



UNIVERSIDADE  
ESTADUAL DE LONDRINA

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VIVIANE DE FÁTIMA MESTRE

**PREGABALINA ALTERA A PERFORMANCE REPRODUTIVA DE  
CAMUNDONGOS MACHOS E PROMOVE MALFORMAÇÕES NA  
PROLE DE CAMUNDONGOS FÊMEAS E RATAS**

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Tese apresentada ao Programa de Pós-graduação  
em Ciências da Saúde da Universidade Estadual de  
Londrina, como requisito para a obtenção do título de  
Doutor.

Orientadora: Profa. Dra. Maria José Sparça Salles

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**BANCA EXAMINADORA**

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Londrina, 23 de fevereiro de 2023.

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*"Você não pode esperar construir um mundo melhor sem melhorar os indivíduos. Para esse fim, cada um de nós deve trabalhar para o seu próprio aperfeiçoamento e, ao mesmo tempo, compartilhar uma responsabilidade geral por toda a humanidade".*

Marie Curie

MESTRE, Viviane de Fátima. **Pregabalina altera a performance reprodutiva de camundongos machos e promove malformações na prole de camundongos fêmeas e ratas.** 2023. 113 f. Tese (Doutorado em Ciências da Saúde) Universidade Estadual de Londrina, Londrina, 2023.

## RESUMO

A pregabalina é um fármaco pertencente ao grupo gabapentínicos neuromodulador com ação antiepiléptica e analgésica. Tem sido administrada em dor neuropática, fibromialgia, transtorno de ansiedade generalizada e como adjuvante para epilepsia. O efeito analgésico da pregabalina se dá pela sua capacidade de ligar-se à subunidade da proteína  $\alpha 2\text{-}\delta$  dos canais de cálcio voltagem-dependentes. Considerando a escassez de estudo sobre o tratamento de pregabalina em homens em idade fértil e mulheres gestantes, este trabalho teve como objetivo avaliar as possíveis alterações causadas pelo fármaco quando administrado em camundongos durante a espermatogênese; após exposição pré-natal na prole de camundongos fêmeas e após exposição pré-natal na prole de ratas sobre a odontogênese e osso trabecular da mandíbula. Para a análise da fertilidade masculina e malformação da prole, camundongos machos receberam diariamente via gavagem, 200 mg/kg de pregabalina por um período de 45 dias; após este período esses animais foram acasalados com fêmeas não tratadas. Para a avaliação dos parâmetros maternos e malformações na prole, camundongos prenhes foram tratadas com pregabalina (200 mg/kg), via gavagem do 5º dia ao 17º dia de prenhez. No 18º dia de prenhez as fêmeas foram submetidas a eutanásia e realizada a laparotomia para a avaliação do desenvolvimento intrauterino. Os fetos foram analisados quanto às malformações congênitas externas, viscerais e esqueléticas. Para a análise da odontogênese e do osso trabecular da mandíbula, ratas prenhes foram tratadas com pregabalina (200 mg/kg) do 5º dia de prenhez ao dia antecessor ao parto (20º dia). No pós-natal dia 30, foram selecionados aleatoriamente 8 filhotes de cada grupo para análise do primeiro molar inferior direito e do osso trabecular adjacente utilizando a microtomografia computadorizada. A administração da pregabalina em camundongos machos mostrou um aumento significativo da medida dos testículos ( $P = 0,009$ ), alterações morfológicas dos espermatozoides ( $P < 0,0001$ ), diminuição no escore de Johnsen ( $P = 0,0002$ ), aumento das células de Leydig ( $P = 0,0002$ ) e diminuição do nível da testosterona ( $P = 0,0152$ ). Além disso, a análise dos parâmetros da prole mostrou diminuição significativa do peso da placenta ( $P = 0,0001$ ), do peso ( $P = 0,0035$ ) e comprimento dos fetos ( $P = 0,003$ ), da taxa de viabilidade fetal ( $P = 0,0458$ ) e aumento significativo do número de reabsorções ( $P = 0,003$ ) e da perda pós-implantação ( $P = 0,0458$ ). As anomalias significativas observadas na prole foram alteração do tamanho dos rins ( $P = 0,0058$ ), metacarpos e falanges ausentes ( $P < 0,0001$ ), alteração do esterno ( $P < 0,0001$ ) e vértebras torácicas supranumerárias ( $P = 0,003$ ). A administração da pregabalina em camundongos fêmeas prenhes apontou aumento significativo na taxa de reabsorção ( $P = 0,041$ ) e diminuição significativa do peso da placenta ( $P < 0,0001$ ), do comprimento ( $P = 0,0249$ ) e peso dos fetos ( $P = 0,0484$ ) e da viabilidade fetal ( $P = 0,0038$ ). Foi verificado que 67,5% dos fetos eram pequenos para a idade de prenhez ( $P < 0,0001$ ). As malformações observadas foram ventriculomegalia fetal ( $P < 0,0001$ ) e ossificação incompleta do osso supraoccipital ( $P < 0,0001$ ). Quanto a análise da administração da pregabalina em ratas prenhes, houve diminuição significativa de ganho de peso materno ( $P = 0,0139$ ) e de filhotes vivos ( $P = 0,0213$ ). Durante o acompanhamento pós-natal, houve uma diminuição significativa do peso ( $F=2,67$ ;  $P=0,048$ ) e do comprimento ( $F=9,49$ ;  $P<0,0001$ ) da prole e atraso da erupção dos dentes incisivos ( $P = 0,001$ ). A análise do primeiro molar inferior direito, utilizando a microtomografia computadorizada, mostrou diminuição significativa do esmalte ( $P = 0,0043$ ). A avaliação do tecido ósseo trabeculado adjacente ao primeiro molar mostrou que a

pregabalina foi capaz de aumentar significativamente o percentual de volume ósseo ( $P = 0,0208$ ) e diminuir o volume ( $P = 0,0266$ ) e percentual de poros abertos ( $P = 0,0208$ ). Na análise histológica, nenhuma alteração na organização celular foi observada. Em conclusão, o tratamento com pregabalina produziu efeitos tóxicos sobre a função reprodutiva de camundongos machos, efeito embriotóxico na prole durante a gestação nos dois modelos animais além de potencial efeito teratogênico.

**Palavras-chaves:** Pregabalina; Toxicidade reprodutiva; Malformação congênita; Microtomografia computadorizada; Odontogênese; Osso trabecular da mandíbula

MESTRE, Viviane de Fátima. **Pregabalin alters the reproductive performance of male mice and promotes malformations in the offspring of female mice and rats.** 2023. 113 f. Thesis (Doctorate in Health Sciences) State University of Londrina, Londrina, 2023.

## ABSTRACT

Pregabalin is a drug belonging to the gabapentinoid neuromodulator group, with antiepileptic and analgesic action. It has been used in neuropathic pain, fibromyalgia, generalized anxiety disorder, and as an adjunct in epilepsy. The analgesic effect of pregabalin is due to its ability to bind to the  $\alpha 2\text{-}\delta$  protein subunit of voltage-gated calcium channels. Considering the scarcity of studies on the treatment of pregabalin in men of childbearing age and pregnant women, the current work aimed to evaluate the possible alterations caused by the drug when administered to male mice during spermatogenesis, the offspring of female mice after prenatal exposure, and the offspring of female rats after prenatal exposure on odontogenesis and trabecular bone of the mandible. For analysis of male fertility and malformations in the offspring, male mice received 200 mg/kg of pregabalin daily via gavage for a period of 45 days; after this period these animals were mated with untreated females. For the evaluation of maternal parameters and malformations in the offspring, pregnant mice were treated with pregabalin (200 mg/kg) via gavage from the 5th to the 17th day of pregnancy. On the 18th day of pregnancy, females were euthanized and laparotomy was performed to assess intrauterine development. Fetuses were analyzed for external, visceral, and skeletal congenital malformations. For the analysis of odontogenesis and trabecular bone of the mandible, pregnant rats were treated with pregabalin (200 mg/kg) from the 5th day of pregnancy to the day before delivery (20th day). On postnatal day 30, 8 pups from each group were randomly selected for analysis of the lower right first molar and adjacent trabecular bone using computed microtomography. Pregabalin administration in male mice led to a significant increase in testicular size ( $P = 0.009$ ), morphological alterations in spermatozooids ( $P < 0.0001$ ), a decrease in the Johnsen score ( $P = 0.0002$ ), an increase in Leydig cells ( $P = 0.0002$ ), and a decrease in testosterone level ( $P = 0.0152$ ). In addition, the analysis of offspring parameters showed a significant decrease in placental weight ( $P = 0.0001$ ), fetal weight ( $P = 0.0035$ ) and length ( $P = 0.003$ ), and fetal viability rate ( $P = 0.0458$ ), and a significant increase in the number of resorptions ( $P = 0.003$ ) and post-implantation losses ( $P = 0.0458$ ). The significant anomalies observed in the offspring were alteration in the size of the kidneys ( $P = 0.0058$ ), absent metacarpals and phalanges ( $P < 0.0001$ ), alteration in the sternum ( $P < 0.0001$ ), and supernumerary thoracic vertebrae ( $P = 0.003$ ). Administration of pregabalin in pregnant female mice led to a significant increase in the rate of resorption ( $P = 0.041$ ) and a significant decrease in placental weight ( $P < 0.0001$ ), length ( $P = 0.0249$ ), and fetal weight ( $P = 0.0484$ ) and viability ( $P = 0.0038$ ). It was found that 67.5% of fetuses were small for pregnancy age ( $P < 0.0001$ ). The observed malformations were fetal ventriculomegaly ( $P < 0.0001$ ) and incomplete ossification of the supraoccipital bone ( $P < 0.0001$ ). With respect to the analysis of pregabalin administration in pregnant rats, there was a significant decrease in maternal weight gain ( $P = 0.0139$ ) and live offspring ( $P = 0.0213$ ). During postnatal follow-up, there was a significant decrease in weight ( $F=2.67$ ;  $P=0.048$ ) and length ( $F=9.49$ ;  $P<0.0001$ ) of the offspring and delayed eruption of incisor teeth ( $P = 0.001$ ). Analysis of the lower right first molar, using computed microtomography, demonstrated a significant decrease in enamel ( $P = 0.0043$ ). The evaluation of the trabecular bone tissue adjacent to the first molar showed that pregabalin was able to significantly increase the percentage of bone volume ( $P = 0.0208$ ) and decrease the volume ( $P = 0.0266$ ) and percentage of open pores ( $P = 0.0208$ ). In the histological analysis, no alterations in cellular organization were observed. In conclusion, treatment with pregabalin produced toxic effects on the reproductive function of male mice, and an embryotoxic effect on the offspring during pregnancy in both animal models, in addition to a potential teratogenic effect.

