Reproductive Toxicity of Eugenol in Wistar Rats

Toxicidade Reprodutiva do Eugenol em Ratas Wistar

Toxicidad para la Reproducción del Eugenol en Ratas Wistar

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ABSTRACT

Objective: To analyze the effects of eugenol oral administration during pre-implantation and organogenesis periods in pregnant Wistar rats. **Methodology:** The animals were divided into eight experimental groups (4 groups for each period) subdivided into control groups and daily treated with eugenol at doses of 37.5, 187.5 and 375 mg/kg/day (n= 9) during pre-implantation or organogenesis phases. **Results:** At all tested doses of eugenol, the groups treated during the pre-implantation presented alterations in the maternal organs, increased pre-and post-implantation loss and stillbirth records. The groups treated during organogenesis also presented alterations in the maternal organs and reduction of the placental indexes, added to the skeletal alterations, in all treatment doses. There were no significant fetal visceral changes. **Conclusions:** Oral administration of eugenol reduced maternal reproductive capacity in the pre-implantation phase and promoted toxic effects and fetal malformations in organogenesis.

Keywords: Eugenol, Organogenesis, Reproduction, Toxicity.

RESUMO

Objetivo: Analisar os efeitos da administração oral do eugenol realizada durante os períodos de pré-implantação e organogênese em ratas Wistar. **Metodologia:** Os animais foram divididos em oito grupos experimentais (sendo quatro grupos para cada período) subdivididos em grupos controle e tratados com eugenol nas doses de 37,5, 187,5 e 375 mg/kg/dia (n = 9) diariamente durante as fases de pré-implantação ou organogênese. **Resultados:** Em todas as doses testadas de eugenol, os grupos tratados durante a pré-implantação apresentaram alterações nos órgãos maternos, aumento da perda pré e pós-implantação e registros de natimortos. Os grupos tratados durante a organogênese também apresentaram alterações nos órgãos maternos e redução dos índices placentários, somados às alterações esqueléticas, em todas as doses de tratamento. Alterações viscerais fetais não foram significativas. **Conclusões:** A administração oral do eugenol reduziu a capacidade reprodutiva materna na pré-implantação e promoveu efeitos tóxicos e malformações fetais na organogênese.

Palavras-chave: Eugenol, Organogênese, Reprodução, Toxicidade.

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RESUMEN

Objetivo: Analizar los efectos de la administración oral de eugenol durante los períodos de preimplantación y organogénesis en ratas Wistar. **Metodología:** Los animales se dividieron en ocho grupos experimentales (4 grupos para cada período) subdivididos en grupos de control y tratados con eugenol a dosis de 37,5, 187,5 y 375 mg/kg/día (n = 9) diariamente durante fases de preimplantación u organogénesis. **Resultados:** en todas las dosis probadas de eugenol, los grupos tratados durante la preimplantación presentaron alteraciones en los órganos maternos, aumento de la perdida pre y postimplantación y registros de mortinatos. Los grupos tratados durante la organogénesis también presentaron alteraciones en los órganos maternos y reducción de los índices placentarios, sumado a las alteraciones esqueléticas, en las tres dosis de tratamiento. No hubo cambios viscerales fetales significativos. **Conclusiones:** La administración oral de eugenol redujo la capacidad reproductiva materna en la fase de preimplantación y promovió los efectos tóxicos y las malformaciones fetales en la organogénesis.

Palabras clave: Eugenol, Organogénesis, Reproducción, Toxicidad.

INTRODUCTION

Essential oils are characterized by their volatility and aroma, exploited in the food, pharmaceutical, and cosmetic industries, cleaning products, among others (NAEEM A, et al., 2018). Eugenol (4-allyl-2-methoxyphenyl) is a naturally occurring aromatic compound in essential oils of several plants, especially clove (*Syzygium aromaticum*) (SANTIN JR, et al., 2011). This monoterpene is used as a synthetic food flavoring and has biological properties of medicinal interest, with extensive pharmaceutical and therapeutic use (CHATTERJEE D and BHATTACHARJEE P, 2015).

Among the properties of eugenol are recognized the actions such as antioxidant (GÜLÇIN I, 2011), antiemetic (CHARANTIMATHS and OSWAL R, 2011), antimicrobial (SILVA FFM, et al., 2018), anxiolytic (WANG X, et al., 2017), neuroprotective (SAID MM and RABO MM, 2017), antinociceptive (BÓ WD, et al., 2013), anticancer (SHARMA UK, et al., 2016), among others.

Exposure to eugenol commonly occurs in industrial and dental environments, through contact with tobacco, wood and marijuana smoke, in soaps, lotions, detergents, perfumes, and the consumption of many foods and beverages (NTP, 1999). Although eugenol is considered a safe compound (FDA, 2018), studies on its toxicological potential are limited, especially about interferences in the animal reproductive period.

In an *in vivo* model study, the inclusion of eugenol in mice diet two weeks before mating and from the day-0 to day-4 of pregnancy, promoted difficulty in implantation of the blastocyst in the uterus, increasing the incidence of cell death (DOMARACKÝ M, et al., 2007).

Liu H, et al. (2017) observed the embryotoxicity of eugenol *in vitro* (ID_{50}: 5.43 ± 0.72 µg/ml) on embryonic stem cell culture. Despite the large pharmacological contribution of eugenol, there is not enough data in the literature to discuss the safety of using this compound during gestational phases in rats.

