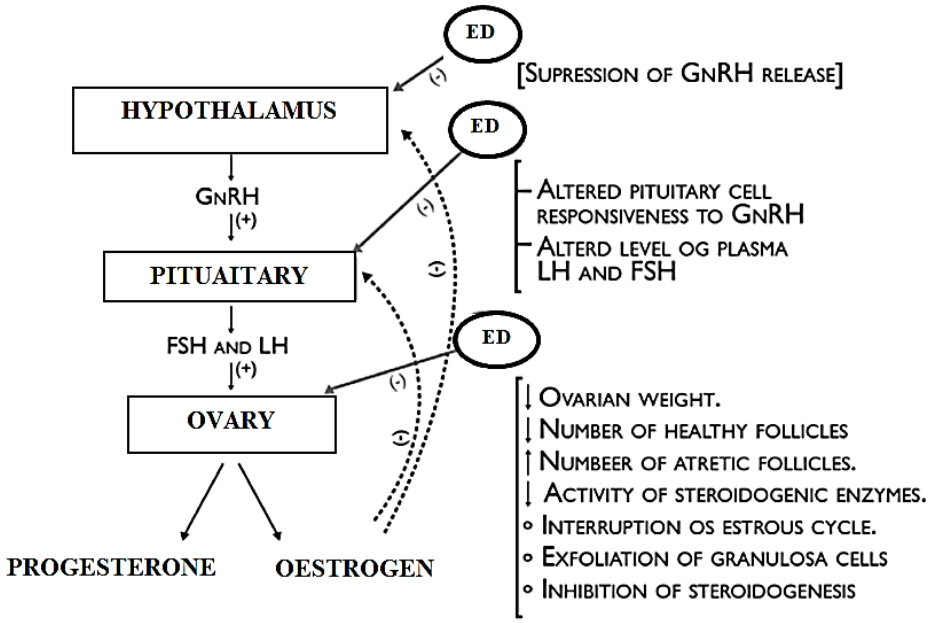


Figure 3: Schematic explanation of the endocrine disruptor potential target sites of action in the regulation of reproductive female functions through the HPG axis (Adapted from Silva *et al*., 2023).

PCOS is a hormonal imbalance (LH and FSH), associated with an excess of testosterone production by the ovaries. It is characterized by ovulatory dysfunction (anovulation or dysovulation), hyperandrogenism, multiple ovarian cysts and metabolic changes (Karami and Fooladi, 2015). The ovulatory dysfunction hypothesis is associated with an excess of testosterone production by the ovaries, which inhibits primordial follicle recruitment and reduces the follicle recruitment response of FSH, thus preventing selection of the dominant follicle. Women are born with a stock of follicles likely to lead to the formation of mature oocytes. This stock decreases during their life, more or less quickly depending on the woman, until menopause.

Premature ovarian failure is defined by an abnormally large follicular loss associated with the absence of a menstrual cycle, with an early menopause occurring in women aged younger than 40 years. The decrease in the follicle stock is more often accompanied by an alteration in oocyte quality, with an increase in the rate of spontaneous miscarriage and fatal chromosomal anomalies. In many cases, its origin is not identified. It probably has at least a partially genetic origin. For example, several mutations in the forkhead box L2 (FOXL2) gene (a major player in the function of ovarian cells known as "granulosa cells") are associated with premature menopause, starting at age 30. Some causes of ovarian or spermatic abnormalities are common to both women and men, such as hypothalamic-pituitary pathologies. These are responsible for an alteration in the production of hormones that can lead to the absence of ovulation (such as hypersecretion of prolactin). It can result from tumor or genetic disease, functional abnormality, especially in women, in response to a deficit in lipid intake or to intense physical activity (Jankowska, 2017; Ding *et al*., 2022).

I.2.1.6. Certain treatments

Many treatments such as anti-cancer treatments (chemotherapy) (Fleetwood and Campo-Engelstein, 2010), contraception can lead to infertility (Inhorn and Buss, 1994; Chasan-Taber *et al*., 1997; Abebe *et al*., 2020). Many studies have shown that, female offsprings of pregnant women exposed to diethylstilbestrol presented defected reproductive tract structure and vaginal clear cell adenocarcinomas at a young age (Reed and Fenton, 2013; Belcher *et al*., 2019). Recent studies suggest that certain drugs that are normally well tolerated, such as analgesics, antihistamines or anti-reflux drugs may also have an impact on reproductive function under certain conditions (Gurunath *et al*., 2011). However, researchers are trying to better understand the underlying environmental factors in order to reduce the risk of exposure.

I.2.1.7. Environmental factors

There is a growing interest in the effects that environmental factors can induce in reproductive female health since they are likely to play a negative role at each stage of reproduction, in both women and men (Ericksen and Brunette, 1996; Abebe *et al*., 2020; Silva *et al*., 2023). Human studies have showed that the general population, including children is rarely exposed to a single compound, but to chemical mixtures present in the environment (air, water, soil), in food (food additives), in consumer products (cosmetic, daily use, etc.) and food packages (EFSA *et al*., 2019; Hashem *et al*., 2019; Hasson, 2020; Tassinari *et al*., 2021; Silva *et al*., 2023). It appears that, recent changes in the lifestyle of individuals and societies seem to contribute to the higher exposure to these harmful chemicals. According to the literature, the number and volume of environmental pollutants have gradually and significantly increased as industrialization has accelerated throughout the world. These environmental substances with significant impacts on the endocrine system are the source of a lively debate about the release of toxins into the environment (Kabir *et al.,* 2015; Silva *et al*., 2023). Many concerns such as early puberty, declining fertility, cancers, and polycystic ovaries are emerging about the long-term effects on human health following chronic exposure to these substances (Gore *et al*., 2015; Zingue *et al*., 2021; Silva *et al*., 2023). Other substances such as persistent organic pollutants, paints, or certain endocrine disruptors such as phthalates, bisphenol A, have shown disrupting effects of the reproductive function (Tassinari *et al*., 2021; Fletcher *et al*., 2022; Silva *et al*., 2023). Psychic factors are also incriminated, in particular stress. Oxidative stress (OS) has been identified as a significant component that can negatively affect fertility outcomes (Mehranjani *et al*., 2010; Hasson, 2020; Chukwuebuka *et al*., 2020; Agarwal *et al*., 2022). It is defined as an imbalance between the reactive oxygen species (ROS) and the total amount of antioxidants (AOXs) in favor of the oxidants**.** It could act at the level of the brain by altering the production of neurohormones and/or gonadotropic hormones in the hypothalamo-hypophysis-ovarian system (Hasson, 2020). ROS act depending of the concentration. At low concentrations, ROS act physiologically as signaling molecules in several processes such as ovulation. Despite this importance role, their excess in stress cause damage during follicles maturation and consequently affects ovulation (Agarwal *et al*., 2005; Hasson, 2020).

I.2.2. Consequences of infertility

In many African nations, a woman's capacity for childbirth determines whether a marriage will be successful or not. In that environment, infertility results in serious psychological trauma such as depression and anxiety, reduced self-esteem and social humiliation. In some cases, infertility can lead to exclusion, verbal and physical abuse, marriage

violence, and breakup. Especially for women, infertility decreases their quality of life and exposes them to multiple sexual partners, sexually transmitted diseases, and increased sexual dysfunction (Monga *et al*., 2004; Fleetwood and Campo-Engelstein, 2010; Abebe *et al*., 2020; Silva *et al*., 2023). In addition, dealing with infertility results in a loss of privacy surrounding the couple's sexual life and frequently leads in having sex for reproduction rather than pleasure (Onat and Kizilkaya, 2012; Esselstrom, 2014; Bokaie *et al*., 2015).

I.2.3. Diagnostic of infertility

Every couple who wishes to receive advanced treatment at a fertility clinic must undergo a deep diagnostic interview. It represents all programs recording data on mental health, marital status, *Human Immunodeficiency Virus* (HIV) status, drug use, the stability of the marriage and/or other romantic connection (Esselstrom, 2014). Other data collected, includes any previous pregnancies and any associated complications, evaluation of physical symptoms, duration of infertility and sexual history. After the clinical interview and physical examination, couples begin the process of diagnostic testing, which may be followed sessions to discuss results and more testing until a final diagnosis is attained (Esselstrom, 2014). Usually in woman, the frequency and regularity of the menstrual period, pelvic pain, abnormal vaginal bleeding and records of pelvic infection are asked during the medical visits (Jose-Miller *et al*., 2007; Esselstrom, 2014). However, it should be noted that between a third and half of pregnancies occur after six months of attempts. Compared to other animal species, part of the reason for this low "reproductive performance" is a high rate of spontaneous miscarriages which appears very early or during the pregnancy. This phenomenon is believed to affect half of the conceptions and is explained by very early developmental defects in many human embryos. Many factors such as health and lifestyle seem to increase this risk of infertility. Therefore, it has been shown that infertility is mostly due to female-related causes (Fleetwood and Campo-Engelstein, 2010; Abebe *et al*., 2020).

I.2.4. Treatment of infertility

It is well knwon that, infertility diagnosis and treatment cost has a detrimental impact on the economy (Macaluso *et al*., 2010; Land *et al*., 2022). This is intensified by the fact that infertility treatments are typically not covered by insurance (Land *et al*., 2022). Infertility treatment represents one of the most stressful events in a couple’s life and its a long-term process which require many sacrifices (Nagórska *et al*., 2019). Depending on the underlying reason of the infertility, numerous treatment options are available. Treatment options for males include changing behavioral and lifestyle factors, such as avoiding alcohol and smooking (Esselstrom , 2014; Moridi *et al*., 2019), starting a regular exercise routine, and increasing the

timing and frequency of intercouse (Esselstrom , 2014). Some conditions may required surgery. For women with cervical factor infertility, intrauterine insemination also knwon as artificial insemination, may be used (Esselstrom, 2014; Nagórska *et al*., 2019). However, when these treatments are either unsuccessful or inavailable, many turn to assisted reproductive technology (ART) (Nagórska *et al*., 2019). It is then clear that more is known about treating infertility than preventing the causes leading to it. Nevertheless, prevention strategies are preferable since they can avert the psychological and financial cost associated with the treatment and prevent some cases of infertility that would otherwise be completely untreatable (Gwet-Bell *et al*., 2018). Given the negative health outcomes, the economic burdens, and the potential for EDCs to disrupt ovulation and fertility, there is a need to further understand the effects and mechanisms by which EDCs impair ovarian function and ovulation (Land *et al*., 2022). Further understanding the impacts and processes by which EDCs affect ovarian function, ovulation, and fertility is necessary (Land *et al*., 2022).

I.3. Female endocrine system

I.3.1. Generality

Beside the immune system and the nervous system, the endocrine system represents a major communication network in the human body that regulates several biological processes via the interaction of glands, hormones, and receptors (Figure 4) (Marieb and Hoen, 2010; Stiefel and Stintzing, 2023). Whereas the nervous system acts via nerve impulses (electrical signals) triggered by neurons, resulting in immediate (millisecond), brief and localized effects, the endocrine system acts via hormones (chemical messengers secreted by the endocrine glands, released into the bloodstream, transported throughout the body, and regulating the metabolic activity of the body's cells). The response in this case, once initiated, tends to last much longer than the reactions in the former (Kent and Rhees, 2001; Marieb and Hoen, 2010; Stiefel and Stintzing, 2023). The endocrine system is one of the regulatory systems (Scanlon and Sanders, 2007) working in close association (synergy) with the nervous system to regulate the body's various functions (Kent and Rhees, 2001). Once produced, hormones are transported through the bloodstream until they reach their target cell or tissue, where they coordinate various functions in the body. Depending on the receptor and its localization, the hormone concentration and the developmental state of the cell or tissue, hormones regulate and maintain a balanced organ function, by control of the overall metabolism, development and growth and reproductive functions. Hence, a well-balanced endocrine system is essential for the maintaining of human health, any disturbance can impair development and growth, disrupts the metabolic processes or the brain function, and induces reproductive disorders or infertility. The

distinct glands and cell systems responsible for the synthesis and secretion of hormones are located at different body sites (Figure 3). The main relevant are hypothalamus, pineal gland or epiphysis, pituitary gland or hypophysis, parathyroid, thyroid, adrenal glands, pancreas, ovaries (female) and testes (male) (Scanlon and Sanders, 2007; Stiefel and. Stintzing, 2023). However, other organs or tissues have the ability to produce, release and/or bind hormones such as white adipose tissue which secrete leptin and adiponectin (Gaillard, 2003), and the digestive tract responsible for the secretion of serotonin and cholecystokinin. In the female reproductive system, the ovary is known to be a key endocrine female reproductive organ, essential for fertility and normal hormone production (Fletcher *et al*., 2022). His activity is under the control of anterior hypophysis. The gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) through their ability to regulate the synthesis of the sex steroids (androgens and estrogens), are indispensable for proper gonadal development and function (Fletcher *et al*., 2022).

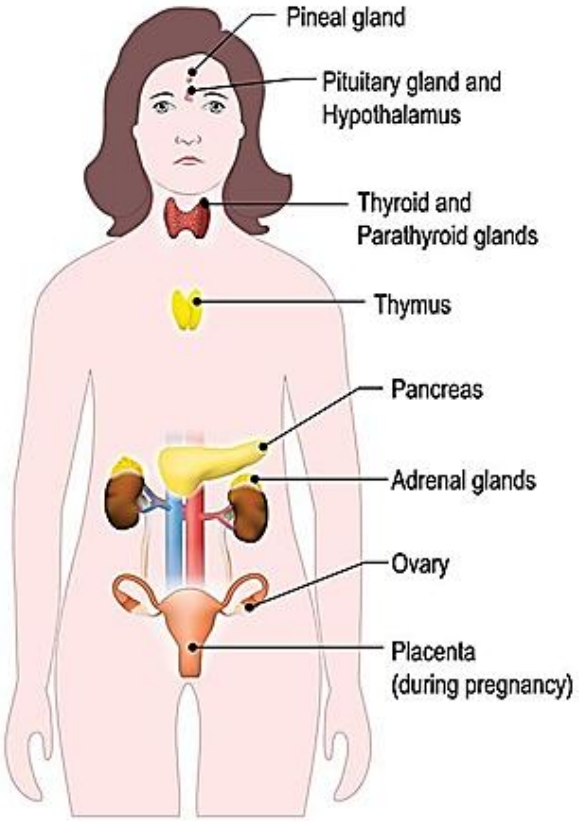


Figure 4: Location of the main endocrine glands (modified from Scanlon and Sanders, 2007).

I.3.2. Female reproductive hormones

Understanding the relationships between the various reproductive hormones and the female reproductive organs is critical when evaluating adverse effects of chemicals on the reproductive system. Derived from different biochemical precursors, reproductive hormones

may be classified in two different classes: amino acid derivatives and steroids (Nussey and Whitehead 2001; Marieb and Hoehn, 2010; Boudalia, 2012; Fletcher *et al*., 2022). Hydrophilic hormones or amino acid derivatives include prolactin, adrenalin, somatotropin, insulin, glucagon, FSH, LH, melatonin while lipophilic hormones consist of steroids that are derived from cholesterol (Example of glucocorticoids, mineralo-corticoids and sex steroids such as oestrogens and progesterone). Sex steroids like estrogen and progesterone are known to play a central role during development and function of the reproductive system (Fletcher *et al*., 2022).

I.3.2.1. Pituitary gonadotropins

Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are two glycoprotein hormones synthesized and released by the anterior pituitary gland and placental trophoblast, and regulating the development and mature reproductive functions in mammals (Kochman and Gajewska, 1996; Hasson, 2020). Gonadotrophins induce their effects through specific receptors almost exclusively located in the gonads. In females, they are required for the regulation of various physiological processes including the sexual cycle and ovarian steroidogenesis. FSH is mainly involved in the stimulation of ovarian follicle growth (oocyte production, selection and development of the follicular cavity) and in the expression of LH receptors on the granulosa cells while LH induces ovulation and subsequent luteinization (formation of the corpus luteum from the Degraaf follicle having expelled its oocyte). LH and FSH regulate follicular steroidogenesis, androgen and oestradiol secretion and LH itself regulates progesterone secretion from the corpus luteum (Considine, 2004; Kochman and Gajewska, 1996; Hasson, 2020).

I.3.2.1.1. Biosynthesis of pituitary gonadotropins

LH and FSH are glycoprotein hormones derived from amino acids, which are almost similar in structure as they all comprise two subunits (α and β) linked by non-covalent bonds. The members of this family are heterodimeric glycoprotein hormones that share structural an identical α subunit (Pierce and Parsons, 1981; Kochman and Gajewska, 1996; Ulloa-Aguirre and Timoss, 2000). In the α/β dimer, the α-subunit of gonadotropic hormones is a 92 amino acid chain that contains two N- linked oligosaccharides in positions α52 and α78. Whatever, LHβ is a 121 amino acid chain possessing a β30 N-glycosylation site, and FSHβ, is a 111 amino acid chain possessing two N-glycosylation sites at residues β7 and β24. These hormones are stored in vesicles and released by exocytosis when needed. Gonadotrophins release can be directly regulated by ovarian steroids, influencing the transcription of their different subunit genes and the GnRH receptor gene. In the hypothalamus, their effects are indirectly triggered by GnRH ("Gonadotropin releasing hormone") -producing cells. GnRH is a potent stimulator

of pituitary gonadotropin by promoting both the synthesis of its mRNA and the apoprotein and its post-translational processing (glycosylation). It acts through Adenosine Monophosphate (cAMP) and diacylglycerol (DAG) (Starzec *et al*., 1989; Ulloa-Aguirre and Timoss, 2000).

I.3.2.1.2. Transport and metabolism

The concentration of gonadotropins in serum is low and varies in a pulsatile manner. These hormones, both secreted by the pituitary are not able to reach their target receptors by crossing the vascular barrier. Therefore, rapid and specific trans-endothelial transport is essential. To cross the endothelial barrier, serum proteins use fluid phase diffusion via plasmalemmal vesicles, which is a non-specific transport mechanism. The vascular endothelium will therefore express their receptors, thus making their transport by transcytosis possible (Misrahi *et al*., 1990; Kobe and Deisenhofer, 1993).

I.3.2.1.3. Gonadotrophin receptors

Gonadotropin receptors are located on the membrane of target cells and act primarily by stimulation of cellular adenylyl cyclase. Theses hormones belong to a family of G-protein coupled receptors (Misrahi *et al*., 1990). The LH and FSH receptors comprise 699 and 695 amino acids respectively with a signal peptide at their amino terminus which is secondarily cleaved. They contain a domain with seven transmembrane passages characteristic of G protein coupled receptors. However, this domain has no homology with other older receptors (adrenergic, muscarinic, cholinergic receptors, etc.). In contrast, it is the most conserved domain between gonadotropin receptors and (Figure 5). The hormone-binding domain, the most voluminous and leucine-rich domain, constitutes the amino-terminal part of the receptor and includes several potential glycosylation sites. It contrasts with the truncated extracellular portion of other receptors with seven transmembrane segments binding small ligands (Kobe and Deisenhofer, 1993; Ghinea, 2002). However, there are several forms of gonadotropin receptors detected at the target organs, an 85 kDa form, which shows complete glycosylation and corresponds to the mature form of the receptor, present at the cell surface. Unusually, there is also in the endoplasmic reticulum and Golgi apparatus of the target cells a high accumulation of an incompletely glycosylated 68 kDa precursor, rich in mannose residues, unable to bind the hormone and of unknown function. This could correspond to an intracellular receptor reserve capable of being matured and then rapidly addressed at the surface in certain physiological situations (Vu-Hai *et al*., 1990).

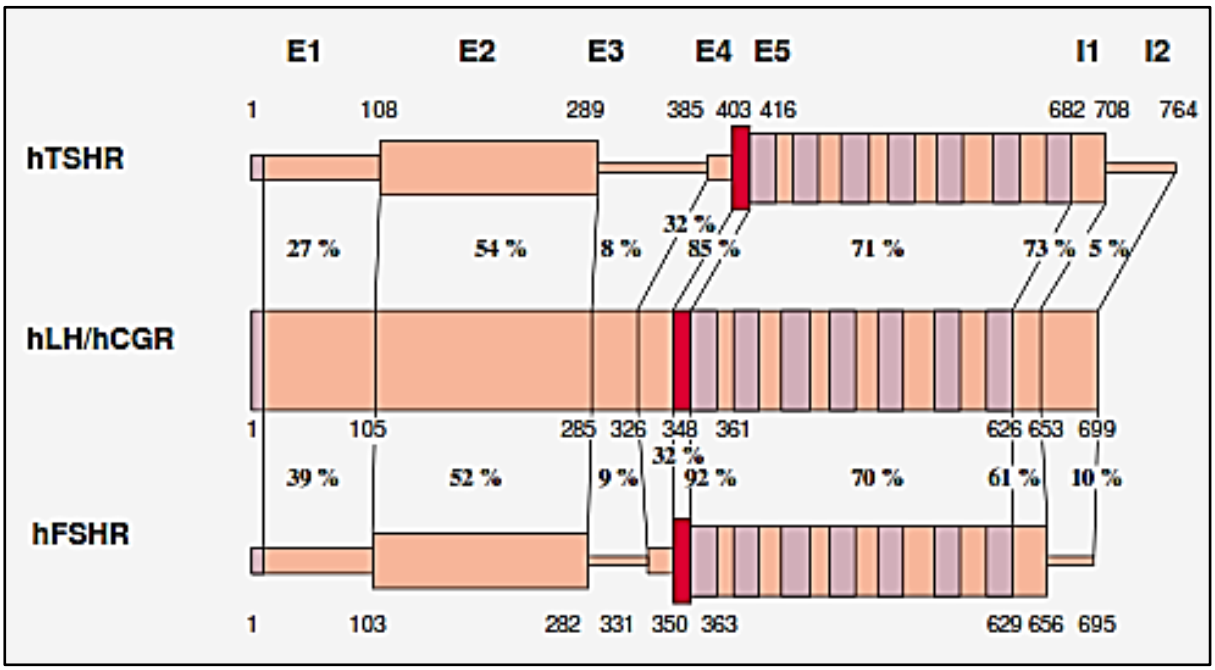


Figure 5: Structure of pituitary gonadotropin receptors (Misrahi *et al*., 1999).

I.3.2.1.4. Mechanism of action

Water-soluble hormones are unable to penetrate the target cells; they must bind to receptors located on the plasma membrane of these cells. Following receptor activation by the ligand, the hormone-receptor complex stimulates the conversion of adenosine triphosphate (ATP) into cAMP (cyclic adenosine monophosphate) via adenylate cyclase. The cAMP thus produced stimulates a protein kinase A, which triggers phosphorylation (P) cascade and activating intracellular proteins leading to the hormonal response (Figure 6) (Marieb and Hoehn, 2010; Ulloa-Aguirre and Timossi, 2000; Silbergnagl and Despopoulos, 2004).

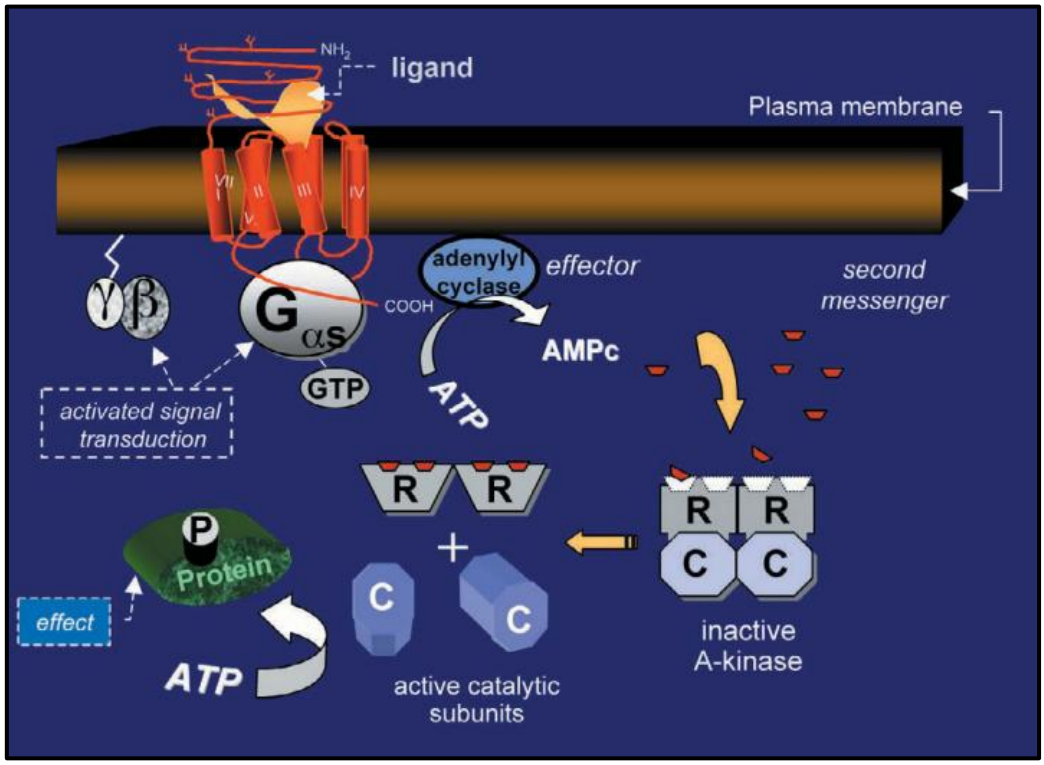


Figure 6: Mechanism of action of pituitary gonadotropins (Ulloa-Aguirre and Timossi, 2000).

I.3.2.2. Sex steroids

Sex steroids (oestrogens, androgens and progesterone) are hormones that play an important role in the regulation of various physiological processes such as development, growth, cell differentiation, maturation of the genitalia and the appearance of secondary sexual characteristics (Nussey and Whitehead 2001).

I.3.2.2.1. Biosynthesis of sex steroids

Sex steroids are derived from cholesterol and are distinguished from each other by side groups. Produced mainly by the gonads (ovaries and testes), these hormones can also come from other organs capable of producing them completely or by conversion of circulating precursors which is stimulated by an enzymatic equipment such as aromatase, thus transforming androgens into oestrogens in many tissues and cells (Examples: Adrenocortical, fetoplacental unit, brain, adipose tissue, liver, skin fibroblasts, chondrocytes, osteoblasts, endothelial and smooth muscle cells) (Bélanger *et al*., 2002; Schiffer *et al*., 2019). The development of the hypothalamic-pituitary-ovarian axis during puberty triggers the onset of ovarian steroidogenesis. It has been demonstrated that the entire reproductive cycle depends on the ability of somatic cells inside the ovary to secrete sexual steroids (Fletcher *et al*., 2022). In the ovary, aromatase activity takes place in the granulosa cells, under the control of FSH (Olson *et al*., 2007; Schiffer *et al*., 2019). The synthesis of sex steroids is achieved by enzymatic

modifications of the cholesterol core (Figure 7) which is stored in esterified form in intracellular lipid vesicles. The response to all these enzymatic modifications leads to steroid formation called steroidogenesis. After internalization by the adrenal and endocrine cells of the gonads, cholesterol is transferred from the cytosol to the inner membrane of the mitochondria. It is then transformed into pregnenolone through cleavage of the side chain by CYP11A1. This critical step is subject to hormonal control by LH in the gonads and is facilitated by the intracellular sterol transport protein called STAR (Steroidogenic Acute Regulatory protein) (Gruber *et al*., 2002; Fletcher *et al*., 2022). Pregnenolone is transported from the inner mitochondrial membrane to the smooth endoplasmic reticulum where it is then converted to progesterone (Δ4 pathway) or 17-α-hydroxypregnenolone (Δ5 pathway) by 3-β-hydroxysteroid dehydrogenase type 2 (3β-HSD 2) and 17-α-hydroxylase (CYP 17) respectively. 17α-hydroxypregnenolone and 17α-hydroxyprogesterone which serves as the preferred substrate for the 17,20-lyase activity of CYP17A1 will be convert in androgens. The resulting androstenedione and testosterone enter the granulosa cells where they are converted to estrogen by the expression of CYP19A1 (Gruber *et al*., 2002; Hannon and Flaws, 2015; Schiffer *et al*., 2019; Fletcher *et al*., 2022).

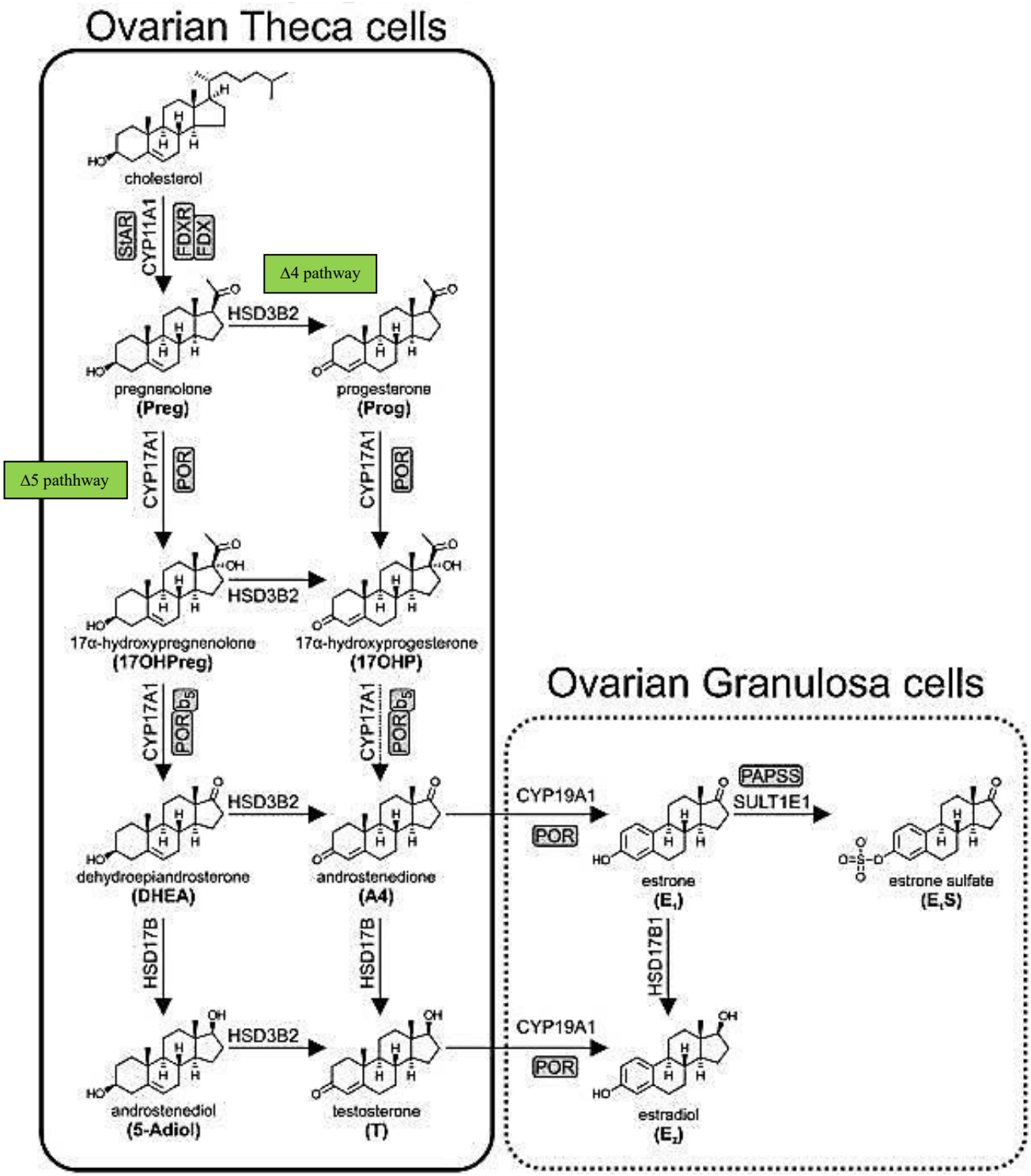


Figure 7: Biosynthetic pathway of sex steroids (Schiffer *et al*., 2019).

I.3.2.2.2. Transport and metabolism

Compared to gonadotropins, sex steroids diffuse the bloodstream where they are bound with specific plasma proteins such as sex hormone binding globulin, Corticosteroid-Binding Globulin (CBG) and albumin. Only 2-3% of testosterone is free, 30 to 40% is bound to albumin and the rest to the Sex Hormone Binding Globulin (SHBG) while about 2% of estradiol is free,

60% bound to albumin, 37% bound to SHBG and less than 0.1% bound to CBG. Approximately 80% of progesterone is bound to albumin, 17% to CBG and less than 3% is free (Gruber *et al*., 2002). All these steroids bind with low affinity but high capacity to albumin and high affinity and low capacity to SHBG. Sex steroids are inherently lipophilic hormones. Their metabolic conversions required their inactivation by several reactions including oxidation, reduction, hydroxylation, sulfation and glucuronidation. This increases their water-solubility and enables efficient excretion in urine and bile (Gruber *et al*., 2002; Schiffer *et al*., 2019). The catabolites are conjugated in the liver and kidney by a condensation with an acid, glucuronic or sulfuric acid, a molecule that is brought in activated form with a nucleotide (UDP-glucuronate, PAPS). Made hydrophilic in this way, the conjugated catabolites are eliminated in the urine (exception: oestrogens, part of which is eliminated in the faeces) (Osawa *et al*., 1993; Schiffer *et al*., 2019).

I.3.2.2.3. Sexual steroid receptors

Sex hormone receptors are members of the nuclear receptor gene superfamily and function as transcription factors that regulate the expression of target gene (Axon *et al*., 2012). These receptors are structurally similar and consist of five functional domains (Mangelsdorf *et al*., 1995): N-terminal domain A/B involved in the activation of transcription, DNA-binding domain "Hormone Response Element" which contains a specific sequence allowing the binding to specific DNA sequences (Mader *et al*, 1989) (C domain), hinge region (D domain) that appears to possess a nuclear localization signal (Pasqualini *et al*., 2001), ligand-binding domain (E domain) and a C-terminal variable region (F) whose presence decreases the receptor's dimerization capacity and therefore decreases its transcriptional activity (Yang *et al*., 2008) (Figure 8) . This high homology suggests a potential interaction of these receptors with the same DNA target sequences. However, their A/B and E domains are very different, with only 24 and 56% homology, and the transcriptional activity of the target genes may vary depending on the type of receptor and ligand (Dahlman-Wright *et al*., 2006). Given the distinct transcriptional activity of the two types of receptors, their differential expression could play a determining role in the sensitivity and response of the target organs, especially since ERβ appears able to modulate the activity of ERα (Weihua *et al*., 2002) through potential heterodimerization mechanisms. Activated oestrogen receptors recognize the same "Estrogen Response Element" sequence. However, it is difficult to predict exactly what type of response is generated. The term "Selective Estrogen Receptor Modulator" is therefore used to describe these ligands whose action varies according to the molecular context of each tissue (Diel, 2002). The tissue distribution of these two receptors is ubiquitous in the body. However, ERα activity is dominant in the liver, adipose tissue, genitalia, mammary gland, and kidney, whereas ERβ

activity predominates in the bladder, prostate, lung, bone, intestine, and salivary glands (Kuiper *et al*., 1997; Enmark and Gustafsson 1999; Dahlman-Wright *et al*., 2006).

There are also two types of progesterone receptors (PRs), PR-A and PR-B, which consist of 769 and 933 amino acids, respectively, and are derived from a single gene (Li *et al*., 2004). As with ERs, the transcriptional activity of PRs is complex and regulated by post-translational modifications of many cofactors. But overall, PR-B is more active and would rather activate the expression of target genes, whereas PR-A would have an inhibitory role towards PR but also towards other nuclear receptors (Graham and Clarke 1997). On the other hand, estrogens induce the expression of PR in many tissues such as the vagina and its represents a good marker of estrogenic stimulation (Flototto *et al*., 2004).

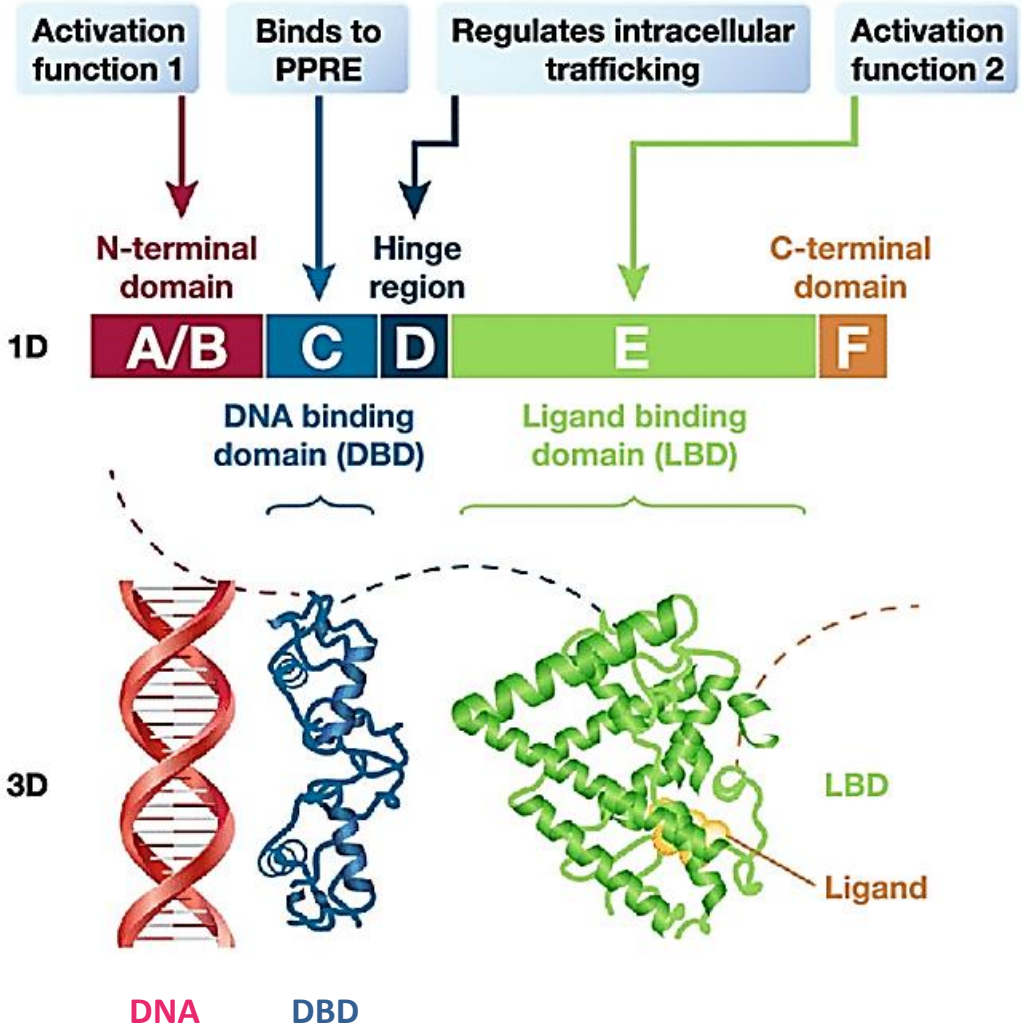


Figure 8: General structure of nuclear receptors (Fruchart *et al.*, 2019).

I.3.2.2.4. Mechanism of action

The physiological effects of sexual steroid hormones result from different modes of action of action of at the cellular level (Figure 9). These receptors act through genomic and non-

genomic mode of action that enable the transcription of target genes responsible for a quick signalling events.

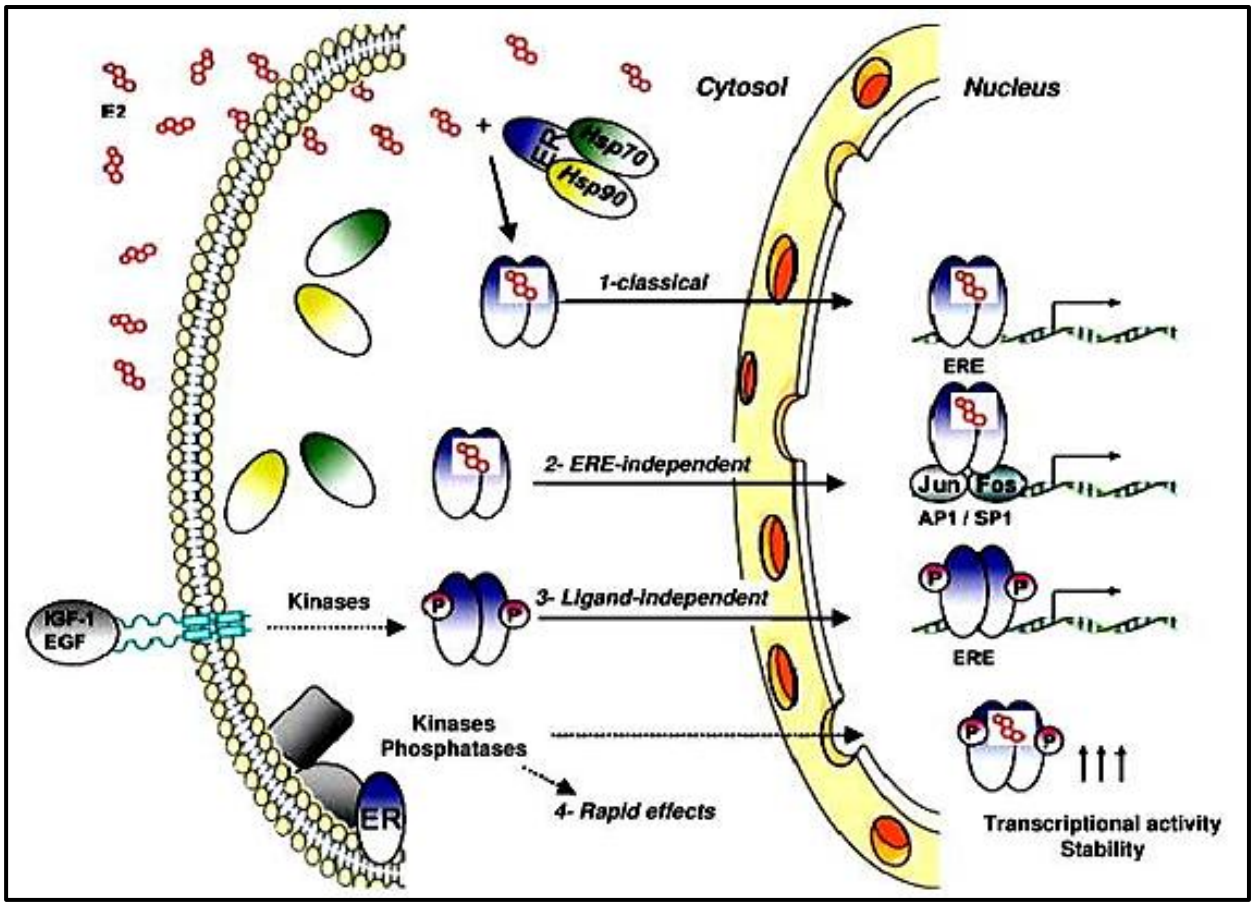


Figure 9: Mechanism of action of sexual steroids receptors (modified from Heldring *et al*., 2007).

a. The genomic pathway

In the cytoplasm, steroid receptors are found in the form of an inactive complex consisting of receptor monomers bound to Heat Shock Protein (HSP) 90 proteins (Pratt *et al*., 2004). The binding of the hormone to its receptor is followed by dissociation of the receptors, formation of dimers (twin ligand-receptor complex systems), followed by translocation of the complex into the cell nucleus through the action of NLS ("Nuclear Localization Signal"). These dimers then bind to specific DNA sequences Hormone Response Element (HRE) and induce or not the transcription of target genes (Hall and Couse, 2001; Nelson *et al*., 2002; Prescott and Coetzee, 2006). The recruitment of co-regulators and transcription factors to the promoter regions initiating RNA polymerase II activation and the synthesis of new mRNAs modulates gene transcription. This mechanism commonly referred to as classical or HRE-dependent, can be mediated in the presence or absence of ligand. In the latter case phosphorylation of serine residues in the AF-1 domain of nuclear steroid receptors stimulated growth factors (Nilsson *et al*., 2001), leading to dissociation of HSP receptors, dimerization of the latter and translocation

of the dimers into the cell nucleus, where they bind to the HRE region of the DNA to activate or not the transcription of target target genes (Joel *et al*., 1998; Martin *et al*., 2000).The HRE-independent pathway is used for genes lacking HRE sequences. Here, the DNA-binding domain (C domain) of the receptor does not bind DNA directly, but is involved in protein-protein interactions with transcription factors such as the AP1 ("Activator Protein 1") or Sp1 ("Specificity Protein 1"). The formed complex can bind to "Thyroid Response Element" (TRE) nucleotide sites (in the case of the AP1-linked receptor), or to a specific regulatory sequence (in the case of the Sp1-linked receptor) receptor linked to the Sp1 protein) of the promoter region of target genes and induce expression of genes (Safe and Kim, 2008).

b. The non-genomic pathway

It is also corresponding to the rapid mode of action. Their effects occur few second or minutes after hormone binding without modifying gene expression. They are based on the presence of receptors localized on the plasma membrane and include 12 the involvement of secondary messenger's characteristic of cellular signalling pathways (Baldi *et al*., 2009; Furukawa and Kurokawa, 2007). After binding to the membrane-associated receptors, estrogen activates the phospholipase C and kinase protein C for the release of calcium as well as PI3K that phosphorylates Akt/PKB leading to rapid production of NO in vascular endothelial cells (Simoncini *et al*., 2004). Competitive or non-competitive interaction with the catalytic sites of various enzyme complexes enables steroids to exert cellular effects on a wide range of enzymes (metabolic enzymes) and cellular including growth factors (Watson *et al*., 2007).

I.4. Endocrine disruptors

Endocrine-disrupting chemicals (EDCs) are ubiquitous environmental contaminants (Figure 10) that alter hormone-dependent functions, including behaviors controlled by the brain’s neuroendocrine systems (Gore *et al*. 2014). Current research has shown that expositions to some of these environmental contaminants are toxic to female reproductive health. Many of them are known to target the ovary at all stages of development and adulthood, causing premature ovarian failure, anovulation, infertility, and decreased steroidogenesis (Hannon and Flaws, 2015; Fletcher *et al*., 2022; Silva *et al*., 2023).

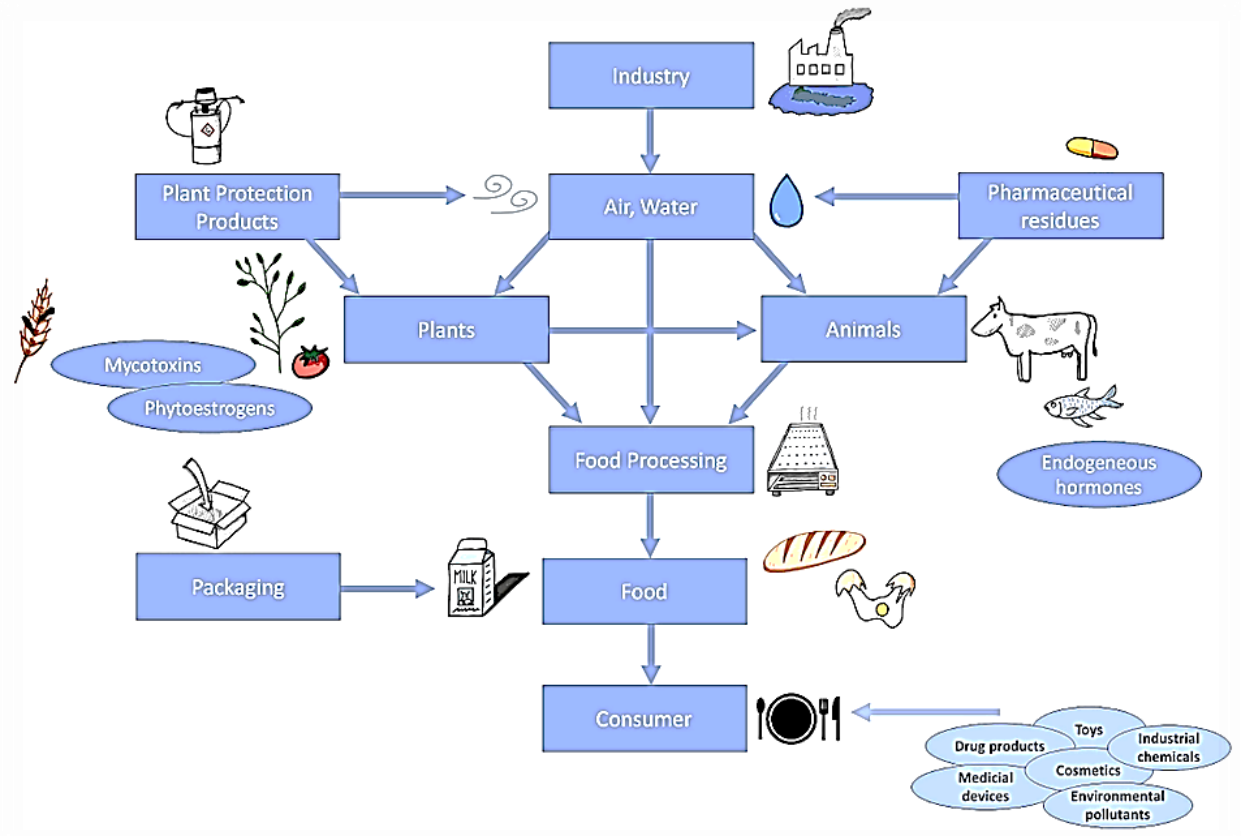


Figure 10: Pathways of endocrine disruptor exposure within the food chain (Stiefel and Stintzing, 2023).