**Key-words:** Pregabalin; Reproductive toxicity; Congenital malformation; Computerized microtomography; Odontogenesis; Trabecular bone of the mandible

## LISTA DE FIGURAS

<b>Figura 1</b> – Estrutura química da PGB (a) e do GABA (b).....	18
<b>ARTIGO 1</b>	
<b>Figura 1</b> – <i>Morphological alterations found in spermatozoids from mice treated with PGB (200 mg/kg) during the period of spermatogenesis</i> .....	40
<b>Figura 2</b> – <i>Photomicrographs of the testes of adult mice</i> .....	41
<b>ARTIGO 2</b>	
<b>Figura 1</b> – <i>Correlation of fetal weight with pregnancy age of offspring exposed to PGB and offspring not exposed during pregnancy</i> .....	58
<b>Figura 2</b> – <i>Frontal section of the cerebral hemisphere</i> .....	59
<b>ARTIGO 3</b>	
<b>Figura 1</b> – <i>Weight gain (a) and body length (b) of the offspring of female rats from PND1 to PND 21 of group C and group PGB</i> .....	73
<b>Figura 2</b> – <i>Reconstructed <math>\mu</math>CT images</i> .....	74
<b>Figura 3</b> – <i>HE-stained histological sections of the RMFM region of the offspring of rats after <math>\mu</math>CT analysis</i> .....	77

## LISTA DE TABELAS

<b>Tabela 1</b> – Vendas de anticonvulsivantes e antiepiléticos no Brasil nos anos de 2017 a 2020 .....	20
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### ARTIGO 1

<b>Tabela 1</b> – <i>Effects of PGB in adult male mice treated with 200 mg/kg for 45 days</i> .....	38
<b>Tabela 2</b> – <i>Effects of PGB on intrauterine development of offspring of treated males during spermatogenesis (200 mg/kg)</i> .....	38
<b>Tabela 3</b> – <i>Malformations observed in fetuses of offspring of males treated with 200 mg/kg of PGB during the period of spermatogenesis and mated with untreated females</i> .....	39
<b>Tabela 4</b> – <i>Malformations observed in spermatozoids from mice treated with 200 mg/kg of PGB during the period of spermatogenesis</i> .....	40
<b>Tabela 5</b> – <i>Analysis of the testes of mice treated with 200 mg/kg of PGB during the spermatogenesis period</i> .....	42

### ARTIGO 2

<b>Tabela 1</b> – <i>Effects of treatment with PGB (200 mg/Kg) in female mice from the 5th to the 17th day of pregnancy</i> .....	56
<b>Tabela 2</b> – <i>Effects of PGB (200 mg/kg) treatment on intrauterine development in offspring of female mice</i> .....	57
<b>Tabela 3</b> – <i>Malformations observed in fetuses after exposure to PGB (200 mg/kg) from the 5th to the 17th day of pregnancy</i> .....	58

### ARTIGO 3

<b>Tabela 1</b> – <i>Effect of treatment with PGB (200 mg/kg) in pregnant rats</i> .....	72
<b>Tabela 2</b> – <i>Monitoring the eruption of incisor teeth in the offspring of animals exposed to PGB (200 mg/kg) during gestational development</i> .....	73
<b>Tabela 3</b> – <i>Evaluation of RMFM enamel and dentin in the offspring of rats treated with PGB (200 mg/kg) during the gestational period</i> .....	75
<b>Tabela 4</b> – <i>Evaluation of the adjacent trabecular bone tissue by RMFM of the offspring of rats treated with PGB (200 mg/kg) during the gestational period</i> .....	75

## LISTA DE ABREVIATURAS E SIGLAS

2D	Bidimensional
3D	Tridimensional
$\alpha 2-\delta$	Alfa-2-delta
AED	<i>Antiepileptic drugs</i>
AGA	<i>Adequate for gestational age</i>
AMBN	Ameloblastina
AMEL	Amelogenina
cAMP	<i>Cyclic adenosine monophosphate</i>
BV	Volume do osso ( <i>Bone volume</i> )
BS	Superfície óssea ( <i>Bone surface</i> )
BS/BV	Superfície/volume ( <i>Bone surface-to-volume ratio</i> )
BV/TV	Volume ósseo/volume tecidual ( <i>Bone volume-to-tissue ratio</i> )
C	Controle
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CONCEA	Conselho Nacional de Controle de Experimentação Animal
DNA	Ácido Desoxirribonucleico
DP	Desvio padrão
DPN	Dia pós-natal
DS	Superfície da dentina ( <i>Dentin surface</i> )
DS/DV	Superfície/volume da dentina ( <i>Dentin surface/volume ratio</i> )
DV	Volume da dentina ( <i>Dentin volume</i> )
ENAM	Esmalteína
EV	Volume do esmalte ( <i>Enamel volume</i> )
ES	Superfície do esmalte ( <i>Enamel surface</i> )
ES/EV	Superfície/volume do esmalte ( <i>Enamel surface/volume ratio</i> )
FDA	<i>Food and Drug Administration</i>
FGR	<i>Fetal growth restriction</i>

FSH	Hormônio folículo estimulante
GABA	Ácido gama aminobutírico
GBP	Gabapentina
HE	Hematoxilina-eosina ( <i>Hematoxylin-eosin</i> )
<i>IGF2</i>	<i>Insulin-like growth factor type II</i>
kV	Quilovolt
KOH	Hidróxido de potássio
LGA	<i>Large for Gestational Age</i>
LH	Hormônio luteinizante
μA	Microampere
μCT	Microtomografia computadorizada ( <i>Computed microtomography</i> )
μm	Micrômetro
n	Número absoluto
OMS	Organização Mundial da Saúde
OPG	Osteoprotegerin
<i>PEG1/MEST</i>	<i>Paternally expressed gene 1/mesoderm-specific transcript</i>
<i>PEG3</i>	<i>Insulin-like growth factor type II</i>
PGB	Pregabalina
<i>PLAG1</i>	<i>Pleomorphic adenoma gene 1</i>
PMID	Primeiro molar inferior direito
PND	<i>Postnatal day</i>
Po(cl)%	Porosidade fechada
Po(op)mm <sup>3</sup>	Volume do espaço poroso aberto
Po(op)%	Porosidade aberta
Po(tot)%	Porosidade total
Po.V(cl)mm <sup>3</sup>	Volume de poros fechados
Po.V(tot)mm <sup>3</sup>	Volume total do espaço poroso
<i>RANKL</i>	<i>Receptor activator of nuclear fator Kappa-B ligand</i>
RMFM	<i>Right mandibular first molar</i>

ROI	<i>Region of Interest</i>
SD	<i>Standard deviation</i>
SGA	<i>Small for the gestational age</i>
Tb.N	Número trabecular
Tb.Sp	Distância entre as trabéculas
Tb.Th	Média da espessura trabecular
UEL	Universidade Estadual de Londrina
VOI	<i>Volume of interest</i>

## SUMÁRIO

<b>1</b>	<b>INTRODUÇÃO</b> .....	<b>18</b>
<b>2</b>	<b>OBJETIVOS</b> .....	<b>22</b>
2.1	Objetivo Geral .....	22
2.2	Objetivos Específicos .....	22
<b>3</b>	<b>MATERIAIS E MÉTODOS</b> .....	<b>23</b>
3.1	Fármaco.....	23
3.2	<b>Artigo 1</b>	
3.2.1	Animais.....	23
3.2.2	Delineamento experimental.....	23
3.2.3	Toxicidade.....	24
3.2.4	Eutanásia.....	24
3.2.5	Análise da espermatogênese.....	25
3.2.6	Concentração sérica de testosterona.....	25
3.2.7	Análise do desenvolvimento intrauterino e embriofetotoxicidade.....	25
3.2.8	Avaliação das malformações congênicas da prole dos camundongos machos tratados e acasalados com fêmeas não tratadas.....	25
3.3	<b>Artigo 2</b>	
3.3.1	Animais.....	26
3.3.2	Delineamento experimental.....	26
3.3.3	Toxicidade.....	26
3.3.4	Eutanásia.....	27
3.3.5	Análise do desenvolvimento intrauterino e embriofetotoxicidade.....	27
3.3.6	Avaliação das malformações congênicas.....	27
3.4	<b>Artigo 3</b>	
3.4.1	Animais.....	27
3.4.2	Delineamento experimental.....	28
3.4.3	Pós-natal.....	28
3.4.4	Eutanásia.....	28
3.4.5	Microtomografia computadorizada.....	29
3.4.6	Análise histológica.....	29
3.5	Análise estatística.....	29
<b>4</b>	<b>RESULTADOS E DISCUSSÃO</b> .....	<b>30</b>