The maternal-fetal toxicological evaluation plays a fundamental role in the knowledge of the possible risks of eugenol in pregnancy. Thus, the present study aimed to analyze the effects of eugenol oral administration during pre-implantation and organogenesis periods in Wistar rats.

METHODS

Animals

The animals were maintained under controlled conditions of temperature (22 ± 3°C), humidity (50-60%), and lighting (12/12h light-dark cycle). They were fed with commercial dry food (Presence®, Purina, Brazil)

and water "*ad libitum*". Were utilized adult Wistar rats (*Rattus norvegicus* albinus) of both sexes, weighing between 250 and 300g; approximately 12 weeks old obtained from the Department of Physiology and Pharmacology of the Federal University of Pernambuco (UFPE). All experimental protocols were submitted to the Animal Experimentation Ethics Committee (CEUA) of the UFPE and approved under process nº 0041/2017.

Compounds and reagents

Eugenol (CAS # 97-53-0, 99% purity, Sigma Aldrich®, St. Louis, EUA) was dissolved in aqueous solution containing 2% Tween 80 (CAS # 9005-65-6, ≥ 99% purity, Sigma Aldrich®, St. Louis, EUA) and both diluted in drinking water.

For general anesthesia of the animals was used 2% (air/O_2) Isoflurano (CAS # 26675-46-7, Roche, SP, Brazil) by inhalation. For visceral and skeletal analyses were used: acetone (CAS # 67-64-1, ≥ 99.62% purity, Merck, SP, Brazil); glacial acetic acid P.A. (CAS # 64-19-7, Química Moderna, SP, Brazil); 95% ethyl alcohol P.A. (CAS # 64-17-5, Química Moderna, SP, Brazil); alizarin red P.A. (CAS # 72-48-0, Dinâmica Química Contemporânea, SP, Brazil); formaldehyde (CAS # 50-00-0, 37% P.A., Neon Comercial, SP, Brazil); pure bidistilled glycerin (CAS # 56-81-5, Merck, SP, Brazil); and potassium hydroxide P.A. (CAS # 1310-58-3, Dinâmica Química Contemporânea, SP, Brazil).

Mating and experimental groups

According to OECD Guideline 421 (OECD, 2016), nulliparous females were mated with adult males (1:1) at the beginning of the dark phase cycle. After 12 hours (at the beginning of the light phase) vaginal lavage was collected with 0.9% (w/v) NaCl for further analysis under optical microscopy. The observation of spermatozoa on slides associated with the presence of estrous phase cells characterized the mating and determined pregnancy day-zero (D0) (COOPER RL, et al., 1993).

After pregnancy identification, the female rats were randomly divided into eight experimental groups (n = 9/group), with four groups treated during the pre-implantation phase (0 to the 5th day of pregnancy) and four treated during the organogenesis phase (6th to 15th day of pregnancy).

The animals received, at specific periods, either vehicle as control 2% Tween 80 in water (5 ml/kg) or eugenol at doses of 37.5 (E1), 187.5 (E2) and 375 mg/kg/day (E3). The doses were chosen based on a previous study of our research group.

Analysis of maternal toxicity

Female rats were observed daily during pregnancy for possible behavioral changes, mortality, water, and food intake, piloerection, diarrhea, and vaginal bleeding (OECD, 2016).

Evaluation of maternal reproductive performance

On the 21st day of pregnancy, the rats were anesthetized according to the National Council for Animal Experimentation Control (CONCEA) euthanasia practice guidelines (BRASIL, 2015), with2% isoflurano (air/O_2) by inhalation, laparotomized and euthanized by cardiac incision. Then, ovariectomy was performed to count the number of corpora lutea. In the uterine horns, macroscopic evaluations were performed for the presence of live or dead fetuses, the number of implantation sites and early or late reabsorption.

Fetuses, placentas, reproductive and other maternal organs (thymus, heart, lungs, liver, kidneys, adrenals, stomach, pancreas and spleen) were weighed and analyzed for macroscopic malformations, determinations of fetal-maternal relationship, relative mass of maternal organs and reproductive implantation indexes, pre- and post-implantation losses and also the reabsorption indexes, according to Costa-Silva JH, et al. (2006).

Evaluation of embryo-fetal toxicity in pre-implantation and organogenesis phases

After the hysterectomy, the fetuses were weighed, sexed, after that the placental index and sex ratio were calculated. For calculation, the litters were considered as an experimental unit.

Visceral analysis in organogenesis

Half of each litter was fixed in Bouin solution (50 ml formaldehyde, 50 ml acetic acid, 752 ml 95% ethyl alcohol and 148 ml distilled water) for one week for subsequent visceral examination according to serial section method by Wilson J (1965).

Skeletal analysis in organogenesis

The other half of the litter was stained with alizarin red for skeletal analysis following an adapted protocol from Staples RE and Schnell VL (1964).

The fetuses were immersed in acetone for 24h, 1% (w/v) aqueous potassium hydroxide (KOH) solution for 24h and alizarin red solution (0.50 mg) in 200 ml of 1% (w/v) KOH every 24h for four days.