I.4.1. History

There is concern that certain chemicals may have the potential to disturb normal sexual differentiation and development in animals and humans. An endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, its offspring, or (sub) populations. EDCs could disrupt hormone action via direct or indirect pathways. It can directly act on the hormone receptor protein complex, or on a specific protein that controls hormone delivery to the right place at the right time. Because the affinity of EDCs to hormone receptors are not equivalent to its potency, EDCs can disrupt the proper functioning of the endocrine system and, consequently, cause adverse health effects (WHO, 2012; Fletcher *et al*., 2022; Silva *et al*., 2023). Several chemical substances of natural or artificial origin foreign to the organism interfere with the functioning of the endocrine system, thus inducing deleterious effects on the individual or on his descendants. This is referred to as endocrine disruptor (Chaussinand, 2015). Given the invasion of the environment by compounds capable of having such activity, a multidisciplinary group of experts met from July 26-28, 1991 in Wingspread. The concept of endocrine disruptor was born as a result of the synthesis of the observations made on the endocrine system and raised at this conference (Colborn and Clement, 1991). Whatever, the

potential risk of contaminants depends on various factors, such as toxicity, exposure route, and exposure time (Rahmani *et al*., 2018; Shahrbabki *et al*., 2018). EDCs may act as single chemical agents or as chemical mixtures; they can influence puberty, accelerating and or delaying the processing of maturation of secondary sexual characteristics (Lucaccioni *et al*., 2020).

I.4.2. Classification and sources of endocrine disruptors

Depending on their origin, EDCs can be classified into three classes: natural, synthetic, and anthropogenic substances (Figure 11) (Chaussinand, 2015).

I.4.2.1. Natural substances

This class represents hormones synthesized by the body. However, these types of hormones are not only present in humans but are also found in the gonads of animals and in plants (in the form of phytoestrogens). So, the question of whether consuming soy or pork might boost hormone levels and play the role of EDCs by increasing sex hormone levels in humans are arising.

I.4.2.2. Synthetic hormones

Whose chemical structure is identical or similar to natural hormones, are used therapeutically to compensate hormonal deficiency by modulating the endocrine system. They include oral contraceptives such as synthetic estrogens, progestins, and steroids.

I.4.2.3. Anthropogenic substances

Certain chemicals represent a wide variety of endocrine disruptors. They are used in industry (cleaning products), in agriculture (pesticides), in consumer foods (additives in plastics), in medicines (preservatives) and in cosmetics (ingredients) (Figure 11). Growing evidence shows that these human-made products can negatively impact health. It has also been shown that, they can directly or indirectly harm reproductive systems, affecting development and fertility (Gonsioroski *et al*., 2020; Hassan *et al*., 2024).

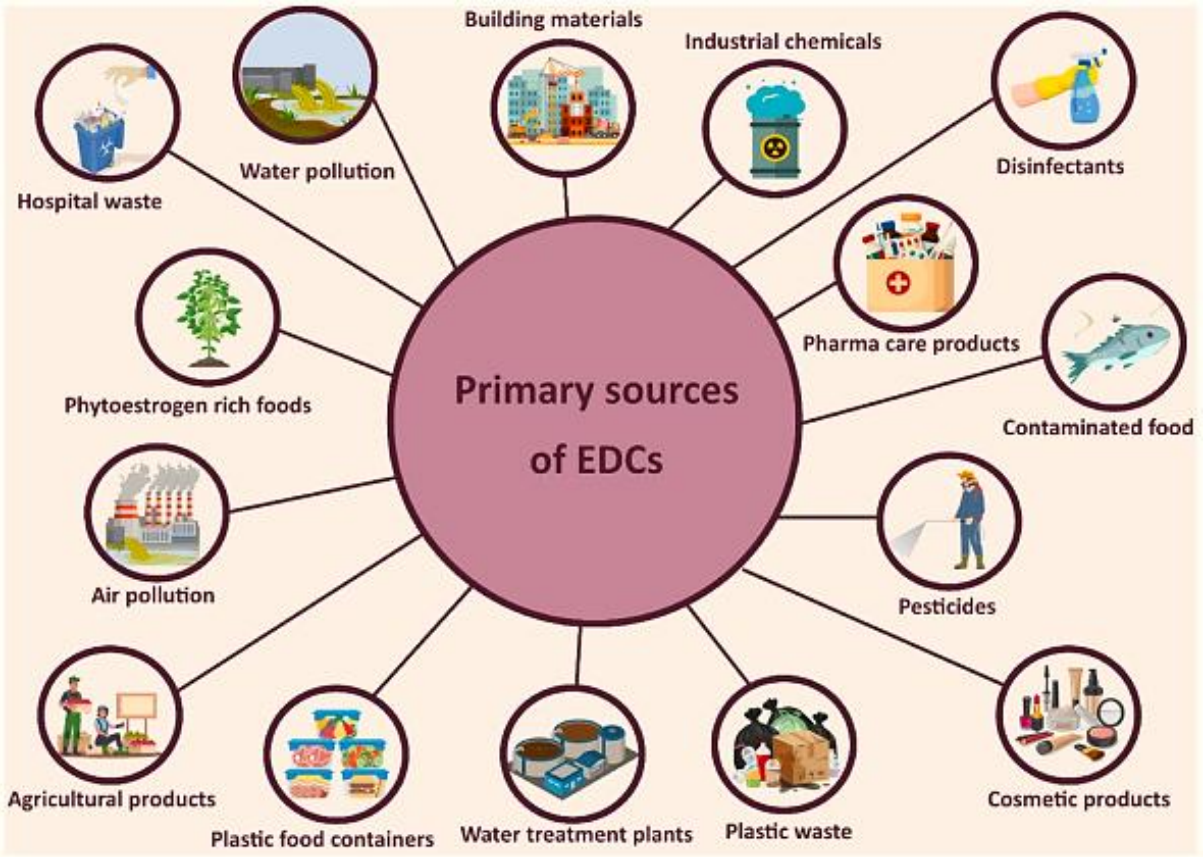


Figure 11: Exposure sources of endocrine disruptors in human (Hassan *et al*., 2024)**.**

I.4.3. Routes of exposure

There are multiple sources of exposure to endocrine disruptors, respiratory, dermal, oral, and transgenerational (Figure 12). They may be present in the different compartments of the environment (air, water), but also in food (of animal or plant origin). EDCs are often come from food packaging, industrial chemicals, combustion products, phytosanitary treatments, detergents and the chemical industry in general. In addition to these anthropogenic substances, some micronutrients are found mainly in plants (Gore *et al*., 2014; Lucaccioni *et a*l., 2020; Silva *et al*., 2023; Hassan *et al*., 2024). However, the oral route represents the main route of exposure to these contaminants (Marques-Pinto and Carvalho, 2013; D'Angelo and St Pier, 2023).

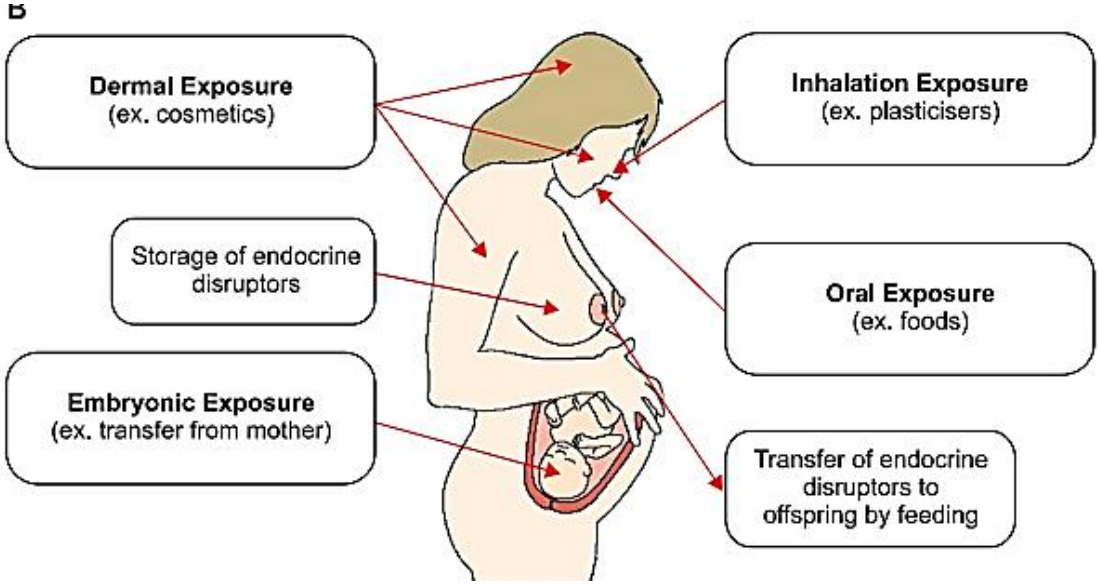
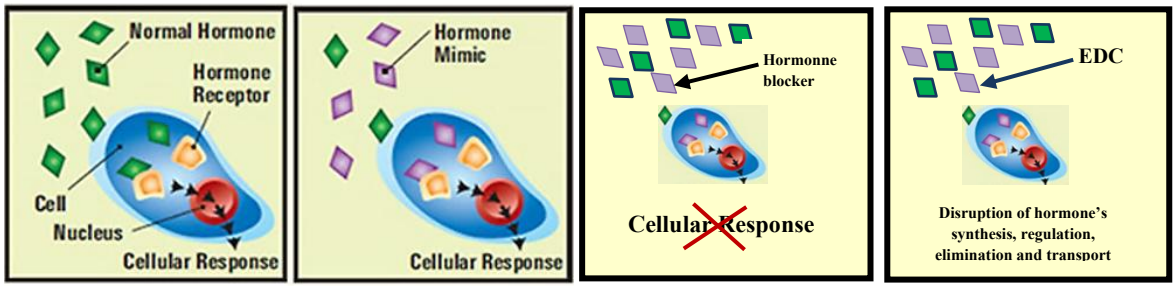


Figure 12: Representation of the different routes of exposure to endocrine disruptors (Yang *et al*., 2015).

I.4.4. Mechanism of action of endocrine disruptors

After their penetration in the body, endocrine disruptors will alter the functioning of the endocrine system according to three mechanisms of action, agonist, antagonist or disruptive (Figure 13).

- Agonist effect: EDCs, by fixing itself on the receptor of the natural hormone will mimic the action of this one thus inducing similar effects but in a deregulated way. This resulted in a normal cellular response.
- Antagonistic effect: here, by binding to the hormone receptor, the EDCs will prevent the emission of the regulation signal.
- Disruptive effect: The EDCs can affect the bioavailability of hormones, by affecting any part of their mechanisms of synthesis, storage, release, degradation or transport. EDCs act at any level in the hypothalamic-pituitary-ovarian axis. Indeed, they can act not only as agonists or antagonists of hormone, but can also target and act against their receptors (Chaussinand, 2015; Schug *et al*., 2016; Combarnous and Nguyen, 2019; Eckert-Lind *et al*., 2020).



A. Normal endocrine signiling B. Endocrine disrupting chemicals signiling

Figure 13: Mechanisms of action of endocrine disruptors (modified from Schug *et al.*, 2016).

I.4.5. Relation between exposure period, dose, effects

Increasing the potential risk is the capability of mixtures of EDCs to produce the same adverse effect as a single EDC. Therefore, exposure to several chemicals, that individually do not produce observable effects, can result in a health risk when combined at low doses (WHO, 2012). In general, hormones act at very low concentrations, thus EDCs as well. There are many examples of low dose effects of endocrine disruptors (Vandenberg *et al.*, 2012). Endocrine disruptors produce nonlinear dose responses, usually of sigmoidal shape, both in vivo and in vitro. Because some hormones are endogenous, minute changes in the exposure to environmental endocrine disruptors could result in a response that is far greater than would be anticipated when only environmental exposure concentrations are taken into consideration (WHO, 2012).

I.4.6. Period of vulnerability

The timing of EDC exposure is a key to determine their outcomes. Their effects on health vary according to the age and physiological state of the exposed individuals. Indeed, during the course of human life and development, there are "windows of susceptibility" or "periods of vulnerability" during which certain populations (pregnant women, early childhood, puberty, for example) are more sensitive to their actions (Chaussinand, 2015; Gore *et al.*, 2015; Schug *et al.*, 2016; Silva *et al.*, 2023). Both epidemiological and animal studies have shown that EDCs exposures during development increase predisposition for disease and dysfunction later in life, including hormone-sensitive neurodevelopmental outcomes (Alonso-Magdalena *et al.*, 2010). It stands to reason, that, due to the interaction of EDCs with hormone actions, exposure during a vulnerable developmental period can have both immediate as well as more latent consequences (Gore *et al.*, 2015). Their effects can also affect the offspring of exposed individuals. We then speak of transgenerational effects. In this case, it is no longer the dose that

makes the poison (as Paracelsus believed at the beginning of the 16th century) but the period that makes the poison (Colborn and Clement, 1991). During early development, fetal programming events can predispose the adult to a number of chronic diseases (Hanson and Gluckman, 2014). The changes produced during this period will last a lifetime (WHO, 2012). Several chronic (non-infectious) diseases and disorders find their cause in abnormal endocrine function and increasing trends for these developmental health issues can be seen worldwide, for instance: Asthma, mental retardation and childhood cancer (WHO, 2012).

I.4.7. Threshold concentration

In general, hormones act at very low concentrations, EDCs as well. There are many examples of low-dose effects of endocrine disruptors (Vandenberg *et al*., 2012). The widely held notion that every compound has a daily acceptable intake has led to the acceptable dogma that exposures to levels below than threshold are safe (Gore *et al*., 2014). Endocrine disruptors produce nonlinear, generally sigmoidal-shaped dose responses both in vivo and in vitro (WHO, 2012).

I.4.8. Latency between exposure and effect

Moreover, recent studies have started to point out how exposure to EDCs during puberty may predispose to breast cancer later in life. In fact, the estrogen-mimicking endocrine disruptors may influence breast tissue development during puberty in two main ways: the first is the action on the proliferation of the breast stromal cells, the second concerns epigenetic mechanisms (Lucaccioni *et al*., 2020). Endocrine-disrupting chemicals (EDCs) influence one or more functions of the endocrine system and cause several adverse health effects in human beings. They can be at the origin of several pathologies with serious effects or delayed immediate effects. In some cases, the symptoms and/or pathologies do not manifest until adulthood, in other cases only after one or more generations. The administration of endocrine disrupting chemicals (EDCs) such as tartrazine, Bisphenol A, Zearalenone during the intrauterine development has been associated with pregnancy loss (Kriszt *et al*., 2015; Hashem *et al*., 2019). Since urbanization and the professionalization of couples, contributing to the growth of consumption of food additives, it becomes urgent not only to identify and quantify each substance used in our diet but also to evaluate their potential endocrine-disrupting activity (Gore *et a*l., 2014; Silva *et al*., 2023).

I.5. Food additives

Food additives are defined as natural or synthetic substances added to a foodstuff for technological or organoleptic purposes. They are commonly used in the food industry where

they are considered as "necessary" or "essential". They are found in the form of colorants, preservatives, antioxidants, emulsifiers, texture agents, sweeteners, and flavors. They are recognized on our labels by their trade name or by the letter E (for Europe) followed by a three-digit number that varies according to the categories of additives (Himri *et al*., 2011).

I.5.1. Potassium sorbate

I.5.1.1. Generality

Potassium sorbate is a potassium salt of sorbic acid (Figure 14). The sorbic acid has been isolated the first time in 1859 but its antimicrobial properties have been discovered in 1940. Practical applications of sorbates include preservation of animal feed, pharmaceuticals, cosmetics products, packeting materials and human food to prevent spoilage, inhibit mold growth and preserve freshness (eg: dairy products, bakery products, fruits and vegetable products) (Sofos and Busta, 1981; Amirpour *et al*., 2015).

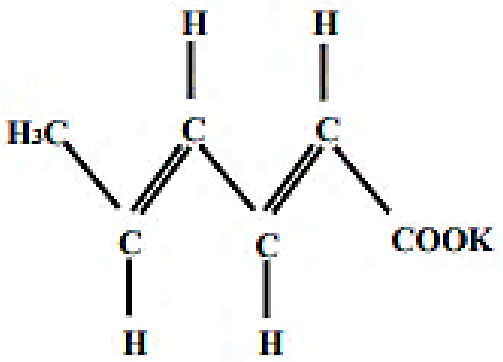


Figure 14: Chemical structure of potassium sorbate (Liebert, 1988).

I.5.1.2. Properties

Potassium sorbate is a water-soluble salt of sorbic acid isolated from the berries of the mountain ash tree (rowan berry) by the German chemist Hoffmann in 1859. His solubility is over 50%, increases with pH and temperature (Sofos and Busta, 1981) and it is more widely used as antimicrobial food additives. However, the stability of sorbic acid and sorbates in food depends on different factors such as pH, water activity, microbiological concentration, composition (organic acids, proteins, other additives such as ascorbic acid in the presence of iron salts, etc.), storage temperature and packaging (Chipley, 2005).

I.5.1.3. Distribution

Practical applications of sorbates include preservation of animal feed, pharmaceutical, cosmetics products, food and packaging materials. Its use as a food preservative includes dairy products, bakery products, fruits and vegetable products (Sofos and Busta, 1981) and according to the Joint FAO/WHO Expert Committee on food additives, the Authorized Daily Intake (ADI)

of potassium sorbate is 0-25 mg/kg body weight/day for sorbic acid (and sorbate salts) (WHO, 2016; Mischek and Krapfenbauer-Cermak, 2012).

I.5.1.4. Previous studies

Potassium sorbate, a water-soluble salt of sorbic acid is one of the food preservatives with oxidative properties which have side effects on the female reproduction system (EFSA *et al*., 2019; Hasson, 2020). Literature have shown that it is responsible for the increase of atresia follicles in rat ovaries, inflammation of oviducts, and degeneration of endometrium in the uterus of female rats (Hasson, 2020).

I.5.2. Tartrazine

I.5.2.1. Generality

From an organoleptic point of view, the appearance of a product is an important factor in the consumer's choice of that product; thus, dyes are used to color or enhance the natural color of food. Tartrazine is an orange dye widely used in the world, in the manufacture of nutrients, drugs, and cosmetics (Himri *et al*., 2011). The acceptable daily intake for humans is 0-7.5mg/kg BW) (Mehedi *et al*., 2009) and its chemical formula is as follow (Figure 15):

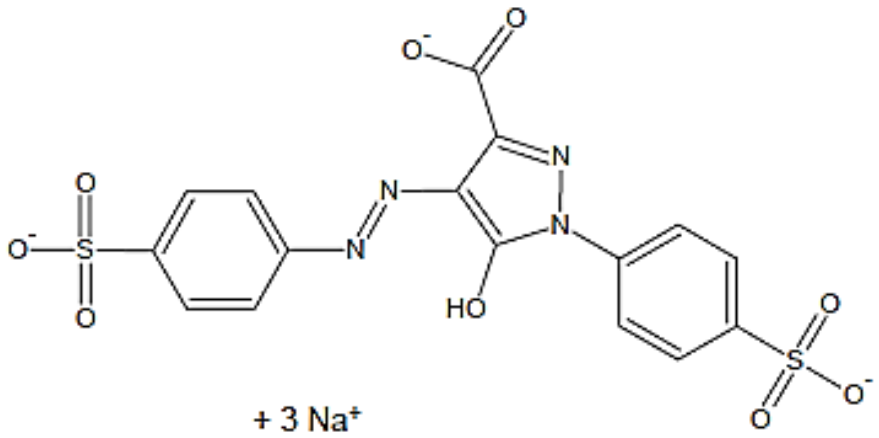


Figure 15: Chemical structure of tartrazine (Himri *et al*., 2011).

I.5.2.2. Properties

Tartrazine is a water-soluble dye, with a high chemical stability and tinting capacity. The speed of the dyeing is high in acidic solutions and at high temperatures (Himri *et al*., 2011).

I.5.2.3. Distribution

Tartrazine is widely used as an ingredient in cosmetics, personal care products, household products and beverages (alcoholic, carbonated, flavored) (Himri *et al*., 2011; Hashem *et al*., 2019). In addition, various type of food, such as juices, cookies, potato chips, cereals (cornflakes), pastries, noodles, canned peas, processed purees, fishery products, confectionery, some ready meals contain tartrazine in order to achieve a yellow, orange, or green hue for easy identification and attraction (Collins *et al*., 1992; Hashem *et al*., 2019).

I.5.2.4. Previous work

Tartrazine is one of the most widely used colorants (Dixit *et al*., 2010; Hashem *et al*., 2019; Zingue *et al*., 2021) by children and adolescents and this makes them the most exposed population (Dixit *et al*., 2010). Several studies showed that in male rats, tartrazine decreases sperm density, mobility, and viability, and serum testosterone concentration (Boussada *et al*., 2017). In female rats, the frequent intake of tartrazine affects thyroid and reproductive hormones (LH, FSH, estrogen, progesterone), (Shakoor *et al*., 2022). According to Nasri and Pohjanvirta (2021), tartrazine is classified as a xenoestrogen that can bind to estrogen receptor α (ERα) in the Michigan Cancer Foundation-7 (MCF-7) cell line and induce a proliferative effect in breast cancer cells (Datta and Lundin-Schiller, 2008). According to literature, tartrazine could be implicated in allergies, tumor diseases, mutagenic, genotoxicity, neuro-behavioral disorders (Engel *et al*., 2015; Khayyat *et al*., 2017; Zingue *et al*., 2021), as well as hepato-nephrotoxicity and alteration of various metabolic aspects in experimental animals (El-Wahab and Moram, 2013; Hashem *et al*., 2019). Several studies have reported the reprotoxicity of tartrazine after administration to pregnant rats. The results suggested that it is responsible for decreasing fertility and teratogenicity (Hashem *et al*., 2019). Some of the side effects of tartrazine have been attributed to his capacity to be metabolized in intestinal microflora into two metabolites, sulfanilic acid and aminopyrazolone (Chung *et al*., 1992; Hashem *et al*., 2019; Faghani *et al*, 2022). Sulfanilic acid and aminopyrazolone are degraded very slowly or not at all, and can be responsible for the production of reactive oxygen species which could induce embryonic malformations (Himri *et al*., 2011; Hashem *et al*., 2019). Teratogenicity, also known as congenital anomalies, is alterations that occur during pregnancy and that affect the body structure or function. These side effects can be identified prenatally, at birth and even later in infancy (Hojo *et al*., 2006; Fraga *et al*., 2022). Since the 1960s, research has been conducted to not only prevent teratogens, but identify a teratogen, define the malformational phenotypes they are connected with, and investigate their mechanisms of action (Fraga *et al*., 2022). Many concerns such as reduce fertility, implantation failure, post-implantation loss are emerging about the long-term effects on human health following chronic exposure to these substances (Ema *et al*., 1999; Ema and Miyawaki, 2002; Adeeko *et al*., 2003; Harazono and Ema, 2003; Grote *et al*., 2009).

I.6. Reproductive toxicity of offsprings

Congenital anomalies, also known as teratogenicity, are alterations that occur during pregnancy and that affect the body structure or function. These side effects can be identified prenatally, at birth and even later in infancy (Hojo *et al*., 2006; Fraga *et al*., 2022). Since the

1960s, research has been conducted to not only prevent teratogenic effects, but also to identify a teratogen, and investigate their mechanisms of action (Fraga *et al*., 2022). A suboptimal in utero environment can harm the pregnancy and have long-term negative "programming" impacts on the offsprings (Supriya *et al*., 2016; Rodprasert *et al*., 2019). Some chemicals used for medical, agricultural, and/or industrial purposes are suspected of having such effects and causing reproductive, neurologic, and immunologic damage, as well as cancers in the intact organism or its progeny as a result of changes in endocrine function (Daston *et al*., 2003; O'Connor and Chapin 2003; Hojo *et al*., 2006). It is estimated that around 10% of congenital anomalies are caused by environmental factors, known as teratogens (Fraga *et al*., 2022). Studies have shown that a suboptimal in utero environment can harm the pregnancy and have long-term negative "programming" impacts on the offsprings (Supriya *et al*., 2016).

CHAPTER II
MATERIAL AND METHODS

II.1. MATERIAL

II.1.1. Food additives (potassium sorbate and tartrazine) and standard substance

Tartrazine (CAS 1934-21-0, Purity≥ 85%) and potassium sorbate (CAS 24634-61-5, 99% of purity) were purchased from Sigma Aldrich (Munich, Germany). The Maca-ginseng-gingembre (PERFORMANCE^{TM}) was purchased from JUVAMINE laboratory (Paris, France).

II.1.2. Animals

The work was carried out with males and female albino Wistar rats (Table I). They were bred in the animal house of the Laboratory of Animal Physiology and Therapeutic Research, University of Yaoundé I (Yaoundé, Cameroon), and had free access to diet and drinking water *ad libitum*. They were housed under natural light/dark cycle. Animals were fed during the experiment with a diet composed of 50% corn, 5% wheat bran, 10% wheat flour, 14% fish, 8.5% peanut, 5% bone meal, 7% palm kernel cake, 0.5% premix, with the only difference that it was soy free because of its phytoestrogen's contribution (Awounfack *et al*., 2018; Yu *et al*., 2021; Liu *et al*., 2024). Animal housing and experiments were carried in conformity with the European Union on Animal Care (CEE Council 86/609) guidelines adopted by the Institutional Ethics Committee of the Cameroon Ministry of Scientific Research and Technological Innovation.

Table I: Age and weight of animals at the beginning of each experiment

Experiment	Ages of rats	Average weight (g)
Sexual maturation	Immature females (21-22 days)	25-40
Sexual behavior	Adult females (21-22 days)	25-40
	Males of proven fertility (84-112 days)	150-180
Fertility	Immature females (21-22 days)	25-40
Reproductive toxicity	Female pups F1, F2 and F3	25-40

II.1.3. Solutions, dye and sodium phosphate buffer

II.1.3.1. Solutions of tested substances

The substances (tartrazine, potassium sorbate, and Maca-ginseng-gingembre) used for the *in vivo* studies were dissolved in distilled water and kept under the temperature conditions -4°C.

II.1.3.2. Doses and concentrations calculation

In the literature, the human equivalent doses (HED) of potassium sorbate and tartrazine are 0-25 mg/kg BW per day (JECFA, 1996; Liebert, 1988; Ferrand *et al*., 2000; EFSA *et al*., 2019) and 0-7.5 mg/kg BW per day (JECFA, 1964; JECFA, 1996; Tanaka, 2006; Elhkim *et al*., 2007; Mpountoukas *et al*., 2010) respectively. The animal equivalent doses (AED: 47 mg/kg BW per day for tartrazine and 78 mg/kg BW per day for potassium sorbate) were calculated based on the body surface area, dividing the HED dose (mg/kg BW) by the ratio (km) provided by the literature (AED= HED/km; km= body weight (kg)/body surface area (m^2). The ratio km used in this work was 0.162 (Shakoor *et al*., 2022). The last doses (27 mg/kg BW per day for tartrazine and 45 mg/kg BW per day or potassium sorbate) used in this work were the means of the HED and AED in order to keep all the doses in the interval of the authorized daily intake.

In order to obtain a mother solution of 7.8 mg/mL of potassium sorbate and 4.7 mg/mL of tartrazine, the two substances were prepared separately by the dilution of 89.622 mg of potassium sorbate and 51.2253 mg of tartrazine in 11.49 mL and 10.899 mL of distilled water respectively. The reconstitution of the remaining solution was obtained by the dilution of the mother solution. In addition, knowing the dose D (mg/kg), weight W (kg) of the animal and the concentrations of each solution, the volume V (mL) to be administered to rat was determined by the following formula:

$$Volume\ (mL) = \frac{\text{Dose (mg/kg) x Poids (kg)}}{\text{Concentration (mg/mL)}}$$

II.1.3.3. Solutions and dye used for the histological stain

The preparation of the solutions was done as follows:

- **10% formaldehyde :**

Ic.Iv. = Fc.Fv:
- **Ic** = initial concentration of formaldehyde
- **Iv** = volume of formaldehyde introduced into a cylinder;
- **Fc** = concentration needed
- **Fv** = final needed volume of formaldehyde

To obtain a final volume (Fv) of 1000 mL, 250 mL of 40% (Ic) formaldehyde (Iv) was added into a cylinder and filled at 1000 mL with distilled water. 10% formaldehyde was used for the conservation of organs.

- **Alcohol at 50, 70 and 80%** used for processing**:** The preparation was done with alcohol 95%, according to the desired concentration and from the alcohol dilution table (confer annex).

- **Harris's hematoxylin** was used to color the nuclei of cells in blue. For the preparation of 200 mL of this solution, 1 mg of hematoxylin, and 20 g of potassium aluminum sulfate were dissolved in warm distilled water. Thereafter, 10 mL of absolute alcohol (ethanol) was added to the mixture for 1 minute. After cooling, 0.5 g of red mercuric oxide was slowly and carefully added to the mixture and the solution was heated again until it became dark purple. After the solution was cooled, 20 mL of glacial acetic acid was added. The resulting mixture was filtered before use.
- **Alcoholic eosin at 0.5%:** Eosin is a solution that stains cell cytoplasm and most connective tissue fibers in varying shades and intensities of orange, pink, and red. For the preparation of this solution, 5g of eosin have been dissolved in 50 mL of distilled water. The mixture has been completed to 1000ml by adding 2 drops of acetic acid and absolute ethanol.
- **Orange G solution at 10%:** The orange G colorant is obtained by dissolving 10 g of Orange G in a small volume of distilled water that will be later completed to 100 mL.
- **Ethanol/xylene solution** used for infiltration**:** This solution results from the mixture of absolute ethanol with xylene in equitable proportions (1:1 v/v).
- **Gelatin water solution:** The gelatin water solution facilitates the retention of tissue on histological sections and improves their adhesion on slides. For the preparation of this solution, 5 mg of gelatin powder was dissolved in 1000 mL of water (45°C). After dissolution, 0.5 g of chromium potassium sulfate dodecahydrate was added to positively charge the slides enabling them to attract negatively charged tissue sections. The solution was filtered before use.
- **Biuret solution**

Biuret solution comes from the mixture of two solutions: Solution A: 0,75 g of hydrated copper sulfate ($CuSO_4$, $5H_2O$) and 3 g of sodium tartrate and potassium were dissolved in 100 ml of distilled water; Solution B: 15 g of soda were dissolved in 100 ml of distilled water. The two solutions have been mixed and the final volume was adjusted to 500 ml by adding water.

- **Sodium phosphate buffer 0,1 M; PH 7,1**

This solution has been prepared as follows: one tablet of saline buffered phosphate (E404-200 TABS/AMRESC0) produced by Biotechnology Grade OH, O, USA was dissolved in 100 ml of distilled water.

II.1.4. Material of evaluation of sexual behavior parameters

For this experiment, animal training and test sessions occurred in an apparatus that permitted sexual interaction while allowing the female to control the rate of the interaction (kops modified apparatus, 2012). The operant response apparatus that was used in this experiment contained two chambers separated by 2 small doors (Figure 16). Three sides of each

chamber were done with wood and separated inside by transparent plexiglass to favor the animal's visibility. The large chamber (operant chamber or male chamber) measures 36 cm large x 40 cm deep and x 45 cm deep high, and the smallest one (non-operant chamber or female chamber) measures 22 cm large x 40 cm deep x 45 cm deep high. The two chambers communicate through 5 cm large doors that also allow the female to get the operant chamber. Kept in their chamber, the male was allowed to move around his side as normal including the ability to perform usual sexual (mount, intromission, ejaculation) and social acts with the female while restraining him from entering the operant chamber due to the reduced high of the doors. Sexual behavior was recorded via a camera (Youcam, Freetalk+) connected to a computer.

Figure 16: Operant response apparatus used for the evaluation of the effects of potassium sorbate and tartrazine on sexual behavior (modified from Kops, 2012**)** (Elisabeth Louise Ndjengue M., in Yaoundé on June 20, 2023), MC = Male compartment, FC= Female compartment, FD = First door, SD = Second door.

II.2. METHODS

II.2.1. Evaluation of the effects of potassium sorbate and tartrazine on the activation of the hypothalamic-hypophysis-ovarian axis, sexual behavior and fertility of parents (F0)

The experimental protocol of the evaluation of the effects of potassium sorbate and tartrazine on the activation of the hypothalamic- hypophysis-ovarian axis, sexual behavior and fertility of parents (F0) is summarized on Figure 17. Ninety-eight rats aged 21- 22-days and weighting 30 g on average were randomly divided into nine groups, seven of 12 animals each and two of 7 animals each. The control group received vehicle (distilled water), three test groups received potassium sorbate at the doses of 12.5, 45, and 78 mg/kg BW, three test groups

received tartrazine at the doses of 7.5, 27, and 47 mg/kg BW and the last two groups PERFORMANCE-1 and PERFORMANCE-2 that have received the Maca-ginseng-gingembre (a substance used to stimulate sexual desire) at the dose of 30 mg/kg BW. PERFORMANCE-1 also received distilled water during the treatment except the day of tests where they received Maca-ginseng-gingembre 2 hours before the test. PERFORMANCE-2 received Maca-ginseng-gingembre for 41 to 51 days, in the same condition as animals treated with potassium sorbate or tartrazine. The volume of water and substance administered was 10 mL/kg BW.

II.2.1.1. Evaluation of the effects of potassium sorbate and tartrazine on the activation of the hypothalamic-pituitary-ovarian axis of F0

The animals receiving potassium sorbate (12.5, 45, and 78 mg/kg BW), tartrazine (7.5, 27, and 47 mg/kg BW), and the control group were orally treated by gavage once daily (between 9 and 10 am) for 40 days from the postnatal days 21 to 22. The animals were weighed twice a week and the vaginal opening which is the marker for puberty onset was daily checked until the day it occurred. From day 36 of treatment (a day when there is a vaginal opening in all the animals) until the fortieth day, 5 animals per group were sacrificed (in estrus) by decapitation after light anesthesia by diazepam-ketamine *i.p.* injection (10 and 50 mg/kg BW, respectively). Blood samples were collected for biochemical analysis in dry tubes. The ovaries, uteri, mammary gland, and vagina, were dissected and weighed (except the vagina and mammary gland which were immersed immediately in 10% formaldehyde). The left ovary and uterus from each animal were also fixed in 10% formaldehyde for histological analysis. The right ovary and right uterus were cut, weighed, and ground separately with the glass potters in sodium phosphate buffer (0.1 M; pH 7.1) to obtain a final homogenate of 20%. After centrifugation at 3000 rpm (Goget Centrifuge, HETTICH, Westphalia, Germany) for 15 min at 5 °C, the collected supernatant was stored at −20 ◦C for subsequent determination of total uterine and ovarian proteins, and ovarian cholesterol. Blood samples collected in dry tubes were also centrifuged at 3000 rpm at 5 °C for 15 min and the serum obtained was kept at −20 ◦C until use (Awounfack *et al*., 2018). Serum and homogenates of the uterus and ovary were used for biochemical analysis. In the serum, follicle Stimulating Hormone (FSH), Luteinizing Hormone (LH), Estradiol, and Progesterone were measured in duplicate using the Enzyme-Linked Immunosorbent Assay (ELISA) technique and reagent kits obtained from Cypress Diagnostics (Langdorp, Belgium) according to the manufacturer's instructions. The results were precise (intra and inter-assay coefficients of variability) with CV ≤ 9.5645% for all the tested samples. The evaluation of ovarian total cholesterol for the parents was measured using reagent kits from Chronolab (Barcelona, Spain).

II.2.1.2. Evaluation of the effects of potassium sorbate and tartrazine on the sexual behavior of F0

The remaining animals per group (7) and the animals receiving maca gensing-gengimbre (30 mg/kg BW) were used for the evaluation of the effects of potassium sorbate (12.5, 45, and 78 mg/kg BW) and tartrazine (7.5, 27, and 47 mg/kg BW) on sexual behavior parameters. The animals were orally treated by gavage once daily (between 9 and 10 am) from the postnatal days 21 to 22 until day 51. Before the test, starting on day 41 until day 51 of the treatment, the receptivity was confirmed by the vaginal smear. All the receptive females were treated one hour before the test (Except the PERFORMANCE groups that were treated two hours before the test) and trained to use the apparatus. During the training session, the female was allowed to roam the apparatus for 5 minutes allowing her the opportunity to get used to the chamber. During the gavage treatment period, a vaginal smear was taken daily (between 9 and 10 a.m.) in all animals. The data obtained from the vaginal smears were used to assess the parameters of the estrous cycle. On test days, animals in the proestrus phase were subjected to an evening sexual behavior test (6-10 p.m.).

One week before the start of the behavioral test, male rats of proven fertility were introduced daily and immobilized (for 5 min) in the operating compartments, to acclimatize them to the apparatus. The females were then individually introduced into the apparatus (for 5 min) to acclimatize them to the apparatus. On the day of the test and one hour after the last administration, the female was placed with the male in a plastic cage for around 30 seconds until the copulatory behavior was initiated. For the test, the male was first introduced into the male compartment of apparatus, then the female in her compartment. The data collection was performed using a recording system consisting of a webcam (Freetalk+) connected to a computer. The sexual behavior was monitored for 30 minutes. At the end of the test, the video recordings of each pair of animals were viewed and analyzed. The parameters were analyzed as follows (Olivier *et al*., 2011; kops, 2012; Yakubu and Olutoye, 2016):

- **The appetitive phasis**: compartment change latency (time taken by the female to move to the male's compartment or operating compartment); compartment change frequency (number of compartment changes/time spent in her compartment or non-operating compartment).
- **The pre-copulatory or proceptive phasis**: number of solicitations (number of times the female makes an orientation with her head towards the male, followed by a flight towards her initial compartment); the number of rejections (number of times the female adopts a defensive posture, boxing, kicking); darting latency (time taken by the female to initiate a forward flight, followed by an abrupt stop with an orientation of her hindquarters towards the male); hopping latency (time taken by the female to make a short jump with frozen legs); darting frequency

(number of darts throughout the observation); hopping frequency (number of hops throughout the observation).

- **The consumption or receptive phasis**: lordosis latency (time taken by the female to adopt a concave curvature of the spinal column, in response to mounting by the male to facilitate intromission); lordosis duration; lordosis frequency (number of lordosis over the observation period); lordosis quotient (number of lordosis received/number of mounts multiplied by 100); number of anogenital grooming (number of times the female cleans her anogenital area).

II.2.1.3. Evaluation of the effects of potassium sorbate and tartrazine on fertility of F0

In the days following the sexual behavior test, the gestation was confirmed by the appearance of a vaginal plug or sperm in a vaginal smear. This day was considered to be gestational day 1. We continued the treatment with the animals receiving potassium sorbate (12.5, 45, and 78 mg/kg BW) and tartrazine (7.5, 27, and 47 mg/kg BW). They were orally treated by gavage once daily (between 9 and 10 am) up to the parturition. Ten days after gestation, a laparotomy under anesthesia (30 mg/kg PC diazepam, i.p + 5 mg/kg PC ketamine, i.p) was undertaken on mating females to count the number of implantation sites in the uterine horns and corpus luteum in the ovaries. These females were then placed in individual cages and monitored until delivery. After delivery, the number, the sex and weight of live fetuses were determined. These data were used to calculate the following parameters:

- The number of resorption sites (number of implantation site – number of viable fetuses),
- Implantation index ([total number of implantation sites/number corpus luteum] x 100),
- Resorption index ([total number of resorption sites/total number of implantation sites] x 100),
- Pre-implantation loss rate ([number of corpus luteum – number of implantations/ number of corpus luteum] x 100),
- post-implantation loss rate ([number of implantations x number of viable fetuses/number of implantations] x 100),
- Gestational rate ([number of females with viable fetuses at birth/total number of gestational females] x 100) (Watcho *et al*., 2009; Telefo *et al*., 2012; Lienou *et al*., 2012).
- Sex-ratio (number of males / number of females) (Vahidi and Sheikhha, 2007).

15 animals from each group were used for the evaluation of the reprotoxicity of the F1, F2, and F3.

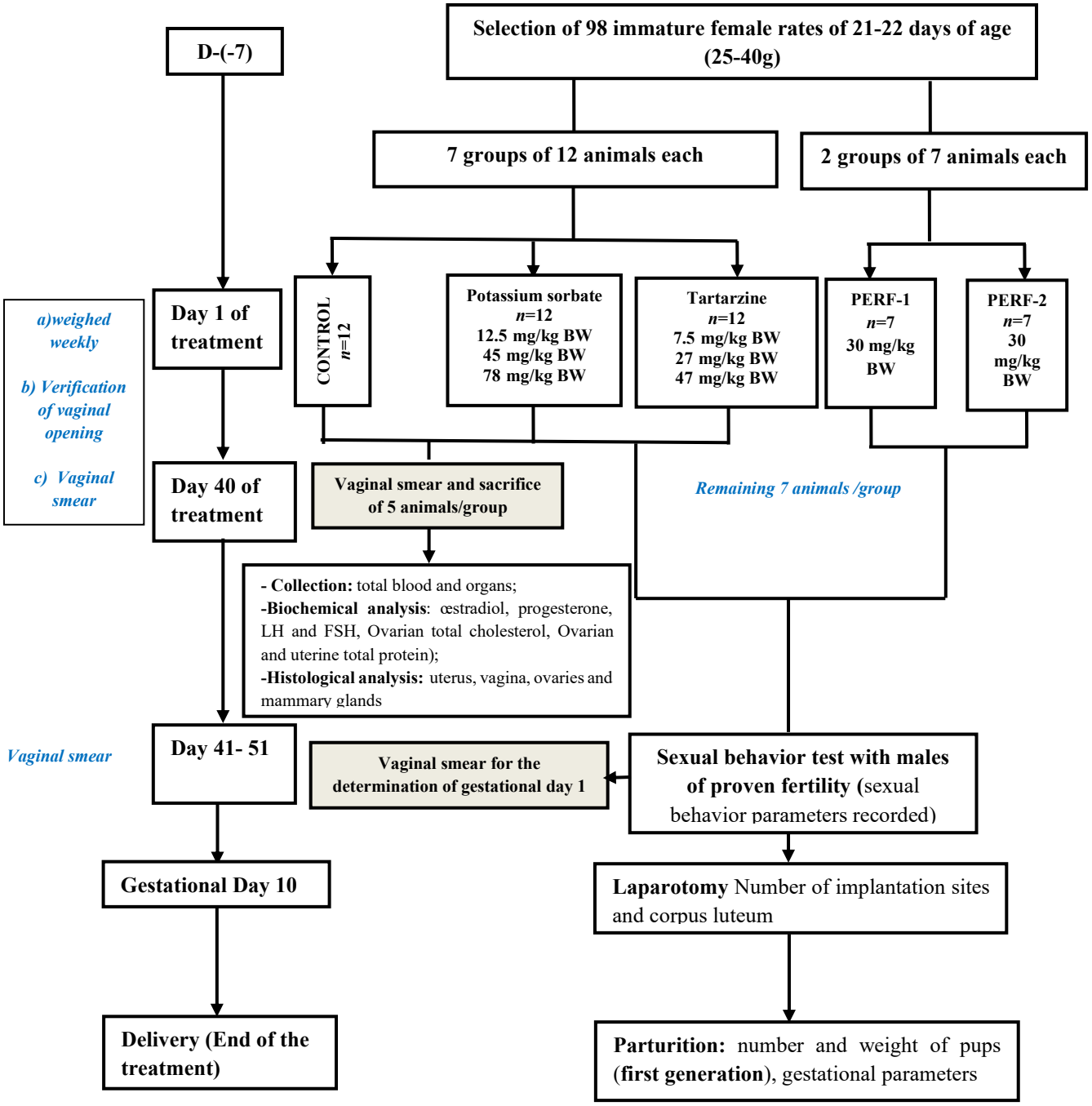


Figure 17: Protocol of the evaluation of effects of potassium sorbate and tartrazine on the activation of the hypothalamic-pituitary-ovarian axis, sexual behavior and fertility of parents (F0).

II.2.1.4. Vaginal smear and evaluation of oestrous cycle

Previously, attempt to evaluate the estrous cycle based on its frequency, regularity, length, estrus phase interval. In this study, we introduced the estrous cycle ratio (ECR) as a reproductive index (Agoreyo and Adeniyi, 2018; Chaitra *et al*., 2020). The animal's vaginal smears were daily taken between 9 and 10 am during 20 days to study the estrous cyclicity and the days after the mating to confirm gestation (Figure 18). For this purpose, a pipette (10 µL)

with a tip containing distilled water was introduced into the vagina (but not d eeply), and the vaginal mucus collected (Paccola *et al*., 2013; Agoreyo and Adeniyi, 2018; Ajayi and Akhigbe, 2020). The smear was then placed on a clean glass slide, air-dried and stained with EOSINE-RAL-555 (Ref: 361640-0109; Lot: 691043). The slide was then examined immediately under a light microscope with 40 x objective lense (Axioskop 40 microscope). The vaginal secretion is made up of three types of cells: round and nucleated ones are epithelial cells; irregular ones without nucleus are the cornified cells; and the little round ones are the leukocytes. Estimation of the phase of estrous cycle was based on the proportion among these cells in the vaginal secretion:

- Diestrus phase shows abundant round nucleated cells with little or no cornified cells.
- The estrus phase was characterized by anucleate cornified cells.
- At metestrus, the vaginal smear is dominated by a round nucleated epithelial cells, and a small number of leukocytes and anucleate cornified epithelial cells are also present.
- Proestrus phase was predominantly characterized by epithelial cells with little or no leucocytes (Marcondes *et al*., 2002; Agoreyo and Adeniyi, 2018; Ajayi and Akhigbe, 2020; Chaitra *et al*., 2020).

The obtained results were used to determine the total number and the percentage of each phase, the 4 to 5 days regular estrous cycles, cycle frequency, number of each phasis, longer regular cycle, irregular cycle, and the estrous cycle ratio. According to the literature, estrous cycle length represents the duration of estrous cycle in the time period between proestrus to diestrus. The cycle frequency can be defined as the total number of estrous cycles during observation period. 4 to 5 days regular estrous cycle is the total number of regular estrous cycles (i.e. 4 -5 days cycles) during observation period (Chaitra *et al*., 2020). ECR was calculated according to the following formula (Agoreyo and Adeniyi 2019):

$$ECR = \frac{(\text{Percentage of proestrus} + \text{Percentage of estrus})}{(\text{Percentage of metestrus} + \text{Percentage of diestrus})}$$

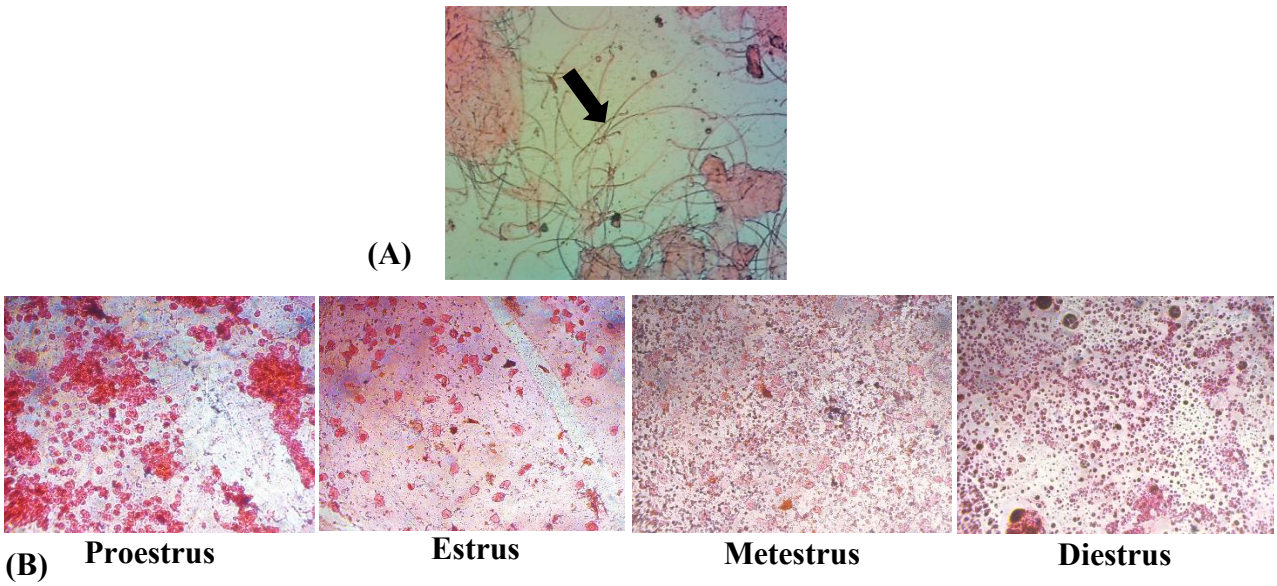


Figure 18: Vaginal smear of a pregnant female at gestational day 1 (A) and at the different phasis of estrus cycle (B) (An arrow indicates the presence of sperms) Elisabeth Louise Ndjengue M, in Yaoundé on June 20, 2023).

II.2.2. Evaluation of the effects of potassium sorbate and tartrazine on the activation of the hypothalamic-pituitary-ovarian axis, on sexual behavior and fertility of F1, F2, and F3

The animals (105 from F1, F2, and F3 generations) were randomly divided into seven groups, of 15 animals each. The abovementioned protocol on the evaluation of the effects of potassium sorbate and tartrazine on the activation of the hypothalamic-pituitary-ovarian axis and sexual behavior of F0 has been used.