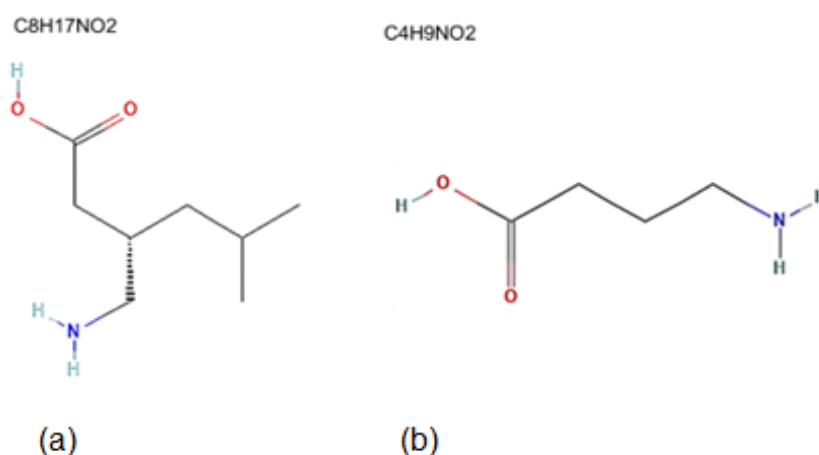
4.1	Artigo 1 - <i>Pregabalin alters reproductive performance in male mice and causes congenital anomalies in offspring</i> .....	31
4.2	Artigo 2 - <i>Pregabalin promotes embryotoxicity and birth defects in mouse fetuses</i> .....	53
4.3	Artigo 3 - <i>Evaluation of the effects of prenatal exposure to pregabalin and postnatal analysis of dental and mandibular bone tissue development in rat offspring</i> .....	67
5	<b>CONCLUSÕES</b> .....	87
6	<b>CONSIDERAÇÕES FINAIS</b> .....	89
	<b>REFERÊNCIAS</b> .....	90
	<b>APÊNDICES</b> .....	101
	APÊNDICE A – Ficha controle de tratamento camundongos machos.....	106
	APÊNDICE B – Ficha controle de tratamento camundongos fêmeas e ratas.....	103
	APÊNDICE C – Ficha de análise esquelética.....	104
	APÊNDICE D – Ficha de análise visceral.....	105
	<b>ANEXOS</b> .....	106
	ANEXO A – Parecer do Comitê de Ética.....	107
	ANEXO B – Parecer da solicitação do uso da Pregabalina no Brasil (ANVISA).....	108
	ANEXO C – Submissão do Artigo 1 à <i>Reproduction, Fertility and Development</i> .....	109
	ANEXO D – Submissão do Artigo 2 à <i>Semina: Ciências Biológicas e da Saúde</i> .....	110
	ANEXO E – Submissão do Artigo 3 à <i>Birth Defects Research</i> .....	111

## 1 INTRODUÇÃO

2 A pregabalina (PGB) é um fármaco de ação anticonvulsivante pertencente à  
3 classe dos gabapentínicos com ação moduladora de canais de cálcio dependentes de  
4 voltagem com indicação terapêutica para dor neuropática, fibromialgia, transtorno de  
5 ansiedade generalizada e como adjuvante para epilepsia (LYRICA®, 2020). Também  
6 tem sido administrada na profilaxia da dor crônica após eventos agudos diminuindo o  
7 consumo de opioides no intraoperatório (HENNEMANN-KRAUSE; SREDNI, 2016).  
8 Frequentemente é prescrita para uso *off label* para insônia, fobia social, enxaqueca,  
9 transtorno de pânico, transtorno bipolar e abstinência de álcool (ALLES; CAIN;  
10 SNUTCH, 2020; EVOY; MORRISON; SAKLAD, 2017; MATHIESON *et al.*, 2020).

11 Estruturalmente, a PGB (Fig. 1-a) é semelhante ao transmissor inibitório do  
12 ácido gama aminobutírico (GABA) (Fig. 1-b); porém, seu mecanismo de ação não está  
13 relacionado à ligação ou degradação do GABA (EVOY *et al.*, 2021). Acredita-se que o  
14 mecanismo de ação da PGB se dá pela modulação de neurotransmissores por meio da  
15 ação pré-sináptica mediada pela subunidade alfa-2-delta ( $\alpha 2\text{-}\delta$ ) dos canais de cálcio  
16 dependentes de voltagem atenuando o influxo de cálcio neuronal (FIELD *et al.*, 2006;  
17 LOTARSKI *et al.*, 2014; SILLS; ROGAWSKI, 2020), diminuindo a liberação de  
18 glutamato, noradrenalina e substância P, contribuindo para suas ações  
19 anticonvulsivante, analgésica e ansiolítica (MATHIESON *et al.*, 2020).

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Figura 1- Estrutura química da PGB (a) e do GABA (b)  
Fonte: Adaptado de PubChem [Internet]

26 O uso da PGB foi autorizado pela *Food and Drug Administration* (FDA) em  
27 2004, com forma farmacêutica em cápsula sólida e com dose recomendada entre 150  
28 a 600 mg/dia (via oral) para a maioria das suas indicações (CROSS; VISWANATH;  
29 SHERMAN, 2022; DERRY *et al.*, 2019, LYRICA®, 2020). Sua absorção é mais rápida  
30 que a gabapentina (GBP) e se dá no intestino delgado, atingindo picos de concentração  
31 sanguínea em 1 h e eliminada pelos rins (BOCKBRADER *et al.*, 2010). A  
32 biodisponibilidade da PGB é de > 90% e de meia vida entre 5,5 e 7 h independente da  
33 dose, sofrendo metabolismo insignificante em roedores; enquanto que em humanos, os  
34 metabólitos representam < 1% da dose (BOCKBRADER *et al.*, 2010; GAJRAJ, 2007).  
35 Além disso, o fármaco mostra relação dose-resposta, isto é, o efeito aumenta com o  
36 aumento da dose e é relativamente livre de interações medicamentosas  
37 (BOCKBRADER *et al.*, 2010; TAYLOR; ANGELOTTI; FAUMAN, 2007).

38 Os efeitos adversos conhecidos do tratamento com PGB são observados em  
39 80 a 90% dos pacientes, principalmente sonolência e tontura (30% a 50%), edema  
40 periférico (8% a 11%) e ganho de peso (14%) (CROFFORD *et al.*, 2005; DERRY *et al.*,  
41 2019; OHTA *et al.*, 2012; PAUER *et al.*, 2011).

42 A prescrição de PGB teve um aumento significativo ocupando o décimo lugar  
43 de medicamentos mais prescritos nos Estados Unidos em 2016 (GOODMAN; BRETT,  
44 2017), e em sexto lugar entre os principais fármacos subsidiados na Austrália em 2016  
45 e 2017 (SEENIYAR; WILKINSON, 2016). De acordo com Spence (2013), entre os anos  
46 de 2007 e 2012, houve um aumento na prescrição da PGB de 350% no Reino Unido.  
47 Goins e colaboradores (2021) sugerem que o aumento do consumo da PGB pode ser  
48 explicado por ser considerado um fármaco relativamente seguro para dor crônica em  
49 comparação aos opioides. Não há dados do uso da PGB no Brasil (ANEXO B - ANVISA).  
50 Contudo, segundo o levantamento realizado pelo Conselho Federal de Farmácia no  
51 Brasil (Tabela 1), houve um aumento de 12% nas vendas de anticonvulsivantes e  
52 antiepiléticos em 2020 em comparação ao ano anterior (CFF, 2021).

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56

Tabela 1 - Vendas de anticonvulsivantes e antiepiléticos no Brasil nos anos de 2017 a 2020

Anticonvulsivantes e antiepiléticos (n)	2017	2018	2019	2020
		64.250.550	65.824.980	68.649.374
Aumento ano a ano (%)	2017-2018	2018-2019	2019-2020	
	2	4	12	

Fonte: Adaptado de Conselho Federal de Farmácia [Internet].  
Dados apresentados em números absolutos e porcentagem.

57

58 Os gabapentinoides (PGB e GBP) são frequentemente prescritos para o  
59 tratamento de dor neuropática (GOINS; PATEL; ALLES, 2021). Estima-se que 30% da  
60 população mundial sofre de dor revelando um importante problema de saúde global com  
61 impacto social e econômico (COHEN; VASE; HOOTEN, 2021; ELZAHAF *et al.*, 2012;  
62 GASKIN; RICHARD, 2012). Pesquisas sobre a prevalência da dor crônica nos Estados  
63 Unidos sugerem que, aproximadamente, 50 milhões de adultos (20,5%) relatam dor  
64 diariamente ou quase todos os dias, afetando a qualidade de vida e causando  
65 sofrimento emocional e limitações das atividades diárias (DAHLHAMER *et al.*, 2018;  
66 YONG; MULLINS; BHATTACHARYYA, 2022). No Reino Unido, a prevalência da dor  
67 crônica variou entre 35% a 51,3% da população geral e 30% em indivíduos entre 18 e  
68 39 anos (FAYAZ *et al.*, 2016).

69 Segundo Templeton (2020), as mulheres possuem maior risco de  
70 desenvolverem condições que levam a estímulos nociceptivos ou dor como fibromialgia  
71 e lombalgia e podem apresentar piores resultados ao tratamento em comparação com  
72 os homens; além disso relatam dor mais intensa e duradoura (LÖVGREN *et al.*, 2022).  
73 Em contrapartida, Raak e colaboradores (2022) não encontraram diferença significativa  
74 na percepção da dor após cirurgia da coluna lombar sugerindo vieses de resposta  
75 específicos de sexo. Os autores ainda afirmam que os homens tendem a subnotificar a  
76 dor enquanto as mulheres superestimam.

77 De acordo com McHugh e Delanty (2008), há uma alta prevalência de mulheres  
78 em idade fértil com dor neuropática e epilepsia. Segundo os critérios da Organização  
79 Mundial da Saúde (OMS), a idade reprodutiva para mulheres é entre 15 e 49 anos. Por  
80 outro lado, a idade reprodutiva masculina se inicia na puberdade e segue durante a vida  
81 (CHRISTIN-MAITRE; YOUNG, 2022). Um estudo realizado na Suíça sobre o uso de  
82 drogas anticonvulsivantes, revelou que a PGB foi o fármaco mais prescrito para

83 mulheres em idade fértil e o terceiro para gestantes (SPOENDLIN *et al.*, 2021). Estudos  
84 pré-clínicos mostraram que a PGB não foi teratogênica em camundongos, ratos e  
85 coelhos apresentando toxicidade fetal acima da exposição humana. (LYRICA®, 2020).  
86 Em contrapartida, estudos sugerem que o fármaco induz alterações no desenvolvimento  
87 embrionário, risco aumentado de alterações esqueléticas e redução da taxa de  
88 ossificação (ETEMAD *et al.*, 2013, SINGH e GUPTA, 2018, WINTERFELD *et al.*, 2016).  
89 O estudo realizado por Margulis (2019) sobre a associação de anticonvulsivantes e  
90 tamanho ao nascer, mostraram que os bebês expostos à PGB durante a gravidez tinham  
91 peso e comprimento menores quando comparados ao grupo exposto à lamotrigina.  
92 Devido à insuficiência de dados sobre o uso da PGB durante a gestação e o risco  
93 potencial para o feto, o fármaco assume a classificação C para o uso na gestação na  
94 escala de teratogenicidade da FDA (quando estudos em modelo animal mostram efeitos  
95 adversos e não há estudos adequados e controlados em humanos, contudo, os  
96 potenciais benefícios justificam o uso do medicamento em gestantes) (MCHUGH;  
97 DELANTY, 2008).