Were analyzed: the number, shape, and location of bones to confirm fetal alterations and malformations according to criteria proposed by Solecki R, et al. (2001). The counting and analysis of ossification points were performed according to Aliverti V, et al. (1979).

Statistical analysis

Samples were subjected to the Shapiro-Wilk normality test to determine normality. Results were expressed as mean ± standard error of the mean (SEM) or median.

Differences between groups were determined by one-way ANOVA followed by Dunnett's post-test or Kruskal-Wallis test followed by Dunn's post-test when applicable.

For the analysis of skeletal and visceral malformations, Pearson's chi-square test was used. All statistical analyses were performed by GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, CA, USA) and $p<0.05$ was accepted as statistically significant.

RESULTS

Oral administration of eugenol at doses of 37.5 (E1); 187.5 (E2) and 375 mg/kg (E3) in the reproductive toxicity experiment did not cause death, behavioral changes or clinical signs of toxicity in the treated progenitors during the pre-implantation period (0 to 5th day of pregnancy) nor during organogenesis (6th to 15th day of pregnancy).

On day-1 of pre-implantation, water consumption was significantly increased by 48.8% in E3 group (57.0 ± 4.4 ml/day/animal) compared to control group (C) (38.3 ± 4.7 ml/day/animal).

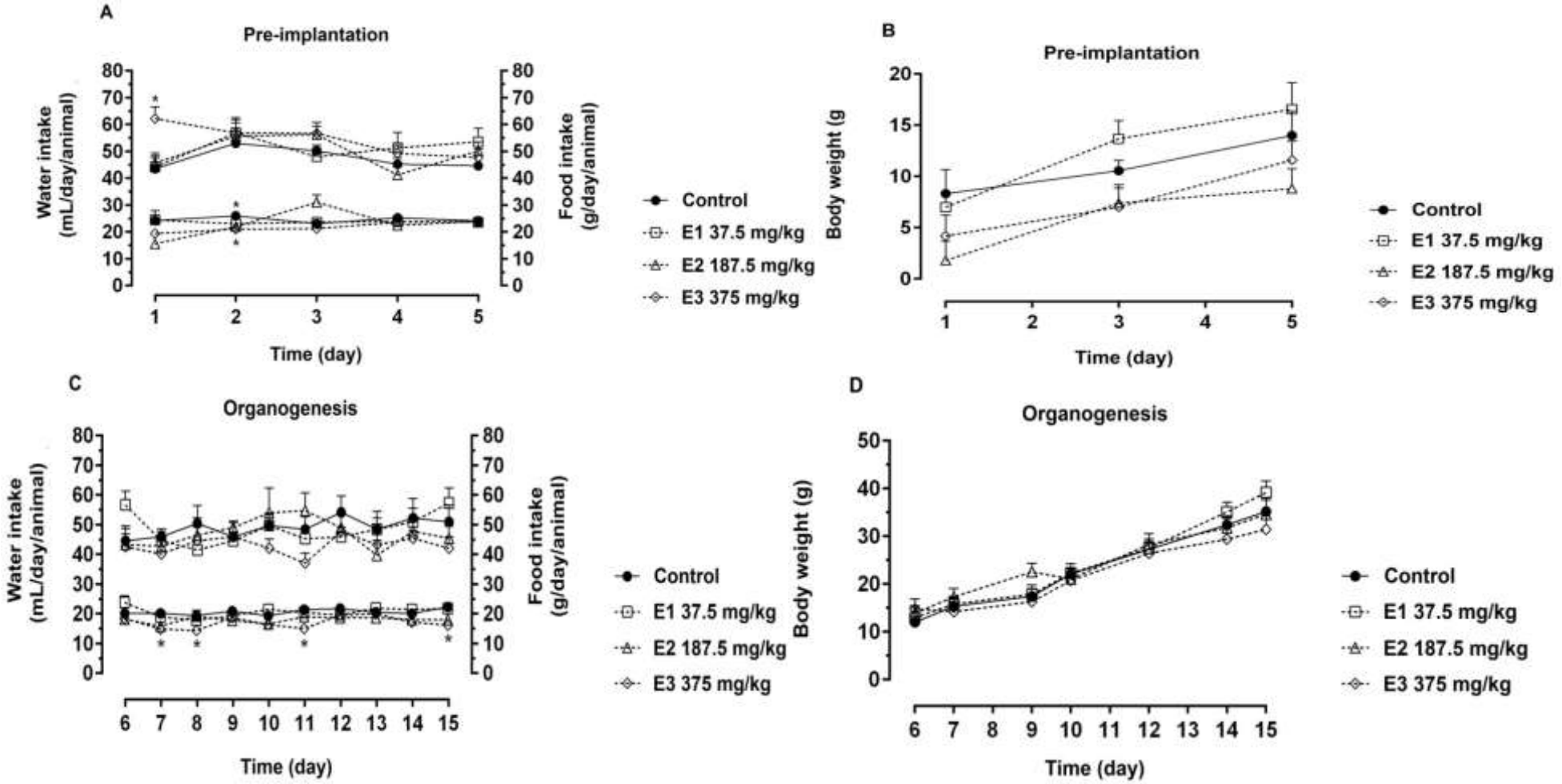
On day-2, food intake was significantly reduced by 19.7% and 24% in E2 (16.7 ± 1.3 g/day/animal) and E3 (15.8 ± 1.0 g/day/animal) groups, respectively, compared to the control group (20.8 ± 1.2 g/day/animal).