II.2.2.1. Evaluation of the effects of potassium sorbate and tartrazine on the activation of the hypothalamic-pituitary-ovarian axis of F1, F2 and F3

To evaluate the effects of potassium sorbate and tartrazine on the activation of the hypothalamic-pituitary-ovarian axis of F1 and F2, F3, 35 animals obtained from each of the three generations were divided into seven groups of 5 animals each, a control group that received distilled water, three test groups which received potassium sorbate at the doses of 12.5, 45 and 78 mg/kg BW and the last three groups which received tartrazine at the doses of 7.5, 27 and 47 mg/kg BW. They were orally treated by gavage once daily (between 9 and 10 am) for 40 days from the postnatal days 21 to 22 and weighed twice a week. The vaginal opening which is the marker for puberty onset was daily checked until the day it occurred. From day 36 of treatment (a day when there is a vaginal opening in all the animals) until the fortieth day, 5

animals per group were sacrificed (in estrus) by decapitation after light anesthesia by diazepam-ketamine *i.p*. injection (10 and 50 mg/kg BW, respectively). Blood samples were collected for biochemical analysis in dry tubes. The ovaries, uteri, mammary gland, and vagina, were dissected and weighed (except the vagina and mammary gland which were immersed immediately in 10% formaldehyde). The left ovary and uterus from each animal were also fixed in 10% formaldehyde for histological analysis. The right ovary and right uterus were cut, weighed, and ground separately with the glass potters in sodium phosphate buffer (0.1 M; pH 7.1) to obtain a final homogenate of 20%. After centrifugation at 3000 rpm (Goget Centrifuge, HETTICH, Westphalia, Germany) for 15 min at 5 °C, the collected supernatant was stored at −20 °C for subsequent determination of total uterine and ovarian proteins, and ovarian cholesterol. Blood samples collected in dry tubes were also centrifuged at 3000 rpm at 5 °C for 15 min and the serum obtained was kept at −20 ◦C until use (Awounfack et al., 2018). Serum and homogenates of the uterus and ovary were used for biochemical analysis. In the serum of F3, follicle Stimulating Hormone (FSH), Luteinizing Hormone (LH), Estradiol, and Progesterone were measured in duplicate using the Enzyme-Linked Immunosorbent Assay (ELISA) technique and reagent kits obtained from Monobind, inc. (Lake Forest, United States of America), and according to the manufacturer's instructions. The evaluation of ovarian total cholesterol was measured using reagent kits from Labkit (Barcelona, Spain) (Figure 19).

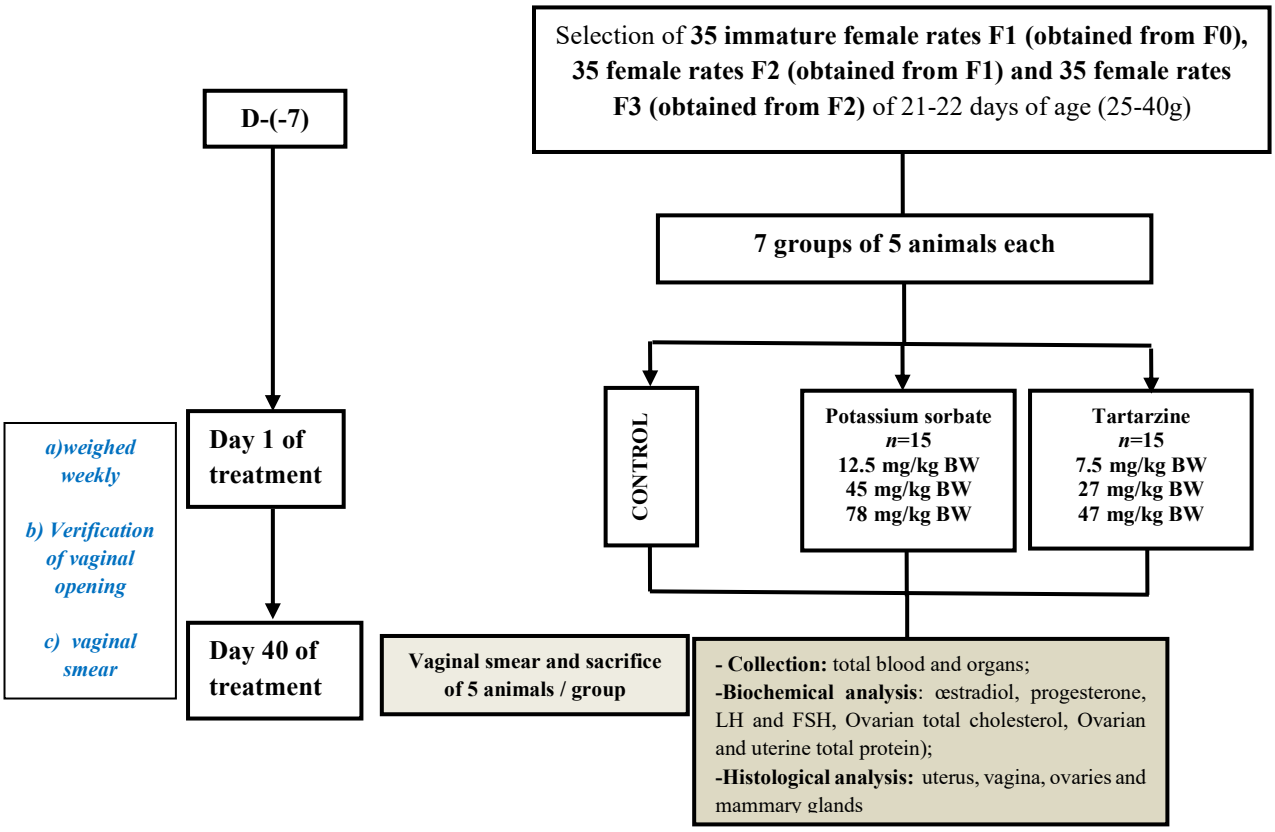


Figure 19: Protocol of the evaluation of effects of potassium sorbate and tartrazine on the activation of the hypothalamic-pituitary-ovarian axis of F1, F2, and F3.

II.2.2.2. Evaluation of the effects of potassium sorbate and tartrazine on sexual behavior and fertility of F3

The remaining 70 animals of the third generation were divided into 7 group of 10 animals according to each treatment. They were orally treated (with potassium sorbate at the doses 12.5, 45 and 78 mg/kg BW and tartrazine at the doses of 7.5, 27 and 47 mg/kg BW) by gavage once daily (between 9 and 10 am) from the postnatal days 21 to 22 until day 51. The last group received distilled water during the period of treatment. Before the test, starting on day 41 until day 51 of the treatment, the receptivity was confirmed by the vaginal smear. All the receptive females were treated one hour before the test and trained to use the apparatus as previously mentioned. During the training session, the female was allowed to roam the apparatus for 5 minutes allowing her the opportunity to get used to the chamber. On test days, animals in the proestrus phase were subjected to an evening sexual behavior test (6-10 p.m.).

One week before the start of the behavioral test, male rats of proven fertility were introduced daily and immobilized (for 5 min) in the operating compartments, to acclimatize them to the apparatus. The females were then individually introduced into the apparatus (for 5 min) to acclimatize them to the apparatus. On the day of the test and one hour after the last administration, the female was placed with the male in a plastic cage for around 30 seconds until the copulatory behavior was initiated. For the test, the male was first introduced into the male compartment of apparatus, then the female in her compartment. The data collection was performed using a recording system consisting of a webcam (Freetalk+) connected to a computer. The sexual behavior was monitored for 30 minutes. At the end of the test, the video recordings of each pair of animals were viewed and analyzed. The appetitive phasis, the proceptive phasis and receptive phasis were then analyzed (Olivier *et al*., 2011; kops, 2012; Yakubu and Olutoye, 2016).

Starting from the day of sexual behavior test, successful pregnancy was determined by the appearance of a vaginal plug or through microscopic observation in which sperms were present in the vaginal smear. This day was considered as day one of pregnancy. On gestational day 20, five females of each group were anesthetized under anesthesia (30 mg/kg PC diazepam, i.p + 5 mg/kg PC ketamine, i.p) and submitted to laparotomy. The uterine horns with fetuses and ovaries were collected and weighted. The uterine horns were then incised and the number, size, and weight of live fetuses were determined. The placentae were also harvested, blotted free of blood and weighed immediately. The uterine horns were exposed and fetuses were examined for any external malformation, including the event of resorptions and the number of live fetuses. The remaining five females were isolated and followed-up till delivery. After delivery, the implantation index ([total number of implantation sites/number corpus luteum] x 100), and gestation rate ([number of females with viable fetuses at birth/total number of gestational females] x 100) were calculated (Sukandar and Safitri, 2016; Ezeuko et al., 2019) (Figure 20).

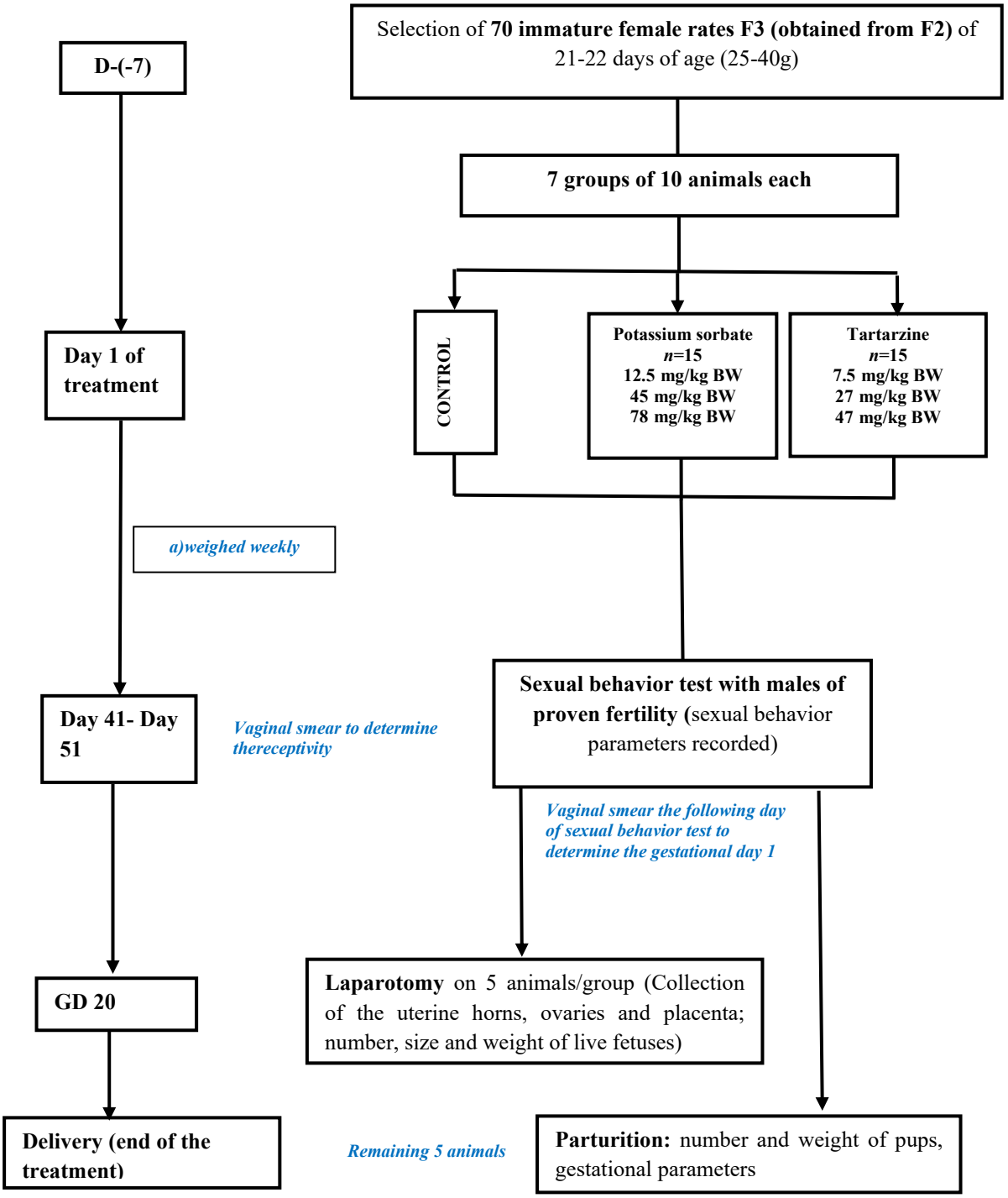


Figure 20: Protocol of the evaluation of effects of potassium sorbate and tartrazine on sexual behavior and fertility of F3.

II.2.2.3. Evaluation of the effects of potassium sorbate and tartrazine on fertility of F1, F2 and F3

Seventy animals of the first and second generation were divided according to the respective treatments into seven groups of 10 animals each. We had a control group that received distilled water, three test groups which received potassium sorbate at the doses 12.5, 45 and 78 mg/kg BW and the last three groups which received tartrazine at the doses of 7.5, 27 and 47 mg/kg BW. They were orally treated by gavage once daily (between 9 and 10 am) from the postnatal days 21 to 22 to the parturition. On days 41, they females were allowed to mate with vigorous males of proven fertility. Starting from the mating day, successful mating was determined by the appearance of a vaginal plug or through microscopic observation in which sperms were present in the vaginal smear. This day was considered as day one of pregnancy. On gestational day 20, five females of each group were anesthetized under anesthesia (30 mg/kg PC diazepam, i.p + 5 mg/kg PC ketamine, i.p) and submitted to laparotomy. The uterine horns with fetuses and ovaries were collected and weighted. The uterine horns were then incised and the number, size, and weight of live fetuses were determined. The placentae were also harvested, blotted free of blood and weighed immediately. The uterine horns were exposed and fetuses were examined for any external malformation, including the event of resorptions and the number of live fetuses. The remaining five females were isolated and followed-up till delivery. After delivery, the implantation index ([total number of implantation sites/number corpus luteum] x 100), and gestation rate ([number of females with viable fetuses at birth/total number of gestational females] x 100) were calculated (Sukandar and Safitri, 2016; Ezeuko *et al*., 2019) (Figure 21).

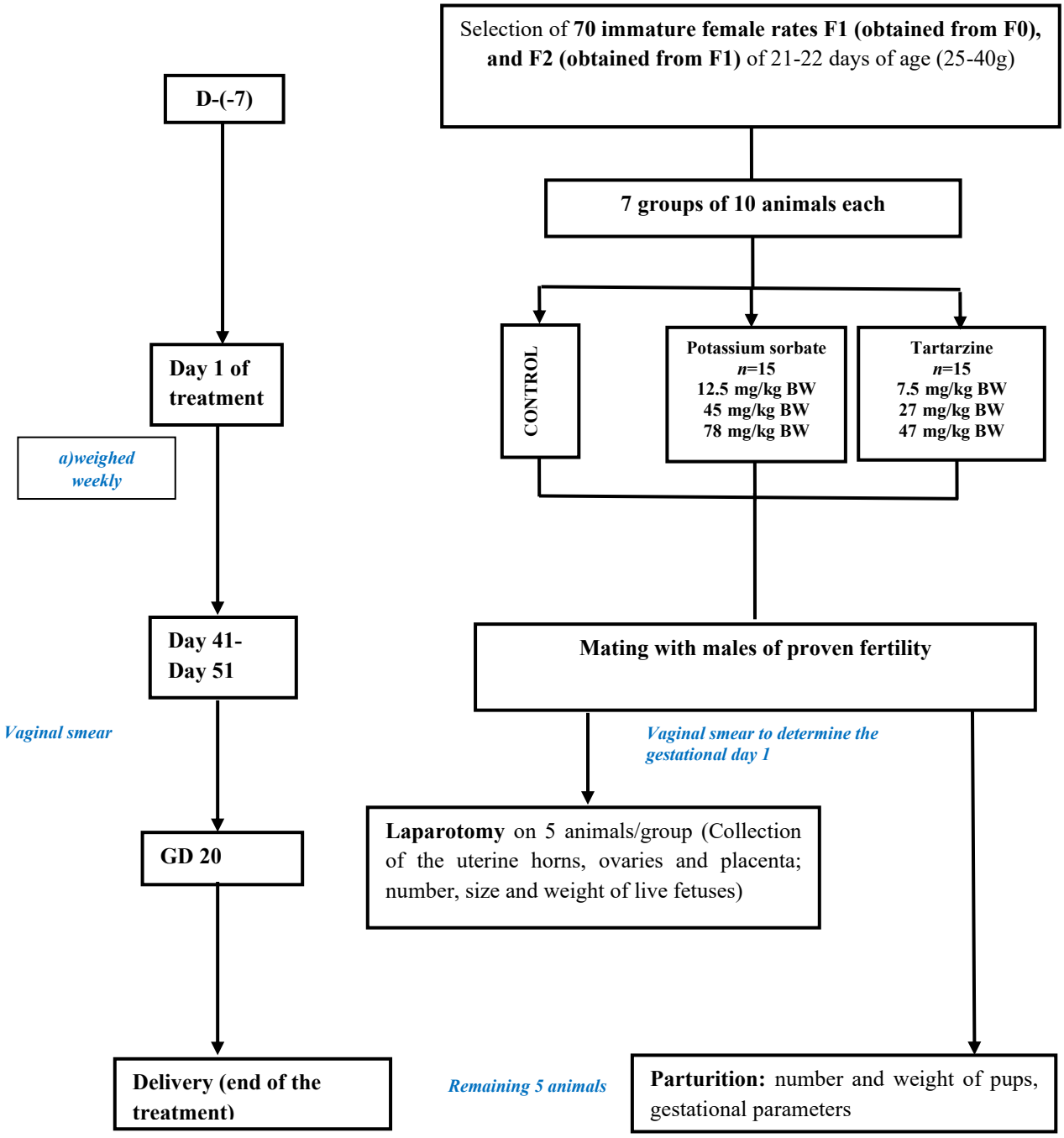


Figure 21: Protocol of the evaluation of effects of potassium sorbate and tartrazine on fertility of F1, F2.

II.2.3. Histological procedure

II.2.3.1. Fixation

Fixation aims to preserve tissue, prevent postmortem alterations (autolysis and putrefaction), and allow tissue to go through the subsequent processing steps without histological change or distortion. The reproductive organs (ovaries, uterus) except the vagina

and mammary gland were immersed in 10% formaldehyde. Series of alcohols used are prepared according to the alcohol dilution table (annex 1).

II.2.3.2. Trimming

The fixed organs were trimmed to obtain 3–5 mm thick sample size and arranged in labeled plastic cassettes.

II.2.3.3. Processing

This step is carried out to remove water from the organ or tissue before embedding. Poor dehydration results in poor processing. Processing involves the immersion of tissue or organs in ascending grades of alcohol prepared according to the alcohol dilution table (annex 1). For this purpose, sections were immersed in a series of alcohols: 80% ethanol (2 hours), 95% ethanol (4 times for 2 hours each), and 100% ethanol (3 times for 2 hours each). Many organic solvents are known as clearing agents. The most common clearing agent that enables the removal of alcohol and facilitates the transition between dehydration and infiltration steps is xylene. After dehydration, samples were immersed in xylene two times (2 hours each), the one increased the refractive ability of the tissue by giving it a transparent appearance. Thus, after this step, the tissue sample was infiltrated with paraffin at 60° for 4 hours.

II.2.3.4. Infiltration and embedding

Tissues must be embedded as soon as processing is finished. This enables to secure tissues in an oriented manner in a solid block in such a way that the hardened material is transparent to the optical method used for viewing the finished samples. During this step, tissue cavities or cells of processed organs were infiltrated and saturated by paraffin through immersion (2 x 2 hours each) in this medium at 60°C. Thereafter organs were placed in molds together with liquid paraffin (60°C) and refrigerated on cold surfaces (4°C).

II.2.3.5. Sectioning

This step consists of sectioning the tissue by using a microtome. Once embedded, organs were sliced into 5 µm sections. It produces sufficiently thin slices of tissue that the details can be observed using microscopy techniques. After cutting, sections floated on a warm gelatin water solution that helps to remove wrinkles. Then, sections were picked up on a glass microscopic slide and dried in the oven at 45°C for 24 hours.

II.2.3.6 Deparaffinization and staining

Staining sections with different colored dyes, having affinities of specific components of tissues, favorites identification and study of their morphology possible since the sections, as

they are prepared, are colorless and different components cannot be appreciated. For this routine histological work, the most common stains hematoxylin and eosin were used. Most of the staining solutions are aqueous, so it is essential to rehydration the section before staining. To remove paraffin from tissues (deparaffinization), the paraffin-coated sections were first heated in oven (55-60°C) for 20 minutes. The slides were positioned to allow drainage of melting paraffin. After that sections are passed progressively through xylene (3 times), 100% ethanol (3 times), 95% ethanol (once), 80% ethanol (once), and distilled water (once). For each time the incubation period was 5 minutes. Staining was accomplished by passing the sections through Harris's hematoxylin (2 times), water (2 times), 70% ethanol (once), 95% ethanol (once), 0.50% alcoholic eosin (once), and water (once). For each solution, the incubation period was 5 minutes.

II.2.3.7 Permanent mounting of sections

This step was accomplished by covering the section in an ideal resin medium such as Canada balsam that hardens and produces a clear binder between the slide and cover slip. For this purpose, 2-3 drops of balsam of Canada were used for each section avoiding entrapping air bubbles. This medium is non-miscible in water and thus, the sections must be dehydrated before being mounted. This was done in two steps: Passing the sections through three different solutions of pure ethanol (5 min x 3 times) and the same procedure was repeated in xylene (5 min x 3 times).

II.2.3.8. Histological analysis

The histomorphological analyses of ovaries, mammary glands, uterine and vaginal epithelial thickness were assessed from 5 µm section of paraffin-embedded organs. Organs were stained with hematoxylin-eosin and micrographs were observed and analyzed on slides using a Zeiss equipment consisting of a microscope Axioskop 40 linked to a computer where the image is transferred with the MRGrab1.0 and AxioVision 3.1 software, all provided by Zeiss (Hallbermoos, Germany). As concerns folliculogenesis, the tenth section of each ovary were selected five times placed on different glass slides. We considered as primary the follicles composed of oocytes surrounded with one layer of cuboidal follicular cells, secondary preantral follicles those with more than one follicular cell layer, and antral follicles those with present antrum of follicular fluid. Ruptured follicles with hypertrophied follicular, cell cavities, and cavities filled with blood were considered as corpus luteum.

II.2.3.8.1. Epithelial thicknesses

The epithelial thickness was measured and expressed in µm on the microphotographs using MRGrab 1. O and AxioVision 3.1 software.

II.2.3.8.2. Mammary gland analysis

This was carried out by observing the number and size of acini, differentiation of the acinar lumen, and the degree of proliferation of adipose and connective tissue.

II.2.4. Biochemical analysis

Serum and homogenates of the uterus and ovary were used for biochemical analysis. In the serum, follicle Stimulating Hormone (FSH), Luteinizing Hormone (LH), Estradiol, and Progesterone were measured in duplicate using the ELISA technique and reagent kits obtained from Cypress Diagnostics (Langdorp, Belgium) according to the manufacturer's instructions, and precise (intra and inter essay coefficients of variability) with CV ≤ 9.5645% for all the tested samples. The total cholesterol in ovaries was measured using reagent kits from Chronolab (Barcelona, Spain).

II.2.4.1. Total proteins

Uterine and ovary total protein were evaluated according to the Biuret method (Gornall *et al.,* 1949).

- **Principle**

Serum protein reacts with copper ions in an alkaline medium to form a violet color. The intensity of the color, which has maximum absorption at 540 nm, is proportional to the concentration of peptide bonds participating in the reaction and thus in the amount of protein present.

- **Procedure**

Reagents were added as indicated in Table II. Ten microliters of uterine or ovarian homogenate were introduced into the respective sample tubes, while in the control tubes, the homogenate was replaced by distilled water. After the introduction of the various reagents into the tubes, the whole was homogenized and incubated for 15 minutes at room temperature. Absorbance was read at 540 nm using a spectrophotometer (SPEKOL1300) against the blank. Protein concentration in the test tubes was determined using the calibration curve (Figure 22).

Table II: Procedure of the dosage of uterine and ovarian total protein

	Blanck	Standards						Samples	
Tubes's number	0	1	2	3	4	5	6	X1 …	X2
BSA (mg/mL	0	0.25	0.5	0.75	1	1.25	1.5	-	-
Distilled water (mL)	3	2.75	2.5	2.25	2	1.75	1.5	2	2
Biuret solution (mL)	2	2	2	2	2	2	2	2	2
Samples (mL)	-	-	-	-	-	-	-	1	1
Quantity of proteins (mg/mL)	0	0.25	0.5	0.75	1	1.25	1.5	-	-

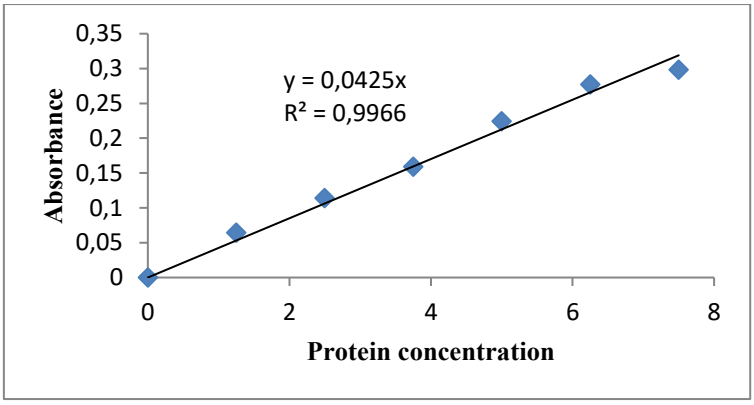


Figure 22: Calibration curve for the determination of uterine and ovarian total proteins.

II.2.4.2. Total cholesterol

- **Principle**

Ovarian total cholesterol was measured enzymatically. In presence of cholesterol esterase, cholesterol esters are hydrolyzed and the total cholesterol is measured by oxidizing with cholesterol oxidase to form hydrogen peroxide. Hydrogen peroxide reacts with phenol and 4-aminoantipyrine to form a red quinoneimine dye. The intensity of the solution is directly proportional to the level of cholesterol present in the sample.

$$\text{Cholesterol ester} + H_20 \xrightarrow{\text{cholesteryl ester hydrolase}} \text{Cholesterol} + \text{Fatty acid}$$

$$\text{Cholestérol ester} + 0_2 \xrightarrow{\text{cholesterol oxidase}} \text{Cholest-4-en-3-one} + H_20_2$$

$$2H_20_2 + \text{Phenol} + \text{4-aminophenazone} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine} + 4H_20_2$$

- **Procedure**

The determination of ovarian total cholesterol for the parents (F0) and the third generation (F3) was measured using reagent kits from Chronolab and Labkit respectively (Barcelona, Spain). The different reagents were added as presented in Table III. After

completion, the mixture was homogenized and incubated for 10 minutes at room temperature. Absorbance was read at 505 nm using a spectrophotometer (SPEKOL1300) against the blank (table III).

Table III: Procedure of the dosage of ovarian total cholesterol

	Blank	Standard	Sample
Reagents (mL)	**1.0**	**1.0**	**1.0**
Standard (µL)	-	**10**	-
Samples (µL)	-	-	**10**
Distilled water (mL)	**10**	-	-

Ovarian total cholesterol concentration, expressed in mg/dL, is determined according to the

$$\text{Concentration of (mg/dL)} = \frac{(\text{Abs sample} - \text{Abs blank})}{(\text{Abs standard}) - (\text{Abs blank})} \times \text{Standard conc.}$$

Abs=Absorbance

II.2.4.3. Hormones concentration

Serum concentrations of pituitary gonadotropins (LH and FSH) and sex steroids (estrogen and progesterone) were measured in duplicate using the ELISA technique and reagent kits obtained from Cypress Diagnostics (Langdorp, Belgium) and Monobind, Inc. (Lake Forest, United stated) according to the manufacturer's instructions.

II.2.4.3.1. Pituitary gonadotropins

The Cypress Diagnostics and Monobind, Inc. FSH and LH assay (96-wells microplates) is a solid phase sandwich ELISA used for their quantitative determination in serum or plasma.

- **Principle**

The principle of the following enzyme immunoassay test follows a typical two-step capture or 'sandwich' type assay. Samples are first incubated together with a mixture of anti-LH-enzyme conjugate and anti-LH-biotin, in streptavidin-lined microtiter wells. Streptavidin has a very high affinity for biotin. As a result, anti-LH-biotin conjugate molecules bind strongly to the microwell surface. The LH molecules in the sample bind simultaneously to the anti-LH-biotin and anti-LH-enzyme conjugates like a sandwich. Whatever, the FSH ELISA essay uses two highly specific antibodies: one polyclonal anti-FSH antibody for solid phase (microtiter wells) immobilized on the surface of microwells and another region monoclonal anti-FSH antibody that is coupled to horse radish peroxidase (HRP). The test sample is then allowed to react simultaneously with the antibodies, resulting in the FSH molecules being sandwiched between the solid phase and enzyme-linked antibodies. A subsequent washing step removes

unbound LH and FSH and conjugate molecules. An enzymatic reaction with the bound enzyme conjugate is initiated by the addition of the 3,3',5,5'-Tetramethylbenzidine (TMB) based substrate and stopped by the addition of the stop solution, resulting in color formation. The absorbance is measured on a microtiter plate reader at 450 nm. The intensity of the color is proportional to the concentration of LH and FSH in the sample.

- **Procedure**

The hormone concentration was measured in duplicate according to the protocol supplied by the company. The first step consisted of assigning microplate wells for controls, standards, and samples. Thereafter, 25 µL of each solution was dispensed into appropriate wells. Subsequently, 100 µL of enzyme conjugate solution was added to each well. The microplates were then covered and incubated for 30 min at room temperature (20-25°C). After being emptied of their contents, the microplate wells were washed simultaneously (3 times) with 300 µL of pre-prepared wash buffer, three times in succession, then patted on clean absorbent paper to remove residual wash solution.

The second incubation was carried out for 15 min at room temperature, after adding 100 µL of substrate to each well. At the end of incubation, 50 µL of stop solution was added to each well in the same order and at the same rate as the substrate was added. The mixture was shaken to mix the solution and effectively stop the reaction. 15 min later, absorbance was measured in an ELISA microtiter plate reader at 450 nm and optical densities (ODs) were obtained. The standard curve was made by plotting the mean absorbance obtained from each standard against its concentration with absorbance value on the vertical(Y) axis and concentration on the horizontal (X) axis. Serum LH concentrations in pg/ml were then extrapolated for each sample.

The microtiter wells were pre-assigned for the controls, standards, and samples to be assayed and the hormone was measured in duplicate according to the protocol supplied by the manufacturer. 25 µL/well of each solution, 100 µL of enzyme conjugate were added to each well. The mixture was incubated for 30 min at room temperature. At the end of incubation, the microwells were emptied and tapped on absorbent paper to remove all liquid. The microwells were then washed (3 times) with 300 µL of wash buffer (supplied by the manufacturer) diluted 1:10 with distilled water and patted with absorbent paper to remove all liquid. The plate was then incubated with 100 µL/well of previously prepared FSH conjugate working solution for 30 min at room temperature. After the second wash, the well was incubated with 100 µL/well of substrate (supplied by the manufacturer) until the standard had turned dark blue. The reaction was then stopped by adding 50 µL/well of stop solution in the same order and at the same rate as the substrate had been assigned. The wells were gently shaken to mix the solution and effectively stop the reaction. 20 min later, absorbances were read in an ELISA microtiter plate

reader at 450 nm. A set of standards is used to plot a standard curve from which the amount of LH and FSH in samples and controls can be directly established (Figure 23). By plotting the OD of standards and controls against their concentrations, we were able to extrapolate serum FSH concentrations in ng/ml for each sample.

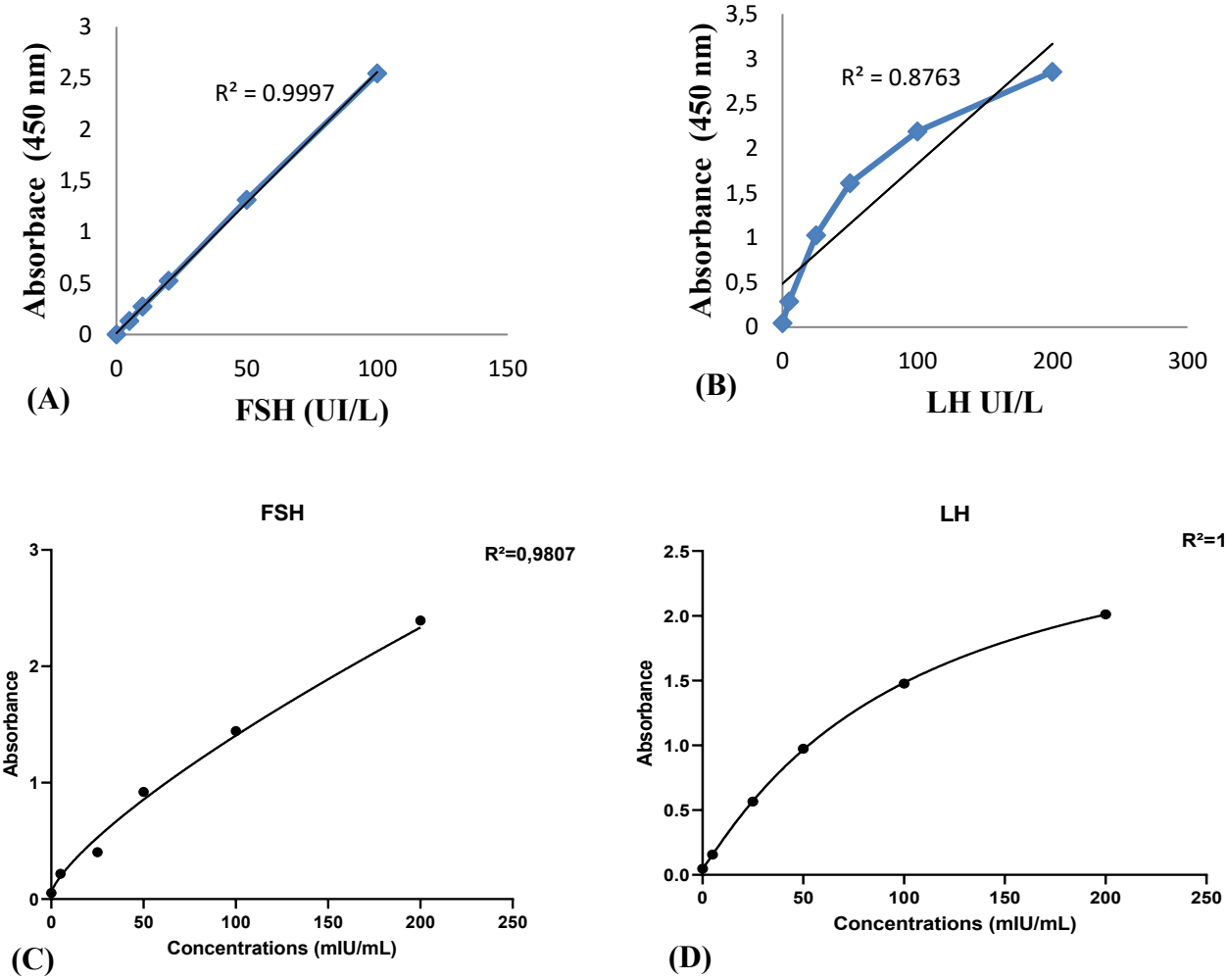


Figure 23: Calibration curves for the determination of FSH and LH of F0 (A, B) and F3 (C, D).

I.2.4.3.2. Sexual steroids

The Estradiol and Progesterone ELISA Kit (96-well microplates) is a solid-phase enzyme-linked immunosorbent assay (ELISA), based on the principle of competitive binding.

- **Principle**

Samples are first incubated with an estradiol-enzyme conjugate or progesterone-enzyme conjugate in microtiter wells coated with a polyclonal antibody directed towards an antigenic site on the estradiol molecule. Competition occurs between total hormones present in standards, controls, and samples and an enzyme-labeled antigen (conjugate) for a limited number of anti-estrogen antibody binding sites on the microplate wells. A subsequent washing step removes

unbound estradiol and conjugate molecules. An enzymatic reaction with the enzymatic conjugate is initiated by the addition of the TMB-based substrate and stopped by the addition of the stop solution, resulting in color formation. Absorbance is then measured at 450 nm. Color intensity is inversely proportional to estradiol concentration in the sample. A calibration curve relating absorbance to estradiol and progesterone concentration is then established (Figure 24).

- **Procedure**

The hormone was measured in duplicate according to the protocol supplied by the manufacturer. After assigning the microplate wells for controls, 25 µL of each solution (calibrator, standards, and specimen) was pipetted and introduced into planned wells. Thereafter, 100 µL/well of estradiol-enzyme conjugate or progesterone-enzyme conjugate was added. The mixture was incubated for 60 min at room temperature (20-25°C). At the end of incubation, the microwells were emptied and patted on absorbent paper to remove all liquid. The microwells were then washed (3 times) with 300 µL of wash buffer (supplied by the manufacturer) diluted 1:20 with distilled water, and patted with absorbent paper to remove all liquid. The plate was then incubated with 100 µL/well of substrate (supplied by the manufacturer), for 30 min at room temperature (20-25°C). After adding 50 µL/well of stop solution in the same order and at the same rate as the substrate had been assigned, the wells were gently shaken to mix the solution and effectively stop the reaction. 15 min later, absorbances were determined with an ELISA microtiter plate reader at 450 nm. By plotting the absorbance of standards and controls against their concentrations, we extrapolated serum estradiol or progesterone concentrations in IU/L for each sample (Figure 24).

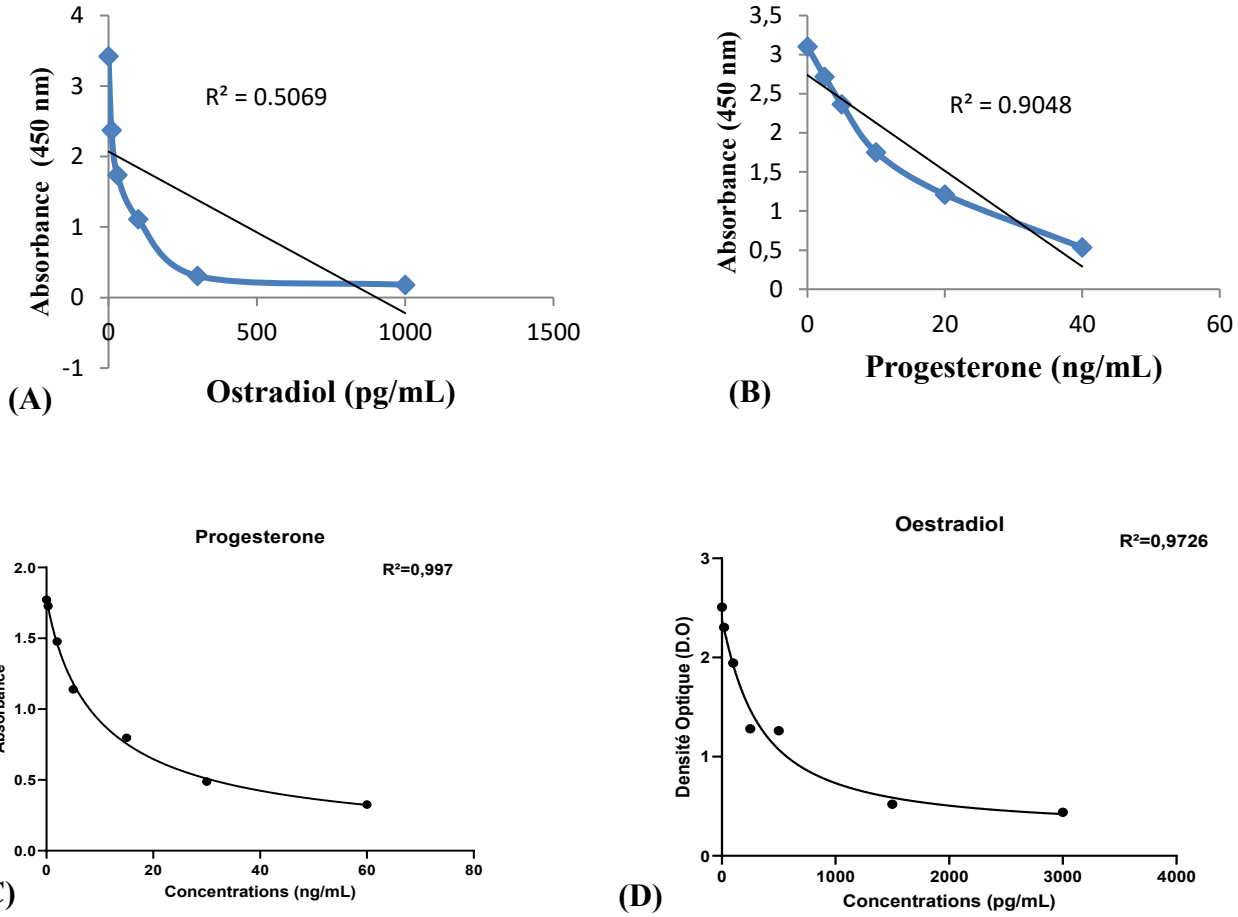


Figure 24: Calibration curve for the determination of estradiol and progesterone of F0 (A, B) and F3 (C, D).

II.2.5. Statistical analysis

Data were expressed as mean ± standard error on the mean (SEM). A two-way ANOVA repeated measures followed by Bonferroni post-hoc tests was used to compare the effect of tartrazine on body weight and the percentage of animals with vaginal opening. The fixed effects or factors were treatment (each dose of potassium sorbate and tartrazine vs. control group), time or periods of analysis, and their interaction. ANOVA one-way followed by Dunnet's test (when appropriate) was used for the other data with treatment as a fixed effect. All of these tests were performed using GraphPad Prism 5.03 software (La Jolla, CA, USA, 2009). Differences were considered significant at $p < 0.05$.

CHAPTER III

RESULTS AND DISCUSSION

III.1. RESULTS

III.1.1. Effects of potassium sorbate and tartrazine on the activation of hypothalamic-hypophysis-ovarian axis, sexual behavior and fertility of the parents (F0)

III.1.1.1. Activation of hypothalamic-hypophysis-ovarian axis

III.1.1.1.1. Effects of potassium sorbate and tartrazine on bodyweight of animals

Figure 25 shows the effect of potassium sorbate (**A**) and tartrazine (**B**) exposure on body weight evolution throughout treatment. The results indicated that all doses of potassium sorbate and tartrazine did not affect the body weight of animals as compared to the control group.

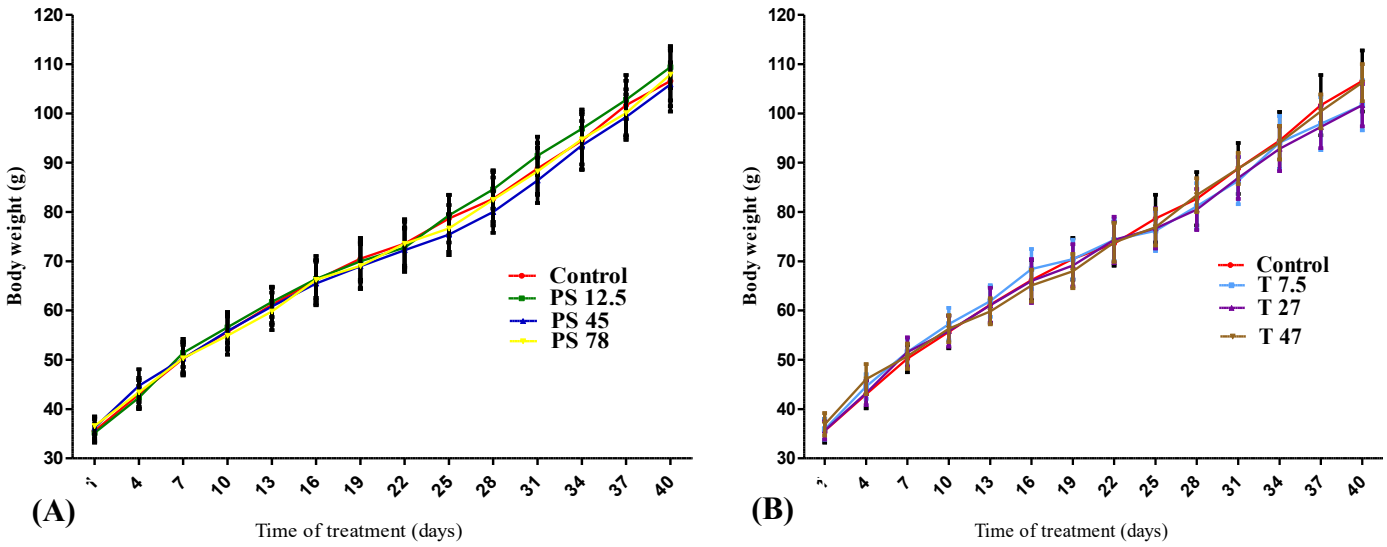


Figure 25: Effects of potassium sorbate and tartrazine on the bodyweight of female Wistar rats during the 40 days of treatment of F0.

Results are shown as a mean ± SEM, *n* = *5*. Control = animals receiving vehicle (distilled water, 10 mL/kg BW); PS= rats treated with potassium sorbate at doses of 12.5, 45, and 78 mg/kg BW; T = rats treated with tartrazine at doses of 7.5, 27, and 47 mg/kg BW.

III.1.1.1.2. Effects of potassium sorbate and tartrazine on vaginal opening

Figure 26 represents the effects of potassium sorbate and tartrazine on the vaginal opening. Compared to the control group, the treatment did not induce any significant modification in the mean age of the vaginal opening (Figure 26**A** and 26**B**). The results on the percentage of animals with vaginal opening showed that potassium sorbate at the dose of 78 mg/kg BW significantly decreased the percentage of animals with vaginal opening compared to control group. On day 46, this group exhibited 25% of vaginal opening vs. 75% for control ($p < 0.001$). On the other side, 47 mg/kg BW tartrazine showed a significant increase ($p < 0.05$) of the percentage of animals with vaginal opening as compared to the Control group. This group

displayed 75 % of vaginal opening vs. 25 % for the control on day 46 and 100 % of vaginal opening vs. 41.66% on day 45.

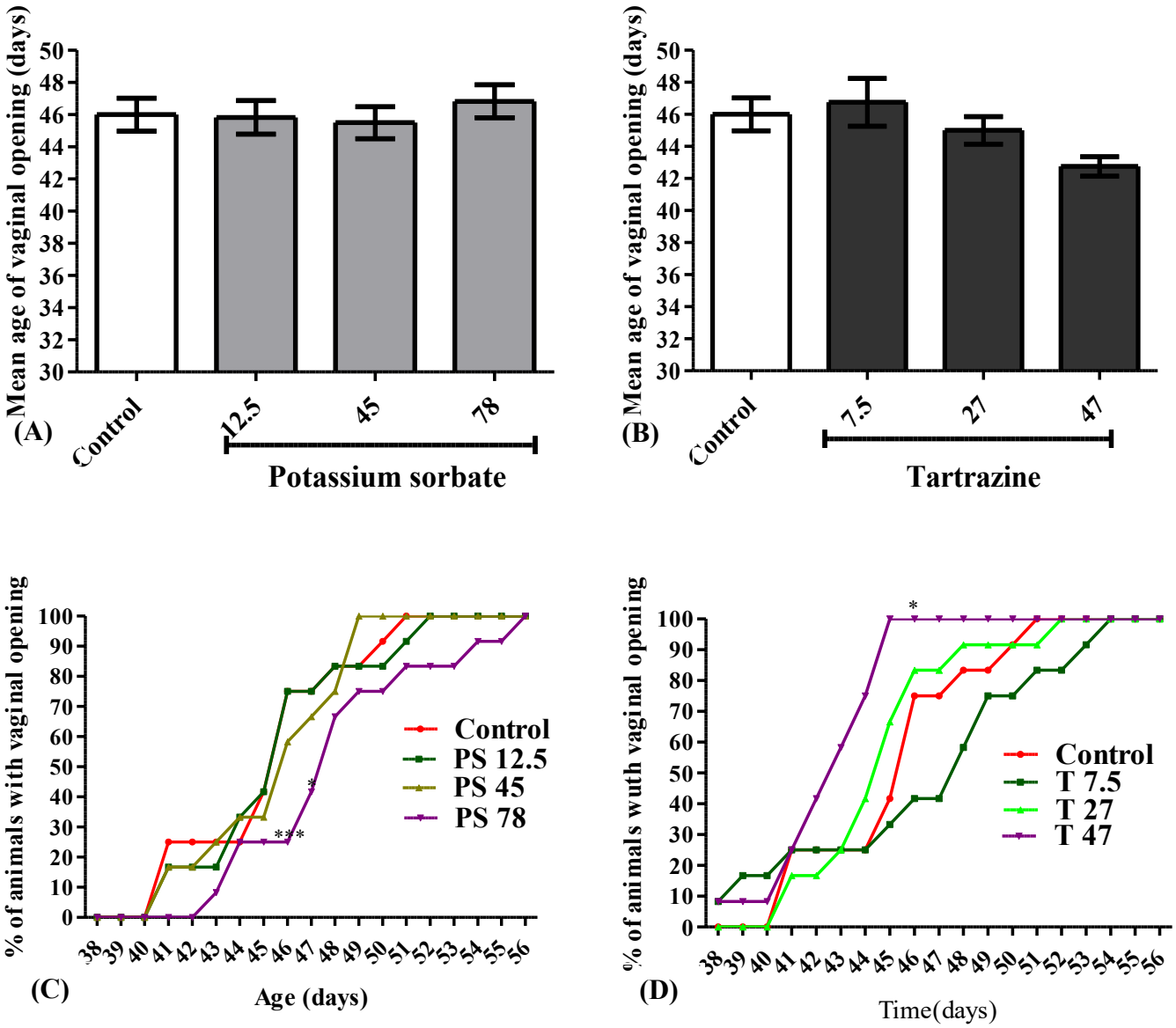


Figure 26: Effects of potassium sorbate and tartrazine on the mean age of the vaginal opening (A, B) and the percentage (%) of rats with a vaginal opening (C, D) during 40 days of treatment of F0.