98 Por outro lado, o tratamento da PGB sobre a fertilidade masculina ainda não  
99 está estabelecido na literatura e a prescrição do fármaco por longos períodos pode  
100 afetar o processo de reprodução (TAHA *et al.*, 2020). Estudos pré-clínicos mostraram  
101 que a PGB pode causar alterações de espermatozoides (DING *et al.*, 2017; ETEMAD *et*  
102 *al.*, 2013; RAYBURN *et al.*, 2018). Outros estudos relataram priapismo contínuo,  
103 anorgasmia, disfunção erétil leve a moderada e epididimite (CALABRÒ *et al.*, 2013;  
104 DING *et al.*, 2017; HITIRIS; BARRETT; BRODIE, 2006; KARANCI, 2020). Não foi  
105 encontrado na literatura estudos objetivando investigar o desenvolvimento intrauterino  
106 e possíveis malformações na prole de camundongos machos tratados com PGB, bem  
107 como o possível impacto do tratamento do macho sobre a prenhez induzida por ele e  
108 sobre a prole.

109 Da mesma forma que a formação dos demais órgãos e processos biológicos,  
110 a odontogênese também está suscetível a passar por alterações devido às perturbações  
111 geradas por agentes nocivos (O'RAHILLY, MÜLLER, 2005). Como um exemplo clássico  
112 de perturbação sistêmica causada por um agente farmacológico, há o padrão de  
113 manchamento dental provocado pela administração de tetraciclina (MOORE,  
114 PERSAUD, TORCHIA, 2004). Outro exemplo é o da ciclofosfamida, agente que, quando  
115 administrado durante a gestação, retardou a formação de molares em fetos de  
116 camundongos e cujos efeitos apresentaram uma proporcionalidade de dose e resposta  
117 (KAWAKAMI; NAKAMURA; KARIBE, 2015; MATHEUS; HETEM, 1990). Nenhum estudo

118 foi encontrado sobre a PGB e seu impacto sobre a odontogênese e sobre o osso  
119 trabeculado mandibular.

120 A revisão da literatura revelou que a segurança do uso da PGB durante a  
121 gravidez ainda não foi estabelecida. Um levantamento realizado por Andrade (2018)  
122 indicou apenas quatro estudos sobre os efeitos da exposição da PGB durante a  
123 gestação. O autor concluiu que, embora os dados disponíveis não identificaram efeitos  
124 adversos associados à exposição da PGB, esses estudos apresentaram vieses como  
125 pequeno tamanho amostral, dosagem e momento da exposição não especificada.

126 Considerando o alto índice de homens e mulheres que necessitam do  
127 tratamento com PGB e a elevada taxa de gravidez não planejada, é alto o potencial de  
128 exposição acidental a esta droga. Portanto, estudos sobre os possíveis efeitos que esta  
129 medicação pode causar sobre a fertilidade masculina e o desenvolvimento embrifetal  
130 podem contribuir para os programas de atenção à saúde a população em idade  
131 reprodutiva, possibilitando estratégias de prevenção contra possíveis danos à prole.

## 132 **2 OBJETIVOS**

133

### 134 2.1 Objetivo Geral

135 Avaliar os possíveis efeitos da PGB sobre a fertilidade quando administrada em  
136 camundongos machos, as possíveis malformações congênitas na prole de  
137 camundongos fêmeas exposta à PGB e as alterações na prole de ratas exposta à PGB  
138 sobre a odontogênese e osso trabecular da mandíbula.

### 139 2.2 Objetivos Específicos

140 Avaliar os possíveis efeitos da PGB sobre a espermatogênese dos  
141 camundongos machos;

142 Avaliar o efeito da PGB no nível sérico da testosterona após a administração em  
143 camundongos machos durante a espermatogênese;

144 Avaliar o desenvolvimento intrauterino da prole de camundongos machos  
145 tratados com PGB durante a espermatogênese;

146 Identificar as malformações estruturais externas, viscerais e esqueléticas nos  
147 fetos de camundongos machos tratados com PGB durante a espermatogênese;

148 Avaliar o desenvolvimento intrauterino da prole de camundongos fêmeas  
149 exposta à PGB durante a gestação;

150 Identificar as malformações congênitas externas, viscerais e esqueléticas de  
151 fetos de camundongos fêmeas expostos à PGB durante a gestação;

152 Avaliar o efeito da PGB na cronologia de erupção dos dentes incisivos da prole  
153 de ratas exposta à PGB durante a gestação;

154 Analisar as possíveis alterações nos processos de amelogênese e  
155 dentinogênese e no desenvolvimento ósseo trabeculado adjacente ao primeiro molar  
156 inferior direito (PMID).

### 157 3 MATERIAIS E MÉTODOS

158 Este estudo foi realizado de acordo com os Princípios Éticos de Experimentação  
159 Animal adotados pelo Conselho Nacional de Controle de Experimentação Animal -  
160 CONCEA sob a Lei n.º 11.794, de 08/out./2008, e aprovado pelo Comitê de Ética em  
161 Experimentação Animal da Universidade Estadual de Londrina (UEL), Londrina, Paraná,  
162 Brasil (CEUA-UEL n. 11174.2018.36).

#### 163 3.1 Fármaco

164 A PGB (LYRICA®, Pfizer) em pó foi diluída em água destilada e administrada  
165 nos grupos tratados na dose de 200 mg/kg (HASANEIN; SHAKERI, 2014; PEGG *et al.*,  
166 2012). Nos grupos controles foi administrada água destilada sob o mesmo delineamento  
167 experimental.

#### 168 3.2 Artigo 1

##### 169 3.2.1 Animais

170 Para a avaliação da exposição da PGB durante a espermatogênese, foram  
171 utilizados 20 camundongos machos e 28 fêmeas adultos da linhagem Swiss (*Mus*  
172 *musculus*), com aproximadamente 35 g de massa corporal provenientes do Biotério  
173 Central do Centro de Ciências Biológicas da UEL (Londrina, Paraná, Brasil). Os animais  
174 foram mantidos em gaiolas de polipropileno forradas com maravalhas e sob condições  
175 de iluminação controlada em ciclo de 12 h claro-escuro a temperatura entre  $22 \pm 2^{\circ}\text{C}$   
176 com água e ração livres.

### 177 3.2.2 Delineamento experimental

178 Os camundongos machos foram distribuídos em dois grupos experimentais,  
179 sendo um grupo controle (C) e um grupo tratado (PGB). Os animais do grupo PGB  
180 receberam diariamente via gavagem, 200 mg/kg de PGB (PEGG *et al.*, 2012), diluída  
181 em água destilada pelo período de 45 dias com volume ajustado a cada três dias de  
182 acordo com a massa corporal do animal. O período de administração foi baseado no  
183 período do ciclo espermatogênico em camundongos (35 dias) (ADLER, 2000). No dia  
184 35, os machos foram colocados para acasalar com fêmeas não tratadas, na proporção  
185 de um macho para duas fêmeas. Os animais continuaram a ser tratados por mais 10  
186 dias, correspondentes a dois ciclos estrais na fêmea. Nos dias seguintes, os  
187 camundongos fêmeas foram examinadas a cada 12 h para verificar a presença do “*plug*  
188 vaginal” considerado o dia zero de prenhez, quando foram identificadas e pesadas. O  
189 grupo C recebeu água destilada sob o mesmo delineamento experimental.

### 190 3.2.3 Toxicidade

191 Para análise da toxicidade, cada animal foi acompanhado e monitorado quanto  
192 à massa corpórea e sinais clínicos como piloereção, olhos avermelhados, diarreia,  
193 alteração da coordenação motora e morte. Após a eutanásia e laparotomia, o coração  
194 pulmão, fígado e rins foram retirados, analisados externamente e pesados (Gehaka AG  
195 200, São Paulo, Brasil).

### 196 3.2.4 Eutanásia

197 No 46º dia, os camundongos machos passaram por eutanásia mediante  
198 decapitação, seguida de coleta de sangue para posterior dosagem de testosterona por  
199 quimioluminescência. Os animais foram laparotomizados seguido de coleta e pesagem  
200 dos órgãos coração, pulmão, fígado, rins, epidídimos e testículos em balança analítica  
201 (Gehaka AG 200, São Paulo, Brasil).

202 Ao 18º dia de prenhez, os camundongos fêmeas foram pesados e passaram por  
203 eutanásia por meio de sobredosagem da associação de 100 mg/g de Dopalen®  
204 (Ketamina) e 10 mg/kg de Anasedan® (Xilazina) via intramuscular seguida de  
205 deslocamento cervical a fim de garantir a anoxia cerebral rápida para o feto. Na  
206 sequência, foram realizadas a laparotomia e a histerectomia para avaliação do  
207 desenvolvimento intrauterino e análise sistemática para detecção de possíveis  
208 malformações nos fetos. Posteriormente, metade dos fetos foram imersos em solução

209 fixadora de Bodian para análise visceral e a outra metade em acetona, seguida de  
210 coloração em KOH 1% e Alizarina (Synth, Diadema, Brasil) para análise esquelética.  
211

### 212 3.2.5 Análise da espermatogênese

213 Para a avaliação da morfologia dos espermatozoides no epidídimo, foi utilizada  
214 a metodologia descrita por Wyrobek *et al.* (1983). Os espermatozoides foram coletados  
215 da cauda do epidídimo e 400 células por animal foram analisadas em microscópio óptico  
216 (Motic BA210, Motic Co, Xiamen, China) com aumento de 1000x. O protocolo de  
217 Johnsen (1970) foi adotado para a análise histológica da espermatogênese no testículo.  
218 Foram analisados 20 túbulos seminíferos por animal e a avaliação histológica foi  
219 classificada em uma escala de escore de 1 a 10, conforme especificados, o escore  
220 mínimo e o máximo, sendo os túbulos que não apresentaram epitélio seminífero  
221 receberam escore 1 e aqueles com presença de todas as células da linhagem  
222 espermatogênica no epitélio incluindo-se a célula de Sertoli, escore 10. Para avaliação  
223 do tecido intersticial, as células de Leydig foram contadas e avaliadas pela quantidade  
224 de células por unidade de área (mm<sup>2</sup>), em 20 imagens por animal, em uma ampliação  
225 de 400X.