In the organogenesis no significant differences were observed in the water intake of the treated groups when compared to the control group.

However, food intake was significantly reduced by 26.4% on day-7 (14.89 ± 1.69 g/day/animal) versus (C: 20.22 ± 0.92 g/day/animal); 25.7% on day-8 (14.44 ± 1.70 g/day/animal) versus (C: 19.44 ± 1.78 g/day/animal); 29.5% on day 11 (15.11 ± 1.28 g/day/animal) versus (C: 21.44 ± 1.69 g/day/animal) and 27.3% on day-15 (16.22 ± 1.40 g/day/animal) versus (C: 22.33 ± 1.72 g/day/animal) in E3 group.

Despite the reported dietary variations, there was no interference in body weight of the treated females, either in the pre-implantation or organogenesis phases (**Figure 1**). During pre-implantation, significant reduction of 21.4% in absolute ovarian mass were observed in the E3 group when compared to the control group. E2 group presented a reduction of 34.6% in the absolute mass of the placentas when compared to the control group. Relative placental masses also showed a significant reduction of 98.1% in the E3 group and 97.5% in E1 and E2 groups, compared with the control group. A significant reduction of 21.3% in the absolute pancreas mass was also observed in the E3 group when compared to the control group (**Table 1**).

Figure 1 – Water and food intake and body weight evolution of eugenol-treated females during pre-implantation and organogenesis.



Subtitle: Effects of eugenol oral administration on water (upper graph) and food intake (lower graph) in pre-implantation (A) and organogenesis (C), and female body weight in the respective gestational phases (B, D). The values represent the mean ± S.E.M. analyzed by one-way ANOVA, followed by Dunnett's test, n = 9/group, *p < 0.05. **Source:** Silva JL, et al., 2019.

Table 1 – Effect of eugenol oral administration on absolute (g) and relative (%) weight of maternal organs during the pre-implantation phase in Wistar rats.

	Control		E1 (37.5 mg/kg)		E2 (187.5 mg/kg)		E3 (375 mg/kg)	
Organs	**Mass (g)**	**Relative (%)**	**Mass (g)**	**Relative (%)**	**Mass (g)**	**Relative (%)**	**Mass (g)**	**Relative (%)**
Ovaries	0.14 ± 0.01	0.04 ± 0.00	0.14 ± 0.01	0.04 ± 0.00	0.12 ± 0.00	0.04 ± 0.00	0.11 ± 0.01**	0.03 ± 0.00
Placentas	5.72 ± 0.44	1.62 ± 0.10	5.09 ± 0.53	0.04 ± 0.00****	3.74 ± 0.40*	0.04 ± 0.00****	5.33 ± 0.60	0.03 ± 0.00****
Pancreas	0.94 ± 0.06	0.27 ± 0.02	0.78 ± 0.04	0.24 ± 0.01	0.94 ± 0.06	0.31 ± 0.03	0.74 ± 0.05*	0.22 ± 0.01
Thymus	0.36 ± 0.04	0.10 ± 0.01	0.36 ± 0.04	0.11 ± 0.01	0.32 ± 0.03	0.10 ± 0.01	0.29 ± 0.02	0.09 ± 0.01
Heart	0.96 ± 0.03	0.27 ± 0.00	0.91 ± 0.03	0.28 ± 0.01	0.94 ± 0.04	0.31 ± 0.02	0.94 ± 0.02	0.27 ± 0.01
Lungs	1.73 ± 0.14	0.49 ± 0.04	1.59 ± 0.08	0.49 ± 0.03	1.68 ± 0.10	0.55 ± 0.03	1.82 ± 0.10	0.54 ± 0.03
Liver	14.81 ± 0.69	4.21 ± 0.09	13.83 ± 0.47	4.26 ± 0.14	13.24 ± 0.41	4.33 ± 0.22	14.04 ± 0.51	4.10 ± 0.11
Kidneys	2.26 ± 0.07	0.64 ± 0.01	2.10 ± 0.05	0.65 ± 0.03	2.04 ± 0.09	0.67 ± 0.04	2.18 ± 0.06	0.64 ± 0.02
Adrenals	0.10 ± 0.01	0.03 ± 0.00	0.09 ± 0.00	0.03 ± 0.00	0.10 ± 0.00	0.03 ± 0.00	0.10 ± 0.00	0.03 ± 0.00
Spleen	0.67 ± 0.09	0.19 ± 0.02	0.72 ± 0.07	0.22 ± 0.02	0.90 ± 0.09	0.29 ± 0.02	0.95 ± 0.15	0.28 ± 0.05
Stomach	1.71 ± 0.05	0.50 ± 0.02	1.57 ± 0.06	0.48 ± 0.01	1.68 ± 0.07	0.52 ± 0.02	1.68 ± 0.03	0.49 ± 0.02
Pregnancy uterus	58.46 ± 4.23	16.49 ± 1.05	50.01 ± 3.49	15.39 ± 1.04	45.05 ± 3.30	13.89 ± 0.89	46.66 ± 8.43	13.30 ± 2.34

Subtitle: The values represent the mean ± S.E.M. analyzed by one-way ANOVA, followed by Dunnett's test (n = 9/group) and the statistical differences represent *p < 0.05, **p < 0.01, ****p < 0.0001, respectively. Relative organ masses were calculated by the ratio of organ weight and body weight on the last day of gestation x 100.
Source: Silva JL, et al., 2019.