Results are shown as a mean ± SEM, $n = 5$. *: $p < 0.05$; ***: $p < 0.001$ in reference to control. Control= animals receiving vehicle (distilled water); PS = rats treated with potassium sorbate at doses of 12.5, 45, and 78 mg/kg BW; T = rats treated with tartrazine at doses of 7.5, 27, and 47 mg/kg BW.

III.1.1.1.3. Effects of potassium sorbate and tartrazine on epithelial thickness of uterus and vagina

The effects of potassium sorbate and tartrazine on the epithelial thickness of uterus are summarized in Figure 27. The results indicated that the treatment with potassium sorbate did not affect the uterine epithelial thickness (Figure 27**A**). As shown in figure 27**B**, tartrazine at the doses of 27 ($p < 0.05$) and 47 ($p < 0.01$) mg/kg BW significantly increased the uterine epithelial thickness as compared to the control group. This difference is also confirmed by the microphotographs presented in Figure 27**C**.

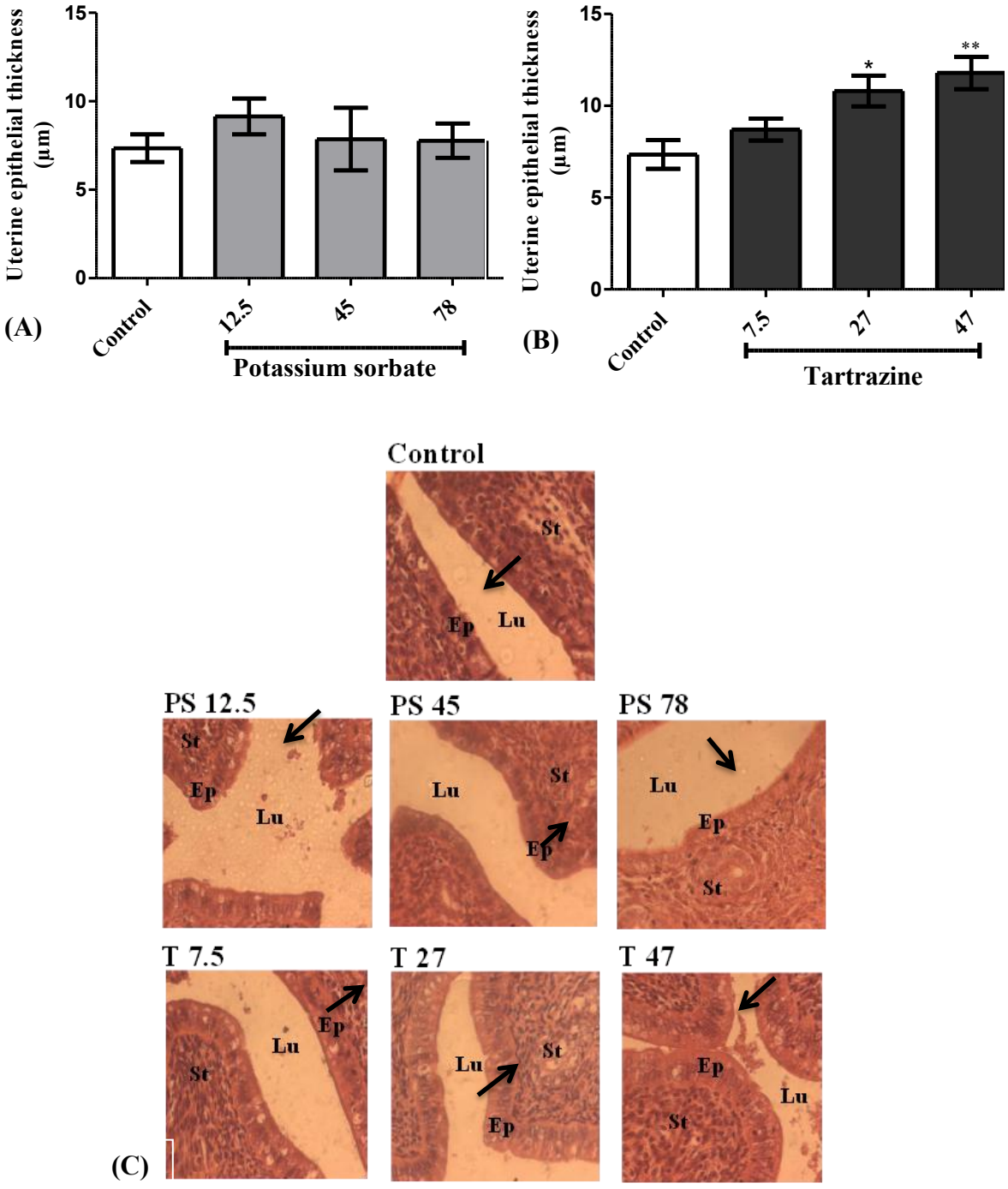


Figure 27: **Uterine (A, B) epithelial thickness as well as microphotographs (40×) of hematoxylin/eosin-stained sections of uteri (C) of female Wistar rats after 40 days of treatment of F0 with potassium sorbate and tartrazine**.

Results are presented as mean ± SEM, $n = 5$. *: $p < 0.05$ **: $p < 0.01$ in reference to control. Lu = Lumen; Ep = Epithelial cell thickness; St = Stroma. Control = animals receiving vehicle (distilled water, 10 mL/kg BW); PS = rats treated with potassium sorbate at doses of 12.5, 45, and 78 mg/kg BW; T = rats treated with tartrazine at doses of 7.5, 27, and 47 mg/kg BW.

III.1.1.1.4. Effects of potassium sorbate and tartrazine on epithelial thickness of vagina

The results presented in Figure 28 showed that potassium sorbate and tartrazine did not induce any significant effect on the vaginal epithelial thickness after 40 days of treatment of F0 (Figure 28).

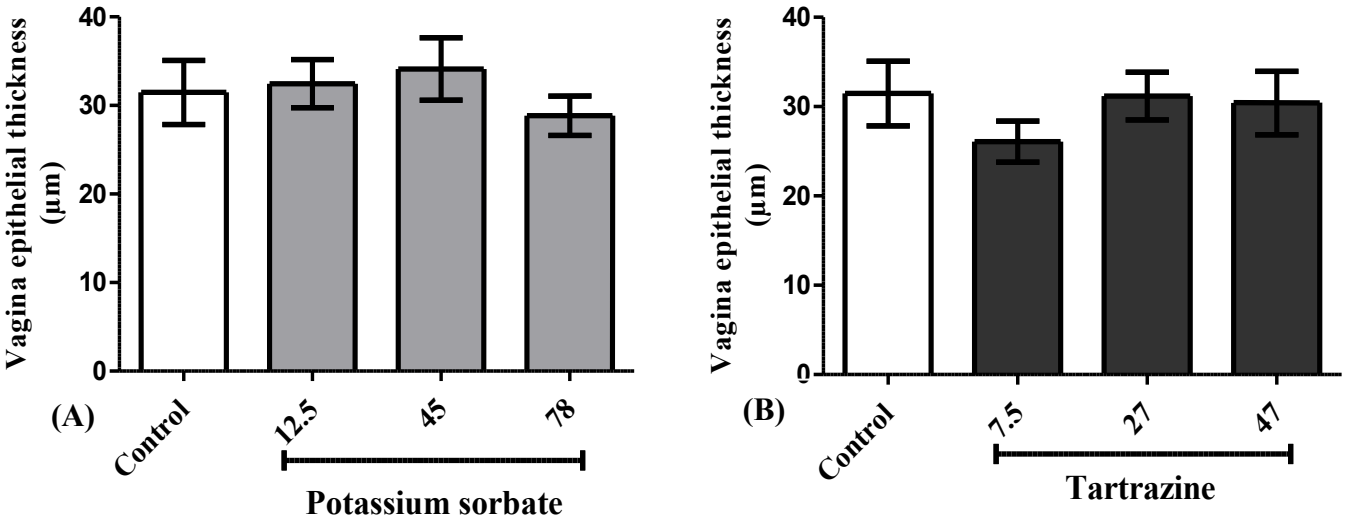


Figure 28: Vagina epithelial thickness of female Wistar rats after 40 days of treatment of F0 with potassium sorbate and tartrazine.

Results are presented as mean ± SEM, $n = 5$. Lu = Lumen; Ep = Epithelial cell thickness; St = Stroma. Control = animals receiving vehicle (distilled water, 10 mL/kg BW); PS = rats treated with potassium sorbate at doses of 12.5, 45, and 78 mg/kg BW; T = rats treated with tartrazine at doses of 7.5, 27, and 47 mg/kg BW.

III.1.1.1.5. Effects of potassium sorbate and tartrazine on the relative weight of reproductive organs

Figure 29 represents the effects of potassium sorbate and tartrazine on the relative weight of ovaries and uterus. Results showed that, 12.5 mg/kg BW potassium sorbate significantly increased ($p < 0.05$) the relative weight of ovaries and decreased ($p < 0.01$) this parameter at the dose of 78 mg/kg BW (Figure 29**A**). Compared to control group, the treatment with tartrazine did not induce any effect on the relative weight of ovaries (Figure 29**B**). Regarding the relative weight of the uterus (Figures 29**C** and 29**D),** the results showed that potassium sorbate at the dose of 45 mg/kg BW ($p < 0.001$), tartrazine at the dose of 47 mg/kg BW ($p < 0.01$) significantly increased the relative weight of the uterus as compared to the control group.

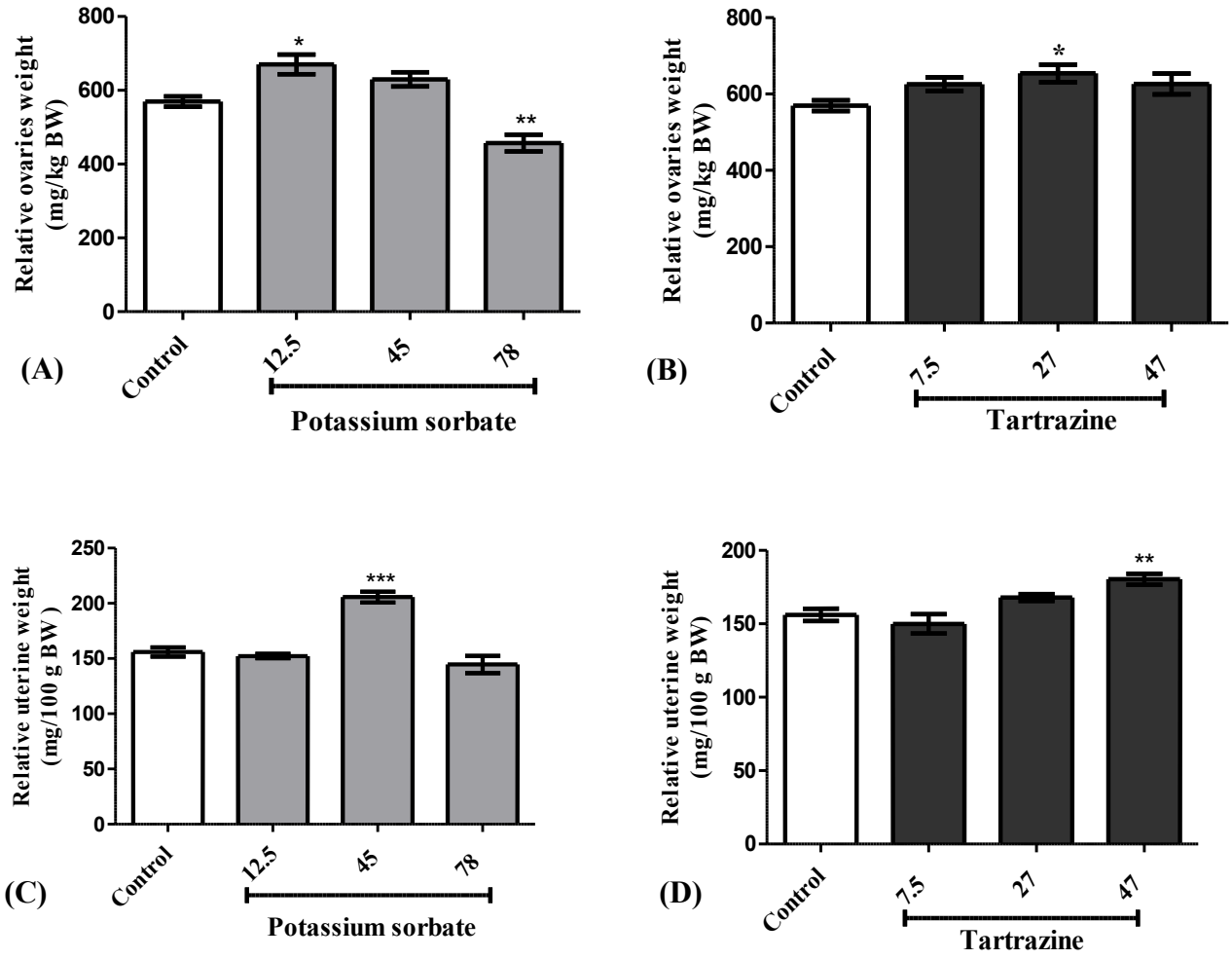


Figure 29: Effects of potassium sorbate and tartrazine on the relative weight of ovaries (A, B) and uterus (C, D) of female Wistar rats after 40 days of treatment of F0.

Results are shown as a mean ± SEM, $n = 5$. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ in reference to control. Control = animals receiving vehicle (distilled water, 10 mL/kg BW); PS = rats treated with potassium sorbate at doses of 12.5, 45, and 78 mg/kg BW; T = rats treated with tartrazine at doses of 7.5, 27, and 47 mg/kg BW.

III.1.1.1.6. Effects of potassium sorbate and tartrazine on mammary glands

The effects of potassium sorbate and tartrazine on mammary glands are summarized in Figure 30. The microphotographs presented showed that, compared to the control group, tartrazine at the dose of 47 mg/kg BW induced eosinophilic secretions in the acinar of the mammary glands.

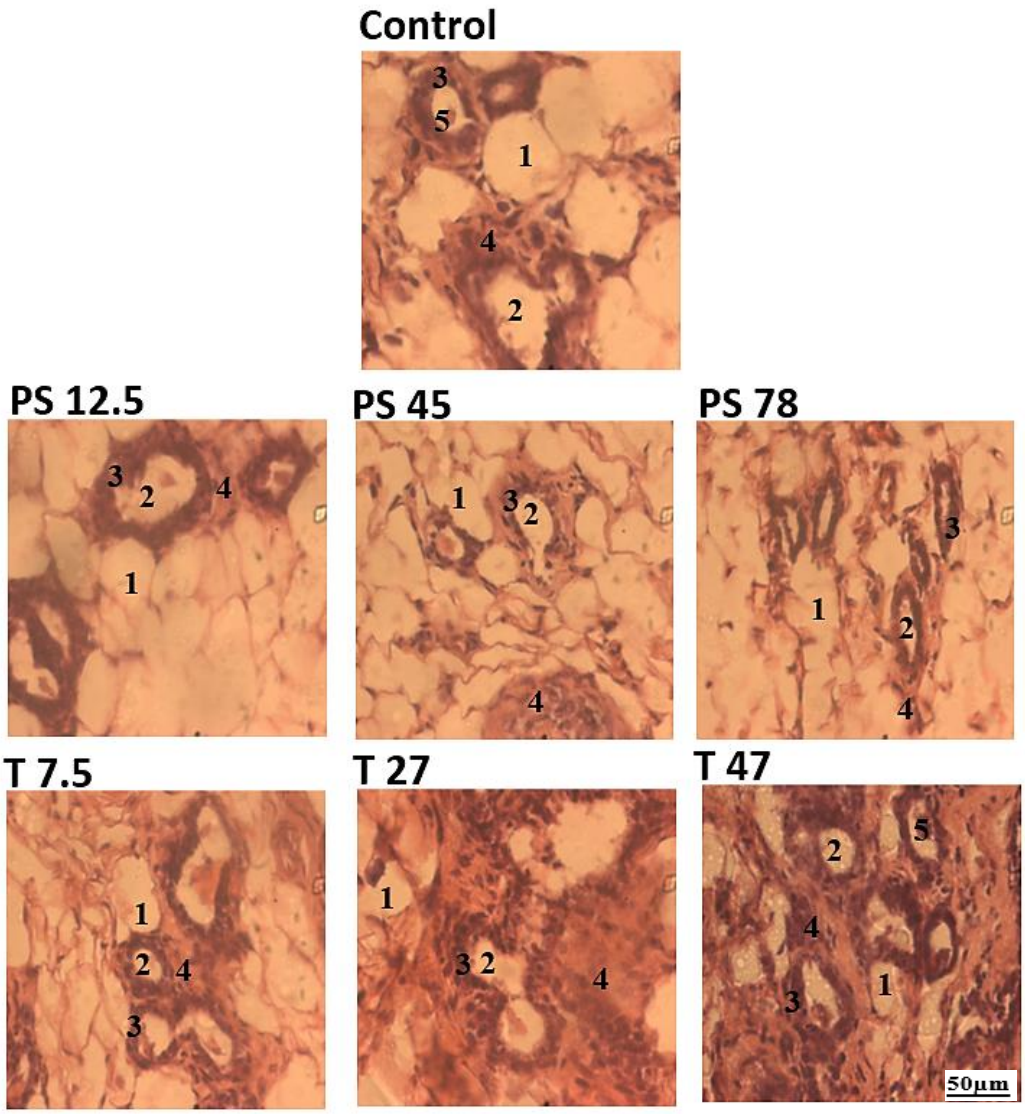


Figure 30: Microphotographs (40×) of hematoxylin/eosin-stained sections of mammary glands of female Wistar rats after 40 days of treatment of F0 with potassium sorbate and tartrazine.

Control = animals receiving vehicle (distilled water, 10 mL/kg BW); PS = rats treated with potassium sorbate at doses of 12.5, 45, and 78 mg/kg BW; T = rats treated with tartrazine at doses of 7.5, 27, and 47 mg/kg BW. 1 = Adipose tissue; 2 = Lumen of alveoli; 3 = Alveoli epithelium; 4 = Gland parenchyma; 5 = Eosinophilic secretion.

III.1.1.1.7. Effects of potassium sorbate and tartrazine on the ovarian total cholesterol and ovarian and uterus total proteins

Figure 31 shows the effects of potassium sorbate and tartrazine on some biochemical parameters. The results indicated that the ovarian total cholesterol, the ovarian total protein and the uterine total protein were not significantly affected by treatments.

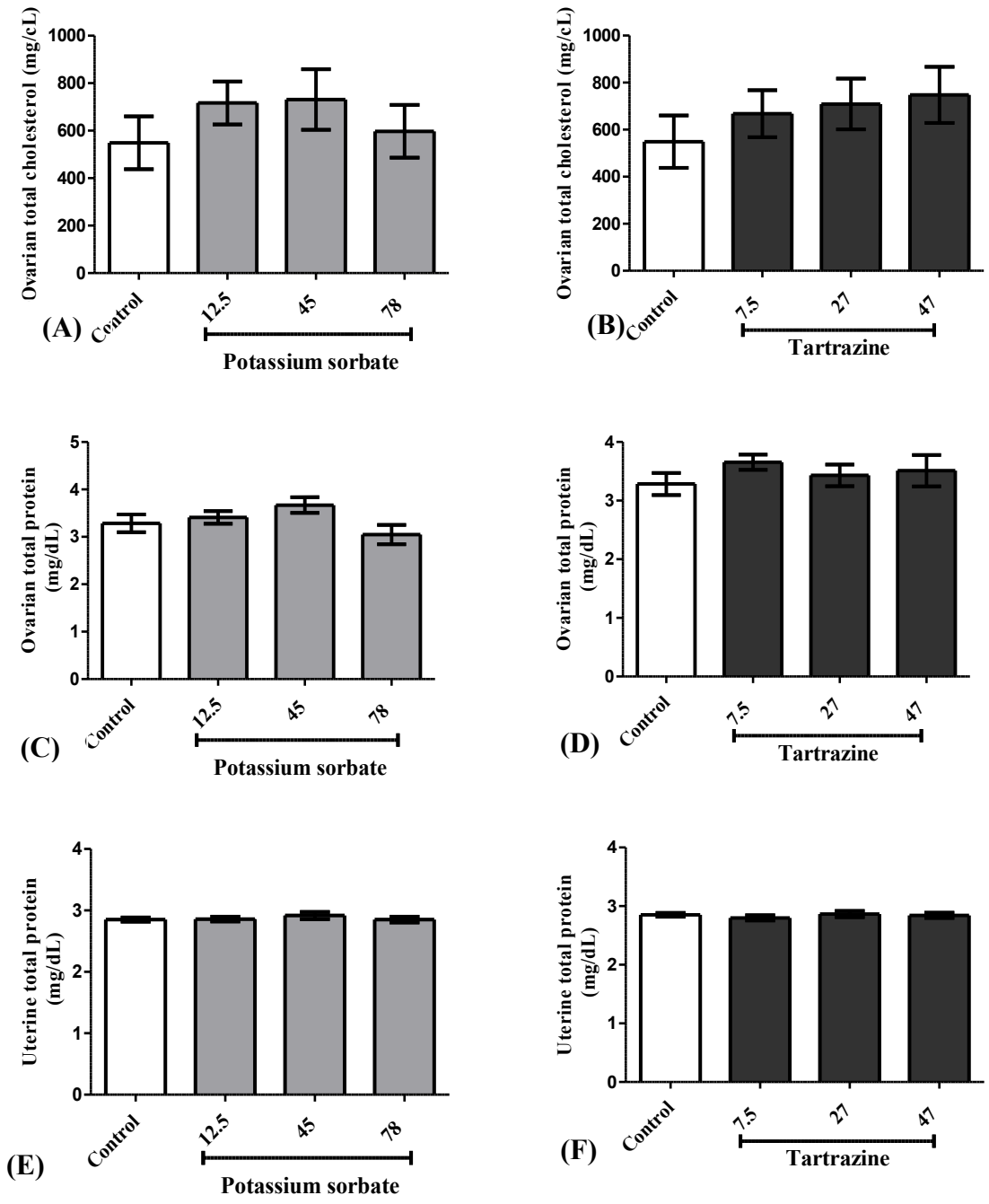


Figure 31: Effects of potassium sorbate and tartrazine on ovarian total cholesterol (A, B), ovarian total protein (C, D), and uterine total protein (E, F) of female Wistar F0.

Results are shown as a mean ± SEM. Control = animals receiving vehicle (distilled water, 10 mL/kg BW); PS = rats treated with potassium sorbate at doses of 12.5, 45, and 78 mg/kg BW; T = rats treated with tartrazine at doses of 7.5, 27, and 47 mg/kg BW.

III.1.1.1.8. Effects of potassium sorbate and tartrazine on folliculogenesis

The effects of potassium sorbate and tartrazine on follicular growth are summarized in Table IV and Figure 32. Table IV and Figure 32 show the number of total follicles and different types of follicles, and the microphotographs of ovaries after 40 days of treatment with potassium sorbate and tartrazine. The results showed a significant increase in the number of total follicles ($p < 0.001$), primary follicles ($p < 0.01$), secondary follicles ($p < 0.001$), and antral follicles ($p < 0.01$) with tartrazine at a higher dose (47 mg/kg BW) as compared to the control. As concerns potassium sorbate, the results showed a significant decrease of primary (PS 78, $p < 0.01$), and total follicles (PS 12.5, $p < 0.01$; PS 78, $p < 0.001$) and an increase of atresia follicles (PS 78, $p < 0.001$), as compared to the control group.

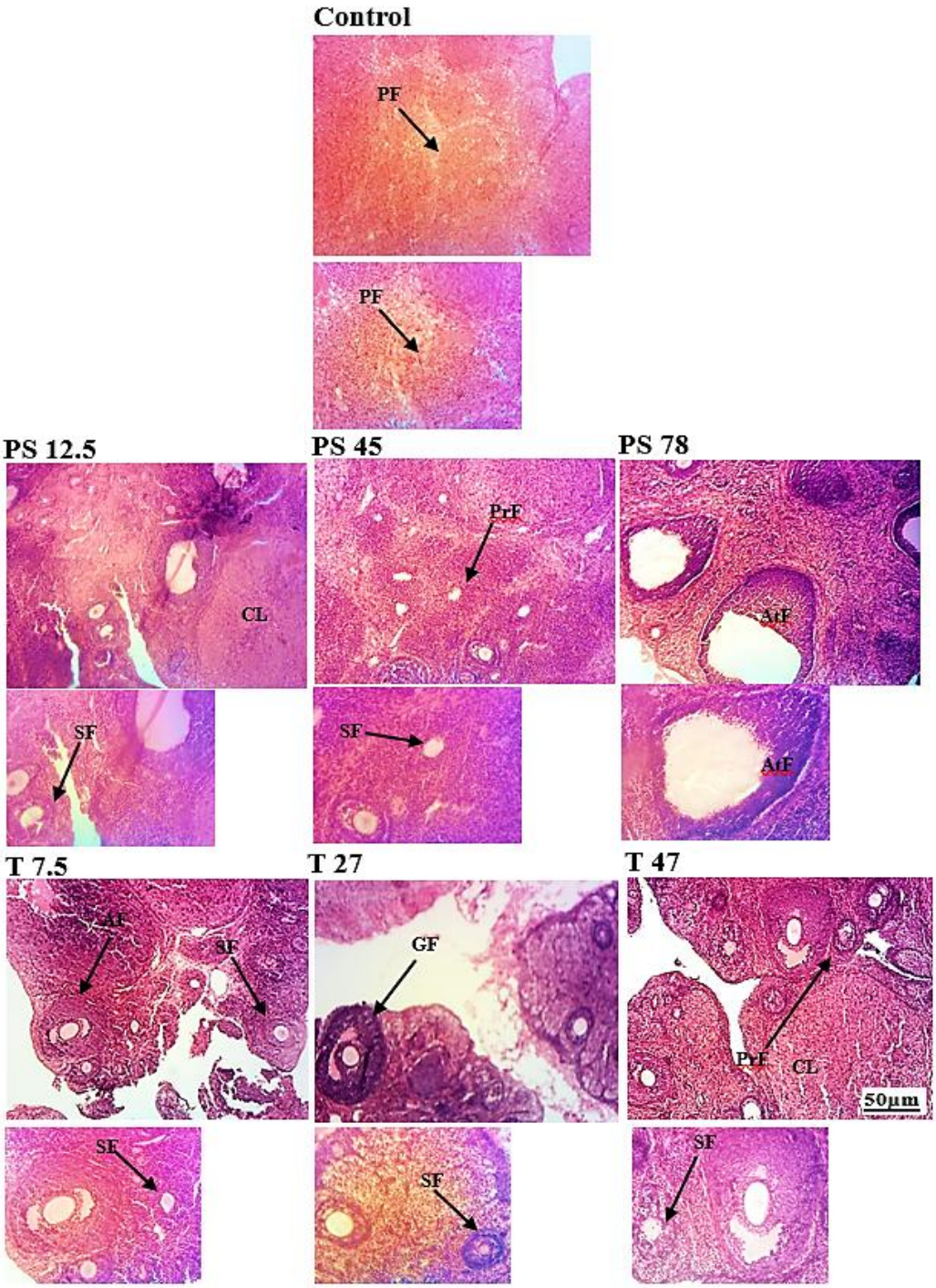


Figure 32: Microphotographs (200× and 25×) sections of ovaries of female Wistar rats after 40 days of treatment of F0.

Control= animals receiving vehicle (distilled water); PS = rats treated with potassium sorbate at doses of 12.5, 45, and 78 mg/kg BW; T = rats treated with tartrazine at doses of 7.5, 27, and 47 mg/kg BW. PF = primordial follicle, PrF = primary follicle, SF= secondary follicle, AF = Antral follicle, GF = Graafian follicle, CL = corpus luteum, AtF = Atresia follicle.

Table IV: Number of different ovarian follicles and corpus luteum of female Wistar rats after 40 days of treatment of F0 with potassium sorbate and tartrazine.

Organs	Control	Potassium sorbate (mg/kg BW)			Tartrazine (mg/kg BW)		
		12.5	45	78	7.5	27	47
Total follicles	100.60 ± 9.14	67.81 ± 9.59**	88.33 ± 3.73	58.16 ± 5.74***	105.39 ± 7.13	83.50 ± 10.61	145.33 ± 1.40***
Primordial follicles	35.33± 7.83	22.00 ± 4.72	42.66 ± 9.62	20.25 ± 2.28	28.00 ± 5.42	21.33 ± 3.59	38.00 ± 3.83
Primary follicles	22.00 ± 2.60	12.75 ± 2.43	15.25 ± 2.51	8.00 ± 2.02**	20.33 ± 2.93	18.66 ± 3.00	37.50 ± 2.53**
Secondary follicles	16.50 ± 1.20	11.50 ± 1.32	21.00 ± 1.58	8.00 ± 1.76	30.40 ± 5.76	28.25 ± 8.29	44.25 ± 2.37***
Antral follicles	4.20 ± 0.96	4.66 ± 0.79	5.50 ± 1.20	3.25 ± 0.91	6.25 ± 1.23	4.80 ± 1.59	10.33± 1.42**
Graffian follicles	5.00 ± 1.04	5.00 ± 1.04	5.50 ± 1.02	3.25 ± 0.91	6.80 ± 0.8	4.80 ± 1.01	6.50 ± 0.92
Atresia follicles	4.75 ± 0.19	6.50 ± 0.74	2.50 ± 0.50	9.00 ± 0.70***	3.80 ± 0.66	3.25 ± 0.58	3.75 ± 0.91
Corpus luteum	5.20 ± 1.20	5.40 ± 2.42	6.75± 1.31	4.00 ± 1.67	3.80 ± 1.11	2.40 ± 0.60	5.00 ± 0.83

Results are presented as mean ± SEM, n=5. (**: $p < 0.01$); (***: $p < 0.001$) in reference to control. Control = animals receiving vehicle (distilled water); PS = rats treated with potassium sorbate at doses of 12.5, 45, and 78 mg/kg BW; T = rats treated with tartrazine at doses of 7.5, 27, and 47 mg/kg BW.

III.1.1.1.9. Effects of potassium sorbate and tartrazine on gonadotropins levels

The results presented in Figures 33 (**A**, **B**, and **C**) indicate a non-significant difference in FSH serum concentration with potassium sorbate and tartrazine as well as a non-significant difference in LH serum concentration with potassium sorbate. As shown in Figure 33**D** the treatment with tartrazine at the dose of 47 mg/kg BW significantly increased the LH ($p < 0.001$) serum concentration as compared to the control group.

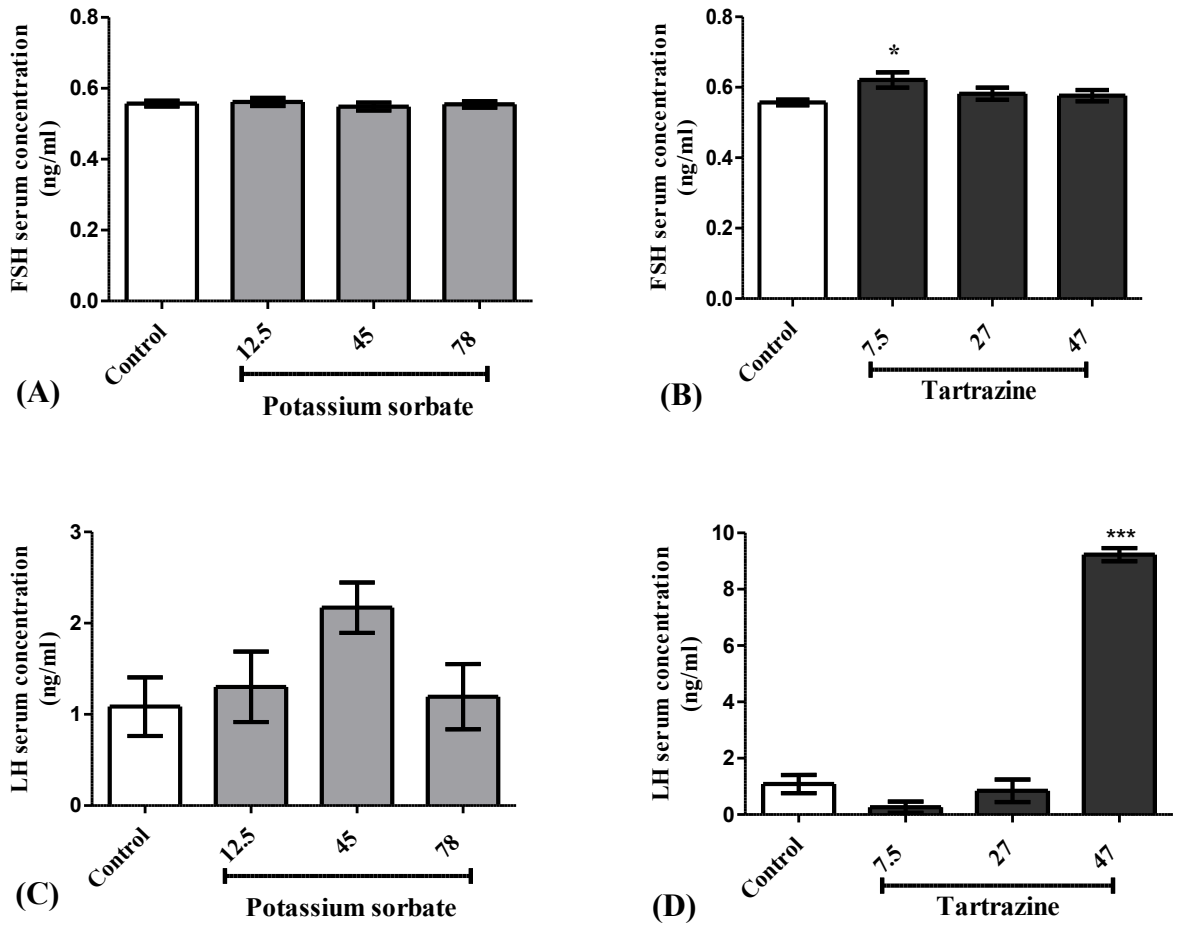


Figure 33: Serum concentration of Follicle Stimulating Hormone (FSH) (A, B) and Luteinizing Hormone (LH) (C, D) of female Wistar rats after 40 days of treatment of F0. Results are shown as a mean ± SEM; $n = 5$. ***: $p < 0.001$ in reference to control. Control = animals receiving vehicle (distilled water, 10 mL/kg BW); PS = rats treated with potassium sorbate at doses of 12.5, 45, and 78 mg/kg BW; T = rats treated with tartrazine at doses of 7.5, 27, and 47 mg/kg BW.

III.1.1.1.10. Effects of potassium sorbate and tartrazine on sexual steroids levels

Figure 34 represent the effects of potassium sorbate and tartrazine on sex steroids concentrations of F0. As compared to the control group, tartrazine at the dose of 47 mg/kg BW (Figure 34**B**) significantly increased ($p < 0.001$) estradiol serum concentration. Regarding potassium sorbate, the treatment was responsible for a decrease of estradiol serum concentration at all the tested doses (Figure 34**A**). No significant effect was observed on progesterone serum concentration (Figures 34**C,** 34**D**).

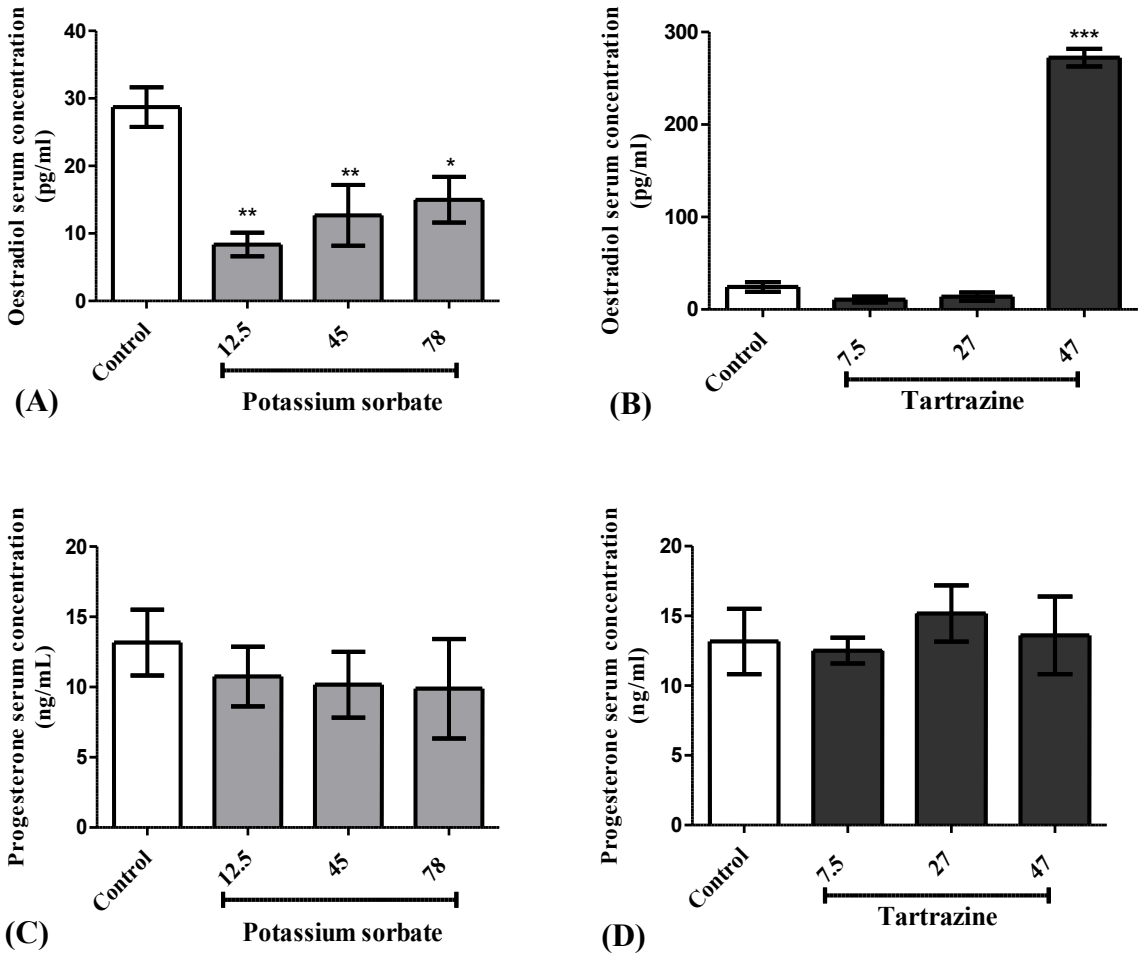


Figure 34: Serum concentration of estradiol (A, B) and progesterone (C, D) of female Wistar rats after 40 days of treatment of F0.

Results are shown as a mean ± SEM, $n = 5$. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ in reference to control. Control = animals receiving vehicle (distilled water, 10 mL/kg BW); PS = rats treated with potassium sorbate at doses of 12.5, 45, and 78 mg/kg BW; T = rats treated with tartrazine at doses of 7.5, 27, and 47 mg/kg BW.

III.1.1.2. Effects of potassium sorbate and tartrazine on sexual behavior and estrous cycle

III.1.1.2.1. Effects of potassium sorbate and tartrazine on estrous cycle

The effects of potassium sorbate and tartrazine on the reproductive cycle rats of F0 are summarized in Table V. Following the treatment, potassium sorbate at the dose of 45 mg/kg BW induced an increase of the number of metestrus ($p < 0.05$) and the number of irregular cycles ($p < 0.01$) at the dose of 78 mg/kg BW as compared to control group. As concerns tartrazine, the results showed a decrease of the number of proestrus ($p < 0.05$) at the dose of 7.5 mg/kg BW, number of dioestrus at the dose of 27 mg/kg BW ($p < 0.001$), and an increase of the number of metestrus ($p < 0.05$) at the dose of 27 mg/kg BW. Compared to the control group, the treatment induced significant effects on estrous cycle ratio (ECR), and irregular cycles. The results showed that tartrazine significantly decreased the percentage of ECR (7.5 mg/kg BW; ($p < 0.05$)), and 4-5 days cycles (7.5 and 47 mg/kg BW; ($p < 0.05$).

Table V: Effects of potassium sorbate and tartrazine on the estrous cycle of F0

Parameters / Groups		Number of cycles	Frequence of estrous cycle (%)	Numbers of each phase of estrous cycle (Days)				Estrous cycle ratio (%)	4-5 days cycles (Days)	Longer regular cycle	Irregular cycle
				Proestrus	Estrus	Metestrus	Diestrus				
Control		3.00 ± 0.31	0.15 ± 0.01	3.80 ± 0.20	3.20 ± 0.20	4.00 ± 1.30	9.00 ± 1.00	0.55 ± 0.08	0.60 ± 0.24	1.25 ± 0.37	1.00 ± 0.31
Potassium sorbate (mg/kg BW)	**12.5**	3.00 ± 0.54	0.15 ± 0.02	3.60 ± 0.74	3.20 ± 0.91	4.40 ± 0.74	7.60 ± 0.81	0.72 ± 0.14	0.40 ± 0.24	1.00 ± 0.44	1.60 ± 0.24
	45	2.40 ± 0.24	0.12 ± 0.01	2.60 ± 0.67	4.60 ± 1.12	7.80 ± 0.91*	5.00 ± 1.34	0.63 ± 0.16	0.20 ±.. 0.2	1.00 ± 0.31	1.20 ± 0.20
	78	2.60 ± 0.40	0.13 ± 0.02	3.40 ± 0.67	3.80 ± 0.37	5.40 ± 0.92	7.40 ± 1.16	0.59 ± 0.11	0.20 ± 0.2	0.60 ± 0.40	2.25 ± 0.19**
Tartrazine mg/kg BW)	**7.5**	2.40 ± 0.24	0.12 ± 0.01	2.20 ± 0.40*	2.80 ± 0.58	5.80 ± 1.39	9.00 ± 0.83	0.35 ± 0.07*	0.00 ± 0.0*	0.40 ± 0.24	2.00 ± 0.31
	27	2.80 ± 0.20	0.14 ± 0.01	3.60 ± 0.50	4.80 ± 0.73	8.40 ± 0.50*	3.30 ± 0.73***	0.73 ± 0.07	0.20 ± 0.2	1.00 ± 0.44	1.60 ± 0.40
	47	2.40 ± 0.67	0.12 ± 0.03	2.60 ± 0.24	5.00 ± 0.77	5.40 ± 0.81	7.00 ± 0.94	0.65 ± 0.14	0.00 ± 0.0*	0.20 ± 0.20	1.60 ± 0.24

Results are presented as mean ± SEM; n=7. *: $p < 0.05$; **: $p < 0.01$ in reference to control. Control = animals receiving vehicle (distilled water); PS = rats treated with potassium sorbate at doses of 12.5, 45, and 78 mg/kg BW; T = rats treated with tartrazine at doses of 7.5, 27, and 47 mg/kg BW.

III.1.1.2.2. Effects of potassium sorbate and tartrazine on the appetitive phasis

The effects of potassium sorbate and tartrazine on the appetitive phasis are presented in Figure 35. The results show that the administration of potassium sorbate did not induce any significant difference in the compartment change latency and frequency (Figures 35**A** and 35**C**), and the time spent in the non-operating compartment (Figures 35**E**). Regarding tartrazine, the results showed a significantly increased in the compartment change frequency at the doses of 27 ($p < 0.01$) and 47 ($p < 0.001$) mg/kg BW as compared to the control group (Figure 35**D**). The results obtained also showed an increase of the compartment change latency in PERF-1 group ($p < 0.01$) and compartment change frequency in PERF-1 and PERF-2 groups ($p < 0.05$) (Figures 35**B** and 35**C**).

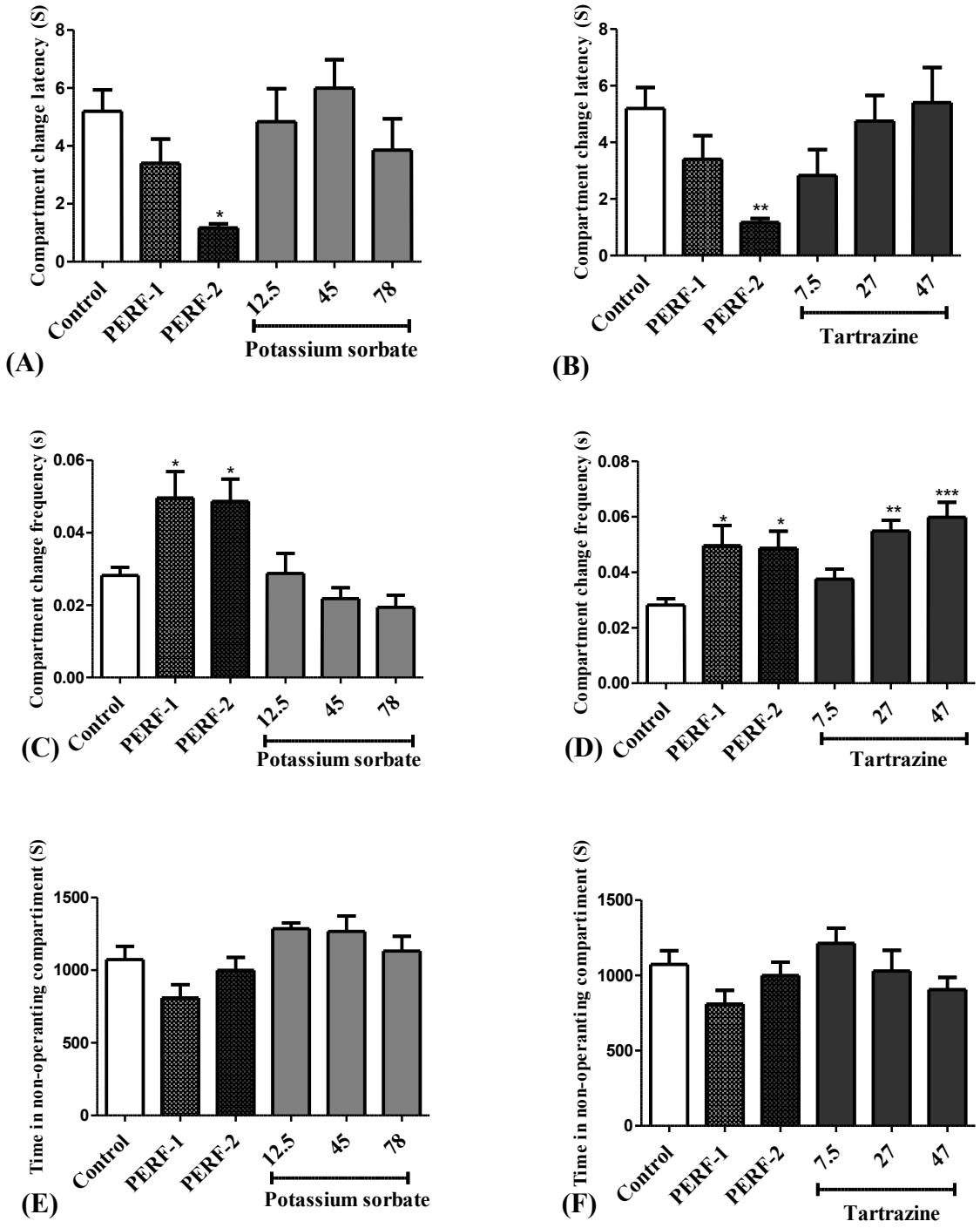


Figure 35: Effects of potassium sorbate and tartrazine on the compartment change latency (A, B), the compartment change frequency (C, D) and the time spent in the non-operant compartment (E, F) of F0.

Results are shown as a mean ± SEM; $n = 7$. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ in reference to control group. Control = animals receiving vehicle (distilled water, 10 mL/kg BW); PERF-1= animals receiving the maca-ginseng-gingembre 2 hours before the experiment; PERF-2 = animals receiving the maca-ginseng-gingembre during 40 days; PS = rats treated with potassium sorbate at doses of 12.5, 45, and 78 mg / kg BW, T = rats treated with tartrazine at doses of 7.5, 27, and 47 mg/kg.

III.1.1.2.3. Effects of potassium sorbate and tartrazine on proceptive phasis

a. Effects of potassium sorbate and tartrazine on the number of rejections

The effects of potassium sorbate and tartrazine on the number of rejections are summarized in Figure 36. The results showed that the number of rejections was significantly increased with potassium sorbate at the dose of 12.5 mg/kg BW ($p < 0.001$) and decreased with PERF-1 and PERF-2 ($p < 0.01$) as compared to the control group (Figure 36**B**). As shown in Figure 36**B**, tartrazine did not induce any significative effect in the number of rejections.