### 226 3.2.6 Concentração sérica de testosterona

227 A análise da concentração sérica de testosterona foi realizada a partir da coleta  
228 do sangue do animal no 46° dia. O sangue coletado foi centrifugado e o soro foi  
229 congelado a -20°C até o momento da análise e medidos por quimioluminescência  
230 (*Architect System, 2Gen Test, Abbott, Illinois, EUA*).

### 231 3.2.7 Análise do desenvolvimento intrauterino e embriofetotoxicidade

232 O conteúdo uterino das fêmeas foi analisado quanto ao número de sítios de  
233 implantações, presença de reabsorções, número de fetos vivos e mortos, peso fetal e  
234 placentário de acordo com o método de Salewski (1964). Os seguintes parâmetros  
235 foram analisados e calculados: taxas de viabilidade fetal, taxa de perdas pós-  
236 implantacionais, taxa de reabsorções embrionárias e índice placentário.

### 237 3.2.8 Avaliação das malformações congênitas da prole dos camundongos machos 238 tratados e acasalados com fêmeas não tratadas

239 Para a análise visceral, os fetos foram examinados pela combinação

240 cortes/microdissecção proposta por Barrow e Taylor (1969) para estudo de tórax e  
241 abdome, e pelos cortes estratégicos propostos por Wilson (1965) para estudo da  
242 cabeça. Esta avaliação foi executada sob microscópio estereoscópio (Motic SMZ 140,  
243 Motic Co, Xiamen, China) com aumento de 10x. Os fetos submetidos à análise  
244 esquelética foram avaliados para detecção de anomalias do crânio, esterno, vértebras,  
245 costelas, bacia, clavícula, falanges, metacarpo e metatarso, conforme descrito por  
246 Taylor (1986).

### 247 3.3 Artigo 2

#### 248 3.3.1 Animais

249 Para a avaliação das possíveis malformações congênitas causadas pela PGB  
250 quando administrada em camundongos fêmeas durante a prenhez, foram utilizados 15  
251 camundongos machos e 30 camundongos fêmeas adultos da linhagem Swiss (*Mus*  
252 *musculus*), com aproximadamente 35 g de massa corporal provenientes do Biotério  
253 Central do Centro de Ciências Biológicas da UEL (Londrina, Paraná, Brasil). Os animais  
254 foram mantidos em gaiolas de polipropileno forradas com maravalhas e sob condições  
255 de iluminação controlada em ciclo de 12 h claro-escuro a temperatura entre  $22 \pm 2^\circ\text{C}$   
256 com água e ração livres.

#### 257 3.3.2 Delineamento experimental

258 As fêmeas foram distribuídas em dois grupos experimentais, sendo um controle  
259 (C) e um grupo tratado (PGB) e o acasalamento se deu na proporção de duas fêmeas  
260 para cada macho não tratado. Nos dias seguintes, com um intervalo de 12 h, as vaginas  
261 das fêmeas foram examinadas para verificar a ocorrência do “*plug* vaginal”, o qual  
262 determinou o dia zero de prenhez, ocasião em que as fêmeas foram identificadas e  
263 pesadas. A partir do quinto dia de prenhez, as fêmeas do grupo PGB receberam  
264 diariamente a dose de 200 mg/kg de PGB (PEGG *et al.*, 2012), diluída em água destilada  
265 e via gavagem com dose reajustada a cada três dias, de acordo com o peso do animal.  
266 O grupo C recebeu água destilada sob o mesmo delineamento experimental. O quinto  
267 dia de prenhez foi o escolhido para o início do tratamento, pois ele marca o término da  
268 implantação (FRITZ; GIESE, 1990), assim consegue-se garantir que o fármaco não  
269 interferirá na implantação dos embriões. Os animais foram tratados do 5º ao 17º dia de  
270 prenhez. Este período de tratamento foi escolhido por compreender o período da  
271 organogênese, no qual ocorre grande proliferação celular e formação dos órgãos.

### 272 3.3.3 Toxicidade

273 Para análise da toxicidade, cada camundongo fêmea foi avaliado quanto ao  
274 acompanhamento da massa corporal durante o período de tratamento e sinais clínicos  
275 como piloereção, olhos avermelhados, diarreia, alteração da coordenação motora e  
276 morte. Após a eutanásia e laparotomia, o coração, pulmão, fígado e rins foram retirados,  
277 analisados externamente e pesados em balança analítica (Gehaka AG 200, São Paulo,  
278 Brasil).

### 279 3.3.4 Eutanásia

280 Ao final do experimento todas as fêmeas com 18 dias de prenhez foram pesadas  
281 e passaram por eutanásia por meio de sobredosagem da associação de 100 mg/kg de  
282 Dopalen® (Ketamina) e 10 mg/kg de Anasedan® (Xilazina) via intramuscular seguida  
283 de deslocamento cervical a fim de garantir a anoxia cerebral rápida para o feto. Em  
284 seguida, foram realizadas a laparotomia e histerectomia para avaliação do  
285 desenvolvimento intrauterino e análise sistemática para detecção de possíveis  
286 malformações nos fetos. Posteriormente, metade dos fetos foram imersos em solução  
287 fixadora de Bodian para análise visceral e a outra metade em acetona, seguida de  
288 coloração em KOH 1% e Alizarina (Synth, Diadema, Brasil) para análise esquelética.

### 289 3.3.5 Análise do desenvolvimento intrauterino e embriofetotoxicidade

290 O conteúdo uterino das fêmeas foi analisado quanto ao número de sítios de  
291 implantações, presença de reabsorções, número de fetos vivos e mortos, peso fetal e  
292 placentário de acordo com o método de Salewski (1964). Os seguintes parâmetros  
293 foram analisados e calculados: taxas de viabilidade fetal, taxa de perdas pós-  
294 implantacionais, taxa de reabsorções embrionárias e índice placentário.

### 295 3.3.6 Avaliação das malformações congênitas

296 Para a análise visceral, os fetos foram examinados pela combinação de  
297 cortes/microdissecção proposta por Barrow e Taylor (1969) para estudo de tórax e  
298 abdome, e pelos cortes estratégicos propostos por Wilson (1965) para estudo da  
299 cabeça. Esta avaliação foi executada sob microscópio estereoscópio (Motic SMZ 140,  
300 Motic Co, Xiamen, China) com aumento de 10x. Os fetos submetidos à análise  
301 esquelética foram avaliados para detecção de anomalias do crânio, esterno, vértebras,  
302 costelas, bacia, clavícula, falanges, metacarpo e metatarso, conforme descreve Taylor  
303 (1986).

### 304 3.4 Artigo 3

#### 305 3.4.1 Animais

306 Para a avaliação das possíveis malformações congênitas causadas pela PGB  
307 sobre a odontogênese e osso trabecular da mandíbula, foram utilizados 24 ratas e 12  
308 ratos Wistar adultos e pesando aproximadamente 250 g - 350 g, provenientes do  
309 Biotério Central do Centro de Ciências Biológicas da UEL (Londrina, Paraná, Brasil). Os  
310 animais foram mantidos em gaiolas de polipropileno forradas com maravalhas e sob  
311 condições de iluminação controlada em ciclo de 12 h claro-escuro a temperatura de  
312 22°C ± 2°C com água e ração livres.

#### 313 3.4.2 Delineamento experimental

314 As ratas identificadas em estros foram acasaladas na proporção de duas fêmeas  
315 para um macho. A confirmação da prenhez se deu pela presença de espermatozoides  
316 no esfregaço vaginal e considerado o dia zero da gestação. As ratas prenhes foram  
317 identificadas, pesadas e distribuídas aleatoriamente em grupo controle (C) e grupo  
318 tratado (PGB). As ratas do grupo PGB receberam diariamente via gavagem, 200 mg/kg  
319 de PGB (HASANEIN; SHAKERI, 2014), diluída em água destilada do quinto dia de  
320 prenhez ao dia antecessor ao parto (20° dia). A dose foi ajustada a cada 3 dias de acordo  
321 com a massa corporal do animal. O grupo C recebeu água destilada sob o mesmo  
322 delineamento. No 21° dia de prenhez o parto ocorreu de forma natural e os animais  
323 recém-nascidos foram identificados como idade pós-natal, dia zero (DPN0).

#### 324 3.4.3 Pós-natal

325 No primeiro dia pós-natal (DPN1), as ninhadas foram reduzidas para quatro  
326 filhotes, sendo dois machos e duas fêmeas. Os quatro animais de cada ninhada foram  
327 acompanhados quanto ao peso, comprimento e erupção dos dentes incisivos (ALDER  
328 E ZBIEN, 1977).

#### 329 3.4.4 Eutanásia

330 No dia 30 pós-natal (DPN30), foram selecionados 8 animais de cada grupo (4  
331 fêmeas e 4 machos) para análise do PMID e do osso trabecular adjacente. Os animais  
332 foram eutanasiados por meio de sobredosagem da associação de 100 mg/g de  
333 Dopalen® (Ketamina) e 10 mg/kg de Anasedan® (Xilazina) via intramuscular. As

334 hemimandíbulas direitas foram dissecadas e acondicionadas em formalina neutra  
335 tamponada a 10% por 24 h e mantidas em álcool 70% até o momento da análise por  
336 microtomografia computadorizada ( $\mu$ CT).