The visceral analysis showed the presence of evident changes in the female organs after the pre-implantation and organogenesis phases. At the doses of 187.5 (E2) and 375 mg/kg (E3), a higher number of affected organs and alterations were observed. The control and E1 groups showed similar results between themselves, without any alterations during the pre-implantation phase (**Frame 1**).

Frame 1 – Changes in maternal organs after daily treatment with eugenol in pre-implantation and organogenesis phases.

Pre-implantation						
Groups	**Adrenals**	**Liver**	**Pancreas**	**Lungs**	**Kidneys**	**Thymus**
Control	-	-	-	-	-	-
37.5 mg/kg	-	-	-	-	-	-
187.5 mg/kg	Far from the kidney (1)	Adhered to Stomach (1)	Nodules (1)	Nodule and/or edema (2)	Nodules (1)	-
375 mg/kg	Adhered to the kidney with nodules over and near the organ (1)	-	-	Nodule and/or edema (3)	-	-
Organogenesis						
Groups	**Adrenals**	**Liver**	**Pancreas**	**Lungs**	**Kidneys**	**Thymus**
Control	-	-	-	-	Nodules (2)	Nodules (1)
37.5 mg/kg	-	-	Nodules (2)	-	Nodules (2)	Nodules (3)
187.5 mg/kg	-	-	Nodules (1)	-	Nodules (7)	-
375 mg/kg	-	-	Nodules (1)	-	-	Nodules (8)

Subtitle: The numbers in parentheses represent the number of animals carrying the changes, and the symbol (-) represents no organ changes.
Source: Silva JL, et al., 2019.

The maternal organs evaluated in the organogenesis indicated a significant reduction of 21 and 24% of absolute pancreas masses in the E2 and E3 groups, respectively, when compared to the control group. Also, the relative pancreas masses showed a significant reduction of 25.8% in the E3 group and 16% in the E1 and E2 groups, when compared to the control (**Table 2**).

Table 2 - Effects of oral administration of eugenol on the absolute (g) and relative (%) masses of maternal organs during the organogenesis phase in Wistar rats.

	Control		E1 (37.5 mg/kg)		E2 (187.5 mg/kg)		E3 (375 mg/kg)	
Organs	**Mass (g)**	**Relative (%)**	**Mass (g)**	**Relative (%)**	**Mass (g)**	**Relative (%)**	**Mass (g)**	**Relative (%)**
Ovaries	0.11 ± 0.01	0.04 ± 0.00	0.12 ± 0.00	0.04 ± 0.00	0.12 ± 0.01	0.04 ± 0.00	0.09 ± 0.01	0.03 ± 0.00
Placentas	4.93 ± 0.66	1.53 ± 0.17	5.17 ± 0.35	1.64 ± 0.07	5.05 ± 0.19	1.66 ± 0.07	4.74 ± 0.45	1.46 ± 0.10
Pancreas	0.99 ± 0.07	0.31 ± 0.02	0.89 ± 0.02	0.26 ± 0.01*	0.78 ± 0.04*	0.26 ± 0.01*	0.75 ± 0.05**	0.23 ± 0.01**
Thymus	0.36 ± 0.02	0.11 ± 0.01	0.34 ± 0.03	0.01 ± 0.00	0.31 ± 0.02	0.10 ± 0.01	0.31 ± 0.03	0.10 ± 0.01
Heart	0.86 ± 0.03	0.27 ± 0.01	0.84 ± 0.03	0.25 ± 0.01	0.78 ± 0.03	0.26 ± 0.01	0.80 ± 0.01	0.25 ± 0.01
Lungs	1.39 ± 0.09	0.44 ± 0.02	1.46 ± 0.08	0.44 ± 0.02	1.33 ± 0.05	0.44 ± 0.02	1.28 ± 0.05	0.40 ± 0.01
Liver	12.14 ± 0.48	3.83 ± 0.09	12.78 ± 0.30	3.82 ± 0.08	12.22 ± 0.75	3.97 ± 0.14	12.69 ± 0.40	3.97 ± 0.06
Kidneys	1.92 ± 0.09	0.61 ± 0.02	1.9 ± 0.08	0.56 ± 0.02	1.85 ± 0.08	0.61 ± 0.02	1.83 ± 0.05	0.57 ± 0.02
Adrenals	0.10 ± 0.01	0.03 ± 0.00	0.09 ± 0.00	0.03 ± 0.00	0.19 ± 0.09	0.07 ± 0.03	0.08 ± 0.00	0.03 ± 0.00
Spleen	0.57 ± 0.03	0.18 ± 0.01	0.53 ± 0.02	0.16 ± 0.01	0.54 ± 0.03	0.18 ± 0.01	0.60 ± 0.03	0.19 ± 0.01
Stomach	1.49 ± 0.06	0.47 ± 0.01	1.56 ± 0.06	0.47 ± 0.01	1.37 ± 0.11	0.45 ± 0.03	1.54 ± 0.06	0.49 ± 0.03
Pregnancy uterus	59.37 ± 7.25	18.38 ± 1.85	68.32 ± 3.93	21.63 ± 0.57	59.2 ± 7.59	19.46 ± 2.46	68.25 ± 6.94	20.97 ± 1.56

Subtitle: The values represent the mean ± S.E.M. analyzed by one-way ANOVA, followed by Dunnett's test (n=9/group) and the statistical differences represent *p<0.05, **p<0.01, ****p<0.0001, respectively. Relative organ masses were calculated by the ratio of organ weight and body weight on the last day of gestationx100.
Source: Silva JL, et al., 2019.