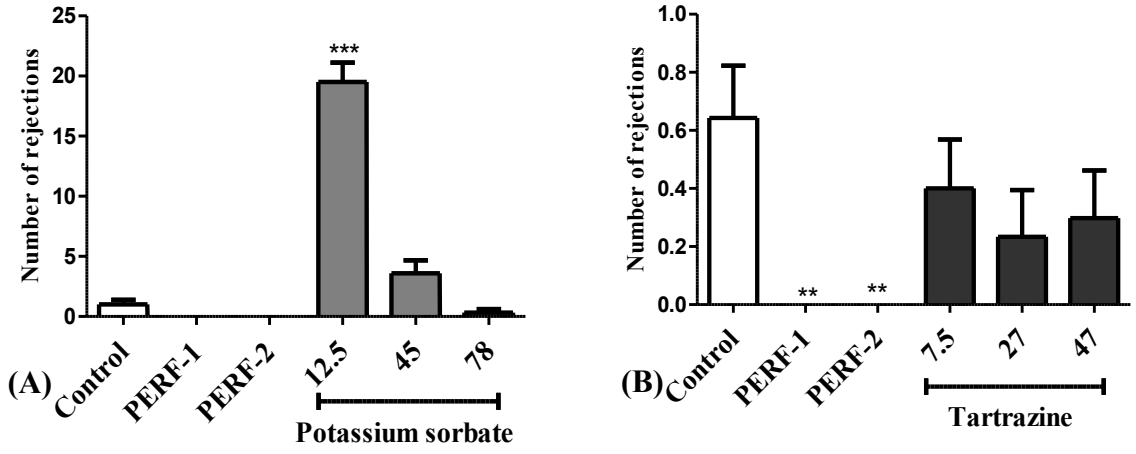


Figure 36: Effects of potassium sorbate and tartrazine on the number of rejections of F0. Results are shown as a mean ± SEM, $n = 7$. ***: $p < 0.001$ in reference to control. Control = animals receiving vehicle (distilled water, 10 mL/kg BW); PERF-1= animals receiving the maca-ginseng-gingembre 2 hours before the experiment; PERF-2 = animals receiving the maca-ginseng-gingembre during 40 days; PS = rats treated with potassium sorbate at doses of 12.5, 45, and 78 mg / kg BW, T = rats treated with tartrazine at doses of 7.5, 27, and 47 mg/kg.

b. Effects of potassium sorbate and tartrazine on ‘‘Darting’’ and ‘‘Hopping’’

The results presented in Figures 37**B** indicated that, 7.5 mg/kg BW tartrazine ($p < 0.01$) as well as PERF-1 group ($p < 0.05$) significantly decreased the darting latency as compared to the control group. As shown in Figure 37**D**, tartrazine significantly decreased the hopping latency at all the tested doses as compared to the control group (T7.5, $p < 0.01$; T27, $p < 0.01$; T47, $p < 0.05$). The same result was obtained with PERF-1 ($p < 0.001$) and PERF-2 groups ($p < 0.01$). Figure 37**C** shows that, the administration of potassium sorbate induced a significant increase of the hopping latency at the dose of 45 mg/kg BW ($p < 0.05$) and not adverse effects on the darting latency as compare to the control group (Figure 37**A**).

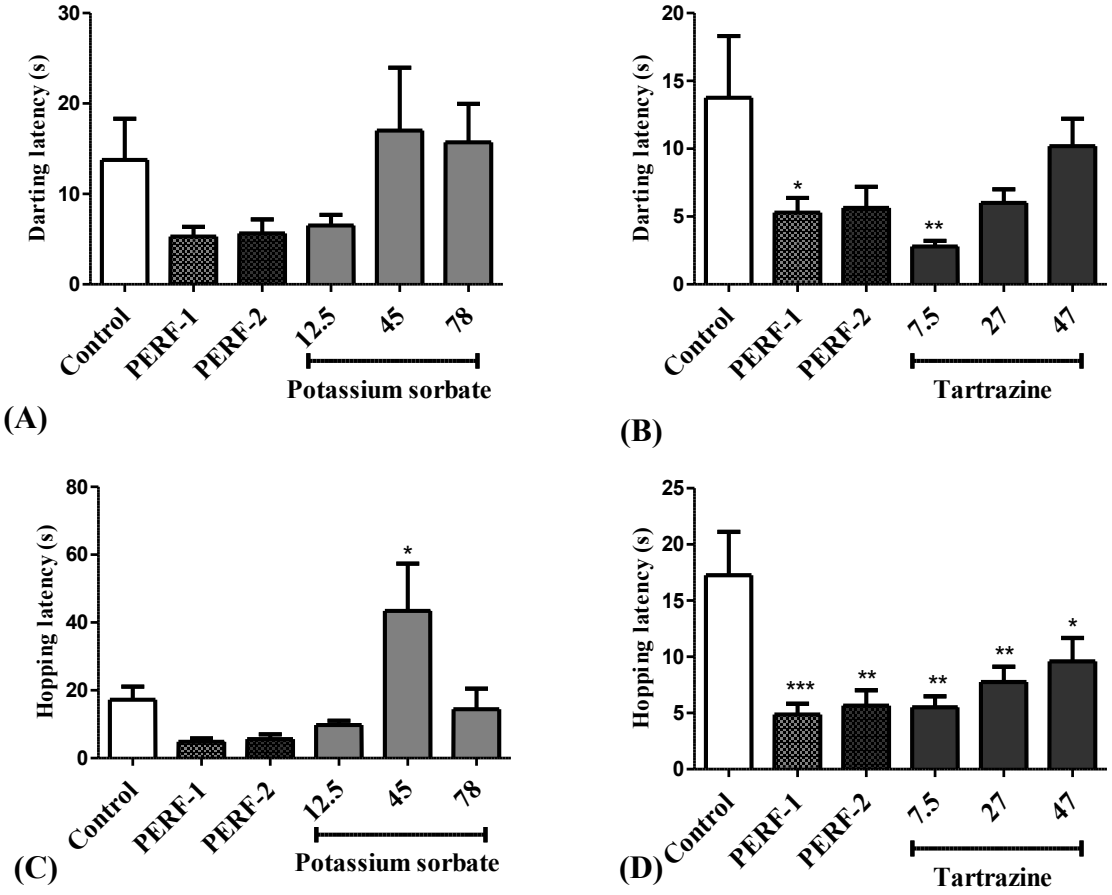


Figure 37: Effects of potassium sorbate and tartrazine on the darting (A, B) and hopping (C, D) of F0.

Results are shown as a mean ± SEM; $n = 7$. *: $p < 0.05$; **: $p < 0.01$ ***: $p < 0.001$ in reference to control. Control = animals receiving vehicle (distilled water, 10 mL/kg BW); PERF-1= animals receiving the maca-ginseng-gingembre 2 hours before the experiment; PERF-2 = animals receiving the maca-ginseng-gingembre during 40 days; PS = rats treated with potassium sorbate at doses of 12.5, 45, and 78 mg / kg BW, T = rats treated with tartrazine at doses of 7.5, 27, and 47 mg/kg.

III.1.1.2.4. Effects of potassium sorbate and tartrazine on receptive phasis

The effects of potassium sorbate and tartrazine on receptive parameters are presented in Figure 38. As shown in Figure 38**A**, 78 and mg/kg BW potassium sorbate significantly increased ($p < 0.001$) the lordosis latency and decreased the lordosis frequency at the doses of 12.5 and 45 ($p < 0.01$) mg/kg BW (Figure 38**C**). Tartrazine at the dose of 27 mg/kg BW significantly increased the lordosis latency ($p < 0.05$) (Figure 38**D**), and decreased the lordosis frequency ($p < 0.001$) (Figure 38**D**). As concerns the number of anogenital grooming, the treatment did not induce any significant effect (Figures 38**E** and 38**F**).

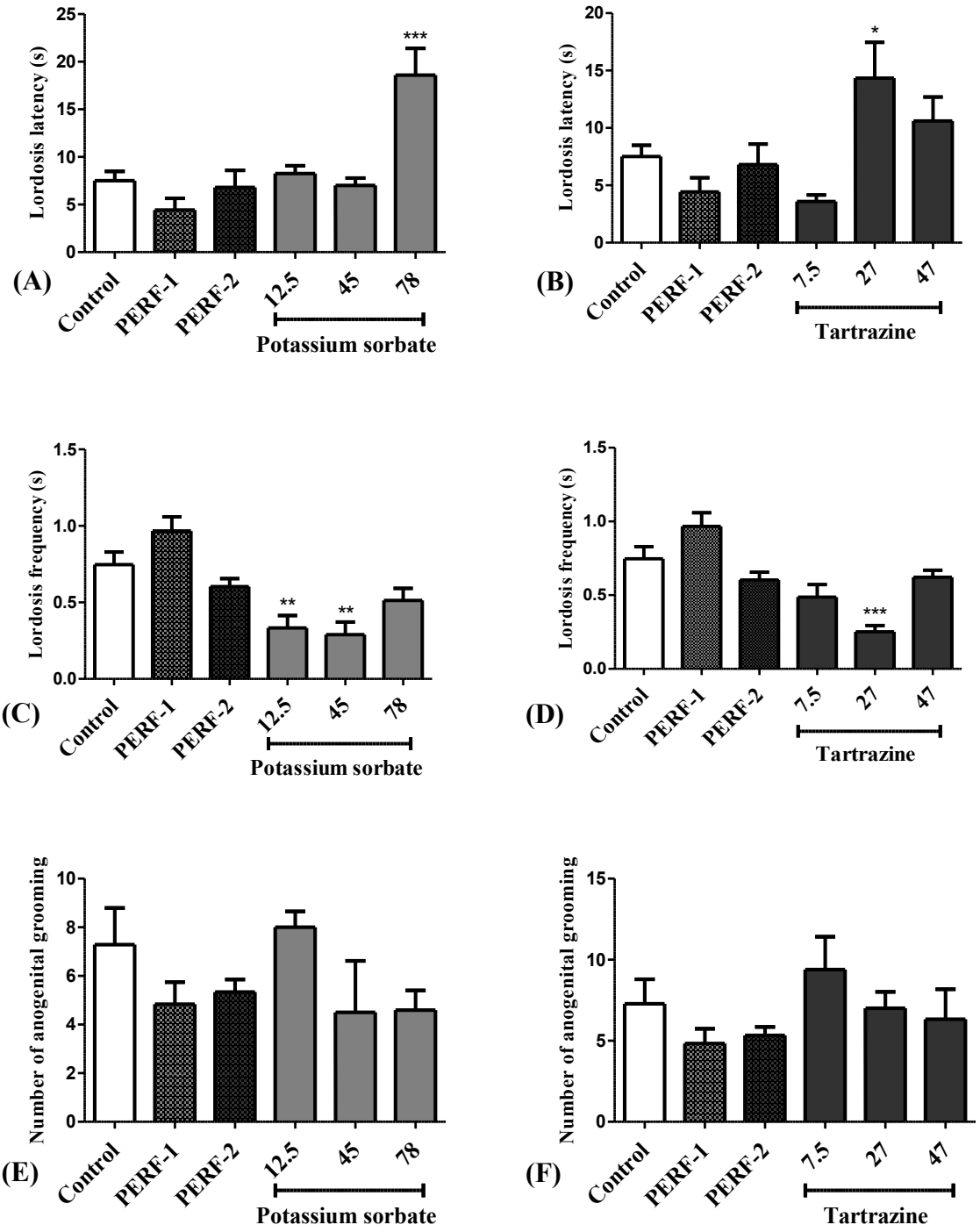


Figure 38: Effects of potassium sorbate and tartrazine on the lordosis latency (A, B) and frequency (C, D), and the number of anogenital grooming (E, F) of F0. Results are shown as a mean ± SEM; $n = 7$. *: $p < 0.05$; **: $p < 0.01$ ***: $p < 0.001$ in reference to control. Control = animals receiving vehicle (distilled water, 10 mL/kg BW); PERF-1= animals receiving the maca-ginseng-gingembre 2 hours before the experiment; PERF-2 = animals receiving the maca-ginseng-gingembre during 40 days; PS = rats treated with potassium sorbate at doses of 12.5, 45, and 78 mg / kg BW, T = rats treated with tartrazine at doses of 7.5, 27, and 47 mg/kg.

III.1.1.3. Effects of potassium sorbate and tartrazine on fertility and gestational parameters of F0

The table VI represents the effects of potassium sorbate and tartrazine on fertility and gestation after 40 days of treatment F0. The fertility index decreased with potassium sorbate at the doses of 45 (75%) and 78 (88.88%) mg/kg BW and with tartrazine at the doses of 27 (75%) and 47 (88.88%) mg/kg BW. Potassium sorbate at the doses of 45 (66.66%) and 78 (87.5%) mg/kg BW, and tartrazine at all the tested doses (T7.5, 88.88%; T, 83.33%; T7.5, 87.5%) decreased the gestational rate (Table VI).

Table VI: Effects of potassium sorbate and tartrazine on the fertility index and gestational rate of F0.

Experimental groups	Fertility index (%)	Gestational rate (%)
Control (Distilled water 10 mL/kg)	100	100
PS 12.5 (mg/kg BW)	100	100
PS 45 (mg/kg BW)	75	66.66
PS 78 (mg/kg BW)	88.88	87.5
Tartrazine 7.5 (mg/kg BW)	100	88.88
Tartrazine 27 (mg/kg BW)	75	83.33
Tartrazine 47 (mg/kg BW)	88.88	87.5

III.1.1.4. Effects of potassium sorbate and tartrazine on gestational parameters

After the treatment, a non-significant adverse effect was observed on the body weight of fetuses, the number of implantation and resorptions at the parturition. Potassium sorbate at all the tested doses significantly increased both the resorption index (from 7.77% to 23.64%) and post-implantation loss rate (from 13.64% to 35.13%) compared to the control group. As compared to the control group (91.62%), the implantation index decreased significantly in the group of rats treated with tartrazine at the dose of 27 mg/kg BW (54.02) and the resorption index increased with tartrazine at all the tested doses (from 7.77% to 29.41%). The treatment with tartrazine at the dose of 47 mg/kg BW reduced (0%) the pre-implantation loss rate and increased the post-implantation loss rate at the doses of 7.5 and 47 mg/kg BW (from 13.64% to 60.94%). Compared to the control group, potassium sorbate and tartrazine reduced significantly the sex ratio at all the tested doses ($p < 0.001$) (Table VII).

Table VII: Effects of potassium sorbate and tartrazine on gestational parameters of F0

Parameters	Control 10 mL/kg BW	Potassium Sorbate (mg/kg BW)			Tartrazine (mg/kg BW)		
		12.5	45	78	7.5	27	47
Number of live fetuses	6.33 ± 0.44	4.60 ± 0.85	2.75 ± 0.80*	4.80 ± 0.85	5.20 ± 0.87	4.00 ± 0.70	3.33 ± 1.03
Body weight of fetuses (g)	4.99 ± 0.44	5.26 ± 0.16	5.30 ± 0.32	5.26 ± 0.17	5.08 ± 0.33	4.94 ± 0.23	4.89 ± 0.09
Number of implantation site	7.33 ± 0.53	7.08 ± 0.72	5.16 ± 1.37	7.40 ± 0.88	7.07 ± 0.43	4.50 ± 1.62	8.50 ± 0.33
Number of resorption site	0.57 ± 0.34	1.00 ± 0.28	0.80 ± 0.37	1.75 ± 0.51	2.00 ± 0.84	1.20 ± 0.31	2.50 ± 1.03
Number of corpus luteum	8.00 ± 0.73	7.40 ± 0.85	5.33 ± 1.42	7.75 ± 0.85	7.20 ± 0.51	8.33 ± 0.11	8.50 ± 0.33
Index of implantation (%)	91.62	95.67	96.81	95.48	98.19	54.02	100
Index of resorption (%)	7.77	14.12	15.50	23.64	28.28	26.66	29.41
Pre-implantation loss rate (%)	8.37	4.32	3.18	4.72	1.80	45.97	0
Post-implantation loss rate (%)	13.64	36.44	46.70	35.13	26.44	11.11	60.94
Sex ratio	2.27 ± 0.16***	0.69 ± 0.16***	1.22 ± 0.12***	1.27 ± 0.11***	0.78 ± 0.14***	1.03 ± 0.22***	0.49 ± 0.05***

Results are presented as mean ± SEM, $n = 7$. ***: $p < 0.001$ in reference to control. Control = animals receiving vehicle (distilled water); PS = rats treated with potassium sorbate at doses of 12.5, 45, and 78 mg/kg BW; T = rats treated with tartrazine at doses of 7.5, 27, and 47 mg/kg BW.

III.1.2. Effects of potassium sorbate and tartrazine on the activation of hypothalamic-hypophysis-ovarian axis, sexual behavior and fertility of the first (F1), second (F2) and third (f3) generations

III.1.2.1. Effects of potassium sorbate and tartrazine on the activation of hypothalamic-hypophysis-ovarian axis of F1, F2, F3

III.1.2.1.1. Bodyweight of animal

Figure 39 shows the effect of potassium sorbate and tartrazine exposure on body weight evolution throughout the period of treatment of the F1, F2, and F3. Despite the non-significant effect observed on the body weight gain in F0, the results indicated that the administration of potassium sorbate or tartrazine to F1, F2, and F3, affected this parameter. As compared to the control groups F1, we have observed an increase (PS 12.5, F1, $p < 0.001$) and decrease (PS 78, F1, $p < 0.01$; T 7.5, F1, $p < 0.01$; T 47, F1, $p < 0.001$) of the body weight gain during the period of treatment (Figures 39**A** and 39**B**). The results also demonstrated that the body weight of animals of F2 was lower (PS 45, F2, $p < 0.01$; P; T 7.5, F2, $p < 0.01$) than the control groups F2 (Figures 39**C** and 39**D**). In the third generation, 40 days oral administration of potassium sorbate or tartrazine induced an increase (PS 45, F3, $p < 0.001$) and decrease (PS 12.5, F3, $p < 0.05$; PS 78, F3, $p < 0.001$; T 7.5, F3, $p < 0.001$; T 27, F3, $p < 0.001$; T 47, F3, $p < 0.001$) of the body weight gain during the period of treatment as compared to the control groups F3 (Figures 39**E** and 39**F**).

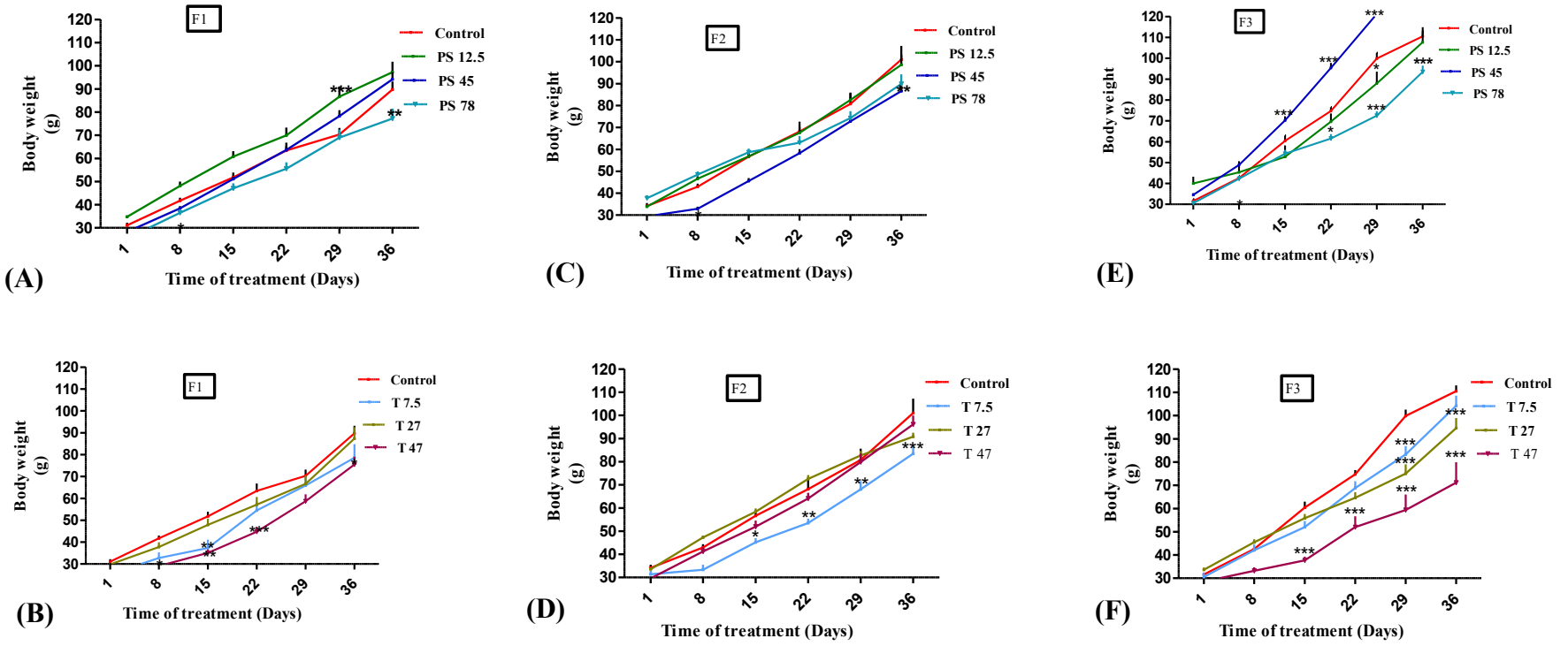


Figure 39: Effects of potassium sorbate and tartrazine on the bodyweight of the first (A, B), second (C, D) and third (E, F) generations of female Wistar rats during the treatment.

Results are shown as a mean ± SEM, $n = 5$. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ in reference to control. Control = animals receiving vehicle (distilled water, 10 mL/kg BW); SP= rats treated with potassium sorbate at doses of 12.5, 45 and 78 mg/kg BW; T = rats treated with tartrazine at doses of 7.5, 27, and 47 mg/kg BW.

III.1.2.1.2. Vaginal opening

Figure 40 represents the effects of potassium sorbate and tartrazine on the mean age of vaginal opening of the F1, F2, and F3 generations. The results observed in F0 demonstrated that the administration of potassium sorbate and tartrazine did not affect the mean age of vaginal opening. Despite this result, Figure 40**A** indicated that, 12.5 mg/kg BW potassium sorbate significantly increased the mean age of vaginal opening at the F1 ($p < 0.001$) and F2 ($p < 0.05$) as compared to control groups F1 and F2 respectively. Regarding tartrazine at F1, a significant decrease of the mean age of vaginal opening was observed at the dose of 47 mg/kg BW ($p < 0.01$) as compared to the control group F1 (Figure 40**B**).

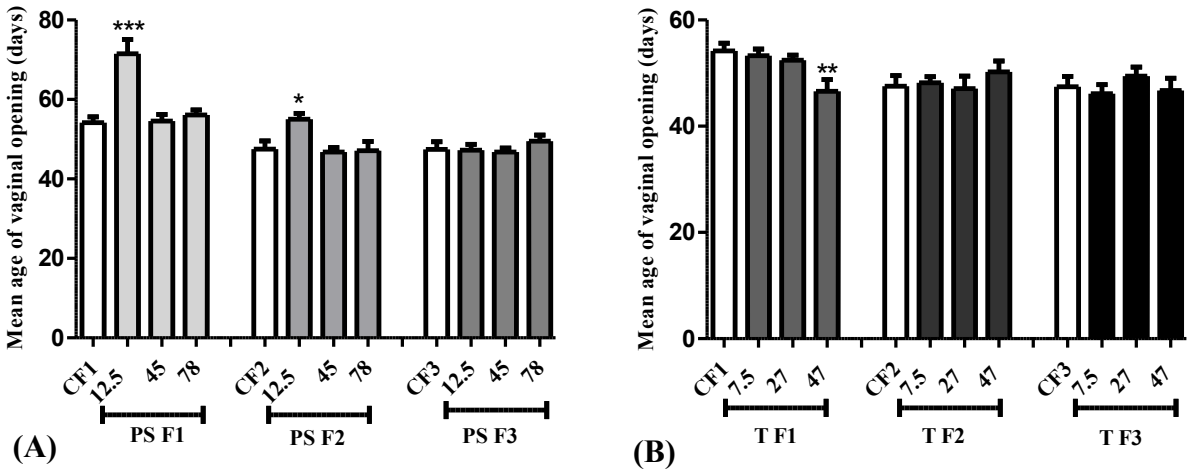
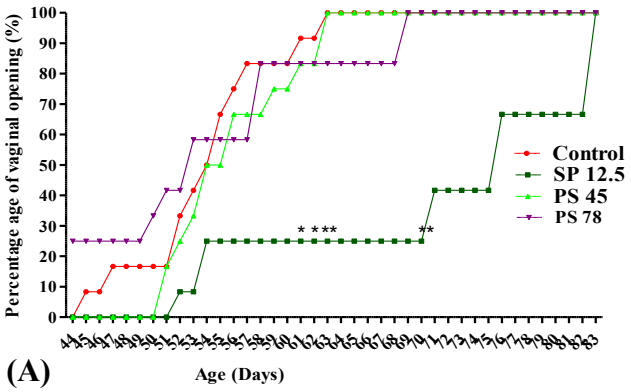


Figure 40: Effects of potassium sorbate (A) and tartrazine (B) on the mean age of the vaginal opening of rats from the first, second and third generations.

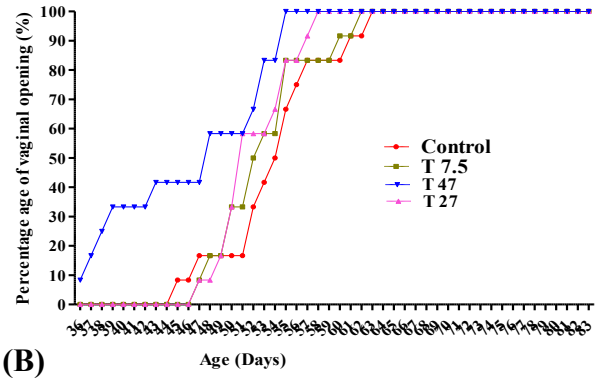
Results are shown as a mean ± SEM, n = 5. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ in reference to control. Control= animals receiving vehicle (distilled water); PS = rats treated with potassium sorbate at doses of 12.5, 45, and 78 mg / kg BW; T = rats treated with tartrazine at doses of 7.5, 27, and 47 mg/kg BW.

III.1.2.1.3. Percentage of rats with vaginal opening

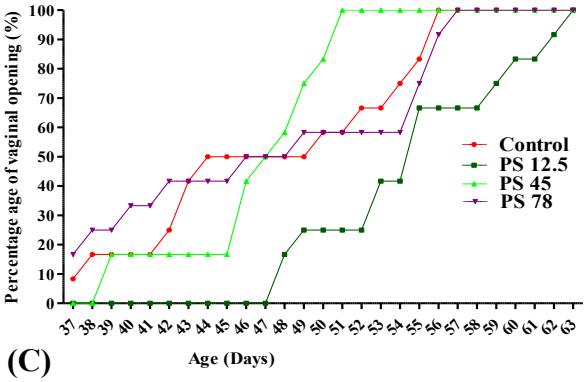
The effects of potassium sorbate and tartrazine on the percentage of animals with vaginal opening is represented in Figure 41. As observed in the 40 days treatment of F0, potassium sorbate affected the percentage of animals with vaginal opening. The results showed that, 12.5 mg/kg BW potassium sorbate significantly decreased the percentage of animals with vaginal opening as compared to control group F1. From day 61 to 70, this group exhibited 25% of vaginal opening vs. 91.67 to 100% for control ($p < 0.05$) (Figure 41**A**). As shown below, not significant effect was observed on the percentage of animals with vaginal opening with potassium sorbate at the second (Figure 41**C**), and third (Figure 41**E**) generations, and with tartrazine at the first (Figure 41**B**), second (Figure 41**D**) and third (Figure 41**F**) generations.



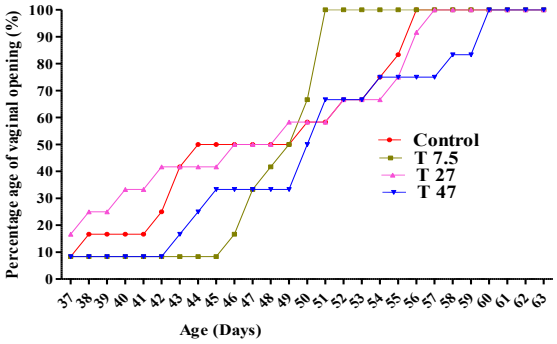
(A)



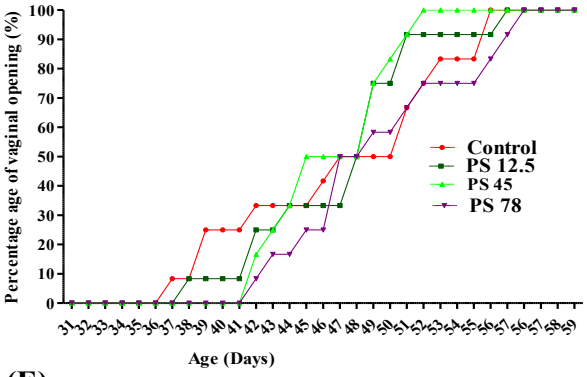
(B)



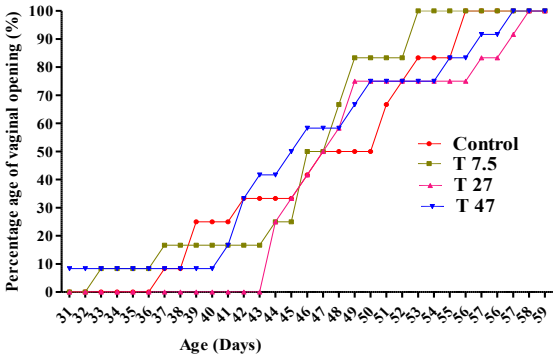
(C)



(D)



(E)



(F)

Figure 41: First (A, B), second (C, D) and third (E, F) generations percentage (%) of rats with vaginal opening during 40 days of treatment.

Results are shown as a mean ± SEM, n = 5. **: $p < 0.01$; **: $p < 0.01$; ***: $p < 0.001$ in reference to control. Control= animals receiving vehicle (distilled water); PS = rats treated with potassium sorbate at doses of 12.5, 45, and 78 mg / kg BW; T = rats treated with tartrazine at doses of 7.5, 27, and 47 mg/kg BW.

III.1.2.1.4. Effects of potassium sorbate on epithelial thickness of uterus of the F1, F2, and F3 generations

Figure 42 summarizes the effects of potassium sorbate on uterine epithelial thickness in the F1, F2, and F3 generations. Despite the non-significant effect observed in F0, the results indicated that the administration of potassium sorbate to F1, F2, and F3, affected the uterine epithelial thickness. As compared to the control group F1, 12.5 mg/kg BW potassium sorbate significantly increased ($p < 0.001$) the uterine epithelial thickness at F1. The same effect was observed at the F2 and F3. Potassium sorbate significantly increased the uterine epithelial thickness at F2 (PS 12.5 F2, $p < 0.01$; PS 45 F2, $p < 0.05$) and F3 (PS 12.5 F3, $p < 0.001$; PS 45 F3, $p < 0.001$) as compared to the control groups F2 and F3 respectively (Figure 42**A**). This difference is also confirmed by the microphotographs presented in Figure 42**B**.

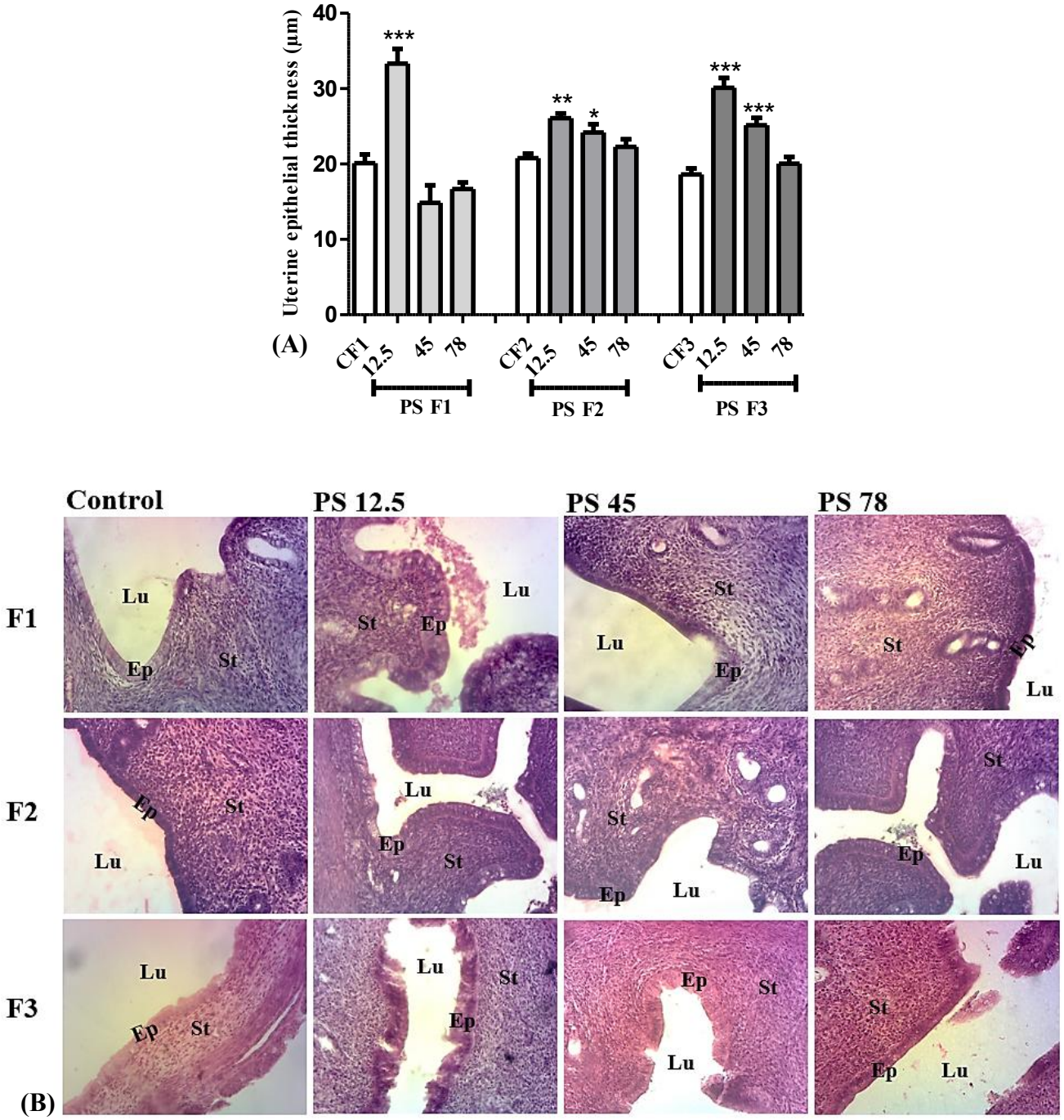


Figure 42: Uterine epithelial thickness (A) as well as microphotographs (40×) of hematoxylin/eosin-stained sections of uteri (B) of the first, second and third generation of female Wistar rats after 40 days of treatment with potassium sorbate.

Results are presented as mean ± SEM, $n = 5$. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ compared to the control. Lu = Lumen; Ep = Epithelial cell thickness; St = Stroma. Control = animals receiving vehicle (distilled water, 10 mL/kg BW); PS = rats treated with potassium sorbate at dose of 12.5, 45, and 78 mg / kg BW.

III.1.2.1.5. Effects of tartrazine on epithelial thickness of uterus of the F1, F2, and F3 generations

The effects of tartrazine on the uterine epithelial thickness are shown in Figure 43. Despite contrary effects obtained in F0, the results showed that tartrazine affected the uterine epithelial thickness in F1 (27 mg/kg BW) and F3 (7.5 mg/kg BW). The administration of tartrazine (7.5, 27 and 47 mg/kg BW) to the F1, and F3 generations, from the PND 21 decreased significantly the uterine epithelial thickness at the doses of 27 mg/kg BW (F1, $p < 0.05$) and 7.5 mg/kg BW (F3, $p < 0.05$) as compared to the control groups F1 and F3 respectively. However, the results obtained with tartrazine at the doses of 27 and 47 mg/kg BW at F3 were similar to the one obtained with the parents. Tartrazine significantly increased the uterine epithelial thickness at the doses of 27 ($p < 0.01$) and 47($p < 0.01$) mg/kg BW as compared to the control group F3 (Figure 43**A**). This difference is also confirmed by the microphotographs presented in Figure 43**B**.

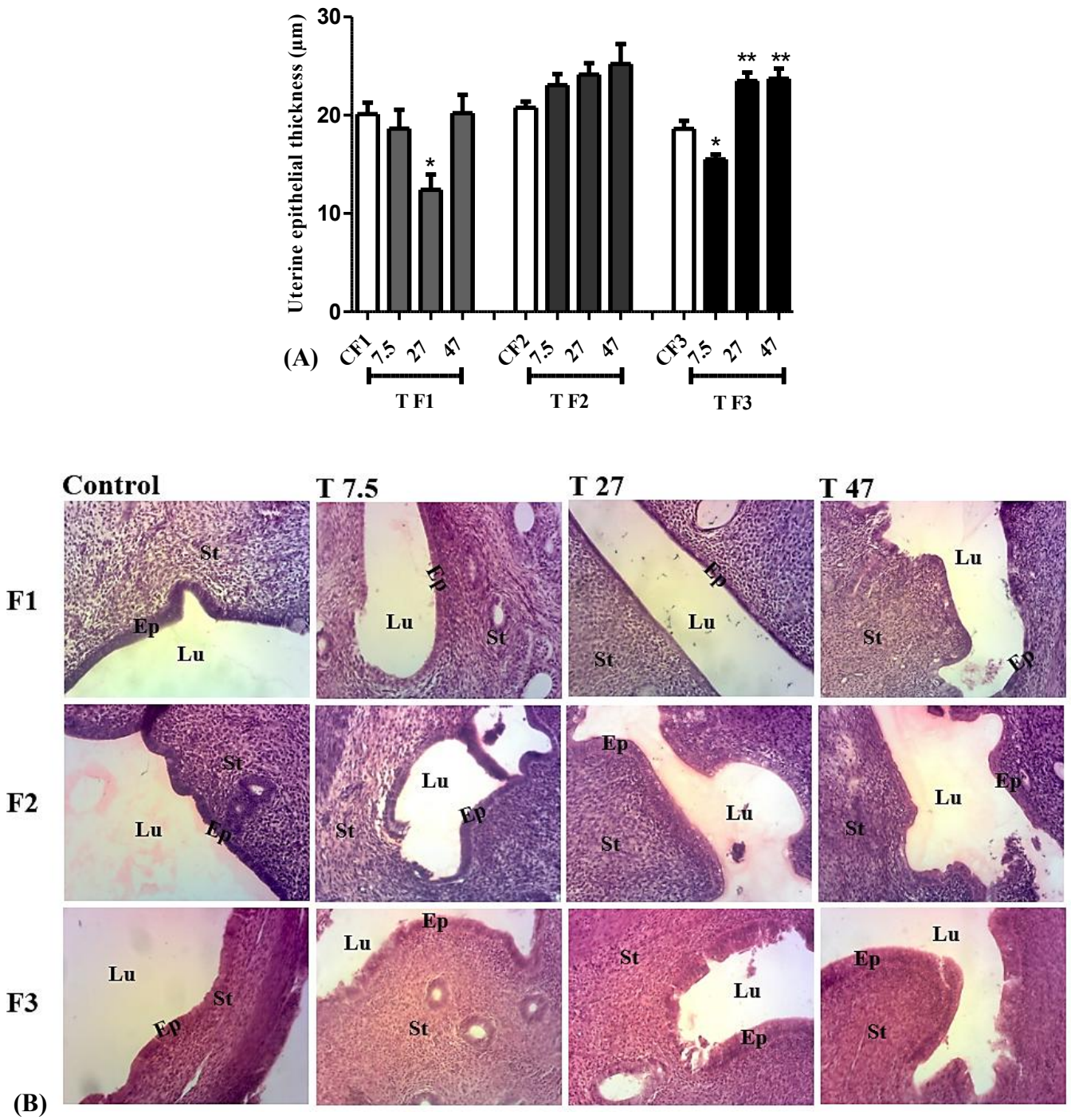


Figure 43: Uterine epithelial thickness (A) as well as microphotographs (40×) of hematoxylin/eosin-stained sections of uteri (B) of the first, second and third generation of female Wistar rats after 40 days of treatment with tartrazine.

Results are presented as mean ± SEM, $n = 5$. *: $p < 0.05$; **: $p < 0.01$ in reference to control. Lu = Lumen; Ep = Epithelial cell thickness; St = Stroma. Control = animals receiving vehicle (distilled water, 10 mL/kg BW); T = rats treated with tartrazine at doses of 7.5, 27, and 47 mg/kg BW.

III.1.2.1.6. Effects of potassium sorbate on epithelial thickness of vagina of F1, F2, and F3 generations

Despite the non-significant effects obtained in F0, the results showed that potassium sorbate affected the vagina epithelial thickness in F1, F2, and F3. As compared to control group F1, 78 mg/kg BW potassium sorbate decreased significantly (F1, $p < 0.01$) the vagina epithelial thickness. Regarding F2, the results demonstrated that the administration of potassium sorbate (12.5, 45 and 78 mg/kg BW) to the F1, F2, and F3, from the PND 21 was responsible for the increase (PS 12.5 F2, $p < 0.001$) and decrease (PS 45 F2, $p < 0.01$; PS 78 F2, $p < 0.001$) of the vagina epithelial thickness as compared to the control group F2. Only potassium sorbate at the dose of 78 mg/kg BW in F3 decreased significantly ($p < 0.01$) the vagina epithelial thickness as compared to the control group F3 (Figure 44**A**). These differences are also confirmed by the microphotographs presented in Figure 44**B**.

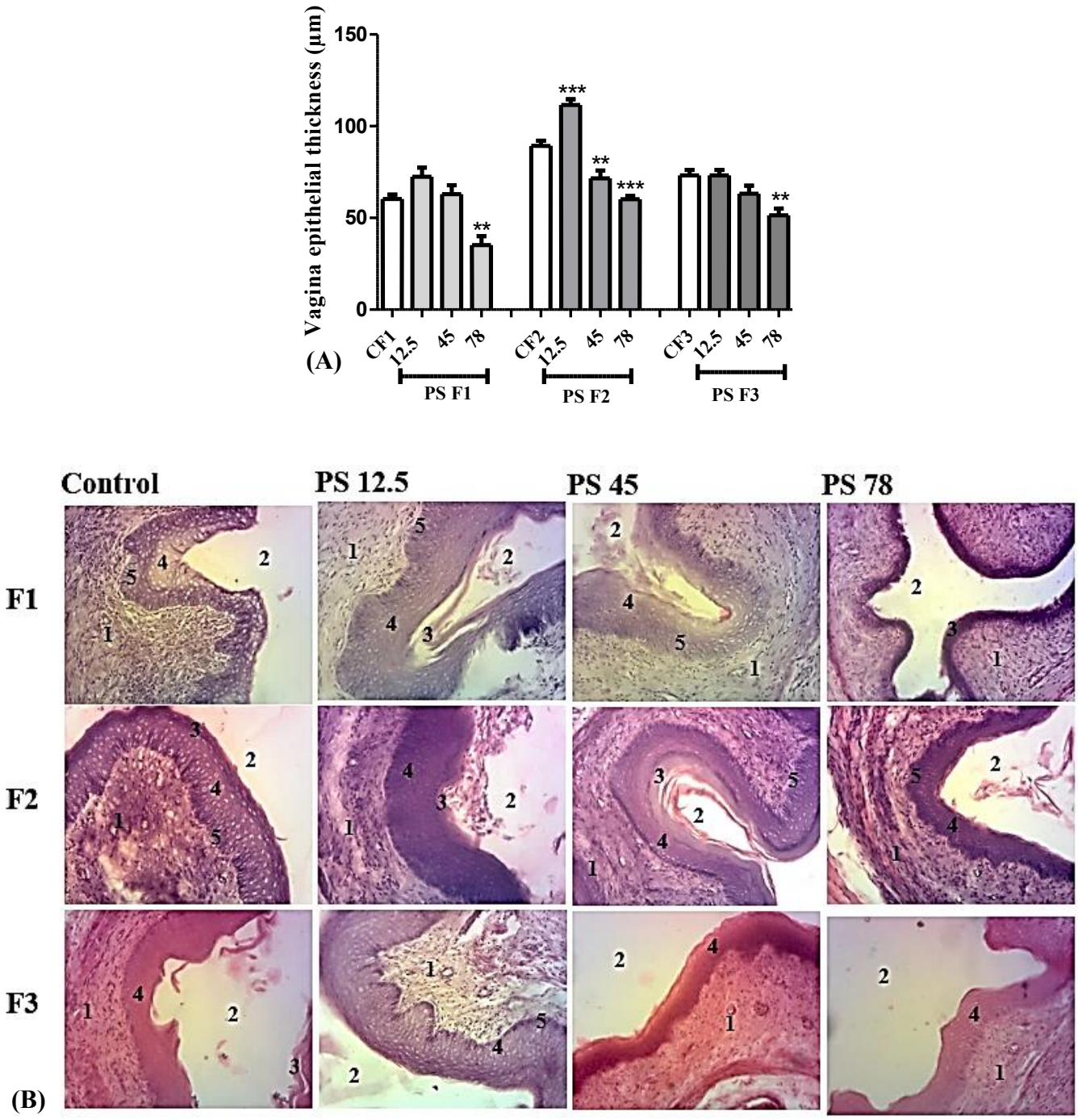
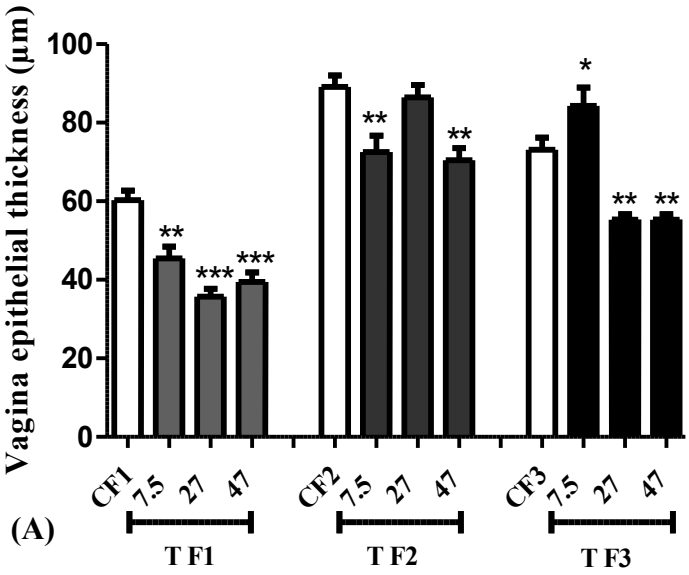


Figure 44: Vagina epithelial thickness (A) as well as microphotographs (40×) of hematoxylin/eosin-stained sections of vagina (B) of the first, second and third generation of female Wistar rats after 40 days of treatment with potassium sorbate.

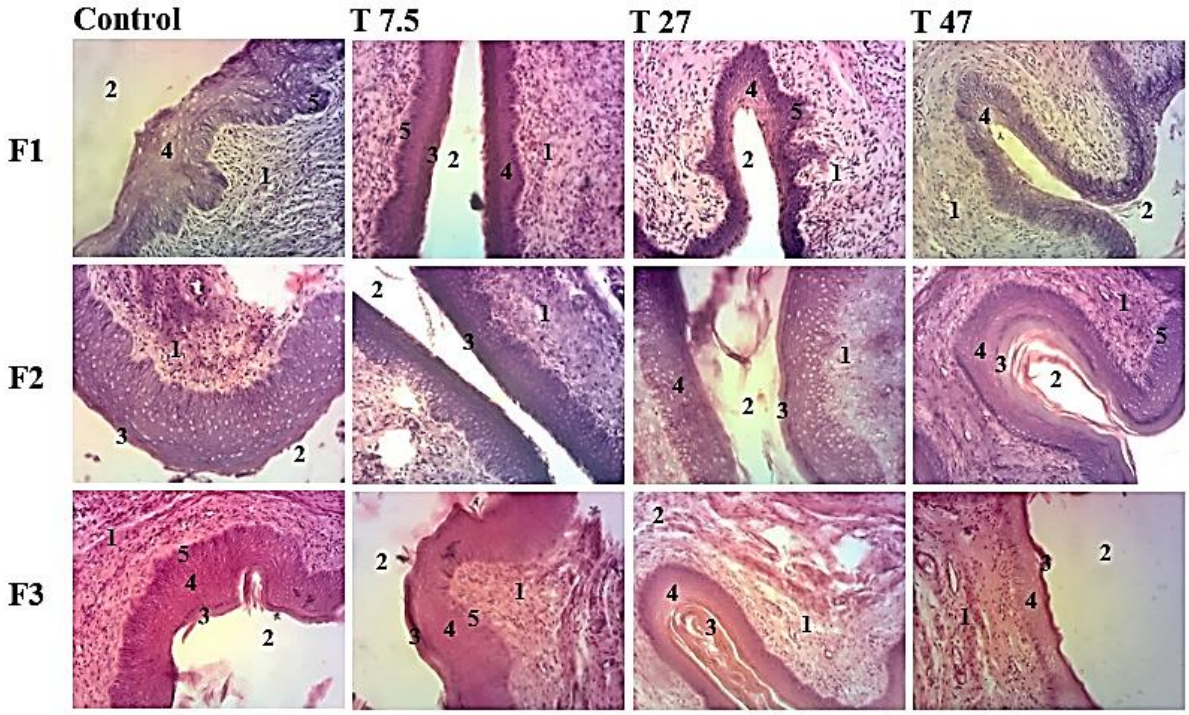
Results are presented as mean ± SEM, $n = 5$. **: $p < 0.01$; ***: $p < 0.001$ compared to the control. Lu = Lumen; Ep = Epithelial cell thickness; St = Stroma. Control = animals receiving vehicle (distilled water, 10 mL/kg BW).