### 337 3.4.5 Microtomografia computadorizada

338 As medidas de  $\mu$ CT foram realizadas utilizando o equipamento *SkyScan-Bruker*,  
339 modelo 1173 (*Bruker BioSpin Corporation, Kontich, Bélgica*). O escaneamento das  
340 amostras foi padronizado usando a tensão de 50 kV e 120  $\mu$ A de corrente. A resolução  
341 das imagens obtidas foi de 6  $\mu$ m. A reconstrução foi realizada pelo programa NRecon  
342 (*Bruker BioSpin Corporation, Kontich, Bélgica, versão 1.7.4.2*) e as análises  
343 quantitativas foram realizadas no programa CTan (*Bruker BioSpin Corporation, Kontich,*  
344 *Bélgica, versão 1.20.8*). As imagens 2D ou 3D foram obtidas pelos softwares  
345 *DataViewer (Bruker BioSpin Corporation, Kontich, Bélgica, versão 1.6.0.0)* e *CTVox*  
346 (*Bruker BioSpin Corporation, Kontich, Bélgica, versão 3.3.1*), respectivamente. Os  
347 parâmetros avaliados no PMID foram o volume do esmalte (EV,  $\text{mm}^3$ ) e dentina (DV,  
348  $\text{mm}^3$ ), superfície do esmalte (ES,  $\text{mm}^2$ ) e dentina (DS,  $\text{mm}^3$ ) e a relação entre  
349 superfície/volume do esmalte (ES/EV,  $\text{mm}^{-1}$ ) e a superfície/volume da dentina (DS/DV,  
350  $\text{mm}^{-1}$ ). Para a análise do osso trabecular adjacente, foram considerados os parâmetros:  
351 volume do osso (BV,  $\text{mm}^3$ ), a superfície óssea (BS,  $\text{mm}^2$ ), razão entre a superfície e o  
352 volume (BS/BV,  $\text{mm}^{-1}$ ), a relação entre o volume ósseo e o volume tecidual (BV/TV, %),  
353 número trabecular (Tb.N,  $\text{mm}^{-1}$ ), distância entre as trabéculas, (Tb.Sp, mm) e média da  
354 espessura trabecular (Tb.Th, mm), volume de poros fechados (Po.V(cl),  $\text{mm}^3$ ),  
355 porosidade fechada (Po(cl), %), volume do espaço poroso aberto (Po(op),  $\text{mm}^3$ ),  
356 porosidade aberta (Po(op), %) volume total do espaço poroso (Po.V(tot),  $\text{mm}^3$ ) e  
357 porosidade total (Po(tot), %).

### 358 3.4.6 Análise histológica

359 Após a obtenção das imagens microtomográficas, as amostras foram  
360 encaminhadas para a análise histológica seguindo o protocolo de preparação, coloração  
361 em hematoxilina-eosina (HE) e montagem das lâminas (AMENDOEIRA; CAPUTO;  
362 MOLINARO, 2009). Os cortes foram feitos com 7 $\mu$ m de espessura e plano de corte  
363 paralelo ao longo eixo do primeiro molar.

### 364 3.5 Análise estatística

365 Os dados referentes a cada análise foram tabulados e, posteriormente,

366 analisados no programa *GraphPad Prism 5* (*GraphPad Software, Inc., La Jolla, CA,*  
367 *EUA*) por meio do teste t de Student ou ANOVA para dados paramétricos e o teste de  
368 Mann-Whitney ou Kruskal-Wallis para dados não paramétricos. Para os dados de  
369 frequências foi utilizado o teste exato de Fisher ou o teste do qui-quadrado. O teste de  
370 normalidade adotado foi Shapiro-Wilk. As variáveis categóricas foram expressas pelo  
371 número absoluto (n) e porcentagem (%). As variáveis contínuas foram expressas pela  
372 média e desvio padrão (DP). O nível de significância adotado foi de 5%.

#### 373 **4 RESULTADOS E DISCUSSÃO**

374 Os resultados obtidos na tese estão descritos em três artigos que foram  
375 submetidos às revistas científicas.

376 *4.1 Pregabalin alters reproductive performance in male mice and causes congenital*  
377 *anomalies in offspring*

378 *4.2 Pregabalin promotes embryotoxicity and birth defects in mouse fetuses*

379 *4.3 Evaluation of the effects of prenatal exposure to pregabalin and postnatal analysis*  
380 *of dental and mandibular bone tissue development in rat offspring*

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392 **4.1 Artigo 1 – Submetido à *Reproduction, Fertility and Development***

393

394 **Pregabalin alters reproductive performance in male mice and causes congenital**  
395 **anomalies in offspring**

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405

406 **Abstract:**

407 **Context:** Pregabalin is an anticonvulsant drug with analgesic activity for the treatment of  
408 neuropathic pain. **Aims:** To evaluate the toxicity of pregabalin in reproductive parameters,  
409 spermatogenesis, and teratogenicity in the offspring of mice. **Methods:** Treated mice  
410 groups PGB and control C (n=10 per group). Administration of pregabalin (PGB) and  
411 distilled water (C) during 45 days. After drug administration, the animals were placed to  
412 mate with untreated females. **Key results:** In the paternal parameters of the PGB group,  
413 there was a significant increase in the size of the testicles, morphological alterations in  
414 the spermatozoids, a decrease in the Johnsen Score, an increase in the Leydig cells,  
415 and a decrease in the serum level of testosterone. In the intrauterine development  
416 parameters of females mated with males from the PGB group, a significant decrease in  
417 placental weight, weight and length of fetuses, and fetal viability rate was observed.  
418 There was a significant increase in the number of resorptions and post-implantation  
419 losses. The significant anomalies observed in the offspring were alteration in the size of  
420 the kidneys, absent metacarpals and phalanges, alteration in the sternum, and  
421 supernumerary thoracic vertebrae. **Conclusion:** Results suggest that pregabalin had  
422 toxic effects on the reproductive function of male mice, and teratogenic potential.

423 **Implications:** The findings of this study may provide new hypotheses, taking into  
424 account the risk-benefit ratio for male reproduction and offspring health.

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426 **Key-words:** Pregabalin, reproductive performance, spermatogenesis,  
427 teratozoospermia, spermiogenesis, pregnancy, congenital malformations, infertility.

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## 451 **Introduction**

452 Pregabalin (PGB) is an anticonvulsant drug belonging to the gabapentinoids  
453 class, with modulating action on voltage-dependent calcium channels, and therapeutic  
454 indication for neuropathic pain, fibromyalgia, generalized anxiety disorder, and as an  
455 adjuvant for epilepsy (Lyrica®, 2020). Its analgesic effect is due to its ability to bind to  
456 the alpha 2 delta protein subunit ( $\alpha 2\text{-}\delta$ ) of voltage-gated calcium channels in the central  
457 nervous system (Di Guilmi et al., 2011). It has also been administered in the prophylaxis  
458 of chronic pain after acute events, decreasing intraoperative opioid consumption  
459 (Hennemann-Krause and Sredni, 2016). PGB is often prescribed for off-label use for  
460 insomnia, social phobia, migraines, panic disorder, bipolar disorder, and alcohol  
461 withdrawal (Evoy et al., 2017; Alles et al., 2020; Mathieson et al., 2020).

462 PGB was the first drug approved by the US Food and Drug Administration (FDA)  
463 for the treatment of fibromyalgia in 2007 and for the management of chronic pain  
464 associated with diabetic neuropathy and post-herpetic neuralgia (Häuser, 2018; Black et  
465 al. 2019). It has been shown to be effective in generalized and social anxiety disorders  
466 and for the treatment of some psychoses (Shneker and McAuley 2005; Davanzo et al.  
467 2013).

468 Breivik et al. (2006), who studied the prevalence and impact of chronic pain in  
469 European countries and Israel, reported that 19% of adults suffer from moderate to  
470 severe chronic pain. In addition, the authors observed that approximately 60% of the  
471 study participants reported having had pain for 2 to 15 years. In turn, Yang et al. (2015),  
472 evaluating individuals diagnosed with diabetic peripheral neuropathic pain, reported that  
473 52.3% were male and among the prescribed drugs, anticonvulsants had a prescription  
474 frequency of 66.6%, with PGB being the second most commonly prescribed (21.6%),  
475 only exceeded by gabapentin (GBP).

476 In 2017, sales of Lyrica® (PGB) ranked 10th in global pharmaceutical sales  
477 (Urquhart, 2018) and demonstrated an increase in use of 350% in the UK in the previous

478 5 years (Torjesen 2019). Goins et al. (2021) suggest that the increased consumption of  
479 PGB may be explained by its being considered as a relatively safe drug for chronic pain  
480 compared to opioids.

481 The known adverse effects of PGB treatment are seen in 80 to 90% of patients,  
482 mainly drowsiness, dizziness, peripheral edema, and weight gain (Crofford et al. 2005;  
483 Pauer et al. 2011; Ohta et al. 2012; Derry et al. 2019).

484 On the other hand, the effects of exposure to PGB on male fertility are not yet  
485 established in the literature. A study carried out by Sikka and collaborators (2015)  
486 showed that individuals who received PGB did not present significant differences in  
487 relation to the level of follicle stimulating hormone (FSH), testosterone, morphology, or  
488 sperm motility when compared to the placebo group. On the other hand, prescription of  
489 the drug for long periods can affect the reproduction process (Taha et al., 2020). Some  
490 studies have reported ongoing priapism, anorgasmia, mild to moderate erectile  
491 dysfunction, and epididymitis (Hitiris et al. 2006; Calabrò et al. 2013; Ding et al. 2017;  
492 Karancı 2020).

493 Considering the increased use of PGB and the lack of knowledge about the drug's  
494 effects on male fertility, the current study aimed to evaluate the possible alterations in  
495 reproductive performance caused by PGB when administered to male mice, as well as  
496 the effect on the offspring of these treated males.

497

## 498 **Materials and methods**

499 All the procedures described for the experimental study in question were  
500 approved by the Ethics Committee on the Use of Animals, at the State University of  
501 Londrina (UEL), Londrina, Paraná, Brazil (CEUA-UEL n. 11174.2018.36).

### 502 *Animals*

503 To evaluate the effects of exposure to PGB during spermatogenesis, 20 male  
504 and 28 female Swiss mice were used (*Mus musculus*), with a body mass of

505 approximately 35 g, from the Central Vivarium of the Center for Biological Sciences of  
506 the UEL (Londrina, Paraná, Brazil). The animals were kept in polypropylene cages lined  
507 with wood shavings and under controlled lighting conditions, in a 12 h light-dark cycle, at  
508 a temperature between  $22 \pm 2^\circ\text{C}$ , with free access to food and water.

#### 509 *Experimental design*

510 Male mice were divided into two experimental groups, a control group (C) and a  
511 treated group (PGB). The animals in the PGB group received, via gavage, 200 mg/kg of  
512 PGB diluted in distilled water daily, for a period of 45 days (35 days corresponding to  
513 one spermatogenesis cycle plus ten days corresponding to two estrous cycles of the  
514 females), with a dose adjusted each three days according to the animal's body mass.  
515 Group C received distilled water under the same experimental design. The dose of 200  
516 mg/kg of PGB was defined as it demonstrated the absence of genotoxicity in a previous  
517 study (Pegg et al., 2012).