Regarding reproductive parameters during the pre-implantation phase, there was a significant reduction in implantations in the E1 (85.7%) and E2 (86.8%) groups compared to control (100%). Also, were significantly increased the pre-implantation loss in the E1 (28.6%) and E2 (25%) groups, the reabsorption index (20.2%) and post-implantation loss in the E3 group (20.2%) when compared to their respective control groups (8.3%) (**Table 3**).

Table 3 - Reproductive parameters of eugenol-treated Wistar rats in pre-implantation.

Reproductive parameters	Control	E1 (37.5 mg/kg)	E2 (187.5 mg/kg)	E3 (375 mg/kg)
Pregnant rats	9	9	9	9
Number of live fetuses	95 (100%)	77 (81.05%)	77 (81.05%)	78 (82.1%)
Number of stillbirths	0	1	1	2
Litter/dam relationship[a,c]	36.58 ± 2.41	31.48 ± 2.57	26.08 ± 2.17	26.93 ± 5.31
Placental index (%)[b,d]	20	10****	15**	20
Sex ratio (M/F)[e]	1:1.3	1:1.2	1:1.5	1:1
Number of implantation sites	102 (100%)	91 (89.21%)	94 (92.15%)	108 (105.88%)
Number of reabsorption sites	8 (100%)	14 (175%)	15 (187.5%)	29 (362.5%)
Number of corpora lutea[a,c]	12.22 ± 0.46	11.89 ± 0.73	11.30 ± 0.73	13.30 ± 0.98
Implantation index (%)[b,d]	100	85.7****	86.8**	100
Reabsorption index (%)[b,d]	8.3	16.7	4.5	20.2***
Pre-implantation loss (%)[b,d]	8.3	28.6****	25***	20.2
Post-implantation loss (%)[b,d]	8.3	16.7	4.5	20.2***

Subtitle: Placental indexes [viable placental mass (g) / litter mass (g) x 100], implantation index [total number of implantation sites / total number of corpora lutea x 100], reabsorption index [total number of reabsorption / total number of implantation sites x 100], pre-implantation loss [(number of corpora lutea minus number of viable implantation sites) / number of corpora lutea x 100], post-implantation loss [(number of implantations minus number of live fetuses / number of implantations x 100] and the sex ratio [total number of male fetuses / total number of female fetuses]. Values were expressed as mean[a] ± S.E.M. or median[b]. Statistical analyses were performed using one-way ANOVA, followed by Dunnett's[c] post-test, Kruskal-Wallis test followed by Dunn's[d] post-test and Pearson's[e] chi-square test. Statistically different values of the control group represent *p < 0.05, **p < 0.01, ****p < 0.0001).

Source: Silva JL, et al., 2019.

External analysis of the maternal organs revealed the marked presence of stiffened nodules in the kidneys, thymus, and pancreas of the progenitors treated during organogenesis (**Frame 1**).

During organogenesis, the reproductive parameters showed an increase in the number of implantation sites in the E1 (114.9%) compared to control (100%).The pre-implantation loss was lower in the E1 (10%), E2 (9.1%) and E3 (10%) groups when compared to control (27.3%). There was no post-implantation loss in the E2 and E3 groups, and the treated E1 group (5%) did not differ significantly from the control (16.7%) (**Table 4**).

Table 4 - Reproductive parameters of eugenol-treated Wistar rats in organogenesis.

Reproductive parameters	Control	E1 (37.5 mg/kg)	E2 (187.5 mg/kg)	E3 (375 mg/kg)
Pregnant rats	9	9	9	9
Number of live fetuses	75 (100%)	96 (128%)	90 (120%)	85 (113.3%)
Number of stillbirths	0	0	0	0
Litter/dam relationship[a,c]	0.18 ± 0.02	0.21 ± 0.01	0.23 ± 0.02	0.19 ± 0.01
Placental index (%)[b,d]	12	11*	11*	11*
Sex ratio (M/F) [e]	1:0.6	1:1.2	1:1	1:1.5
Number of implantation sites	94 (100%)	108 (114.89%)	92 (97.87%)	91 (96.8%)
Number of reabsorption sites	19 (100%)	11 (57.89%)	2 (10.52%)	6 (31.57%)
Number of corpora lutea	11,78 ± 0,36	11,60 ± 0,52	11,33 ± 0,69	11,22 ± 0,57
Implantation index (%)[b,d]	90,91	96,43*	100****	90,91
Reabsorption index (%)[b,d]	16,67	0****	0****	0****
Pre-implantation loss (%)[b,d]	27.27	10**	9.09****	10**
Post-implantation loss (%)[b,d]	16.67	5	0****	0****