III.1.2.1.7. Effects of tartrazine on epithelial thickness of vagina of F1, F2, and F3 generations

Figure 45 represents the effects of tartrazine on vagina epithelial thickness of the F1, F2, and F3 generations. The results obtained in F0 showed that tartrazine did not induce any significant effect on vagina epithelial thickness. Despite these results, the figure below demonstrates that tartrazine affected the vagina epithelial thickness at F1, F2, and F3. Tartrazine was responsible for the decrease of vagina epithelial thickness at F1 (T 7.5, $p < 0.01$; T 27, and T 47, $p < 0.001$), F2 (T 7.5 and T 47, $p < 0.01$), and F3 (T 7.5, $p < 0.05$; T 27, and 47, $p < 0.01$) as compared to control groups F1, F2 and F3 respectively (Figure 45**A**). As confirmed by the microphotographs presented in Figure 45**B**, vagina epithelial thickness increased significantly with tartrazine at the doses 7.5 mg/kg BW (F3 $p < 0.05$).



(A)



(B)

Figure 45: Vagina epithelial thickness (A) as well as microphotographs (40×) of hematoxylin/eosin-stained sections of vagina (B) of the first, second and third generation of female Wistar rats after 40 days of treatment with tartrazine.

Results are presented as mean ± SEM, $n = 5$. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ compared to the control. Lu = Lumen; Ep = Epithelial cell thickness; St = Stroma. Control = animals receiving vehicle (distilled water, 10 mL/kg BW); T = rats treated with tartrazine at doses of 7.5, 27, and 47 mg/kg BW.

III.1.2.1.8. Effects of potassium sorbate and tartrazine on the relative weight of reproductive organs

As observed in F0, the results showed that the treatment with potassium sorbate and tartrazine significantly affected the relative weight of reproductive organs in F1, F2 and F3. The results indicated that, the administration of potassium sorbate to female rats increased significantly the relative ovaries weight at the dose of PS 45 mg/kg BW (F3, $p < 0.05$) as compared to the control group F3 (Figures 46**A**). Regarding tartrazine, the effect was observed at all the generations. Tartrazine increased significantly the relative ovaries weight at F1 (T 7.5 F1, $p < 0.05$), F2 (T 7.5 F2, $p < 0.01$; and T 47 F2, $p < 0.01$), and F3 (T 7.5 and 27 F3, $p < 0.05$) as compared to the control groups F1, F2, and F3 respectively (Figures 46**B**). As shown in Figure 46**C**, potassium sorbate significantly decreased the relative weight of uterus at the doses of 12.5 (F1, $p < 0.001$), 45 (F1, $p < 0.01$), and 78 (F1, $p < 0.01$) mg/kg BW as compared to the control group F1. However, the relative weight of uterus significantly increased at F2 (PS 45, $p < 0.001$; PS 78, $p < 0.001$). As compared to the control group F2, tartrazine at the doses of 27 (F2, $p < 0.001$) and 47 (F2, $p < 0.001$) mg/kg BW significantly increased the relative weight of uterus (Figure 46**D**).

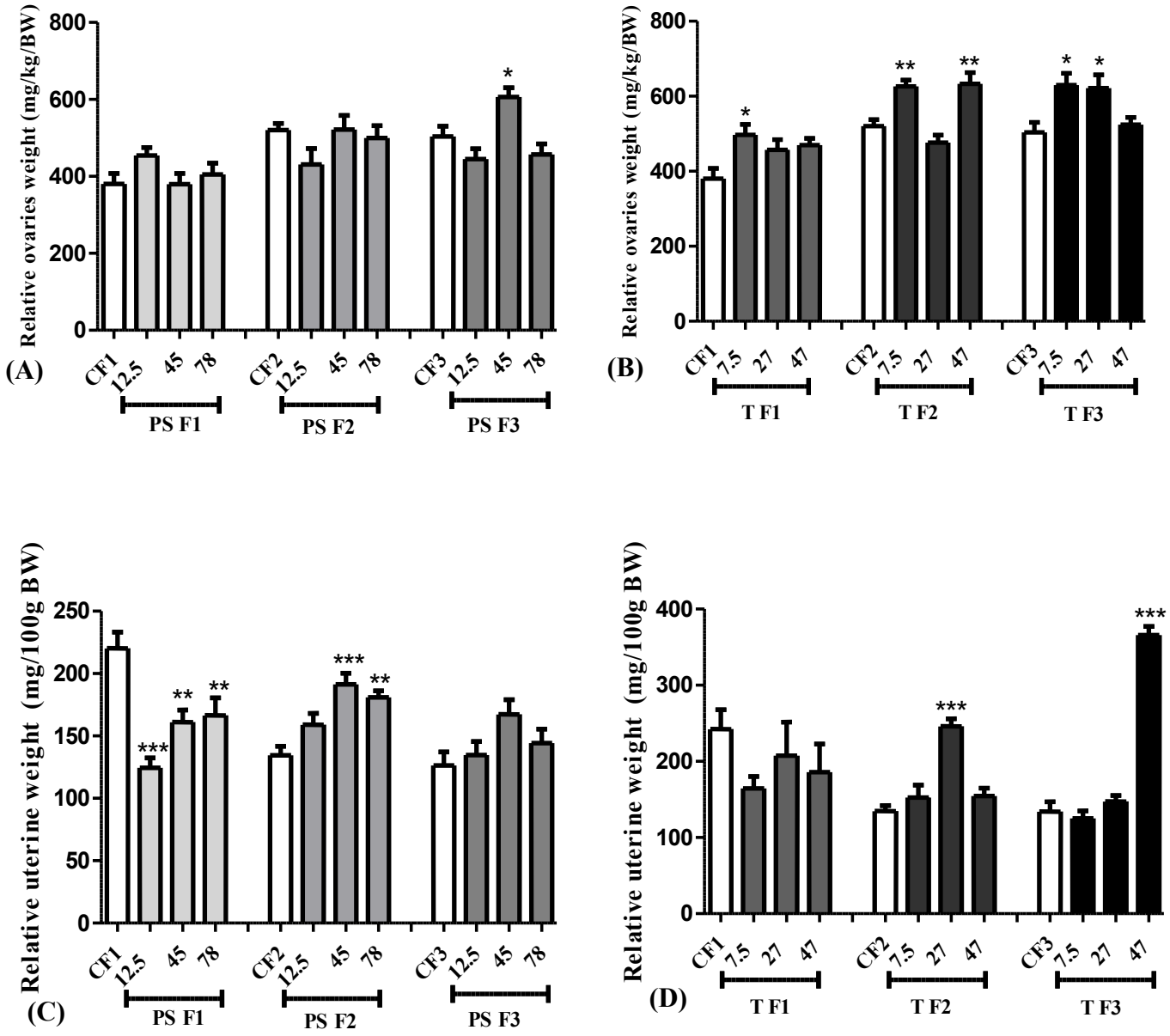


Figure 46: Relative ovaries (A, B) and uterine (C, D) weight of the first, second and third generation of female Wistar rats after 40 days of treatment with potassium sorbate and tartrazine.

Results are presented as mean ± SEM, $n = 5$. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ compared to the control. Control = animals receiving vehicle (distilled water, 10 mL/kg BW); PS = rats treated with potassium sorbate at dose of 12.5, 45, and 78 mg / kg BW; T = rats treated with tartrazine at doses of 7.5, 27, and 47 mg/kg BW.

III.1.2.1.9. Effects of potassium sorbate and tartrazine on the ovarian total cholesterol and ovarian and uterine total protein of the third generation

Despite the non-significant effects observed in F0, the results below indicate that potassium sorbate and tartrazine significantly affected the ovarian total cholesterol and ovarian total protein of the third generation (Figure 47). As compared to the control group F3, the ovarian total cholesterol decreased significantly with potassium sorbate at the dose of 12.5 mg/kg BW ($p < 0.001$) and with tartrazine at the dose of 27 mg/kg BW ($p < 0.001$) (Figure 47**A** and 47**B**). The two treatments also decreased significantly the ovarian total protein as compared to the control group F3 (PS 12.5 and 78, T 7.5 and 47, $p < 0.001$; PS 45, $p < 0.05$) (Figures 47**C** and 47**D**). As shown in Figures 47**E** and 47**F**, the treatment did not affect the uterine total protein.

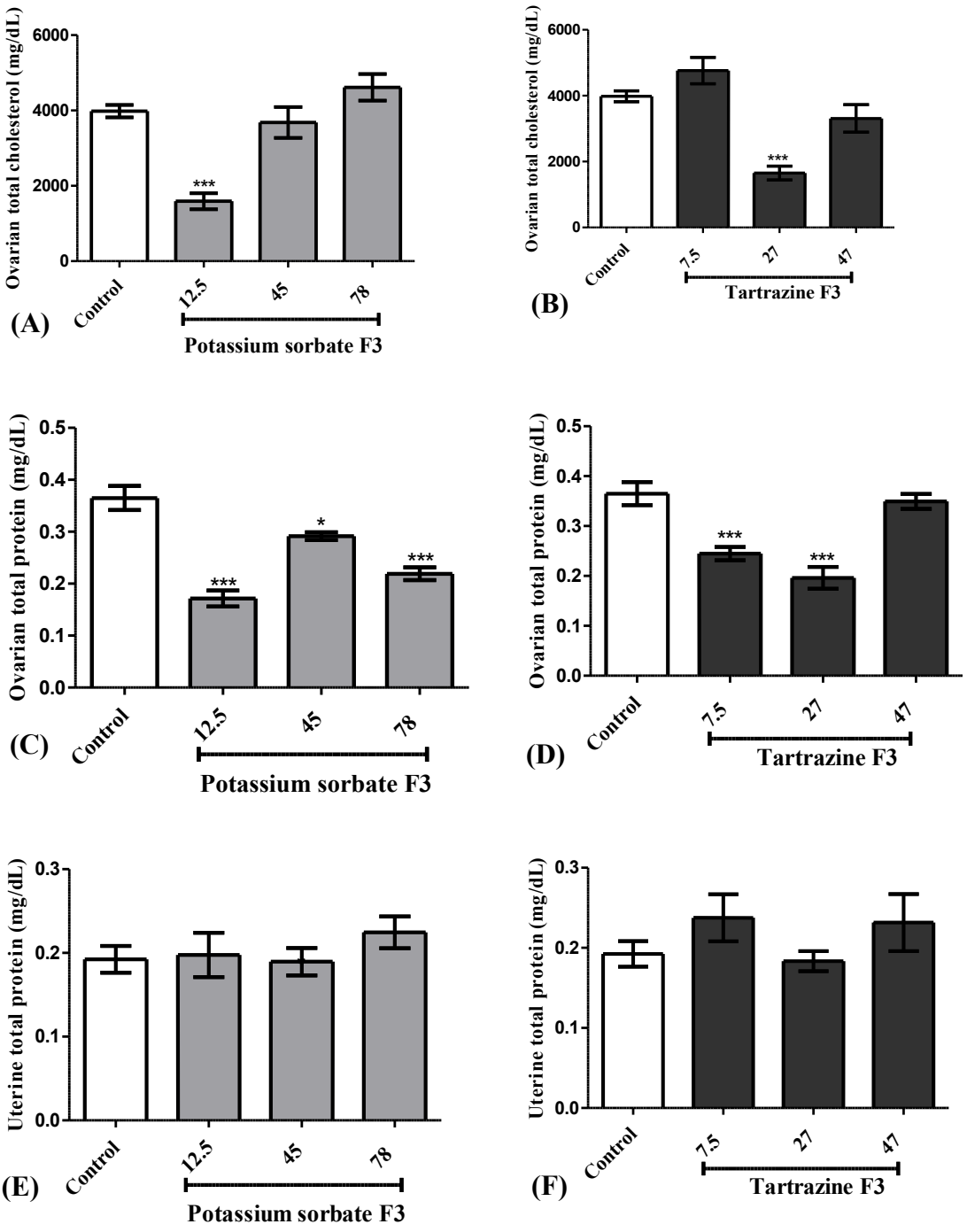


Figure 47: Effects of potassium sorbate and tartrazine on ovarian total cholesterol (A, B), ovarian total protein (C, D) and uterine total protein (E, F) of female Wistar rats of the third generation after 40 days of treatment.

Results are shown as a mean ± SEM, $n = 5$. **: $p < 0.05$; ***: $p < 0.001$* in reference to control. Control= animals receiving vehicle (distilled water); Potassium sorbate = rats treated with Potassium sorbate at doses of 12.5, 45, and 78 mg / kg BW; Tartrazine = rats treated with tartrazine at doses of 7.5, 27, and 47 mg / kg BW.

III.1.2.1.10. Effects of potassium sorbate and tartrazine on the ovarian follicles of the third generation

As observed in F0, the results showed that the treatment with potassium sorbate and tartrazine significantly affected the number of ovarian follicles in F3. Table VIII summarizes the effects of potassium sorbate and tartrazine on the number of follicles in F3. The results showed that the treatment significantly increased the number of primordial of follicles (T 47, $p < 0.001$), secondary follicles (PS 12.5, $p < 0.001$ and T 47, $p < 0.001$), antral follicles (PS 12.5, $p < 0.05$ and T 47, $p < 0.001$), Graafian follicles (T 47, $p < 0.001$) and decreased the number of primary follicles (PS 45, $p < 0.01$ and PS 78, $p < 0.001$), and corpus luteum (PS 78, $p < 0.05$) as compared to the control group F3.

Table VIII. Number of different ovarian follicles and corpora lutea of female Wistar rats after 40 days of treatment of F3.

Organs	Control	Potassium sorbate (mg/kg BW)			Tartrazine (mg/kg BW)		
		12.5	45	78	7.5	27	47
Total follicles	25.68 ± 1.58	36.65 ± 2.14	22.70 ± 0.79	12.66 ± 0.42	26.81 ± 1.21	15.91 ± 0.79	52.32 ± 2.86
Primordial follicles	2.50 ± 0.50	2.25 ± 0,37	3,80 ± 0,37	1.25 ± 0.58	1.66 ± 0.18	1.50 ± 0.74	9.50 ± 0.15***
Primary follicles	11.33 ± 1.31	11.25 ± 0.58	5.50 ± 0.74**	3,25 ± 0.85***	8.50 ± 1.10	6.25 ± 1.11	10.00 ± 1.14
Secondary follicles	6.50 ± 0.22	15.33 ± 1.27***	5.50 ± 0.97	3.50 ± 0.92	8.33 ± 1.59	3.75 ± 1.19	22.66 ± 1.62***
Antral follicles	0.25 ± 0,19	1.33 ± 0.18*	0.50 ± 0.22	1.00 ± 0.31	2.33 ± 079*	0.33 ± 0.18	2.75± 0.37***
Graafian follicles	1.25 ± 0.22	1.50 ± 0.50	2.20 ± 1.01	0.50 ± 0.38	2.33 ± 0.48	1.25 ± 0.48	4.33 ± 0.79**
Atresia follicles	0.00 ± 0.00	1.33 ± 0.48	0.80 ± 0.37	2.00 ± 0.31*	0.66 ± 0.36	0.50 ± 0.38	0.75 ± 0.37
Corpus luteum	3.60 ± 0.50	3.66 ± 0.48	4.40 ± 0.67	1.66 ± 0.18*	3.00 ± 0.54	2.33 ± 0.36	2.33 ± 0.48

Results are presented as mean ± SEM, $n = 5$. **: $p < 0.05$*; *** $p < 0.01$*; ****: $p < 0.001$* in reference to control. Control= animals receiving vehicle (distilled water); Potassium sorbate= rats treated with Potassium sorbate at doses of 12.5, 45, and 78 mg / kg BW; Tartrazine = rats treated with tartrazine at doses of 7.5, 27, and 47 mg / kg BW.

III.1.2.1.11. Effects of potassium sorbate and tartrazine on hormone level of the third generation

a. Effects of potassium sorbate and tartrazine on sex steroids

The effects of potassium sorbate and tartrazine on sexual steroids concentrations of the third generation are presented in Figure 47. As observed in 40 days oral administration of potassium sorbate or tartrazine to F0, the results showed that the treatment of F3with potassium sorbate and tartrazine significantly affected the sex steroids concentration. Oral administration of potassium sorbate and tartrazine significantly decreased the progesterone serum concentration (PS 12.5 and 45, $p < 0.001$ and T 47, $p < 0.05$), estradiol serum concentration (PS 45, $p < 0.05$) and increased the same parameters with tartrazine at the doses of 27 (progesterone, $p < 0.05$) and 7.5 (estradiol, $p < 0.001$) mg/kg BW as compared to the control group.

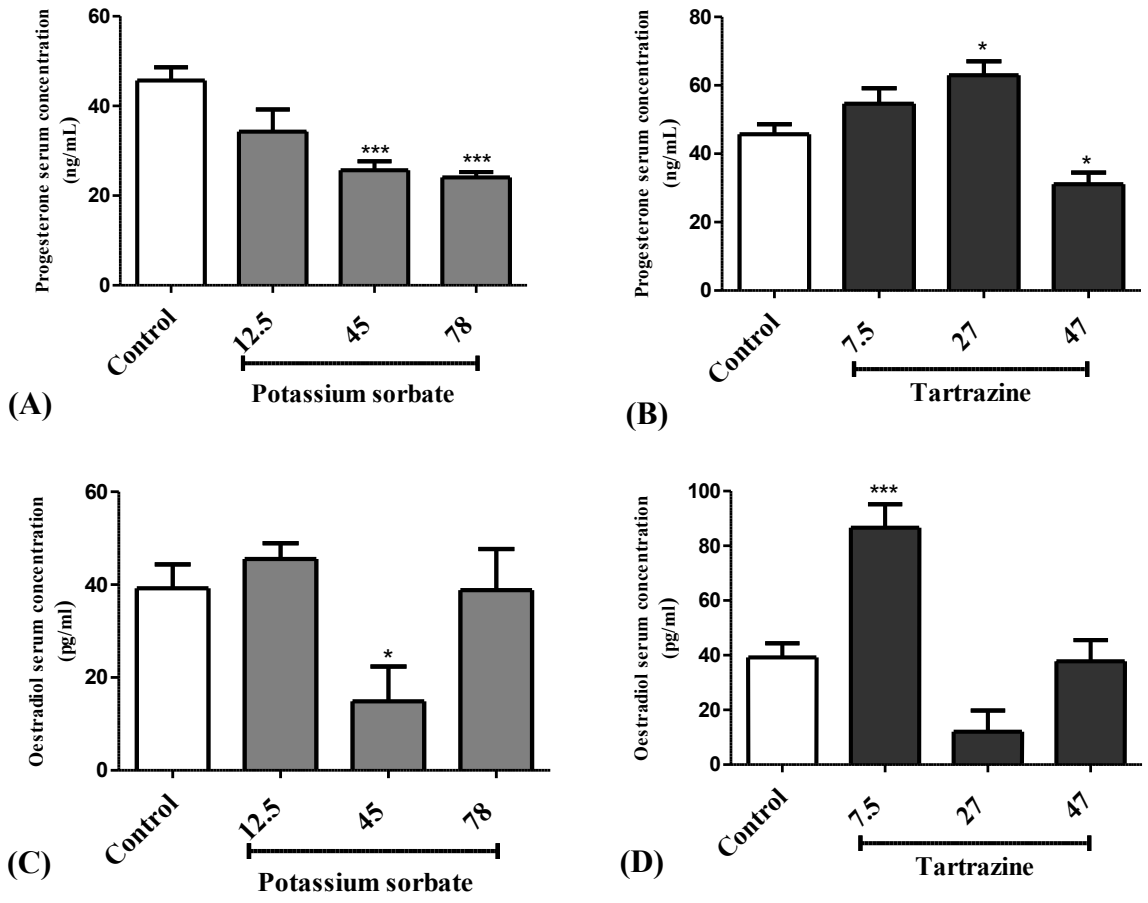


Figure 48: Serum concentration of progesterone (A, B) and estradiol (C, D) of female Wistar rats of the third generation after 40 days of treatment.

Results are shown as a mean ± SEM; $n = 5$. **: $p < 0.05$*; ***: $p < 0.001$ in reference to control. Control = animals receiving vehicle (distilled water, 10 mL/kg BW); PS = rats treated with

potassium sorbate at doses of 12.5, 45, and 78 mg / kg BW, T = rats treated with tartrazine at doses of 7.5, 27, and 47 mg/kg BW.

b. Effects of potassium sorbate and tartrazine on gonadotrophins

As observed in F0, the results indicate that 40 days oral administration of potassium sorbate or tartrazine to F3 significantly affected the LH serum concentration. Figure 49 represents the effects of the administration of potassium sorbate and tartrazine on LH and FSH serum concentrations. Potassium sorbate (PS 12.5, $p < 0.01$; PS 45 and 78, $p < 0.001$), and tartrazine (T 7.5, $p < 0.01$ and T 27, $p < 0.05$) significantly decreased the LH serum concentration as compared to the control group F3 (Figure 49**A**). As shown in Figure 49**B**, FSH serum concentrations of animals of the third generation was not affected by the treatment.

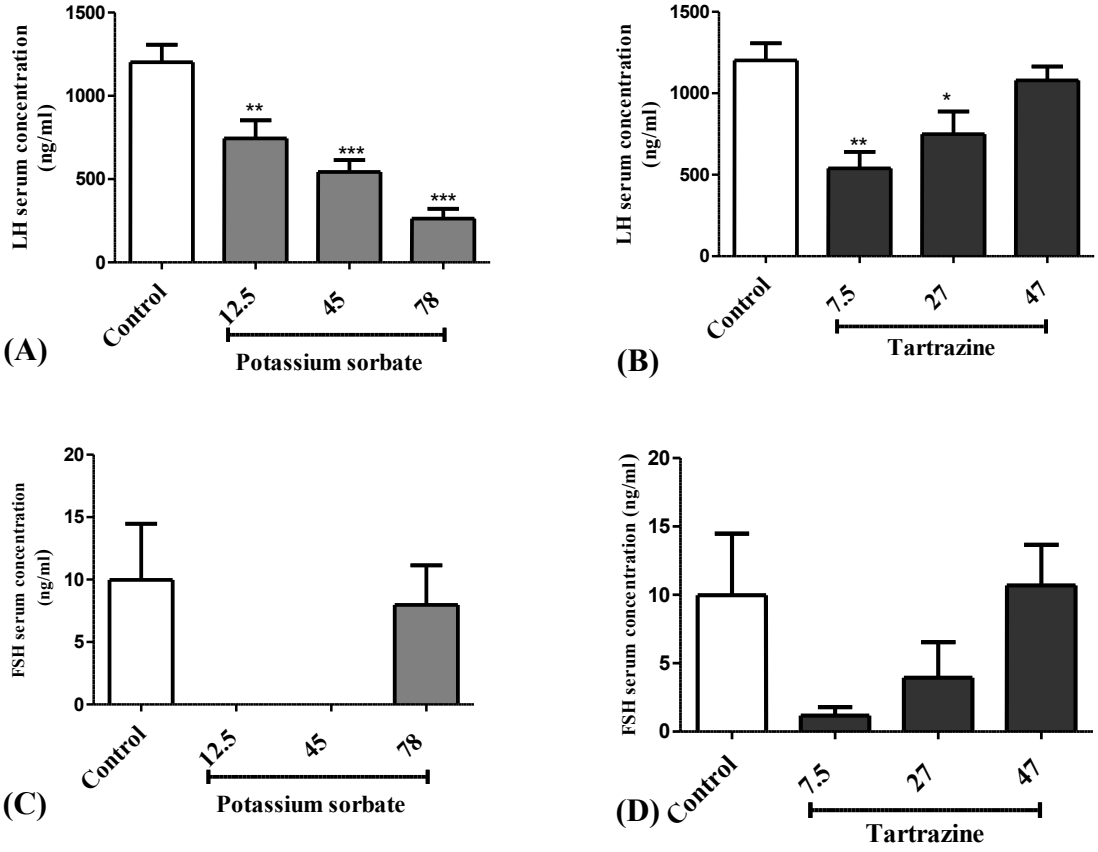


Figure 49: Serum concentration of LH (A, B) and FSH (C, D) of female Wistar rats of the third generation after 40 days of treatment.

Results are shown as a mean ± SEM; $n = 5$. *: $p < 0.05$; ***: $p < 0.01$; **: $p < 0.001$ in reference to control. Control = animals receiving vehicle (distilled water, 10 mL/kg BW); PS = rats treated with potassium sorbate at doses of 12.5, 45, and 78 mg / kg BW; T = rats treated with tartrazine at doses of 7.5, 27, and 47 mg/kg BW.

III.1.2.2. Effects of potassium sorbate and tartrazine on sexual behavior of the third generation

III.1.2.2.1. Effects of potassium sorbate and tartrazine on the appetitive phasis

The effects of potassium sorbate and tartrazine on the appetitive phasis are presented in Figure 48. As observed in F0, the treatment with tartrazine affected the appetitive parameters in the third generation. Tartrazine significantly increased the compartment change latency (T 47 mg/kg BW, $p < 0.01$) (Figure 50**B**), the time spent in the non-operating compartment (7.5 mg/kg BW, $p < 0.001$) (Figure 50**D**), and the compartment change frequency (27 mg/kg BW, $p < 0.01$) (Figure 50**F**), as compared to the control group F3. Regarding Figure 50**D**, 27 mg/kg BW tartrazine significantly reduced ($p < 0.01$) the time spent in the non-operating compartment as compared to the control group F3. As observed in F0, potassium sorbate did not induce any significant effect on the appetitive phasis (Figures 50**A,** 50**C** and 50**E**).

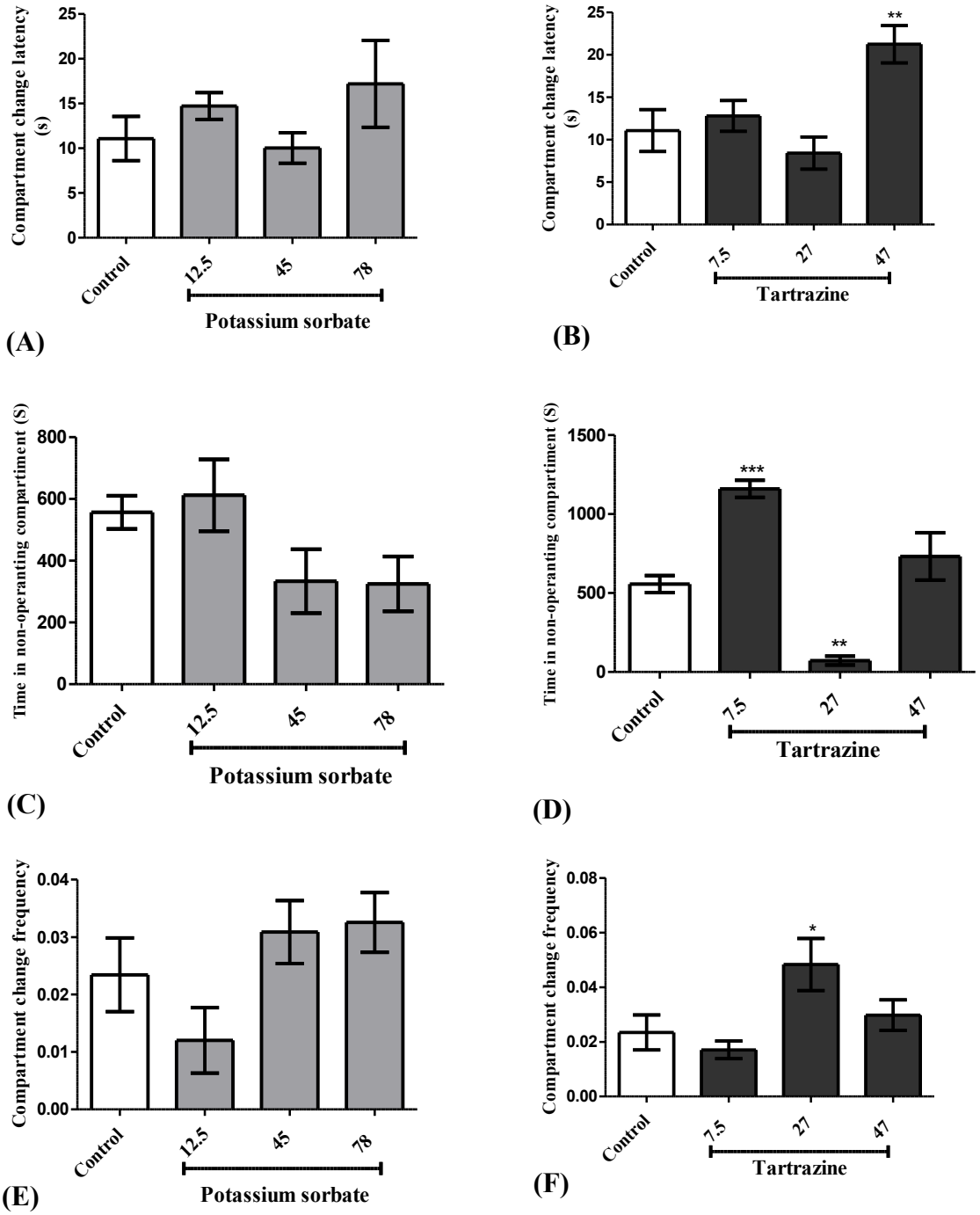


Figure 50: Effects of potassium sorbate and tartrazine on the compartment change latency (A, B), the time spent in the non-operating compartment (C, D) and the compartment change frequency (D, E) of the third generation.

Results are shown as a mean ± SEM, $n = 7$. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ in reference to control. Control = animals receiving vehicle (distilled water, 10 mL/kg BW); PS = rats treated with potassium sorbate at doses of 12.5, 45, and 78 mg / kg BW, T = rats treated with tartrazine at doses of 7.5, 27, and 47 mg/kg BW.

III.1.2.2.2. Effects of potassium sorbate and tartrazine on on proceptive phasis

a. Effects of potassium sorbate and tartrazine on ‘‘Darting’’

Despite the non-significant effect observed in F0, 12.5 and 45 mg/kg BW potassium sorbate significantly decreased ($p < 0.05$) the latency of ‘‘Darting’’ (Figure 51**A**) as compared to control group F3. Contrary to what we have obtained in F0, tartrazine induced any adverse effects on the darting latency in F3 as compared to the control group F3 (Figure 51**B**).

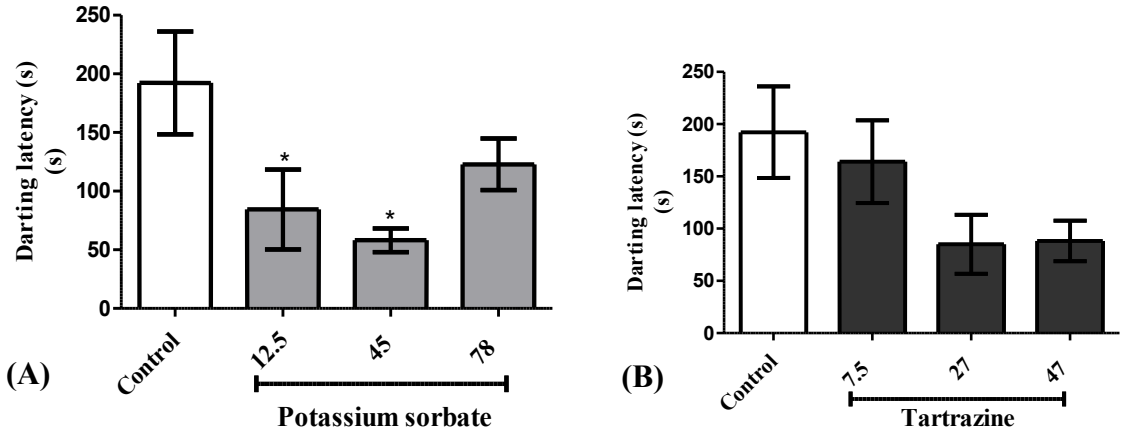


Figure 51: Effects of potassium sorbate and tartrazine on the latency of darting of the third generation.

Results are shown as a mean ± SEM, $n = 7$. *: $p < 0.05$ in reference to control. Control = animals receiving vehicle (distilled water, 10 mL/kg BW); PS = rats treated with potassium sorbate at doses of 12.5, 45, and 78 mg/kg BW, T = rats treated with tartrazine at doses of 7.5, 27, and 47 mg/kg BW.

b. Effects on ‘‘Hopping’’

Contrary to what we have obtained in F0, the results below showed that tartrazine increased the ‘‘hopping’’ latency in F3. Tartrazine at the doses of 7.5 ($p < 0.001$) and 47 ($p < 0.05$) mg/kg BW significantly increased the ‘‘hopping’’ latency as compared to the control group F3 (Figure 52**B**). Figure 52**A** indicates that, the ‘‘hopping’’ latency was neither increased nor decreased with potassium sorbate as compared to the control group F3.

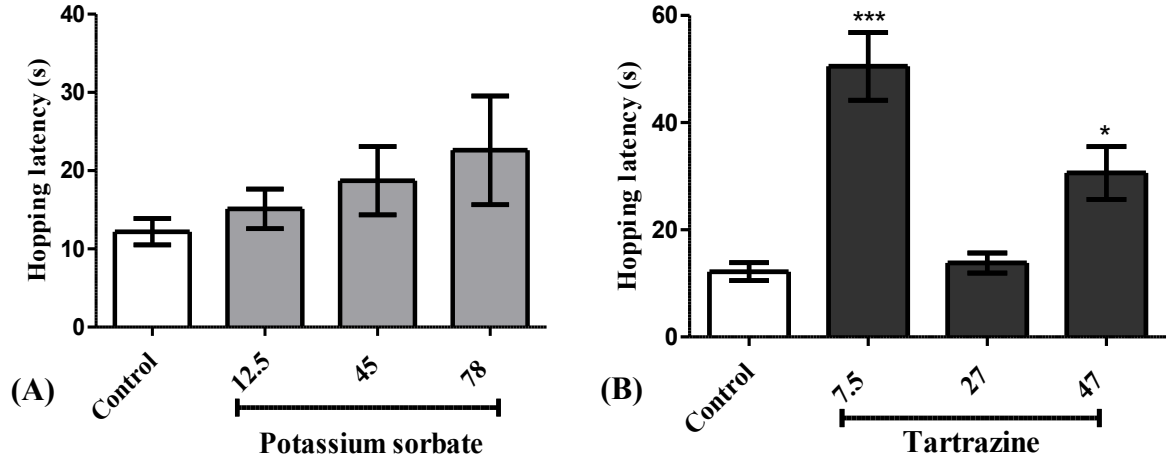


Figure 52: Effects of potassium sorbate and tartrazine on the hopping latency of the third generation.

Results are shown as a mean ± SEM, $n = 7$. *: $p < 0.05$; ***: $p < 0.001$ in reference to control. Control = animals receiving vehicle (distilled water, 10 mL/kg BW); PS = rats treated with potassium sorbate at doses of 12.5, 45, and 78 mg/kg BW; T = rats treated with tartrazine at doses of 7.5, 27, and 47 mg/kg BW.

III.1.2.2.3. Effects of potassium sorbate and tartrazine on the receptive phasis

a. Effects on lordosis

Figure 53 represents the effects of potassium sorbate and tartrazine on lordosis. As observed in F0, the results showed that oral administration of potassium sorbate or tartrazine to F3 significantly affected the receptive phasis. The results indicated an increase of the 'lordosis'' latency (tartrazine at the doses of 7.5 and 47 mg/kg BW ($p < 0.05$) (Figure 53**B**), the lordosis frequency (potassium sorbate at the dose of 78 mg/kg BW and tartrazine at the doses of 27 mg/kg BW ($p < 0.05$)) (Figures 53**C**, 53**D**), and the duration of lordosis (potassium sorbate at the doses of 12.5 ($p < 0.05$), 45 ($p < 0.01$) and 78 ($p < 0.05$) mg/kg BW and tartrazine at the doses of 27 and 47 mg/kg BW ($p < 0.001$)) (Figures 53**E**, 53**F**) as compared to the control group F3. As shown in Figure 53**A**, the lordosis latency was not significantly affected by the treatment with potassium sorbate.

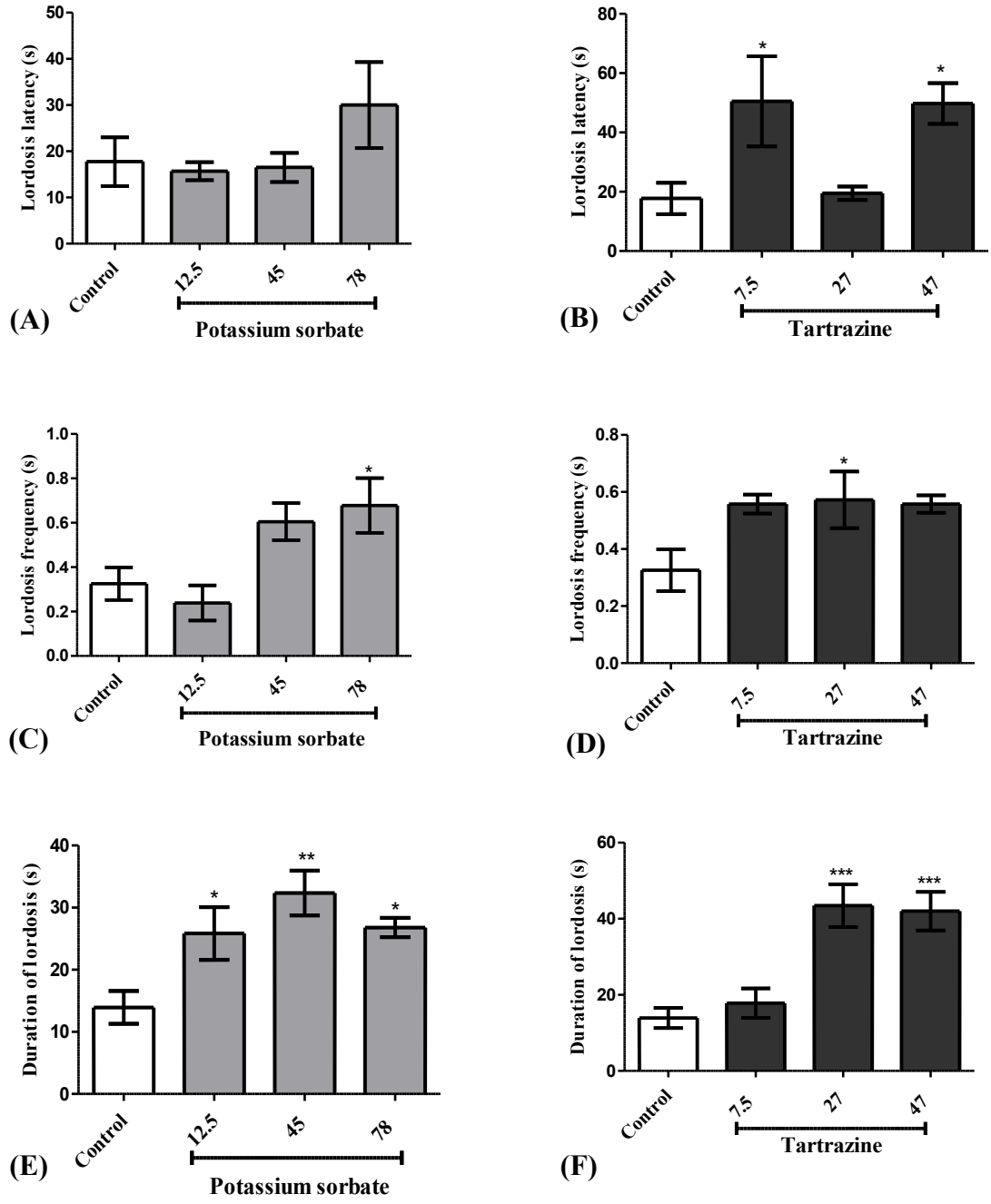


Figure 53: Effects of potassium sorbate and tartrazine on the latency (A), the frequency (B) and the duration (D) of lordosis of the third generation.

Results are shown as a mean ± SEM, $n = 7$. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ in reference to control. Control = animals receiving vehicle (distilled water, 10 mL/kg BW); PS = rats treated with potassium sorbate at doses of 12.5, 45, and 78 mg / kg BW; T = rats treated with tartrazine at doses of 7.5, 27, and 47 mg/kg BW.

b. Effects on anogenital grooming

Despite the non-significant effect obtained in F0, the administration of potassium sorbate or tartrazine to F3 was responsible for a decrease of the number of anogenital grooming (potassium sorbate at the dose of 12.5 mg/kg BW; $p < 0.01$ and tartrazine, at the dose of 27 mg/kg BW; $p < 0.05$) (Figures 54**A**, 54**B**).

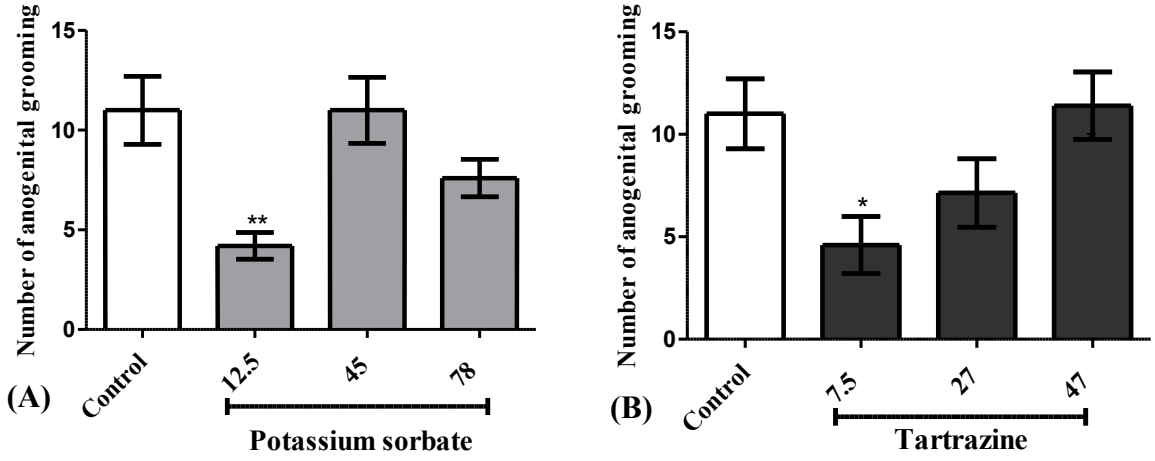


Figure 54: Effects of potassium sorbate and tartrazine on the number of anogenital grooming of the third generation.

Results are shown as a mean ± SEM, $n = 7$. *: $p < 0.05$; **: $p < 0.01$ in reference to control. Control = animals receiving vehicle (distilled water, 10 mL/kg BW); PS = rats treated with potassium sorbate at doses of 12.5, 45, and 78 mg / kg BW; T = rats treated with tartrazine at doses of 7.5, 27, and 47 mg/kg BW.

III.1.2.3. Effects of potassium sorbate and tartrazine on fertility of F1, F2, and F3

III.1.2.3.1. Effects of potassium sorbate and tartrazine on the body weight gain of pregnant rats

Figure 55 shows the effect of potassium sorbate and tartrazine exposure on body weight evolution throughout the period of treatment of pregnant rats of the F1 and F2 generations. The results indicated that the treatment was responsible for the increase (PS 78, F1; T 7.5, 27 and 47, F2) and decrease (PS 12.5, 45, 78, F1; T 27 and 47, F1) in the body weight gain during the period of treatment as compared to the control groups F1 and F2.

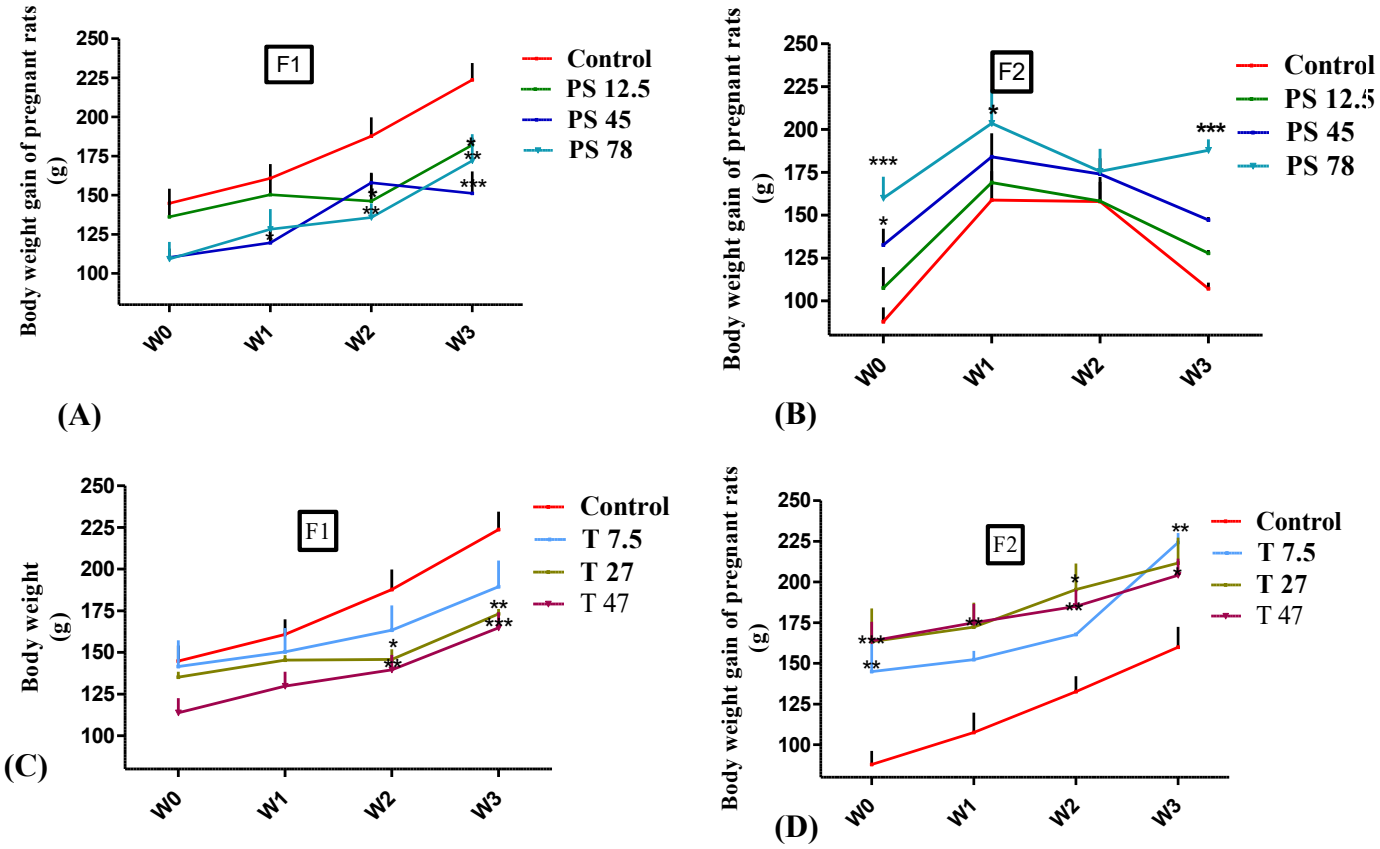


Figure 55: Effects of potassium sorbate and tartrazine on the bodyweight evolution of pregnant rats from the first (A, B) and second (C, D) generations.

Results are shown as a mean ± SEM, $n = 5$. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ in reference to control. Control = animals receiving vehicle (distilled water, 10 mL/kg BW); SP= rats treated with potassium sorbate at doses of 12.5, 45 and 78 mg/kg BW; T = rats treated with tartrazine at doses of 7.5, 27, and 47 mg/kg BW.

III.1.2.3.2. Relative weight of uteri (with pups) and ovaries of pregnant rats of F1, F2, and F3

As shown in Figure 56, the administration of potassium sorbate and tartrazine was responsible for the increase (PS 12.5 and 78, F1, $p < 0.001$; PS 12.5, F2, $p < 0.01$; PS 78, F2, $p < 0.001$; T 27, F1, $p < 0.01$) and decrease (PS 45, F2, $p < 0.001$; PS at the all the tested doses F3, $p < 0.001$; T 47, F1 and T at all the tested doses F3, $p < 0.001$) of the relative uterine weight (Figures 56**A**, 56**B**). Compared to the control group, the treatment significantly increased (PS 78, $p < 0.05$; T 7.5, $p < 0.05$ F1 and T 47, $p < 0.01$) the relative ovaries weight at the F2 (Figures 56**C**, 56**D**).