#### 518 *Mating*

519 After 35 days of PGB administration, male mice were placed to mate with  
520 untreated females in the ratio of one male mouse to two females. During the period of  
521 exposure to PGB, the toxicity of the animal was analyzed by monitoring body mass,  
522 piloerection, diarrhea, motor coordination abnormalities, and death. On the 46th day, the  
523 males were weighed and submitted to euthanasia, followed by blood collection for later  
524 measurement of serum testosterone concentration by chemiluminescence.

525 Female mice were examined every 12 h to check for the presence of the “vaginal  
526 plug”, considered day zero of pregnancy, at which time they were identified and weighed.

#### 527 *Analysis of reproductive parameters and congenital malformations*

528 On the 18th day of pregnancy, the females were euthanized by an overdose of  
529 the combination of 100 mg/g of Dopalen® (Ketamine) and 10 mg/kg of Anasedan®  
530 (Xylazine) intramuscularly. Subsequently, a laparotomy and a hysterectomy were  
531 performed. The evaluation of intrauterine development and embryo-fetal toxicity was

532 performed by analyzing the number of resorptions, number of live and dead fetuses, fetal  
533 weight and length, and placental weight. From these data, it was possible to calculate  
534 the following parameters: fetal viability rate, post-implantation loss rate, embryo  
535 resorption rate, and placental index.

536 To detect possible external structural malformations, the fetuses were evaluated  
537 under a stereoscopic microscope (Motic SMZ 140, Motic Co, Xiamen, China) at 10x  
538 magnification. Analysis of congenital malformations was conducted by fixing half of the  
539 fetuses in Bodian's solution for visceral analysis and the other half in acetone, followed  
540 by staining in 1% potassium hydroxide (KOH) and Alizarin (Synth, Diadema, Brazil) for  
541 skeletal analysis.

542 Visceral analysis was performed through the combination of  
543 sections/microdissection proposed by Barrow and Taylor (1969) for the study of the  
544 thorax and abdomen, and by the strategic sections proposed by Wilson (1965). The  
545 fetuses submitted to skeletal analysis were evaluated for the detection of anomalies of  
546 the bones of the skull, thorax, vertebral column, and upper and lower limbs (Taylor,  
547 1986).

#### 548 *Spermatogenesis analysis*

549 For the evaluation of sperm morphology in the epididymis, the methodology  
550 described by Wyrobek et al. (1983) was used. A total of 400 spermatozooids per animal  
551 were analyzed and counted under an optical microscope (Motic BA210, Motic Co,  
552 Xiamen, China) at 1000x magnification.

553 For the analysis of spermatogenesis, the right testis of each animal was weighed,  
554 the length measured with the aid of a caliper, and fixed in Bouin solution to prepare a  
555 histological slide. Histological sections were made of 5 mm and stained with hematoxylin  
556 and eosin. Twenty seminiferous tubules per animal were analyzed under an optical  
557 microscope with 20x magnification (ZEISS Axio A1, Oberkochen, Germany), following  
558 the Johnsen protocol (1970). Analysis of the germ cells of the seminiferous tubules was

559 performed by classifying the tubules on a scale from 1 to 10, according to the integrity of  
560 the germinal epithelium; with a score of 1 indicating only Sertoli cells without the  
561 presence of germ cells, and a score of 10 indicating complete spermatogenesis and  
562 intact and organized germinal epithelium. The number of Sertoli cells was counted per  
563 seminiferous tubule. Evaluation of the interstitial tissue was performed by counting and  
564 analyzing the nuclei of Leydig cells in 20 images per animal, selecting a unit of area  
565 (mm<sup>2</sup>).

#### 566 *Serum testosterone concentration*

567 The analysis of the serum testosterone concentration was performed from blood  
568 collected on the 46th day. The collected blood was centrifuged and the serum was frozen  
569 at -20°C until the time of analysis and measured by chemiluminescence (Architect  
570 System, 2Gen Test, Abbott, Illinois, EUA).

#### 571 *Statistical analysis*

572 Data were analyzed using the GraphPad Prism 5® program (GraphPad Software,  
573 Inc., La Jolla, CA, USA) using the Student's t test for parametric data and Mann-Whitney  
574 test for non-parametric data. For frequency data, Fisher's exact test or the chi-square  
575 test was used. Categorical variables are expressed by the absolute number (n) and  
576 percentage (%). Continuous variables are expressed as mean and standard deviation  
577 (SD). The normality test adopted was the Shapiro-Wilk test. Differences were considered  
578 significant with a P value <0.05.

579

## 580 **Results**

### 581 *Paternal toxicity*

582 No clinical signs of parental toxicity, such as piloerection, diarrhea, weight  
583 change, motor coordination abnormalities, and death were observed during the  
584 treatment period. Regarding the organs evaluated, there was a statistically significant  
585 increase in the measurement of the testes (Table 1).

Table 1 - Effects of PGB in adult male mice treated with 200 mg/kg for 45 days

Parameters of the males	C (n=10)	PGB (n=10)	P value
Weight of the heart <sup>a</sup> (g)	0.18 ± 0.02	0.18 ± 0.033	0.737
Weight of the lungs <sup>b</sup> (g)	0.25 ± 0.056	0.22 ± 0.049	0.094
Weight of the liver <sup>a</sup> (g)	1.79 ± 0.26	1.87 ± 0.21	0.406
Weight of the kidneys <sup>a</sup> (g)	0.52 ± 0.074	0.54 ± 0.062	0.519
Weight of the epididymis <sup>a</sup> (g)	0.041 ± 0.0047	0.04 ± 0.012	0.796
Seminal vesicle volume <sup>a</sup> (g)	0.074 ± 0.035	0.075 ± 0.043	0.952
Weight of the testicle <sup>a</sup> (g)	0.099 ± 0.009	0.095 ± 0.012	0.498
Size of the testicle <sup>a</sup> (mm)	6.28 ± 0.35	6.82 ± 0.51*	0.009*

Data expressed as mean ± standard deviation. Student's t test<sup>a</sup>. Mann-Whitney U test<sup>b</sup>

\* P<0.05

Legend: Control group (C); Treated group (PGB); Pregabalin (PGB).

586

587 *Effect of PGB on the offspring of treated males mated with untreated females*

588 Treatment of males with PGB significantly impacted intrauterine development.

589 There was a significant decrease in placental weight, fetal weight and length, the

590 placental index, and fetal viability rate, and a significant increase in the number of

591 resorptions, resorption rate, and post-implantation losses (Table 2).

592

Table 2 - Effects of PGB on intrauterine development of offspring of treated males during spermatogenesis (200 mg/kg)

Parameters of the females	C (n=14)	PBG (n=14)	P value
Weight of the uterus <sup>a</sup> (g)	20.2 ± 3.07	19.33 ± 4.06	0.528
Weight of the placenta <sup>a</sup> (g)	0.091 ± 0.013	0.069 ± 0.013*	0.0001*
Weight of the fetuses <sup>a</sup> (g)	1.44 ± 0.054	1.314 ± 0.139*	0.0035*
Size of the fetuses <sup>a</sup> (cm)	2.32 ± 0.16	2.121 ± 0.161*	0.003*
Live fetuses <sup>a</sup>	10.14 ± 2.14	10.79 ± 2.91	0.5119
Dead fetuses <sup>b</sup>	0	0.21 ± 0.58	0.5491
Resorptions <sup>b</sup>	0.43 ± 0.65	3.36 ± 4.05*	0.003*
Implantations <sup>b</sup>	10.57 ± 2.57	14.36 ± 3.74	0.5837
Resorption rate <sup>b</sup> (%)	3.28 ± 4.99	20.63 ± 24.05*	0.003*
Post-implantation loss <sup>b</sup> (%)	3.28 ± 4.99	22.33 ± 23.75*	0.0458*

Placental index <sup>b</sup> (%)	6.33 ± 0.93	4.94 ± 1.19*	0.0016*
Fetal viability rate <sup>b</sup> (%)	96.72 ± 4.99	77.67 ± 23.75*	0.0458*

Data expressed as mean ± standard deviation. Student's t test<sup>a</sup>. Mann-Whitney U test<sup>b</sup>

\* P<0.05

Legend: Control group (C); Treated group (PGB); Pregabalin (PGB).

593

594 The congenital malformations of the offspring observed in the study are shown in  
595 Table 3. In the PGB group, there was a significant change in the size of the kidneys,  
596 shape and size of the sternum, in addition to absent metacarpals and phalanges, and  
597 supernumerary thoracic vertebrae.

598

Table 3 - Malformations observed in fetuses of offspring of males treated with 200 mg/kg of PGB during the period of spermatogenesis and mated with untreated females

<b>Visceral malformations</b>	<b>C (n=75) (%)</b>	<b>PGB (n=72) (%)</b>	<b>P value</b>
Kidneys (change in size)	0	7 (9.72)*	0.0058*
Heart (reduced auricles)	0	1 (1.39)	0.4898
<b>Skeletal malformations</b>	<b>C (n=75) (%)</b>	<b>PGB (n=74) (%)</b>	<b>P value</b>
Supraoccipital (incomplete ossification)	4 (5.33)	5 (6.76)	0.7453
Parietal and interparietal (incomplete ossification)	0	4 (5.41)	0.0583
Metacarpals and phalanges (absent)	0	24 (32.43)*	<0.0001*
Sternum (changes in shape and size)	0	16 (21.62)*	<0.0001*
Thoracic vertebrae (supernumerary)	0	8 (10.81)*	0.003*

Data presented as absolute number and percentage of affected fetuses. Fisher's Exact Test