Subtitle: Placental index[viable placental mass (g)/ litter mass (g) x 100], implantation index[total number of implantation sites/total number of corpora lutea x 100], reabsorption index [total number of reabsorption/total number of implantation sites x 100], pre-implantation loss [(number of corpora lutea minus number of viable implantation sites) / number of corpora lutea x 100], post-implantation loss [(number of implantations minus number of live fetuses) /number of implantations x 100) and the sex ratio [total number of male fetuses / total number of female fetuses]. Values were expressed as meana ± S.E.M. or medianb. Statistical analyses were performed using one-way ANOVA, followed by Dunnett'sc post-test, Kruskal-Wallis test followed by Dunn'sd post-test and Pearson'se chi-square test. Statistically different values of the control group represent *p<0.05, **p<0.01, ****p<0.0001).

Source: Silva JL, et al., 2019.

During pre-implantation, there was a statistically significant reduction in placental index in the E1 (10%) and E2 (15%) groups when compared to the control group (20%) (**Table 3**). While in organogenesis, these indexes showed a statistically significant reduction in all treated groups (11%) compared to their respective controls (12%) (**Table 4**). Regarding embryo-fetal toxicity, no significant differences were observed in fetal masses, sex ratio, or maternal-fetal relationship in both gestational phases (pre-implantation and organogenesis). However, there was a record of stillbirth (1/77) in the E1 and E2 groups and two stillbirths (2/78) in the E3 group in the pre-implantation (**Table 3**).

Visceral analyses showed pulmonary artery dilatation, bladder dilation, and palate grooves of the fetuses analyzed (data not shown). Skeletal analyses identified statistically significant bone malformations such as proximal phalanx agenesis of anterior and posterior paws, reduction of the number of cervical vertebrae, shortening of the ribs, incomplete ossification of the squamous bone of the head. Bone malformations related to incomplete ossification and morphological alteration of the sternum (butterfly shape) were also significant (**Table 5**).

Table 5 - Relationship of skeletal malformations of fetuses of eugenol-treated females during organogenesis.

Skeletal malformations	Control	E1 (37.5 mg/kg)	E2 (187.5 mg/kg)	E3 (375 mg/kg)
Agenesis of proximal phalanges of anterior paws	8/34 (23.5%)	19/45 (42.2%) *	10/44 (18.5%)	6/38 (15.8%)
Agenesis of proximal phalanges of posterior paws	10/34 (29.4%)	26/45 (57.8%)	17/44 (38.6%)	18/38 (47.3%)
Absence of cervical vertebrae	1/34 (2.9%)	45/45 (100%) ****	37/44 (84.1%)	38/38 (100%) ****
Shortened ribs	0/34 (0%)	0/45 (0%)	3/44 (6.8%) *	0/38 (0%)
Incomplete ossification of the squamous	0/34 (0%)	16/45 (35.6%) **	10/44 (22.7%)	8/38 (21.0%)
Incomplete ossification of the sternebrae	19/34 (55.8%)	16/45 (35.6%)	25/44 (56.8%)	29/38 (76.3%) **
Butterfly shape sternum	1/34 (2.9%)	0/45 (0%)	4/44 (9.1%)	16/38 (29.6%) ****
Incomplete ossification of manubrium	1/34 (2.9%)	0/45 (0%)	0/44 (0%)	0/38 (0%)
Absence of xiphoid process	0/34 (0%)	0/45 (0%)	0/44 (0%)	2/38 (5.3%)
Agenesis of the last sternal center	0/34 (0%)	1/45 (2.2%)	0/44 (0%)	0/38 (0%)

Subtitle: The results of fetal skeletal malformations were organized from the relationship between the number of affected fetuses/ total number of fetuses analyzed. Values in parentheses represent the percentage of skeletal malformations observed. Statistical differences between treated and control groups were calculated by Pearson's chi-square test, *p<0.05, **p<0.01, ****p<0.0001).

Source: Silva JL, et al., 2019.

DISCUSSION

The results obtained after treatment with eugenol showed that female rats had a significant reduction in food intake in both gestational phases, but there was no change in body weight. Wang M, et al. (2019) compared the effect of 13 pesticides on female rats body weight during 14, 21, 28, 42 and 70 days and concluded that there is a time-dependent toxicity relationship, where shorter exposures (14 days) had less pronounced effects. Treatment with eugenol lasted six days on pre-implantation and 10 days on organogenesis, both less than 14 days, indicating that the exposure time may not have been sufficient to affect female body weight gain, but the reduction in the ingestion of food and water can be the beginning of systemic toxicity.

The reduction in ovarian mass during pre-implantation observed after oral administration of eugenol at the highest dose may indicate a reduction in ovarian activity. Similar results were observed from the intramuscular administration of eugenol (0.2 ml/day/animal) in female rats, where the significant presence of atretic ovarian follicles concerning healthy follicles, associated with ovarian mass reduction, indicated antiestrogenic activity of this substance (KULKARNI DS, 2011).

There was also a reduction in absolute and relative placental masses after eugenol administration at all evaluated doses. The placenta is an organ capable of interacting with xenobiotics from specific enzyme-mediated oxidation, reduction, or conjugation reactions. This organ is also capable of metabolic reactions involving CYP450 complex enzymes (AL-ENAZY S, et al., 2017). In the presence of these enzymes, eugenol starts to perform a pro-oxidant activity, resulting in a metabolic intermediate with cytotoxic capacity called quinone-methide (THOMPSON DC, et al., 1991).