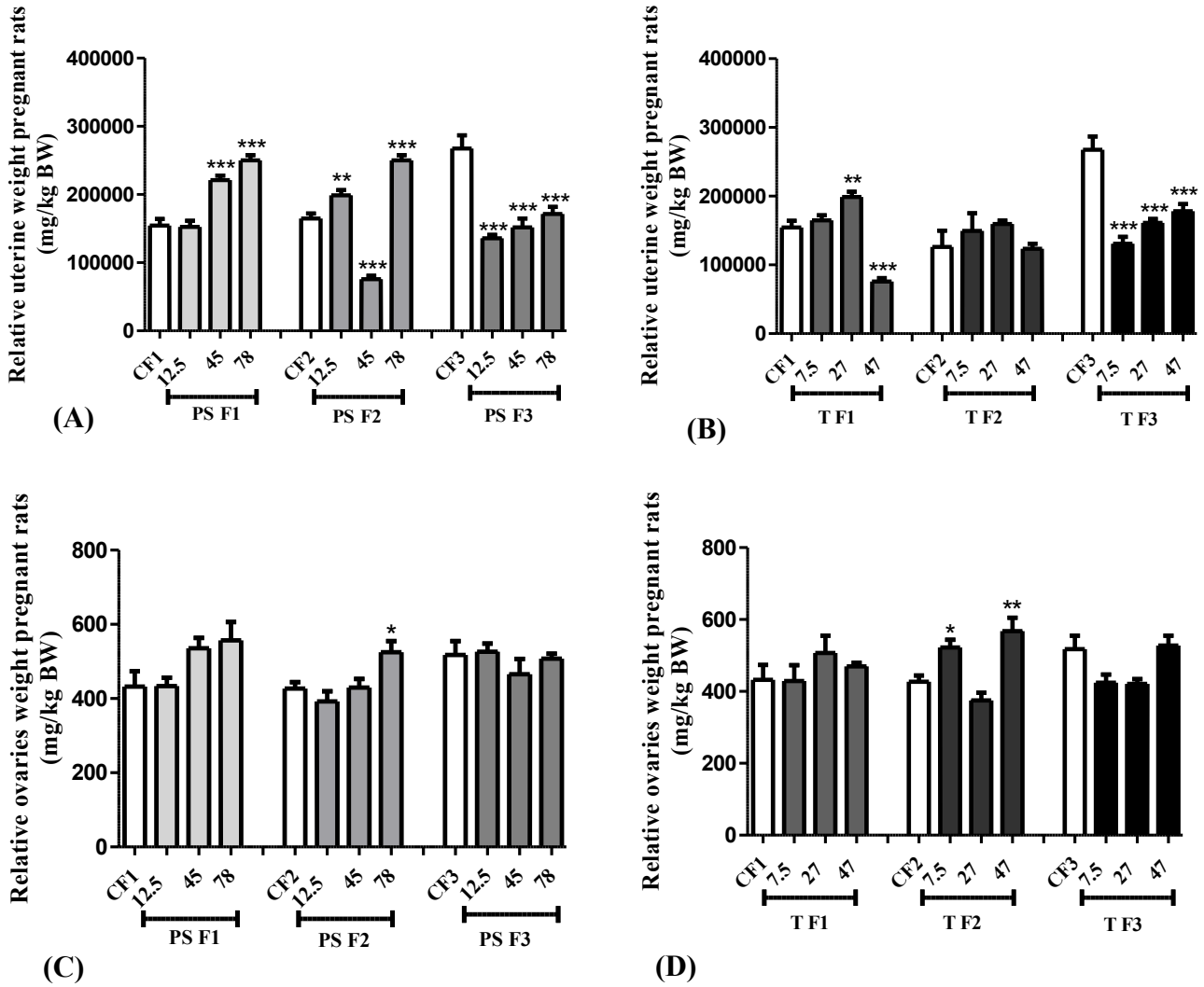


Figure 56: Effects of potassium sorbate and tartrazine on the relative uterine and ovaries weight of pregnant rats of F1, F2, and F3 on gestational day 20.

Results are shown as a mean ± SEM, $n = 7$. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.01$ in reference to control. Control = animals receiving vehicle (distilled water, 10 mL/kg BW); PS = rats treated with potassium sorbate at doses of 12.5, 45, and 78 mg / kg BW; T = rats treated with tartrazine at doses of 7.5, 27, and 47 mg/kg BW.

III.1.2.3.3. Placenta and foetal weight of F1, F2, and F3 at gestational day 20

The Figure 57 represents the effects of the potassium sorbate and tartrazine on placenta and fetuses's weight at GD 20. The results indicate that the treatment was responsible for the increase (PS 12.5 F3, T 7.5 F2, T 27 F3, $p < 0.05$; PS 78, F3, $p < 0.01$; PS 45, F1, T 27 F1, T 47 F3, $p < 0.001$) of the relative weight of placenta as compared to the control group (Figures 57**A**, 57**B**). As shown in Figures 57**D**, tartrazine at the dose of 7.5 mg/kg BW significantly increased (F2; $p < 0.05$) the weight of pups and decreased the same parameter at the third generation (F3; $p < 0.01$). Concerning potassium sorbate, its oral administration was responsible

for the decrease of pups weight at the doses of 45 (F3; $p < 0.05$) and 78 (F3; $p < 0.05$) mg/kg BW as compared to the control group (Figure 57**C**).

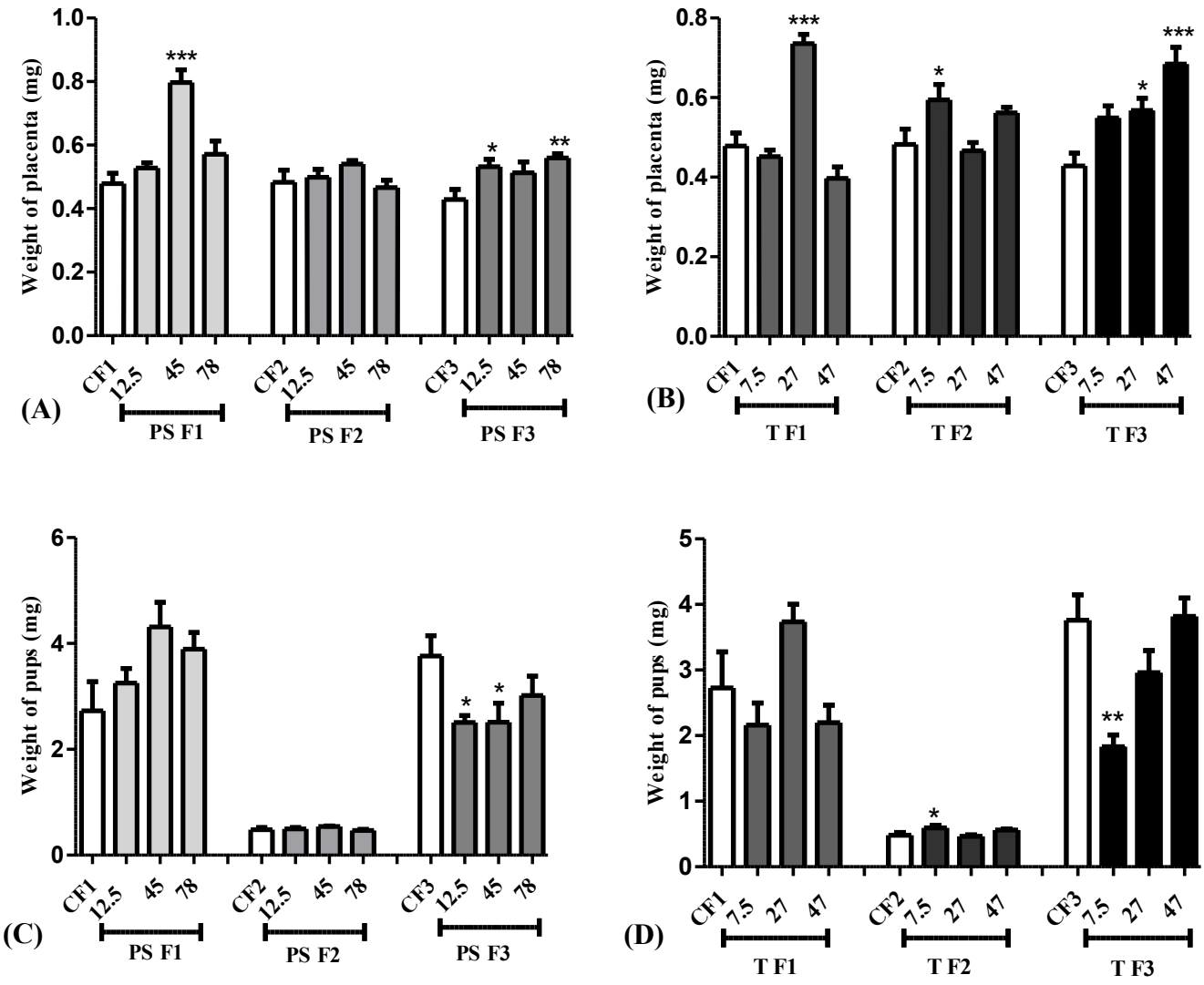


Figure 57: Effects of potassium sorbate and tartrazine on the weight of placenta and pups of F1, F2, and F3 at gestational day 20.

Results are shown as a mean ± SEM, $n = 7$. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.01$ in reference to control. Control = animals receiving vehicle (distilled water, 10 mL/kg BW); PS = rats treated with potassium sorbate at doses of 12.5, 45, and 78 mg / kg BW, T = rats treated with tartrazine at doses of 7.5, 27, and 47 mg/kg BW.

III.1.2.3.4. Number and size of live fetuses on gestational day 20

Figure 58 shows the effect of potassium sorbate and tartrazine exposure on size and number of live pups on GD 20. The results indicated that potassium sorbate at the dose of 45 mg/kg BW was responsible for the increase (F2, $p < 0.01$) of the size of pups at GD 20 as

compared to the control group (Figures 58**A**). Figure 58**B** indicated that, tartrazine did not increase or reduced the size of pup at GD 20. As shown in the Figures 58**C**, 58**D**, the treatment was responsible for the increase (T 47, F2, $p < 0.05$) and decrease (PS 12.5, F3, $p < 0.05$; PS 45, F3, $p < 0.01$; T 27, F3, $p < 0.01$) of the number of live pups as compared to the control groups.

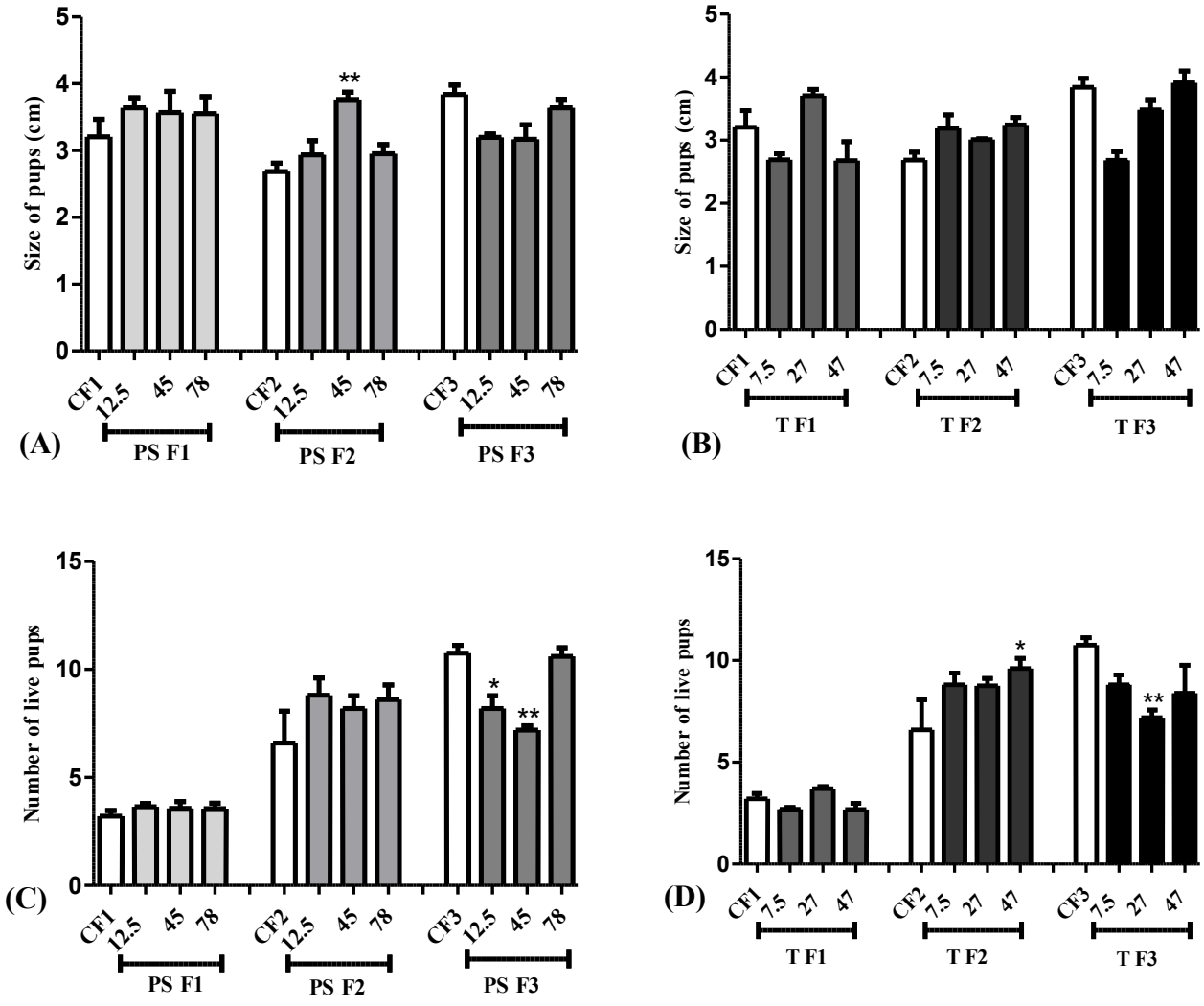


Figure 58: Effects of potassium sorbate and tartrazine on the size and number of live fetuses of F1, F2, and F3 at gestational day 20.

Results are shown as a mean ± SEM, $n = 7$. *: $p < 0.05$; **: $p < 0.01$in reference to control. Control = animals receiving vehicle (distilled water, 10 mL/kg BW); PS = rats treated with potassium sorbate at doses of 12.5, 45, and 78 mg / kg BW; T = rats treated with tartrazine at doses of 7.5, 27, and 47 mg/kg BW.

III.1.2.3.5. fetal resorption after daily oral administration of potassium sorbate and tartrazine to pregnant rats

Figure 59 represents rat uterus showing fetal resorption (arrow) after daily oral administration with potassium sorbate (12.5, 45 and 78 mg/kg BW) and tartrazine (7.5, 27 and 47 mg/kg BW) to pregnant rats, from the PND 21 to GD 20.

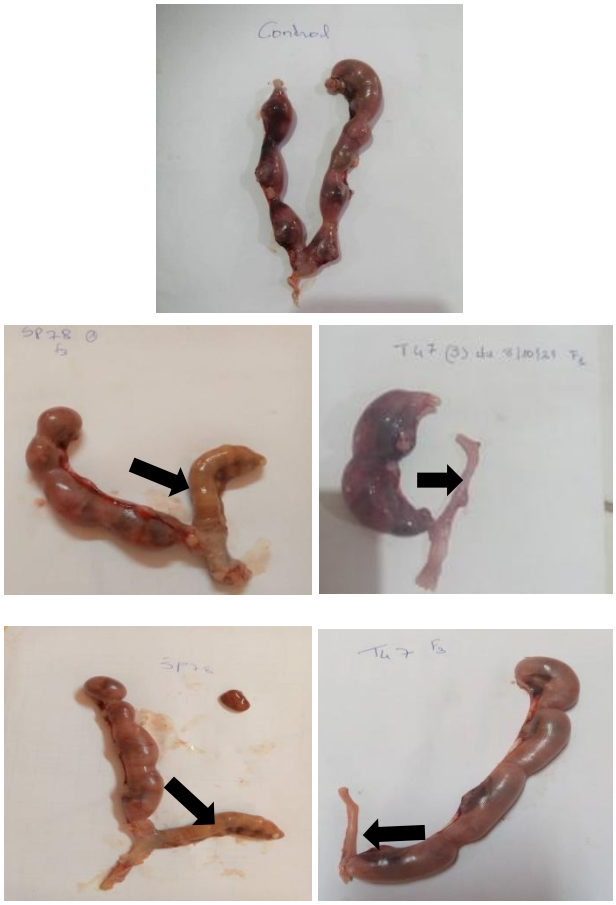


Figure 59: Rat uterus after daily oral administration of potassium sorbate and tartrazine to pregnant rats, from the PND 21 to gestational day 20.

Control = animals receiving vehicle (distilled water, 10 mL/kg BW); PS 78= rats treated with potassium sorbate at dose of 78 mg / kg BW; T 47 = rats treated with tartrazine at dose of 47 mg/kg BW.

III.1.2.3.6. Effects of potassium sorbate and tartrazine on gestational parameters of the F1 and F2 after the treatment

As observed in F0, the treatment with potassium sorbate and tartrazine affected the gestational parameters of offspring. After the treatment, fertility index decreased with potassium sorbate (F2) at the dose of 12.5 mg/kg BW (from 100% to 80.00%) and with tartrazine (F1) at the doses of 7.5 (83.33% to 64.28%) and 47 (83.33% to 75.00%) mg/kg BW.

The results showed that tartrazine (F1) also decreased the gestational rate at the does of 7.5 (80.00% to 69.23%) and 47 (83.33% to 66.66%) mg/kg BW (Table IX).

Table IX: Effects of potassium sorbate and tartrazine on gestational parameters of the F1 and F2 after the treatment

Experimental groups	**Fertility index (%)**		**Gestational rate (%)**		**Sex ratio**
	F1	**F2**	**F1**	**F2**	**F3**
Control (Distilled water 10 mL/kg)	83.33	100	80	100	1.59 ± 0.15
PS 12.5 (mg/kg BW)	90.90	80	83.33	100	1.52 ± 0.05
PS 45 (mg/kg BW)	100	100	100	100	0.74 ± 0.12**
PS 78 (mg/kg BW)	81.81	100	83.33	100	1.36 ± 0.29
Tartrazine 7.5 (mg/kg BW)	64.28	100	69.23	100	1.01 ± 0.34
Tartrazine 27 (mg/kg BW)	87.50	100	83.33	100	0.99 ± 0.19
Tartrazine 47 (mg/kg BW)	75.00	100	66.66	100	1.26 ± 0.16

Results are presented as mean ± SEM, n=7; (*: $p < 0.05$); (**: $p < 0.01$) in reference to control. Control = animals receiving vehicle (distilled water); PS = rats treated with potassium sorbate at doses of 12.5, 45, and 78 mg/kg BW; T = rats treated with tartrazine at doses of 7.5, 27, and 47 mg/kg BW

III.2. DISCUSSION

The onset of secondary sexual characteristics, and the pubertal growth spurt, are markers of the developmental process that lead to sexual and reproductive maturity, the development of mental processes and adult identity. Puberty is defined as the appearance of secondary sexual characteristics during the transition from childhood to young adulthood that prepare the human body for reproduction (Parent *et al*., 2005; Bellis *et al*., 2006; Brito *et al*., 2016; Stagi *et al*., 2020). Most recent data consistently indicate that the food additives present in industrial processed food (in the form of preservatives and dyes) are more and more pointed out as being an endocrine disruptor which can be one of the causes of the precocity or the delay of puberty responsible for the current decline of fertility (Scippo and Maghuin-Rogister, 2007; Ricard, 2011; Gore *et al*., 2014; Faghani *et al*., 2022). Several studies have established its effects on male and female reproductive system (Mehedi *et al*., 2009; Nasri and Pohjanvirta, 2021; Datta and Lundin-Schiller, 2008; Axon *et al*., 2012; Boussada *et al*., 2017; Wopara *et al*., 2021; Shakoor *et al*., 2022). This study began with the determination of the age of onset of sexual maturation in experimental animals, which is characterized by vaginal opening (Kriszt *et al*., 2015; Awounfack *et al*., 2018). Therefore, this work aimed at assessing the effects of potassium sorbate and tartrazine on the female reproductive system in a model of immature female Wistar rats. Sexual maturation also known as puberty is a crucial stage of development. It requires changes in the sensitivity, activity, and functionality of the hypothalamic-pituitary-gonadal axis which can cause a direct maturation of the genital organs. Without these signals, the genital organs maintain the appearance they had during childhood, and the reproductive system remains non-functional. In other words, sexual maturation is a prerequisite for fertility (Marieb and Hoehn, 2010). Although there was no effect on the average age of vaginal opening, the results of this study shown that 45 days after their birth, the rats treated with tartrazine at the dose of 47 mg/kg BW had (100%) vaginal opening versus 41.66% in animals in the control group of the same age. This result showed that tartrazine advanced puberty as measured by the percentage of rats showing vaginal opening and it is in accordance with many researchers who demonstrated that the administration of xenoestrogen such as zearalenone and Bisphenol A to sexually immature rats is responsible of early puberty (Kriszt *et al*., 2015). Thus, early sexual maturation is associated with many adverse effects such as initiation of sexual activity, increase risk of sexual abuse, estrogen-dependent cancer (mainly breast cancer), unprotected sex and, consequently, sexually transmitted infections and teenage pregnancies (Heger *et al*., 2005; Macedo *et al*., 2014; Brito *et al*., 2016). Whatever, sexual maturation has been delayed with potassium sorbate at the dose of 78 mg/kg BW. This group exhibited 25% of vaginal opening

versus 75% for control on day 46 and 41.66% of vaginal opening vs. 75% for control on day 47. Many researchers demonstrated that, environmental toxins with oxidative properties are responsible for the delay sexual maturation in pre-pubertal treated females and female offsprings (Reilly *et al*., 2010; Souza *et al*., 2019). According to the literature, the pathophysiology of subfertility in both males and females has been linked to OS, a condition marked by an imbalance between pro-oxidant molecules, including reactive oxygen and nitrogen species, and antioxidant defenses (Hasson, 2020; Chukwuebuka *et al*., 2020; Faghani *et al*., 2022). It has been demonstrated that, excess of oxidative stress causes damage during steroidogenesis and ovulation (Agarwal *et al*., 2005; Gupta *et al*., 2006; Chukwuebuka *et al*., 2020) leading to the delay puberty. Potassium sorbate decreased significantly the estradiol serum concentration at all the tested doses. This may be related to the inhibition of the hypothalamus, (Spiers *et al*., 2014; Hasson, 2020), or to the OS in a follicular fluid surrounding the developing egg of the ovary. Potassium sorbate may have delayed sexual maturation by inhibiting the hypothalamus through the oxidative stress. The advanced puberty observed with tartrazine at the dose of 47 mg/kg BW is accompanied by a very significant increase in the secretion of estradiol and LH. These results corroborate with the observations made in rats by Ramirez and Sawyer (Ramirez and Sawyer, 1965), which stipulate that the vaginal opening is the initial and external sign of the increase in secretion in estrogens accompanying the beginning of puberty. Tartrazine could have advanced the puberty by activating the release of the reproductive hormones of the hypothalamic-pituitary-ovarian axis. Contrary to the present study, Shakoor and colleagues (Shakoor *et al*, 2022) showed that tartrazine significantly decreases LH, FSH and estrogen levels; and increases progesterone levels after 30 days of treatment at the dose of 9.5 mg/Kg BW to adult female Sprague Dawley rats (6–7 months old). These differences may be due to the differences in species, animal age, and duration of treatment. Literature shows that differences in certain results in studies of the same molecules, substances, plants may arise from differences in the protocols used such as type of studies (in vitro or in vivo); species and age of animal used, duration of treatment, route of administration (Chung *et al*., 1992; Hashem *et al*., 2019; Faghani *et al*, 2022) and the stage of the estrous cycle if the authors used female animals (Milad *et al*, 2009). It is well known that GnRH released in a pulsatile pattern of rhythmic secretory bursts whose amplitude and frequency vary according to cycle stage. The pituitary cells called gonadotropes, responding to GnRH stimulation, synthesize and release LH and FSH, which induce ovarian folliculogenesis, steroidogenesis, ovulation and formation of corpus luteum (Marieb and Hoehn, 2010; Chaitra *et al*., 2020). Furthermore, the increase in serum LH levels after 40 days of treatment observed, testifies the capacity of the rats to ovulate, since the increase in the serum level of gonadotropin to a certain

threshold is a prerequisite for ovulation and subsequent promotion of the luteal phase (production of progesterone). According to Marieb and Hoehn (Marieb and Hoehn, 2010), the production of estrogens increases with follicular growth and when their size (in this case that of the dominant follicle) reaches a certain threshold, the level of estrogen produced briefly exerts a retro activation on the hypothalamus and the adenohypophysis causing a sudden release of LH to a certain extent and FSH, approximately in the middle of the cycle. This hypothesis confirms the results observed on folliculogenesis (47 mg/kg BW). It increased the number of total follicles (primary, secondary, and antral follicles) and the concentration of FSH (non-significant increase). During puberty, the growth and maturation of the ovaries are mainly attributed to the presence of mature follicles (antral and Graafian follicles (Peters *et al*., 1978; Picut *et al*., 2014). Then development and function (size expansion and the number of mature follicles, proliferation of fibrous tissues and antrum) of these ovaries require the presence of estrogens as much as that of pituitary gonadotropins (Picut *et al*., 2014). This implies that tartrazine at this dose stimulated the mechanism intervening in the process of the hormone synthesis in the granulosa cells and its secretion into the blood stream. Meanwhile, the increase of folliculogenesis can lead to the loss of pool of primordial follicles and ovarian follicular reserve is tartrazine is prolonged use from a young age. Literature shows that, when the follicular pool reaches thousand follicles, the ovary cannot maintain the hormonal feedback with the hypothalamus and ovarian ageing also known as menopause is reached (te Velde, 1998; Wilkosz *et al*., 2014, Cruz *et al*., 2017). The delay of sexual maturation observed with potassium sorbate at the dose of 78 mg/kg BW was accompanied with a decrease of primary and secondary follicles, ovarian cholesterol and an increase in the number of atresia follicles. This may be due by the absence of appropriate concentration of LH and FSH caused by the inhibition of the hypothalamus (Hasson, 2020; Spiers *et al*., 2014), by the decrease of estradiol serum concentration or to the oxidative stress effects in the ovary. It is well known that the induction of folliculogenesis is stimulated by the secretion of LH and FSH by the adenohypophysis (Marieb and Hoehn, 2010; Chaitra *et al*., 2020). Such alterations in ovarian tissues agree with the assertion of Agarwal and collaborators who reported that OS can induce the disruption of the corona radiata and zona pellucida and degeneration of granulosa cells in the process of cellular autophagy leading to cell death (Agarwal *et al*., 2005; Chukwuebuka *et al*., 2020; Stanojlovi *et al*., 2023). In addition, severe OS in follicular fluid has been associated with decrease in oocyte quality and fertilization rates, which may be due to DNA damage in oocytes, changes in the follicular microenvironment, and decreased availability of antioxidants (Zaha *et al.,* 2023), which can lead to a decrease of pregnancy rates. This study suggests that potassium sorbate has modified steroidogenesis both locally by altering folliculogenesis and

through the HP axis by inhibiting the hypothalamus. In addition, the abovementioned parameters remain insufficient in the evaluation of the impact of chemicals on the development of the female reproductive system. The relative weight of the uterus, ovaries, and/or the size of the uterine and vaginal epithelia is essential parameters (Njamen *et al*., 2013; Tassinari *et al*., 2021). In addition to the vaginal opening, reproductive maturation is associated with the activation of the hypothalamic-hypophysis-ovarian axis, which helps to induce secretion of the appropriate quantities of gonadotropins (FSH, LH) and ovarian hormones (estrogens and progesterone) responsible for the development and function of primary estrogen targets (uterus, vagina, and mammary gland) (Tassinari *et al*., 2021; Marieb and Hoehn, 2010). The side effects observed on folliculogenesis with potassium sorbate at the dose of 78 mg/kg BW was accompanied by a significant decrease of the relative weight of ovary. This result corroborates with the observations of Hasson, (Hasson, 2020) who shown that environmental stressors with oxidative property (Mehranjani *et al*., 2010; Hasson, 2020; Chukwuebuka *et al*., 2020) such as potassium sorbate (Hasson, 2020) are responsible for the decrease of the relative ovarian weight and the increase the number of atresia follicles. These effects can be due to the injury caused in the ovary by potassium sorbate since the administration of potassium sorbate to female rats is responsible for the increase of MDA (in ovaries) which is one of the important products resulting from lipid peroxidation (Hasson, 2020). Mehranjani and collaborators (Mehranjani *et al*., 2010) reported that increase in the number of atretic follicles is sometimes due to the disturbance of folliculogenesis and oogenesis as a result of oxidative stress and later leads to a decrease in the number of follicles in pre-ovulation stage. These data suggest that potassium sorbate at the dose of 78 mg/kg BW induced injury in rat's ovary, leading the decrease of the ovaries weight and the damage of follicular maturation. On the other hand, tartrazine at the dose of 47 mg/kg BW induced uterine growth (thickness of the uterine epithelium and the relative uterine weight), and eosinophilic secretions in the acinar of the mammary glands. Estrogens have been reported to stimulate proliferation and differentiation of uterine and vaginal epithelial cells, and eosinophilic secretions in the acinar of the mammary glands (Mvondo *et al*., 2012; Njamen *et al*., 2013). In an in vitro study, tartrazine has been identified as a new activator of human estrogen receptors (xenoestrogens). Its mechanism of action is through the activation of ERα receptors expressed in various tissues, but mainly in the uterus, ovary, pituitary gland, vas deferens, adipose tissue; and regulates the expression of more than 2800 genes in mammary gland cell lines (Axon *et al*., 2012; Nasri and Pohjanvirta, 2021).

According to the literature, estrous cycle represents an important marker of sexual maturity or gonadotropin responsiveness, and any alteration in this parameter may be reflected

in the fertility of the females, with reduced fertility as a consequence which exhibited a commitment in several fertility parameters including reduced corpora lutea and implantation numbers may indicate the interference of EDCs in the hypothalamic-pituitary-ovarian axis (Gore *et al*., 2018; Jorge *et al*.; 2021). During the estrous cycle, many physiological, histological and biochemical changes occur. Examples of such changes are pre-ovulatory follicles maturation and ovulation events taking place under ovarian and extra ovarian hormones controls, follicles growth, fluctuations in gonadotropin and steroidal hormones, and sexual receptivity (Adeniyi and Agoreyo, 2019). In addition to the role of OS, (Hasson, 2020) any imbalance or defect of these hormones resulted in irregularity in ovarian functions (Supriya *et al*., 2016; Hasson, 2020) and can lead to the alteration of sexual performances (Supriya *et al*., 2016). Many parameters such as estrous frequency and regularity, estrous cycle length, and estrous cycle ratio appear to be good predictors of luteogenesis and luteolysis intervals (Marcondes *et al*., 2002; Adeniyi and Agoreyo, 2019). The present study showed that the administration of potassium sorbate (12.5, 45, and 78 mg/kg BW) and tartrazine (7.5, 27, and 47 mg/kg BW) to sexually immature rats was associated with the decrease ($p < 0.05$) of estrous cycle ratio (tartrazine at the dose of 7.5mg/kg BW). The decrease in ECR has been linked to acyclicity and the delay in new folliculogenesis. It has been shown that as long as the ECR decreases, as well as the inhibition of corpus luteum regression occurs (Adeniyi and Agoreyo, 2019). The results obtained can be due to the capacity of tartrazine to interfere with corpus luteum regression, preventing them from luteolysis and leading to the alteration of the processes of follicle maturation and selection. In rodents, prolactine is one of the hormones responsible for the maintaining of the luteal phase (Paccola *et al*., 2013). Since compounds with estrogenic capacity such as genistein (Diel, 2002; Nasri and Pohjanvirta, 2021) can stimulate the secretion of prolactin (Nasri and Pohjanvirta, 2021), the xenoestrogen tartrazine might have decreased the ECR by this mechanism. This data suggested tartrazine can alter the physiological dynamics of follicles and corpus luteum delaying the beginning of a new estrus cycle. The results also showed that and tartrazine and potassium sorbate were responsible for the decrease of 4 to 5 days cycles (tartrazine at the doses of 7.5 and 47 mg/kg BW) and an increase ($p < 0.01$) of irregular cycles (potassium sorbate at the dose 78 mg/kg BW). The Study of the estrous cycle in experimental animals is an important parameter used to measure the integrity of the hypothalamic-hypophysis-gonadal axis and the reproductive status of the female reproductive system (Chaitra *et al*., 2020). The acyclicity observed can be due to the hypothalamic-hypophysis-ovarian axis dysregulation (Spiers *et al*., 2014; Hasson, 2020; Shakoor *et al*, 2022) triggered by the administration of potassium sorbate and tartrazine. It is well known that the reproductive cycles are under the control of hypothalamic-pituitary-gonadal (HPG) axis.

Luteinizing hormone (LH) and follicle stimulating hormone (FSH) are released in response to gonadotropin-releasing hormone (GnRH), which causes ovarian folliculogenesis, ovulation, and the development of corpus luteum (CL) (Marieb and Hoehn, 2010; Chaitra *et al*., 2020). Any dysfunction of the hypothalamic-pituitary-ovarian (HPG) axis caused by endocrine disruptors (EDs) may result to inhibition of gonadotropin-releasing hormone (GnRH) secretion, and a decrease in levels of androgens and gonadotropins, significant changes in steroid biosynthesis. All of these alterations may impair folliculogenesis and ultimately result in estrous cycle irregularity, early ovarian failure, and decreased female fecundity (Stanojlović *et al*., 2023). Following to this, the analysis of the videos recorded during the sexual behavior test showed that the treatment was responsible for an increase of the compartment change frequency (T27, $p < 0.01$) and 47, $p < 0.001$), a decrease in the darting (T 7.5, $p < 0.01$), hopping latency (T at all the tested doses), the lordosis frequency (PS 12.5 and 45, $p < 0.01$), and increase of hopping latency (PS 45, $p < 0.05$), the number of rejections (PS 12.5, $p < 0.001$) and the lordosis latency (PS 78, $p < 0.001$ and T 27, $p < 0.05$). The increased of appetitive and proceptive parameters was also observed with animals receiving maca-genseng-gingimbre. These results suggested that female receiving tartrazine at all the tested doses as well as those receiving maca-genseng-gingimbre were sexually attractive and proceptive. The results also demonstrate that animals receiving tartrazine were not receptive at the dose of 27 mg/kg BW when those receiving potassium sorbate presented a reduce proceptivity and receptivity. The results obtained with maca-genseng-gingimbre and tartrazine illustrates the willingness of female rats to approach and sollicitate the males. It can be justified by the capacity of tartrazine to activate estrogen receptor. In an in vitro study, tartrazine has been identified as a new activator of human estrogen receptors (xenoestrogens) (Axon *et al*., 2012; Nasri and Pohjanvirta, 2021). Estrogen participates in a complex central neural circuit that controls and influences sexual motivation at the central level (Ogawa *et al*., 1999; Walf *et al*., 2008a, 2008b; Antal *et al*., 2012) with other neurotransmitters such as dopamine (Amin *et al*., 2005; Micevych and Meisel, 2017; Melis *et al*., 2022). Together with xenoestrogenicity properties of tartrazine, this food dye can also increase the estrogen serum concentration as shown in the evaluation of their effects in sexual maturation parameters. According to the literature, the modulation of estrogen receptor dependent on female sexual behavior by the MPN stimulates the proceptive behavior in female rats. This raises the possibility that, the increase in sexual motivation and desire observed with tartrazine might be due to the increase in estrogen serum concentration and modifications in dopamine activity in ventromedial nuclei and medial preoptic area since estrogen is known to modulate this neurotransmitter (Olivier *et al.,* 2011; Powers, 1972; Amin *et al*., 2005; Micevych and Meisel, 2017; Melis *et al*., 2022). The decrease of receptivity observed with tartrazine at

the dose of 27 mg/kg BW may be associated with excessive of oxidative stress in ovary tissues which negatively reflect on ovarian steroidogenesis led to a change in hormonal balance (Spiers *et al*., 2014; Hasson, 2020). The increase of hopping latency (PS 45, $p < 0.05$) and the number of rejections (PS 12.5, $p < 0.001$) observed with potassium sorbate show that it is responsible for a decrease of pre-copulatory (PS 45 mg/kg BW) and receptive (PS 78) activity in females. This reflects a lack of willingness of female rats to approach males, to solicit them and, later, to show receptive behavior. It is well known that the motivational behavior has been shown to transmit information to the brain, particularly in the ventromedial area of the hypothalamus (VMH), which is essential for controlling lordosis. This reflex requires activation of the estrogen receptor (ERα) (Olivier *et al*., 2011; Powers, 1972). Sexual behavior has been significantly impacted by the potassium sorbate, which suppresses lordosis and the estrous cycle. Additionally, lordosis activation is regulated by various neurotransmitter systems across the brain, which process depending on the differential regulation of the signaling pathways mediated by the various neurotransmitters such as serotonin. Since sexual behavior in rats is organized by brain areas, it has been demonstrated that a decrease of serotonin leads to affecting female sexual behaviors directly such as lordosis (Snoeren *et al*., 2010; Karabaşoğlu and Erbaş, 2021). Lordosis behavior, a measure of sexual receptivity that involves estradiol membrane-initiated signaling in the arcuate nucleus (ARH), activating β-endorphin projections to the medial preoptic nucleus (MPN), which in turn modulate VMH activity the common output from the hypothalamus. Sexual experience sensitizes the response of nucleus accumbens neurons to dopamine signaling through the induction of a long lasting early immediate gene. While estrogen alone increases spines in the ARH, sexual experience increases dendritic spine density in the nucleus accumbens. These two circuits appear to converge onto the medial preoptic area where there is a reciprocal influence of motivational circuits on consummatory behavior and vice versa (Micevych and Meisel, 2017). Present-day data suggests that tamoxifen disrupts females' estrous cyclicity maintaining estradiol levels very low and unable to stimulate the expression of PR in the MPN and VMH, both critical neuronal areas where estradiol and progesterone promote proceptive behavior and lordosis reflex, respectively (Sá *et al*., 2018). The duration of behavioral estrus varies between individuals. Taking into account the importance of the reproductive development in acquisition of fertility, it is important to investigate on the contaminants capable of affecting the hormonal balance of an organism, since they can compromise fertility (Perera *et al*., 2003). Many researches have shown the implication of alterations of sexual behavior to physiological disorders and female infertility (Yakubu and Olutoye, 2016).

In female, ovaries, uterus, fallopian tubes, and a normal endocrine system are essential for fertility and normal hormone production. (Silva *et al*., 2023). To better evaluate the endocrine disrupting effects of potassium sorbate and tartrazine, fertility tests were carried out. Embryo susceptibility to teratogenesis decreases as tissue differentiation proceeds, and the embryo as a whole is resistant to teratogens once organogenesis is completed (McQueen, 1972; Hashem *et al*., 2019). Results from this study showed that exposure to these substances from PND 21 to the parturition caused maternal toxicity and impairment in fetal development. The daily oral administration of potassium sorbate and tartrazine was responsible for the decrease of fertility index, gestational rate, implantation index and the increase of the resorption index, post-implantation loss rate as compared to the control group. Implantation, which is complex step indispensable in the establishment of a successful pregnancy (Hiremath *et al*., 1999; Awounfack *et al*., 2018) occurs 4 to 8 days after fertilization in human and rodents (Oludare and Iranloye, 2016; Awounfack *et al*., 2018). It appears that, oxidative stress can compromise fertility during this critical period and induces pregnancy complications such as spontaneous abortion, recurrent pregnancy loss (Sekhon *et al*., 2010; Hasson, 2020). Many researchers have demonstrated that excessive of oxidative stress in ovary tissues which negatively reflect on ovarian steroidogenesis is responsible for antizygotic, blastocytotoxic or anti-implantation activity leading to the implantation loss (Hashem *et al*., 2019; Hasson, 2020; Spiers *et al*., 2014). Oxidative stress can stimulate the brain secretion of stress hormone in the hypothalamic-pituitary axis and the female reproductive organs altering ovarian functions and causing deleterious effects during gestation period on the developing fetus (Chukwuebuka *et al*., 2020). Such alterations are responsible for altered physiology such as angiogenesis, maturation, blastocysts development, fertilization of ovum, implantation of ovum, and maintenance of pregnancy (Gupta *et al*., 2006; Lu *et al*., 2018; Chukwuebuka *et al*., 2020). The pathophysiology of infertility linked to oxidative stress has shown that increased production of free radical damage the reproductive function of the ovary (Behrman *et al*., 2001; Chukwuebuka *et al*., 2020), uterine tubes and embryos (El-Mouatassim *et al*., 1999; Chukwuebuka *et al*., 2020), increase the embryonic resorption (Gupta *et al*., 2006; Chukwuebuka *et al*., 2020) and irregular cycle (Saraswathi *et al*., 2010; Chukwuebuka *et al*., 2020; Ding *et al*., 2022). Because neuroendocrine changes necessary for initiating the best pregnancy outcome depend on number and timing of intromissions before ejaculation during mating (Erskine 1985), the effects of potassium sorbate on proceptive behavior may influence the reproductive function of females. Potassium sorbate and tartrazine may have altered the rate of conception through the oxidative stress responsible for the puberty disturbance, increased acyclicity cycle and impairment of sexual behavior. In addition to the oxidative stress, the

regulation of reproduction is under the control of the ovarian hormones (estrogens and progesterone) and any dysregulation in the equilibrium level of these hormones may alter implantation resulting infertility (Awounfack *et al*., 2018; Stanojlović *et al*., 2023). All of these changes may impair oocyte maturation, increase the frequency of anovulatory cycles, and ultimately lead to disturbed puberty onset, reduced female fecundity, and premature ovarian failure (Stanojlović *et al*., 2023).The literature has confirmed the ability of xenoestrogen to disrupt the steroid secretion and effects. Xenoestrogen are capable of binding to oestrogen receptors causing hormonal disorders responsible for adverse pregnancy outcomes (Patisaul *et al*., 2006; Siepmann *et al*., 2011) including placental dysfunction, prematurity, and alteration in the intra-uterine growth, which can persist across multiple generations without additional exposure to the toxic (Jorge *et al*., 2021). It has also been demonstrated that during conception, synthesis and release of estrogens is essential for establishment of pregnancy and, endometrial exposure to estrogen before the normal conceptus secretion results in total pregnancy loss (Watcho *et al*., 2009). The increase in post-implantation loss rate observed with tartrazine may be attributed to its oxidative and estrogenic properties. The oestradiol molecule, one of the various compounds implicated in the inductive effects of tartrazine, controls multiple physiological functions not only at ovarian levels but also in the hypothalamus, pituitary, bone and uterus. It plays crucial role in uterine smooth muscle contraction and could thus improve or affect eggs nidation or embryo resorption (Telefo *et al*., 2012). Tartrazine exhibited estrogenic activity as shown by the significant increase percentage of female with vaginal opening, uterine weight and thickness of endometrium and folliculogenesis in immature rats. Significant decreases in the implantation index as well as post implantation losses were recorded in this study could be attributed to the fast migration of fertilized eggs through the oviduct. This fast migration is stimulated by the contraction of oviduct and uterine smooth muscle which are under high oestrogenic milieu, following the induction of ovarian folliculogenesis or steroidogenesis by tartrazine. In such conditions, the eggs will enter the uterine lumen in an immature state and could not easily adhere to the endometrium (Telefo *et al*., 2012). An in vivo study in rat fetuses following daily oral administration of tartrazine to pregnant Wistar rats at the 6th-15th day of gestation indicated that tartrazine induces fetal resorption, fetal mortality and damages preimplantation embryo development and embryo quality through the induction of oxidative stress (Hashem *et al*., 2019). These data corroborate with our findings and reinforce the toxicity of tartrazine to female reproductive system. Endocrine disrupting effects were evident for both potassium sorbate and tartrazine, with the range-finding study showing puberty disturbance, acyclicity, sexual behavior impairment and reduce fertility.

Studies have shown that exposure to synthetic chemicals during the period when the reproductive organs and the CNS are undergoing rapid and irreversible developmental changes can interfere with the endocrine system and other vital systems during (Colborn *et al*., 1993; Vom Saal *et al*., 1995; Chung *et al*., 1992; Hashem *et al*., 2019). It is known that during the period where cellular differentiation occurs (organogenesis), the sensibility of embryo makes it most sensitive to various external and internal factors, the ones decrease as tissue differentiation proceeds (Hashem *et al*., 2019). The results obtained contribute to this evidence and revealed the multigenerational effect of maternal exposure to potassium sorbate and tartrazine with special focus on the reproductive disorders in female offspring. In general toxicity investigation, it has been shown that the body weight (Samuel *et al*., 2014**;** Jorge *et al*., 2021) and organ weight (Samuel *et al*., 2014; Njamen *et al*., 2015; Tassinari *et al*., 2021) are sensitive indicators of potentially toxic chemicals. The maternal exposure to potassium sorbate and tartrazine compromised an important marker related to fetal programming, evidenced by a decrease in the body weight of all female descendants. As a result, oral administration of potassium sorbate and tartrazine to immature female from F1, F2, and F3 generations was responsible for an increase (PS 12.5, F1; PS 45, F3) and decrease (PS 12.5, F3; PS 45, F2; PS 78, F1, and F3; T 7.5 all the generations; T 27, F3; T 47, F1, and F3) in the body weight gain, maternal weight gain and fetuses weight at GD20. Administration of potassium sorbate and tartrazine induced embryotoxicity and teratogenicity as indicated by decrease in maternal and fetuses body weight. In this study, the female's descendants also presented changes in other estrogen-dependent parameters, such as a delay in puberty onset with potassium sorbate (PS 12.5 F1) and an early puberty onset with tartrazine (T 47 F1), demonstrating an alteration in hormonal response of these substances. Potassium sorbate at the third generation was responsible for the decrease of LH serum concentration at all the tested doses. This result confirms potassium sorbate can inhibit the hypothalamus. As observed in F0, these findings obtained with potassium sorbate and tartrazine may have been provoked by the inhibition of the hypothalamus through the oxidative stress (Reilly *et al*., 2010) of potassium sorbate and xenoestrogenicity capacity (Kriszt *et al*., 2015) of tartrazine induced by the maternal exposure to these chemicals. The delay puberty and precocious puberty observed with potassium sorbate and tartrazine at the first generation is similar to the result obtained in the parents. It has been shown that the body weight of an individual is often reported to affect the onset of puberty in both the human and rodent (Reinehr *et al*., 2017). Many researchers have demonstrated that the increasing energy intake can accelerated the events associated with the onset of puberty in female rats. In the same hand, Reinehr and colleagues (2017) demonstrated that the decrease in overweight children is associated with later onset of puberty in girls (Reinehr *et al*., 2017).

Endocrine disruptors can also have an impact on reproductive organs. Maternal exposure to potassium sorbate and tartrazine also resulted in an adverse reproductive effect on the females of F1, F2, and F3 generations, which was manifested by the disruption of the growing of the reproductive organs. The treatment was responsible for a decrease of relative weight of uterus with PS 12.5, F1 and of the vagina epithelial thickness of rats with potassium sorbate at the dose of 78 mg/kg BW and tartrazine at the doses 7.5 and 47 mg/kg BW in all the generations. The treatment decreased the vagina epithelial thickness of rats with potassium sorbate at the dose of 78 mg/kg BW and tartrazine at the doses 7.5 and 47 mg/kg BW in all the generations, disrupted the growing of the reproductive organs. Studies have demonstrated that epigenetic modifications can be changed by environmental factors, can be lost during gametogenesis or affected by gene-environmental interactions, others can be passed on to the next generation (Jorge *et al*., 2021) as occurred in this study. In relation to fertility and reproductive performance, the female offspring of the maternal females exposed to the potassium sorbate and tartrazine showed altered folliculogenesis, reproductive hormone secretion, sexual behavior and fertility. These include decrease and increase in the body weight during pregnancy, number of live fetuses, uterine plus fetus weight, increase in placenta weight and decrease in pup's weight. These results corroborate those obtained by Hashem *et al*., 2019 and EFSA *et al*., 2019 who demonstrated that exposure to potassium sorbate and tartrazine to pregnant females is responsible for reproductive toxicity. Our results also demonstrated that the administration of potassium sorbate and tartrazine to F0, significantly reduces the sex ratio at all the tested doses ($p < 0.001$). We have obtained the same results with potassium sorbate at the dose of 45 mg/kg BW in F3. According to the literature, boys are more numerous than girls at birth and the sex ratio at birth (SRB) is around 105 male births per 100 female births (Vahidi and Sheikhha, 2007). The decrease in sex ratio can then be related to higher male mortality. The results suggest that potassium sorbate and tartrazine are responsible for male mortality; the one can justify the reduced fertility observed. Endocrine disrupting effects were evident for both potassium sorbate and tartrazine, with the range-finding study showing puberty and sexual behavior alterations, post-implantation loss, resorptions, and perinatal death, thus confirming previous studies. Endocrine disrupting effects were evident for both potassium sorbate and tartrazine, with the range-finding study showing puberty and sexual behavior alterations, post-implantation loss, resorptions, and perinatal death, thus confirming previous studies.

CONCLUSION, RECOMMANDATIONS AND PERSPECTIVES

CONCLUSION

The contribution of food additives on the disruption of endocrine system and declining fertility represents a topic of intense debate. Given the widespread use of potassium sorbate and tartrazine in food industry and their adverse effects on some reproductive parameters, it was essential to investigate their potential endocrine disrupting activity following exposure during the "periods of vulnerability" and the consequences on fertility. This work aimed to study the effects of potassium sorbate and tartrazine on the activation of the hypothalamic-hypophysis-ovarian axis, sexual behavior and the fertility of the parents, first, second and third generations. The doses 78 mg/kg BW and 47 mg/kg BW (AED) are corresponding to admissible daily intake in human 12.5 mg/kg BW for potassium sorbate and 7.5 mg/kg BW for tartrazine respectively. In conclusion, the alterations in development, behavior and reproductive performance of female and their progeny by exposure to potassium sorbate and tartrazine in this study, indicated that early life and transplacental exposure to higher doses of potassium sorbate (78 mg/kg body weight) and tartrazine (47 mg/kg body weight) can affect the onset of puberty, resulting in sexual behavior and fertility alteration and can compromise post-natal development, behavior and fertility output of the female progeny. Critical and sensitive windows of susceptibility to potassium sorbate and tartrazine include early life, puberty and pregnancy and should be overlooked to avoid adverse developmental outcomes, deterioration in behavior and decline in fertility in adults. Taken all together, these results might justify in part the increasingly early and delay puberty and decline fertility observed in our populations and provide a substantial scientific prove confirming potassium sorbate and tartrazine as endocrine disruptors.

Recommendations

The current study demonstrated that potassium sorbate and tartrazine can disrupt the normal of endocrine system. The results suggest revising and reducing the current authorized daily intake (ADI) of potassium sorbate to 7.5 mg/kg BW (corresponding to 45 mg/KGBW) of tartrazine to 4.5 mg/kg BW (corresponding to 27 mg/KG BW) as well as ensuring strict respect of these ADI in our community.

Perspectives

Thus, there is an emergent need:

- To assess the effects of combinatory exposure to potassium sorbate and tartrazine on the reproductive system,
- To assess the effects of potassium sorbate and tartrazine on teratogenesis,

- To assess the effects of potassium sorbate and tartrazine on the integrating neural circuit controlling female sexual behavior,
- To assess the effects of potassium sorbate and tartrazine on reproductive system immature males Wistar rats and assess the combinative exposure of males and females,
- To assess the effects of a natural food dye (such as *Beta vulgaris*) and food preservative (such as *Ocimum gratissimum*) on the male and female reproductive system,
- To formulate a food dye and preservative with no adverse effects on the reproductive system. To formulate a food dye and preservative with no adverse effects on the reproductive system.

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ANNEX

ALCOHOL DILUTION TABLE

REDUCTION TABLE [DILUTION OF ALCOHOL TO LOWER STRENGTHS]

Desired strength	Strengths to be Reduced (% v/v)																
% v/v	95%	90%	85%	80%	75%	70%	65%	60%	55%	50%	45%	40%	35%	30%	25%	20%	15%
	Volume Units of Pure Water to Add to Each 100 Volume Units of Alcohol																
90%	6.41																
85%	13.33	6.58															
80%	20.9	13.8	6.83														
75%	29.5	21.9	14.5	7.2													
70%	39.1	31.0	23.1	15.3	7.64												
65%	50.2	41.5	33.0	24.6	16.4	8.15											
60%	63.0	53.6	44.2	35.4	26.5	17.6	8.76										
55%	78.0	67.8	57.9	48.0	38.3	28.6	19.0	9.5									
50%	95.9	84.8	73.9	63.1	52.4	41.7	31.3	20.5	10.4								
45%	118	105	93.3	81.3	69.5	57.8	46.0	34.5	22.9	11.4							
40%	144	131	117	104	90.8	77.6	64.5	51.4	38.5	25.6	12.7						
35%	179	163	148	133	118	103	88.0	73.0	58.3	43.6	29.0	14.4					
30%	224	206	189	171	154	136	119	102	85.0	67.5	50.5	33.5	16.7				
25%	287	266	245	224	204	183	162	142	121	101	80.4	60.2	40.0	20.0			
20%	382	356	330	304	278	253	227	201	176	150.6	125.2	100	75.0	49.9	24.9		
15%	540	505	471	437	403	369	335	301	267	234	200	166.4	133	96.7	66.4	33.2	
10%	855.6	804	753	703	652.2	602	551	500.6	450.2	400	350	300	249.4	199.4	150	100	50

Alcohol:water mixtures are non-volume additive mixtures. This table may be used to determine the volume of water needed to reduce known volume strengths of alcohol solutions (such as distilled spirits for sensory evaluation or the cutting of spirits to desired final strengths) to desired volume/volume strengths.

EXAMPLE CALCULATION OF DATA: [Requires table 6 - or its equivalent - from the US Gov. Gauging Manual: "Respective Volumes of Alcohol and Water".
In reducing 95% (vol.) to 85% (vol) first convert to Proof (US), i.e. x Vol. by 2. Then see US Gauging Manual - Table 6.
190 Proof is 95% (vol.) Ethanol and 6.18% (vol.) H_20. 170 Proof is 85% (vol.) Ethanol and 17.46% (vol.) H_20.
Ratio: 95/85 = 1.1176471*--then-- 17.46% (vol. water in 85% vol/vol alcohol water mixture) x 1.1176471* = 19.514118*
Next: 19.514118* - 6.18 = 13.33; Dilution is now 100 Units [e.g., gallons, mL. etc.] of the initial alcohol solution to 13.33 Units of water. *[Significant figures not accounted for.] ***Prepared by Gary Spedding - Brewing and Distilling Analytical Services, LLC. gspedding@alcbevtesting.com***

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Effects of Tartrazine on Some Sexual Maturation Parameters in Immature Female Wistar Rats

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Abstract: Over the past century, the average age for onset of puberty has declined. Several additives present in our food are thought to contribute significantly to this early puberty which is recognized to also affect people's health in later life. On this basis, the impact of 40-days unique oral administration of the food dye tartrazine (7.5, 27, and 47 mg/kg BW doses) was evaluated on some sexual maturation parameters on immature female Wistar rats. Vaginal opening was evaluated during the treatment period. At the end of the treatments, animals were sacrificed (estrus phase) and the relative weight of reproductive organs, pituitary gonadotrophin and sexual steroids level, cholesterol level in ovaries and folliculogenesis were evaluated. Compared to the control group, animals receiving tartrazine (47 mg/kg BW) showed significantly high percentage of early vaginal opening from day 45 of age, and an increase in the number of totals, primaries, secondaries, and antral follicles; a significant increase in serum estrogen, LH and in uterine epithelial thickness. Our findings suggest that tartrazine considerably disturbs the normal courses of puberty. These results could validate at least in part the global observations on increasingly precocious puberty in girls feeding increasingly with industrially processed foods.

Keywords: food additive; tartrazine; rat; early puberty; folliculogenesis; endocrine disruptor

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1. Introduction

Puberty is a major developmental event at the end of the juvenile stage, with marked physical and psychological changes, which prepare for adulthood [1,2]. It can be seen as a complex sequence of biological events marked by the reactivation of the hypothalamus-pituitary-gonadal axis after a period of quiescence during childhood; followed by an important increase in sex hormone secretion by the gonads, which leads to a gradual maturation of sexual characteristics which culminate into the attainment of full adult reproductive capacity [1–3]. The onset of secondary sexual characteristics, and the pubertal growth spurt, are markers of this developmental process that lead to sexual and reproductive maturity, the development of mental processes and adult identity [4]. Puberty is accompanied by bodily changes, encourages curiosity, promotes interest in sexual activity, increases aggression in adolescents, and can intensify risky behaviors [3]. In the early part of this century, the timing of puberty has received considerable attention because of its associations with health problems such as the increased risk of developing reproductive cancers (breast and prostate cancer), weight gain, obesity and cardiovascular disease later

in life. It is also associated with psychosocial problems (depression) and is a risk factor for teenage pregnancy [5,6].

Precocious puberty, or abnormally early sexual development, is defined as the premature onset of pubertal development or secondary sexual characteristics before the age of 8 years in girls and 9 years in boys [4,7]. In girls, it is manifested by features such as advanced breast and ovarian development and rapid bone growth or maturation. It can be attributed to endocrine disorders, with elevated sex hormone secretion [4]. Most recent data consistently indicate that the age of onset of puberty is becoming earlier in Europe and the USA [1] than in Africa [2]. This decrease concerns both the mean age at menarche and the mean age at onset of breast development, which has decreased in all ethnic groups [7–9]. These geographical variations indicate an earlier onset in the USA (8.8–10.3 years) and later onset in Africa (10.1–13.2 years) [2]. This may be the result of more stable socio-economic conditions, improved nutrition, and hygiene, as has been observed in industrialized populations. The prevalence of precocious puberty is about 10 times higher in girls than in boys [1,6].

The exact mechanisms underlying the reactivation of the hypothalamus-pituitary-gonadal axis are not fully understood; however, it is assumed that genetic, nutritional, stress-related, and environmental factors influence the onset of puberty [2,6,10]. These include environmental factors such as weight, fetal nutrition, childhood eating habits, physical activity, psychological factors, and exposure to electromagnetic fields and/or endocrine disrupting chemicals [7]. It has been reported that dietary habits appear to significantly influence the mechanism of estrogen metabolism, which is inextricably linked to early puberty [11,12]. Some animal studies have suggested that postnatal overfeeding tends to invariably increase secretion of luteinizing hormone (LH), follicle stimulating hormone (FSH), leptin and insulin levels in pubertal females [13]. Overeating, including excessive consumption of processed foods, is considered the main agent responsible for the secular decline in the pubertal age [13,14]. Today, the growth of consumption of these processed foods is the source of a lively debate about the release of toxins (in concentrations and varieties) into the environment, which damages the endocrine system not only of animals but also that of man [15–17]. Thousands of them are widely distributed in food, water, air, and certain industrial products (drugs, cosmetics, and phytosanitary products among other) [15,16,18]. These so-called hormonal (or anti-hormonal) environmental contaminants are designated by the term endocrine disruptors [19].

Many concerns (such as early puberty, declining fertility and cancers) are emerging about the long-term effects on human health following chronic exposure to these substances [16,20–22]. Children are more at risk because of intensive use of food additives [16,20,21]. The increased cases of gynecomastia are mostly attributed to food additives ("fast food", and drinks,) [23]. Unfortunately, few studies on their effects on women's reproductive function are available. In addition, compared to boys, alterations in the female tract are likely to remain invisible until they reach sexual maturity (puberty).

The reproductive maturation which is a prerequisite of fertility [24] is usually marked in female rats by the vaginal opening [25,26]. The estrous cycle following, is divided in four main phases including proestrus, estrus, metestrus and diestrus characterized by different cell types desquamated from the vaginal epithelium, the presence or absence of leukocytes and mucus in vaginal smears [27–29]. Ovulation (sexual receptivity or heat) occurs during the night of the estrus phase after the luteinizing hormone (LH) surge [29,30].

Faced with the hypothesis that food additives contribute to current decline of the age of puberty onset, it becomes urgent not only to identify and quantify each substance that is used as food additives in our diet but also to evaluate the potential endocrine disrupting activity of food additives at concentrations lower than or equal to their threshold of toxicity [31]. In line with this, the present study was designed and carried out to evaluate the potential endocrine disruptor activity on some parameters of sexual maturation in immature female Wistar rats with their hypothalamic-pituitary-ovarian still nonfunctional.

In addition, the animal's diet was soy free, to eliminate any interference with natural phytoestrogens [26,32].

To bring out our contribution, one of the most used dyes (tartrazine) has aroused our interest and justifies its use in this study. Known in some cases as E102, FD and C Yellow 5, C.I 19140, acid Yellow 23, Food yellow 4 or trisodium 1-(4-sulfonatophenyl)-4-sulfonatophenylazo)-5pyrazolonz-3-carboxylate, it is a synthetic lemon-yellow azo dye made by coal tar [33,34], with the chemical formula: 4-5-Dihydro-5-oxo-1-(4-sulfophenyl)4-((4-sulfophenyl) aso) 1H-pyrazole-3 carboxylic acid [35]. In Human, the daily mean exposure of tartrazine is 7.5 mg/kg BW equivalent 47 mg/kg BW in rats [36,37]. Tartrazine is an orange water soluble powder very widespread (drinks, cookies, confectionery, preserves, yogurts, cosmetics, drugs, etc.) used as a dye. It has been shown in previous in vitro studies to be responsible for allergies, tumor diseases, mutagenic and genotoxic effects, and neuro- behavioral disorders (hyperactivity and sleep disturbance in children) [22,38–40]. Prolonged usage of tartrazine increases the number of gastric mucosa lymphocytes and eosinophils [41]. Several studies showed that, this dye has adverse effects on male reproduction especially on sperm parameters (negative impact on sperm maturation process and decrease in sperm density, mobility and viability) [33,42]. These effects are accompanied by a significant decrease in serum testosterone concentration [42]. However, the combined treatment of tartrazine and erythrosine mixture in adult male rats impair testicular architecture and function and is accompanied by an increase in a serum hormone (LH, FSH and testosterone) [43]. In female rats, the frequent intake or increased of tartrazine affect thyroid and reproductive hormones (LH, FSH, estrogen, progesterone) and mineral content in tissues; increases the chances of free radical production, leading to the development of oxidative stress in the body [34]. Tartrazine has also been classified as a xenoestrogen [44,45] that can bind to Estrogen Receptor α (ERα) in the Michigan Cancer Foundation-7 (MCF-7) cell line and induce a proliferative effect in breast cancer cells and increase the expression of an estrogen reporter gene [46]. Despite the multiple effects of tartrazine, especially on reproductive hormones [34,42–45] which are responsible of sex maturation, such as folliculogenesis, ovulation, reproductive behaviors and successful of pregnancy, there is still a lack of available information about its harmful effects on female reproductive function in juvenile. However, no study has evaluated the effects of certain products (tartrazine), considered as potential endocrine disruptors on the parameters of sexual maturation. Therefore, this work aimed at assessing the effects of tartrazine on the physiological parameters allowing the onset of puberty (age of vaginal opening), production of pituitary gonadotropins (FSH and LH), and sex steroids (estradiol and progesterone) to assess their ability to stimulate the hypothalamic-pituitary-ovarian axis; and further, to evaluate their effects on folliculogenesis, on the growth of the reproductive organs (ovaries, vagina, and uterus) in a model of immature female Wistar rats.

2. Materials and Methods

2.1. Chemicals

Tartrazine (CAS 1934-21-0, Purity $\geq$ 85%), was purchased from Sigma Aldrich (Munich, Germany). In this study, the doses of 7.5, 27, and 47 mg/kg BW were extrapolated according to the recommendations of daily doses administered by the World Health Organization [47,48].

2.2. Animals and Housing

In this case, 21 and 22-day-old immature female Wistar rats (average weight of 30 g) were kept in the animal house with a 12 h of the light-dark cycle. These animals were bred in the laboratory of animal physiology, University of Yaoundé I (Yaoundé, Cameroon) under natural conditions and had free access to diet and drinking water *ad libitum*. Animals housing and experiments were carried out according to the guidelines of the Institutional Ethics Committee of the Cameroon Ministry of Scientific Research and Innovation (Reg. no.

FWA-IRD 0001954, 4 September 2006), which has adopted the guidelines established by the European Union on Animal Care CEE Council 86/609).

2.3. Dose and Concentation Calculation

In the literature, the human equivalent dose (HED) of tartrazine is 7.5 mg/kg BW per day [36,37]. The animal equivalent dose (AED: 47 mg/kg BW per day) was calculated on the basis of the body surface area, dividing the HED dose (mg/kg BW) by the ratio (km) provided by the literature (AED = HED/km; km = body weight (kg)/body surface area (m^2). The ratio km used in this work was 0.162 [34,49]. The third dose (27 mg/kg BW per day) used in this work was the mean of the HED and AED.

2.4. Experimental Design

The animals (20) were randomly divided into four groups of 5 animals each, a control group that received distilled water and the three test groups which received tartrazine at 7.5, 27 and 47 mg/kg BW. Tartrazine was dissolved in distilled water. The volume of water and substance administered was 10 mL/kg BW. All animals were orally treated by gavage once daily (between 9 and 10 am) for 40 days from the postnatal days 21 to 22. The animals were weighed twice a week and the vaginal opening which is the marker for puberty onset was daily checked until the day it occurred. From the day 36 of treatment (a day when there is vaginal opening in all the animals) until the fortieth day, the animals were sacrificed (in estrus) by decapitation after light anesthesia by diazepam-ketamine i.p. injection (10 and 50 mg/kg BW, respectively). Blood samples were collected for biochemical analysis in dry tubes. The ovaries, uteri, mammary gland and vagina, were dissected and weighed (except the vagina and mammary gland which were immerse immediately in formol). The left ovary and uterus from each animal, as well as the vagina, and mammary glands were fixed in 10% formaldehyde for histological analysis. The right ovary and right uterus were cut, weighed and ground separately with the glass potter's in sodium phosphate buffer (0.1 M; pH 7.1) to obtain a final homogenate of 20%. After centrifugation at 3000 rpm (Goget Centrifuge, HETTICH, Westphalia, Germany) for 15 min at 5 °C, the collected supernatant was stored at −20 °C for subsequent determination of total uterine and ovarian proteins, and ovarian cholesterol. Blood samples collected in dry tubes were also centrifuged at 3000 rpm at 5 °C for 15 min and the serum obtained was kept at −20 °C until use.

2.5. Measurement of Biochemical Parameters

Serum and homogenates of the uterus and ovary were used for biochemical analysis. In the serum, follicle Stimulating Hormone (FSH), (Luteinizing Hormone (LH), Estradiol, and Progesterone were measured in duplicate using the ELISA technique and reagent kits obtained from Cypress Diagnostics (Langdorp, Belgium) according to the manufacturer's instructions, and precise (intra and inter essay coefficients of variability) with CV $\leq$ 9.5645% for all the tested samples. Whatever, the total cholesterol in ovaries were measured using reagent kits from Chronolab Systems (Barcelona, Spain).

2.6. Histopathological Evaluation

In addition, 5 µm thick sections of paraffin-embedded tissues (uterus, vagina, and ovaries) were prepared and stained with hematoxylin-eosin. The photomicroscopic observation/analysis (uterine and vaginal epithelial thickness, identification of ovarian follicles) was performed using a complete set of Zeiss (Hallbermoos, Germany) equipment (microscope Aioskop 40), the software programs MRGrab 1.0 (Carl Zeis, Hallbermoos, Germany, 2001) and Axio Vision 3.1 (Carl Zeis, Hallbermoos, Germany, 2001) installed in a computer. As concerns folliculogenesis, the tenth section of each ovary was selected. We considered as primary the follicles composed of oocytes surrounded with one layer of cuboidal follicular cells, secondary preantral follicles those with more than one follicular cell layer, and antral follicles those with present antrum of follicular fluid. Ruptured follicles with hypertrophied follicular, cells cavity, and cavity filled with blood were considered as corpora lutea.

2.7. Statistical Analysis

Data were expressed as mean ± standard error on the mean (SEM). A two-way ANOVA repeated measures followed by Bonferroni post-hoc tests was used to compare the effect of tartrazine on body weight and the percentage of animals with vaginal opening. The fixed effects or factors were treatment (each individual dose of tartrazine vs. control group), time or periods of analysis, and their interaction. ANOVA one-way followed by Dunnet's test (when appropriate) was used for the other data with treatment as a fixed effect. All of these tests were performed using GraphPad Prism 5.03 software (La Jolla, CA, USA, 2009). Differences were considered significant at $p < 0.05$.

3. Results

3.1. Bodyweight of Animals

Figure 1 shows the effect of tartrazine exposure on body weight evolution throughout the period of treatment. Two-way ANOVA indicated a significant time effect (F = 108.3; $p < 0.0001$; df = 13), a non-significant treatment effect (F = 0.2327; $p = 0.8736$; df = 3) and a non-significant interaction effect (F = 0.1026; $p > 0.9999$; df = 39). In addition, Bonferroni correction multiple comparison test indicated that all doses of tartrazine were neither effective ($p > 0.9999$) in increasing, nor reducing body weight suggesting that the significant time effect observed is due to the normal growth of animals over time.

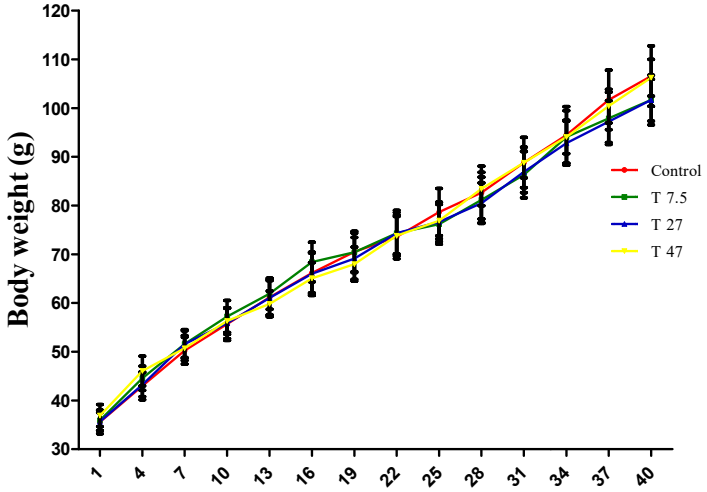

Figure 1. Effects of tartrazine on the bodyweight of immature female Wistar rats during 40 days of treatment. Control = animals receiving vehicle (distilled water, 10 mL/kg BW); T = rats treated with tartrazine at dose of 7.5; 27, and 47 mg/kg BW. Results are shown as a mean ± SEM, $n = 5$.

3.2. Vaginal Opening

The main endocrine effect on the onset of puberty is summarized in Figure 2. Compared to the control group, the treatment did not induce any significant modification in the mean age of the vaginal opening (Figure 2A) as determined by one-way ANOVA (F = 2.748, $p = 0.0541$). With respect to the percentage of animals with vaginal opening (Figure 2B), the two-way ANOVA showed significant time (F = 38.48; $p < 0.0001$; df = 18) and treatment (F = 10.83; $p < 0.0001$; df = 3) effects. Based on Bonferroni post hoc test, 47 mg/kg BW tartrazine showed a significant difference ($p < 0.05$) in the percentage of animals with vaginal opening as compared with the Control group. This group displayed 100% of vaginal opening vs. 41.66% for control on day 45.

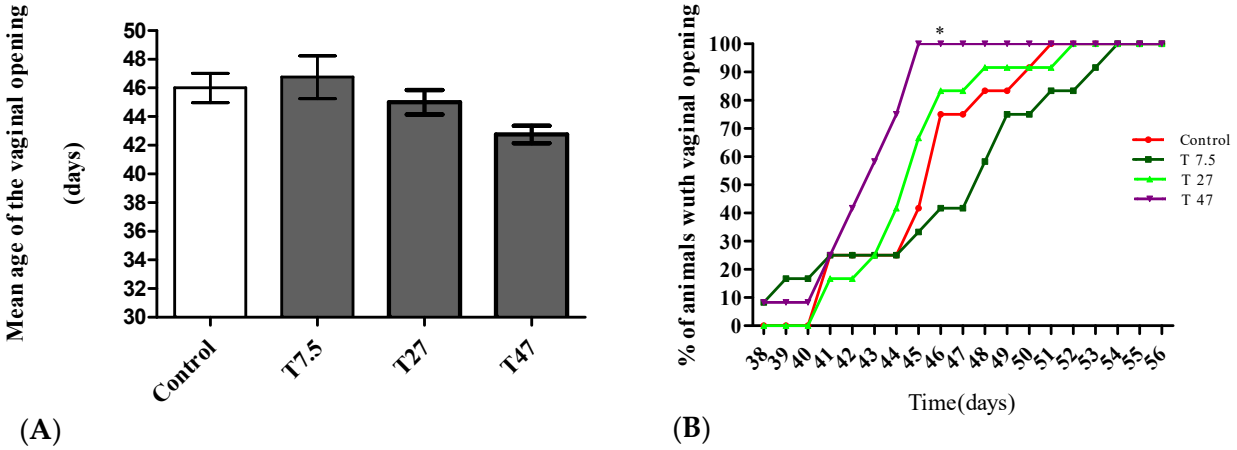

Figure 2. Effects of tartrazine on the mean age of the vaginal opening (**A**), the percentage (%) of rats with a vaginal opening (**B**) during 40 days of treatment. Control = animals receiving vehicle (distilled water, 10 mL/kg BW); T = rats treated with tartrazine at dose of 7.5; 27, and 47 mg/kg BW. Results are shown as a mean ± SEM, $n = 5$. *: $p < 0.05$ in reference to control.

3.3. Relative Weight of Ovary and Uterus

The main effects on reproductive organs are presented in Figure 3. The results of the relative uterine weight indicate that there is a statistically significant difference between groups as determined by one-way ANOVA (F = 6.098, $p = 0.0057$). Dunnett Post Hoc multiple comparisons test showed that the difference between tartrazine at the dose of 47 mg/kg BW (Figure 3B) and the control group is statistically significant ($p < 0.05$). Tartrazine increased significantly the relative uterine weight at the dose of 47 mg/kg BW (Figure 3B). Whatever, after 40 days of treatment, tartrazine had no significant effect on the relative weight of the ovaries (Figure 3A) as determined by one-way ANOVA (F = 2.793, $p = 0.0739$).

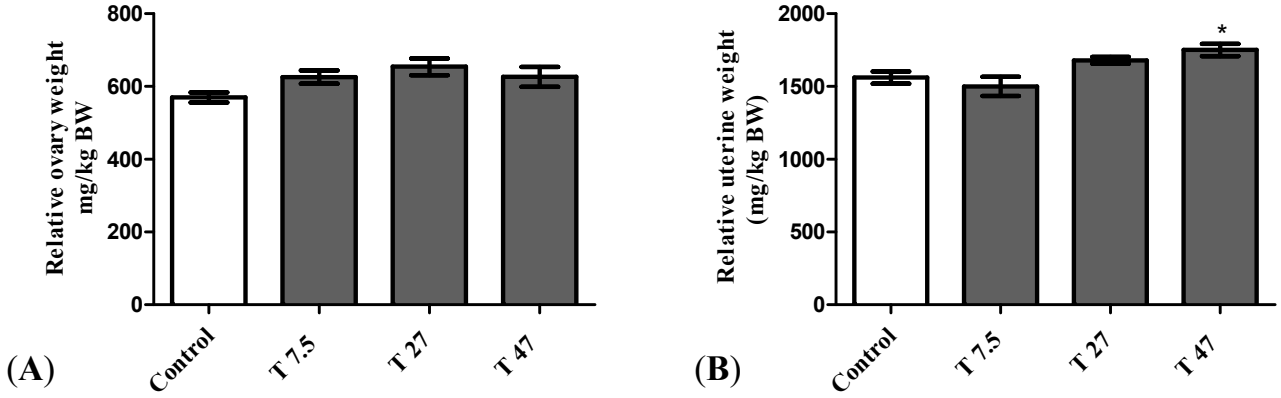

Figure 3. Effect of tartrazine on the relative weight of ovaries (**A**) and uterus (**B**) of immature female Wistar rats after 40 days of treatment. Control = animals receiving vehicle (distilled water, 10 mL/kg BW); T = rats treated with tartrazine at doses of 7.5; 27, and 47 mg/kg BW. Results are shown as a mean ± SEM, $n = 5$. *: $p < 0.05$ in reference to control.

3.4. Epithelial Thickness of the Uterus and Vaginas

The main reproductive effects are summarized in Figure 4. The results of the uterine epithelial thickness indicate that there is a statistically significant difference between groups as determined by one-way ANOVA (F = 6.602, $p = 0.0041$). Dunnett Post Hoc multiple comparisons test showed a significant difference between tartrazine at the dose of 27 ($p < 0.05$) and 47 mg/kg BW ($p < 0.01$) as compared to the control group (Figure 4A). This difference

is also confirmed by the microphotographs presented in Figure 4C. However, one-way ANOVA indicated that sacrificed at the estrus phase, the administration of tartrazine had no significant effect (F = 0.6609, p = 0.5880) on the vaginal epithelial thickness (Figure 4B).

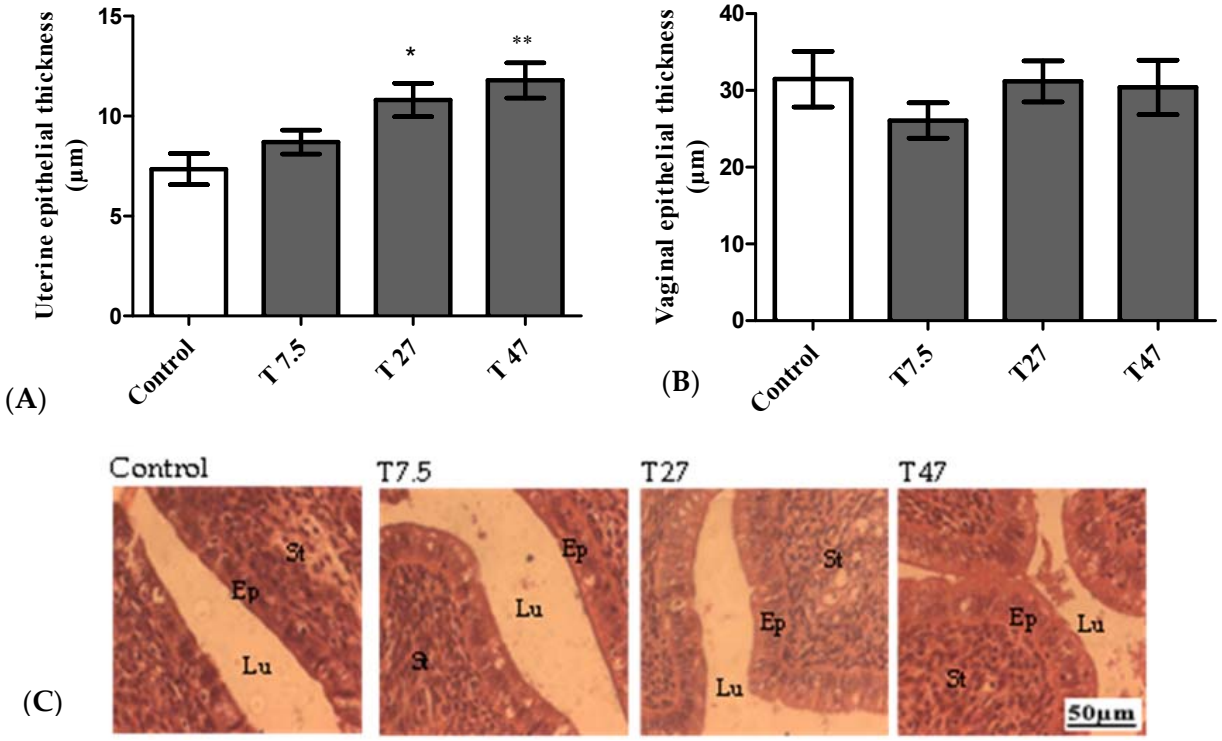

Figure 4. Effect of tartrazine on uterine (**A**) and vagina (**B**) epithelial height as well as microphotographs (25×) of hematoxylin/eosin-stained sections of uteri (**C**) of immature female Wistar rats after 40 days of treatment. Control = animals receiving vehicle (distilled water, 10 mL/kg BW); T = rats treated with tartrazine at doses of 7.5; 27, and 47 mg/kg BW. Results are presented as mean ± SEM; n = 5. *: $p < 0.05$; **: $p < 0.01$ compared to the control. Lu = Lumen; Ep = Epithelial cell thickness; St = Stroma.

3.5. Mammary Glands

The main effects on mammary glands are summarized in Figure 5. The microphotographs presented showed that, compared to the control group, tartrazine at the dose of 47 mg/kg BW induced eosinophilic secretions in the acinar of the mammary glands.

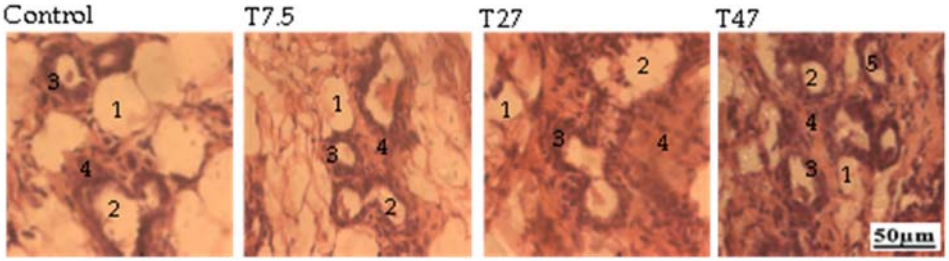

Figure 5. Microphotographs (25×) of hematoxylin/eosin-stained sections of mammary glands of immature female Wistar rats after 40 days of treatment. Control = animals receiving vehicle (distilled water, 10 mL/kg BW); T = rats treated with tartrazine at doses of 7.5; 27, and 47 mg/kg BW. 1 = Adipose tissue; 2 = Lumen of alveoli; 3 = Alveoli epithelium; 4 = Gland parenchyma; 5 = Eosinophilic secretion.

3.6. Ovarian Follicles

The main effects on follicular growth are summarized in Table 1 and Figure 6. Table 1 and Figure 6 show the number of total follicles and different types of follicles, and the microphotographs of ovaries after 40 days of treatment with tartrazine, respectively. One-way ANOVA indicated a significant difference between groups. The difference was reflected on the number of total follicles ($F = 8.831$, $p = 0.0011$), primary follicles ($F = 9.771$, $p = 0.0007$), secondary follicles ($F = 4.744$, $p = 0.0149$), and antral follicles ($F = 4.329$, $p = 0.0205$). Dunnett Post Hoc multiple comparisons test showed a significant increase in the total number of follicles ($p < 0.01$), primary follicles ($p < 0.01$), secondary follicles ($p < 0.01$), and antral follicles ($p < 0.05$) with tartrazine at a higher dose (47 mg/kg BW) as compared to the control group.

Table 1. Number of different ovarian follicles and corpora lutea of immature female Wistar rats after 40 days of treatment.

Organs	Control	Tartrazine (mg/kg BW)		
		T 7.5	T 27	T 47
Total follicles	92.98 ± 11.90	99.38 ± 11.80	83.49 ± 13.74	145.33 ± 2.37 **
Primordial follicles	35.33± 7.83	28.00 ± 5.42	21.33 ± 3.59	38.00 ± 3.83
Primary follicles	22.00 ± 2.60	20.33 ± 2.93	18.66 ± 3.00	37.50 ± 2.53 **
Secondary follicles	16.50 ± 1.20	30.40 ± 5.76	28.25 ± 8.29	44.25 ± 2.37 **
Antral follicles	4.20 ± 0.96	6.25 ± 1.23	4.80 ± 1.59	10.33± 1.42 *
Graafian follicles	5.00 ± 1.04	6.80 ± 0.8	4.80 ± 1.01	6.50 ± 0.92
Atresia follicles	4.75 ± 0.19	3.80 ± 0.66	3.25 ± 0.58	3.75 ± 0.91
Corpora lutea	5.20 ± 1.20	3.80 ± 1.11	2.40 ± 0.60	5.00 ± 0.83

Results are presented as mean ± SEM; (*: $p < 0.05$), (**: $p < 0.01$) in reference to control. Control = animals receiving vehicle (distilled water, 10 mL/kg BW); T = rats treated with tartrazine at doses of 7.5; 27, and 47 mg/kg BW.

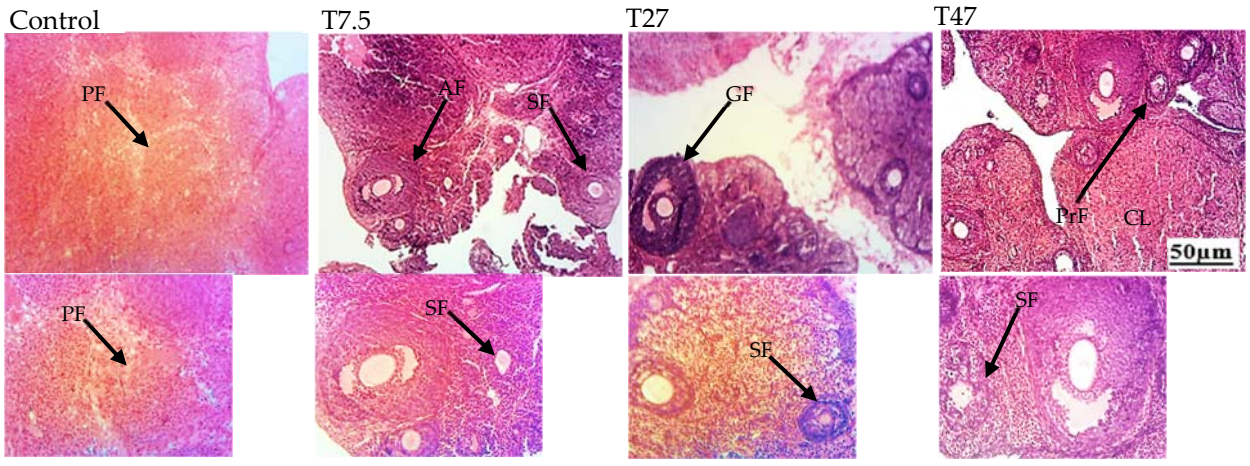

Figure 6. Microphotographs (25× and 200×) of hematoxylin/eosin-stained sections of ovaries of immature female Wistar rats after 40 days of treatment. Control = animals receiving vehicle (distilled water, 10 mL/kg BW); T = rats treated with tartrazine at dose of 7.5; 27, and 47 mg/kg BW. Results are shown as a mean ± SEM, $n = 5$. PF = primordial follicle, PrF = primary follicle, SF = secondary follicle, AF = Antral follicle, GF = Graafian follicle, CL = corpora lutea.

3.7. Ovarian Total Cholesterol and Proteins

Figure 7 represents the ovarian total cholesterol and protein after 40 days of treatment. One way ANOVA indicated that ovarian total cholesterol ($F = 0.6152$, $p = 0.6151$) and protein ($F = 0.6086$, $p = 0.6190$) were not significantly affected following treatments (Figure 7).

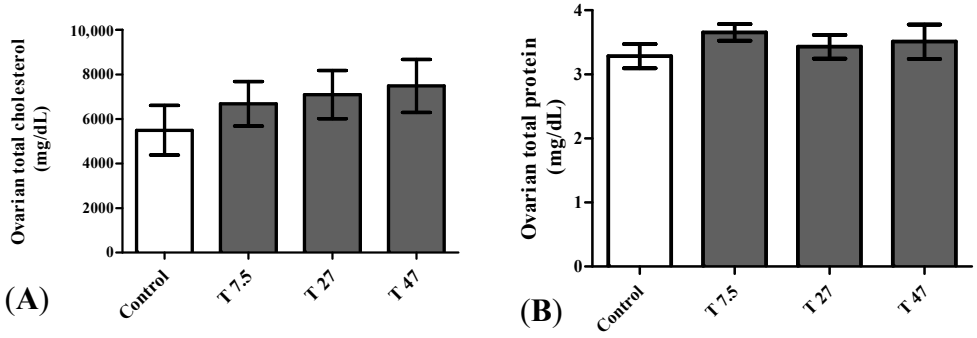

Figure 7. Effect of tartrazine on ovarian total cholesterol (**A**) and ovarian total protein (**B**) of immature female Wistar rats after 40 days of treatment. Control = animals receiving vehicle (distilled water, 10 mL/kg BW); T = rats treated with tartrazine at doses of 7.5; 27, and 47 mg/kg BW. Results are shown as a mean ± SEM; $n = 5$.

3.8. Hormone Levels

The main effects on hormone serum concentrations are summarized in Figure 8. One-way ANOVA indicated a significant difference between groups. The difference was reflected on the LH serum concentration (F = 10.85, $p = 0.0004$), and estradiol serum concentration (F = 130.1, $p < 0.0001$). Dunnett Post Hoc multiple comparisons test showed a significant increase in LH ($p < 0.001$) (Figure 8B) and Estradiol ($p < 0.001$) (Figure 8C) serum concentration at the dose of 47 mg/kg BW as compared to the control group. However, FSH (F = 2.619, $p = 0.0866$) and Progesterone (F = 0.2903, $p = 0.8318$) serum concentrations were not significantly affected by the treatment with tartrazine at all tested doses (Figure 8A,D).

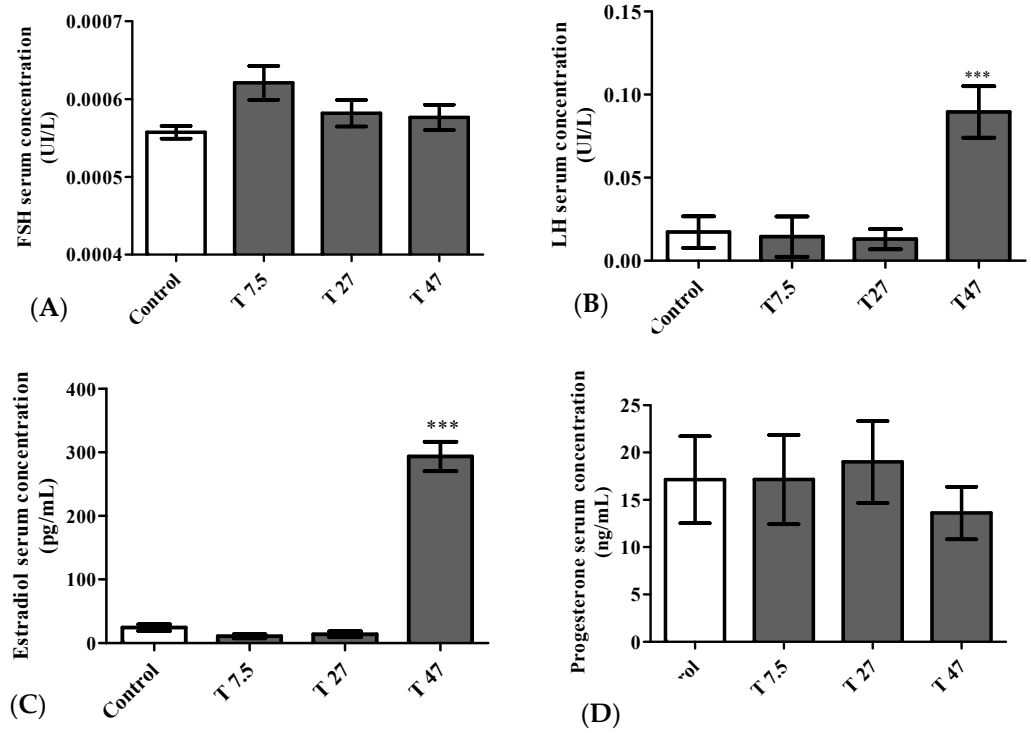

Figure 8. Serum concentration of Follicle Stimulating Hormone (FSH) (**A**), Luteinizing Hormone (LH) (**B**), Estradiol (**C**) and Progesterone (**D**) of immature female Wistar rats after 40 days of treatment. Control = animals receiving vehicle (distilled water, 10 mL/kg BW); T = rats treated with tartrazine at doses of 7.5; 27, and 47 mg/kg BW. Results are shown as a mean ± SEM; $n = 5$. ***: $p < 0.001$ in reference to control.

4. Discussion

The food additives present in industrial processed food (in the form of preservatives and dyes) are more and more pointed out as being an endocrine disruptor which can be one of the causes of the precocity or the delay of puberty responsible for the current decline of fertility in human [20,23,31]. Several studies have established its effects on male and female reproductive system [33,34,42–46]. This study began with the determination of the age of onset of sexual maturation in experimental animals, which is characterized by vaginal opening [25,26,50,51]. Therefore, this work aimed at assessing the effects of tartrazine on sexual maturation and on folliculogenesis in a model of immature female Wistar rats. Sexual maturation also known as puberty is a crucial stage of development. It requires changes in the sensitivity, activity, and functionality of the hypothalamic-pituitary-gonadal axis which can cause a direct maturation of the genitals [24]. Without these signals, the genitals maintain the appearance they had during childhood, and the reproductive system remains non-functional. In other words, sexual maturation is a prerequisite for fertility [24]. Although there was no effect on the average age of vaginal opening, the results of this study shown that 45 days after their birth, the rats treated with tartrazine (47 mg/kg BW) had (100%) vaginal opening versus 41.66% in animals in the control group of the same age. This result showed that tartrazine advanced puberty as measured by the percentage of rats showing vaginal opening and it is in accordance with Kriszt and colleagues [25] who demonstrated that the administration of xenoestrogen to sexually immature rats is responsible of early puberty. The results showed this advanced puberty is accompanied by a very significant increase in the secretion of estradiol and LH in the group treated with tartrazine at a dose of 47 mg/kg BW. These results corroborate with the observations made in rats by Ramirez and Sawyer [50], which stipulate that the vaginal opening is the initial and external sign of the increase in secretion in estrogens accompanying the beginning of puberty. Tartrazine could have advanced the puberty by activating the release of the reproductive hormones of the hypothalamic-pituitary-gonadal axis. Contrary to the present study, Shakoor and colleagues [34] showed that tartrazine significantly decreases LH, FSH and estrogen levels; and increases progesterone levels after 30 days of treatment at the dose of 9.5 mg/Kg BW to adult female Sprague Dawley rats (6–7 months old). These differences may be due to the differences in species, animal age, and duration of treatment. Literature shows that differences in certain results in studies of the same molecules, substances, plants may arise from differences in the protocols used such as type of studies (in vitro or in vivo); species and age of animal used, duration of treatment, route of administration [52] and the stage of the estrous cycle if the authors used female animals [53]. It is well known that GnRH released in a pulsatile pattern of rhythmic secretory bursts whose amplitude and frequency vary according to cycle stage. The pituitary cells called gonadotropes, responding to GnRH stimulation, synthesize and release LH and FSH, which induce ovarian folliculogenesis, steroidogenesis, ovulation and formation of corpus luteum [24,54]. Furthermore, the increase in serum LH levels after 40 days of treatment observed, testifies the capacity of the rats to ovulate, since the increase in the serum level of gonadotropin to a certain threshold is a prerequisite for ovulation and subsequent promotion of the luteal phase (production of progesterone). According to Marieb and Hoehn [24], the production of estrogens increases with follicular growth and when their size (in this case that of the dominant follicle) reaches a certain threshold, the level of estrogen produced briefly exerts a retro activation on the hypothalamus and the adenohypophysis causing a sudden release of LH to a certain extent and FSH, approximately in the middle of the cycle. This hypothesis confirms the results observed on folliculogenesis (47 mg/kg BW). It increased the number of total follicles (primary, secondary, and antral follicles) and the concentration of FSH (non-significant increase). During puberty, the growth and maturation of the ovaries are mainly attributed to the presence of mature follicles (antral and Graafian follicles [55,56]. Then development and function (size expansion and the number of mature follicles, proliferation of fibrous tissues and antrum) of these ovaries require the presence of estrogens as much as that of pituitary gonadotropins [56]. Meanwhile, the increase of folliculogenesis can lead to the loss

of pool of primordial follicles and ovarian follicular reserve is tartrazine is prolonged use from a young age. Literature shows that, when the follicular pool reaches thousand follicles, the ovary cannot maintain the hormonal feedback with the hypothalamus and ovarian ageing also known as menopause is reach [57–59]. However, for some authors, the above-mentioned parameters remain insufficient in the evaluation of the impact of chemicals on the development of the female reproductive system. The relative weight of the uterus and/or the size of the uterine and vaginal epithelia are essential parameters [21,60]. In addition to the vaginal opening, reproductive maturation is associated with the activation of the hypothalamic-hypophysis-ovarian axis, which helps to induce secretion of the appropriate quantities of gonadotropins (FSH, LH) and ovarian hormones (estrogens and progesterone) responsible for the development and function of primary estrogen targets (uterus, vagina, and mammary gland) [21,24]. The effects produced by tartrazine at a dose of 47 mg/kg BW confirm this hypothesis. Tartrazine at this dose induced uterine growth (thickness of the uterine epithelium and the relative uterine weight), and eosinophilic secretions in the acinar of the mammary glands. Estrogens have been reported to stimulate proliferation and differentiation of uterine and vaginal epithelial cells, and eosinophilic secretions in the acinar of the mammary glands [61,62]. In an in vitro study, tartrazine has been identified as a new activator of human estrogen receptors (xenoestrogens). Its mechanism of action is through the activation of ERα receptors expressed in various tissues, but mainly in the uterus, ovary, pituitary gland, vas deferens, adipose tissue; and regulates the expression of more than 2800 genes in mammary gland cell lines [44,45]. In addition, it should be interesting to evaluate the effects of tartrazine on the estrous cycle and sexual behavior which are important parameters of sexual maturation.

5. Conclusions

In this study, tartrazine at a dose of 47 mg/kg BW (AED corresponding to admissible daily intake in human) increased the cumulative number of rats with vaginal opening, the relative epithelial thickness and the relative weight of the uterus, and the production of hormones of the hypothalamic-pituitary-ovarian axis (LH and Estradiol). Then, on the growth and maturation of the ovarian follicles, the results showed that tartrazine at the dose of 47 mg/kg BW increased the total number of ovarian follicles. Taken all together, these results might justify at least in part the increasingly early onset of puberty observed in our populations and provide a substantial scientific prove confirming tartrazine as an endocrine disruptor.

Author Contributions: Conceptualization, methodology, software, validation, formal analysis, investigation, resources, data curation, writing—original draft preparation, writing—review and editing, visualization, project administration, E.L.N.M., C.F.A.; writing—review and editing, visualization, D.T.N.; writing—review and editing, funding acquisition visualization, supervision, R.W.M.K.; conceptualization, methodology, validation, writing—original draft preparation, writing—review and editing, visualization, project administration, supervision, D.N. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Animals housing and experiments were carried out according to the guidelines of the Institutional Ethics Committee of the Cameroon Ministry of Scientific Research and Innovation (Reg. no. FWA-IRD 0001954, 4 September 2006), which has adopted the guidelines established by the European Union on Animal Care CEE Council 86/609).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are not publicly available but available on request from the corresponding author.

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Conflicts of Interest: The authors of this manuscript declare no conflict of interest.

Abbreviations

FSH	Follicle-stimulating hormone
GnRH	Gonadotropin-releasing hormone
LH	Luteinizing hormone
HED	Human equivalent dose
AED	Animal equivalent dose
BW	Body weight
CV	Coefficient of variation

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