Eugenol in the presence of hydrogen peroxide can be metabolized *in vitro* by placental peroxidases, causing placental dysfunction (ZHANG R, et al., 2000). Given this, we can correlate oxidative stress to constriction of blood vessels, with a consequent reduction of placental mass and activity.

In order to evaluate the involvement of eugenol on placental function, we calculated the placental index, a parameter that indicates the relationship of nutrient exchange between mother and fetus, mediated by the placenta (KINGDOM JCP and KAUFMANN P, 1999). Therefore, the reduction in placental indexes, associated with the reduction in placental mass and constriction of blood vessels, suggest that eugenol can cross the transplacental barrier and interfere with maternal metabolism, exerting a toxic action on the fetus.

The observation of stillbirths, reduction on implantation indexes, increase in pre-and post-implantation loss and reabsorption, demonstrated in this study, are indicators of maternal, embryonic, and fetal toxicity resulting from the administration of eugenol. These findings may be correlated with inhibition of estrogen secretion during the pre-implantation, which interferes with the process of uterine epithelial proliferation and disturbs the blastocyst implantation in the uterus (SINGH MM, et al., 1996), increasing embryonic death (DOMARACKÝ M, et al., 2007). These changes reinforce the contraindication for eugenol use during the initial gestational period. The demonstration of pulmonary edema, hepatic, renal, and adrenal alterations observed after the treatment of pregnant rats with eugenol during the pre-implantation is another toxicity indicator of this compound. Similar findings have been reported after prolonged administration of this substance in rats (SOBER HA, et al., 1950), hamsters (LAVOIE EJ, et al., 1986), rabbits (MCDONALD JW and HEFFNER JE, 1991), and frogs (GOULET F, et al., 2011).

The presence of stiffened nodules in the pancreas of eugenol-treated females in the pre-implantation and organogenesis phases may have obstructed pancreatic ducts, impairing secretory activity, deregulating the available calcium concentration and recruiting intrapancreatic enzymes to trigger an inflammatory process in the affected organ. Such inflammation triggers a systemic response, depressing the immune system, affecting various organs such as the liver, lungs, and kidneys (PÉREZ S, et al., 2015). This metabolic imbalance may cause a reduction in the pancreas mass and facilitate the formation of the observed nodules.

Goulet F, et al. (2011) observed dose-dependent lesions on kidneys, liver, and lungs of African frogs (*Xenopus laevis*) immersed in eugenol aqueous solution (375 µl/L) for 24 and 72h. These lesions were reversible after 15 days from exposure interruption. This work suggests that stopping eugenol treatment allowed the reversal of the observed toxic effects.

The reversibility of the toxic action after interruption of exposure to the agent could explain the low presence of nodules in the maternal organs during pre-implantation, whose treatment lasted six days, with a more extended recovery period (15 days) when compared with the groups treated in the organogenesis, where the treatment was longer (10 days) and the recovery time shorter (6 days). Visceral and skeletal analyzes of fetuses were performed only in organogenesis phase to investigate the teratogenic potential of eugenol.

The visceral changes were not statistically significant. On the other hand, skeletal changes were significant when compared to control. Bone tissue homeostasis is known to be related to maintenance of estrogen levels, so the decrease of this hormone can cause bone mass loss (FALONI APS and CERRI PS, 2007).

In this way, the antiestrogenic action of eugenol may have reduced estrogen levels in the body of treated females, resulting in delayed fetal bone development during organogenesis. A previous study indicates that some monoterpenes can inhibit bone reabsorption by an unclear mechanism of action (MÜHLBAUER RC, et al., 2003). This ability may also be present in eugenol and have partially caused the observed skeletal changes.

CONCLUSION

The reproductive toxicological study showed eugenol interferes with maternal reproductive capacity and causes embryo-fetal toxicity, mainly in pre-implantation, and contributes to fetal malformations in the organogenesis phase. However, further studies are needed to establish a safe dose of use of this compound and to elucidate the mechanisms of action associated with the effects of continuous use.

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ABBREVIATIONS

% – percentage
°C – Celsius degree
µg – microgram
µl – microliter
ANOVA – analysis of variance
C – control group
CAS – Chemical Abstracts Service
CEUA – Animal Experimentation Ethics Committee
CONCEA – National Council for Animal Experimentation Control
CYP450 – cytochrome P450
D0 – day-zero
E1 – 37.5 mg/kg/day eugenol dose
E2 – 187.5 mg/kg/day eugenol dose
E3 – 375 mg/kg/day eugenol dose
FDA – Food and Drug Administration
g – gram
h – hour
ID – inhibition of differentiation
kg – kilogram
KOH – potassium hydroxide
L – Liter
M/F – Male/Female
mg – milligram
ml – milliliter
n° – number
NaCl – sodium chloride
NTP – National Toxicology Program
O_2 – oxygen
OECD – Organisation for Economic Co-operation and Development
PA – pro analysis
S.E.M. – standard error of the mean
UFPE – Federal University of Pernambuco
w/v – weight/volume

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