\*P<0.05

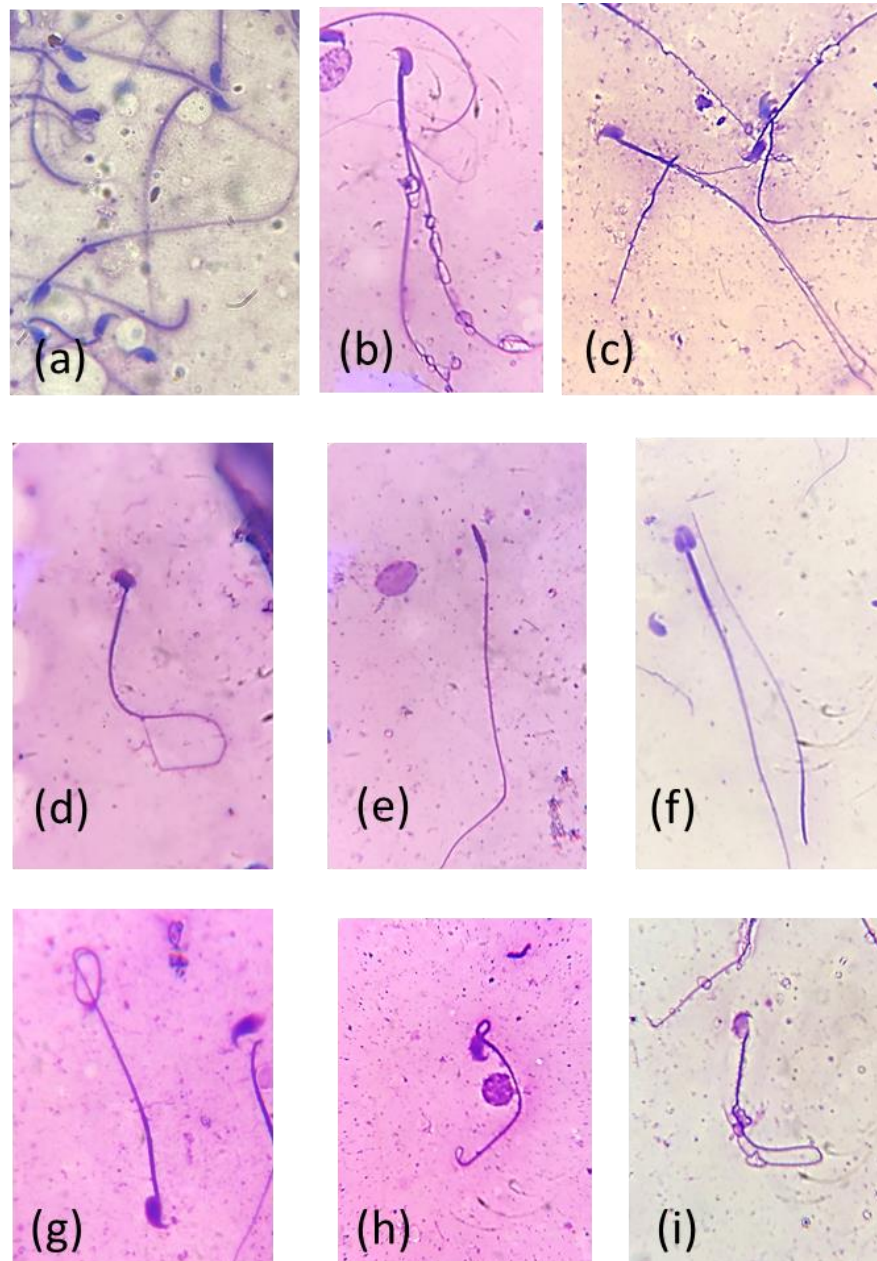
Legend: Control group (C); Treated group (PGB); Pregabalin (PGB).

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600 *Spermatozoids, testicular morphology and serum testosterone concentration*

601 The alterations observed in the spermatozoid analysis are shown in Table 4.  
602 Animals treated with PGB showed significant morphological malformations of  
603 spermatozoids in both tail and head (Fig. 1). The most frequent alterations in the tail  
604 were: curled, knotted, and two-tailed and the most frequent alterations in the head were:  
605 vacuolated, bicephalic, and amorphous.

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 Figure 1. Morphological alterations found in spermatozoids from mice  
 treated with PGB (200 mg/kg) during the period of spermatogenesis.  
 Normal spermatozoids (a). Note two-tailed spermatozoid (b, c).  
 Amorphous head (d, e). Biencephalic spermatozoid (f). Curled tail (g,h).  
 Vacuolated head (i). Legend: Pregabalin (PGB).

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Table 4 - Malformations observed in spermatozoids from mice treated with 200 mg/kg of PGB during the period of spermatogenesis

Alterations	C (n=4000) (%)	PGB (n=4000) (%)	P value
Tail	828 (20.7)	1270 (31.75)*	<0.0001
Head	24 (0.6)	425 (10.62)*	<0.0001

Data presented as absolute number (n) and percentage (%). Chi-square test with Yates correction

\*P<0.05

Legend: Control group (C); Treated group (PGB); Pregabalin (PGB).

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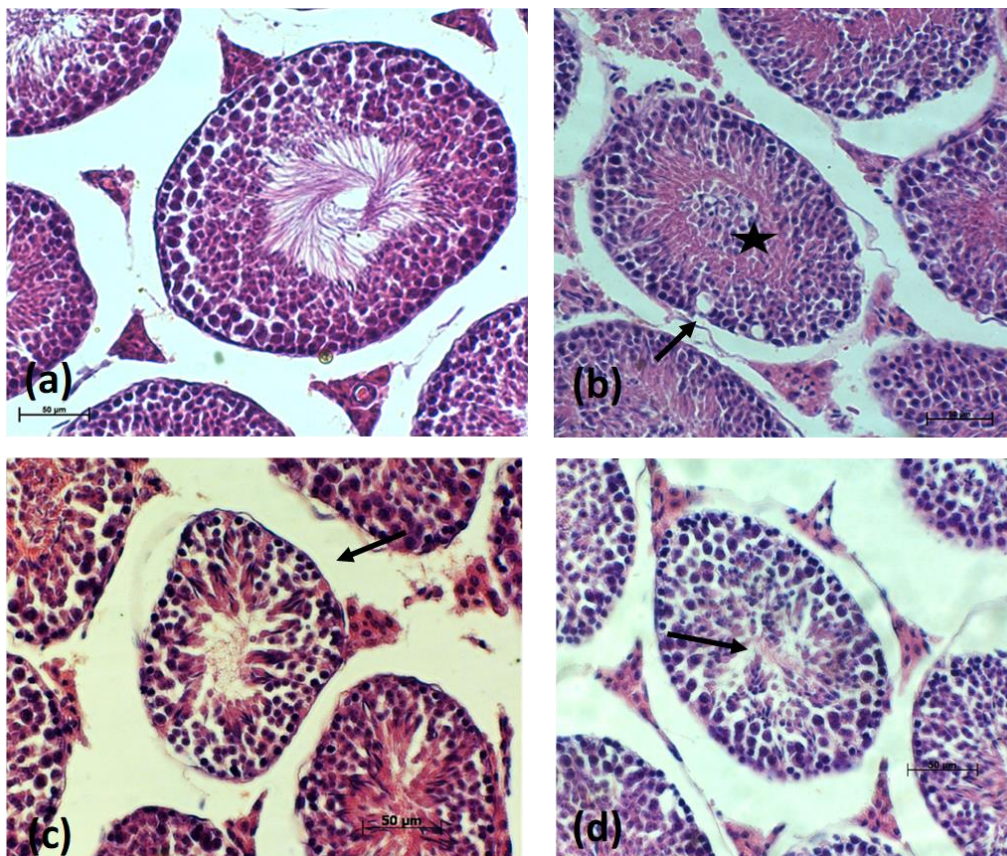
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The evaluation of spermatogenesis and serum testosterone concentration (Table 5) indicated a significant increase in Leydig cells and a significant decrease in the Johnsen Score (Fig. 2). The serum concentration of testosterone was significantly reduced in the group treated with PGB.



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Figure 2. Photomicrographs of the testes of adult mice. Seminiferous tubule of the control group without alterations (a); Seminiferous tubules of the group treated with PGB for 45 days (b, c, d) with alterations. Star indicating intrusion of cells from the

631 germinal epithelium into the lumen and arrow indicating the presence of a vacuole (b);  
 632 arrow indicating seminiferous tubule with disorganized epithelium; (c); Arrow indicating  
 633 spermatozoid count less than 5 (d).

634

Table 5 - Analysis of the testes of mice treated with 200 mg/kg of PGB during the spermatogenesis period

Alterations	C (n=200)	PGB (n=200)	P value
Johnsen score <sup>b</sup>	9.912 ± 0.1018	9.04 ± 0.416*	0.0002*
Sertoli cells <sup>a</sup> (unid./mm <sup>2</sup> )	19.74 ± 2.508	17.94 ± 2.738	0.1317
Leydig cells <sup>a</sup> (unid./mm <sup>2</sup> )	37.92 ± 3.806	61.43 ± 12.33*	0.0002*
Testosterone <sup>b</sup> (ng/ml)	996.7 ± 551.6	355.6 ± 296.0*	0.0152*

Data expressed as means ± standard deviation. Student's t test<sup>a</sup>. Mann-Whitney U test<sup>b</sup>  
 \*P<0.05

Legend: Control group (C); Treated group (PGB); Pregabalin (PGB).

635

## 636 Discussion

637 Some studies suggest that pregnant women exposed to PGB during the first  
 638 trimester may present embryo-fetal alterations and a higher risk of developing congenital  
 639 malformations (Etemad et al. 2013; Morse et al. 2016; Winterfeld et al. 2016; Patorno et  
 640 al. 2017; Singh and Gupta 2018). However, research is limited on the effects of drug  
 641 exposure on spermatogenesis, reproductive performance, and the effects on offspring  
 642 (Ding et al., 2017; Lyrica®, 2020).

643 The intrauterine development of the offspring of male mice exposed to PGB  
 644 during the period of spermatogenesis resulted in decreased placental weight and  
 645 reduced weight and size of fetuses, indicating impaired fetal growth. The study carried  
 646 out by Heinonen et al. (2001) confirms that fetal growth depends on the weight of the  
 647 placenta. Some paternal genes are responsible for placenta-fetal growth such as:  
 648 pleomorphic adenoma gene 1 (*PLAG1*), paternally expressed gene 1/mesoderm-specific  
 649 transcript (*PEG1/MEST*), insulin-like growth factor type II (*IGF2*) and gene 3 expressed  
 650 paternally (*PEG3*) (Vasco et al., 2008). The use of PGB during the period of  
 651 spermatogenesis may have contributed to altering the expression of one or more of these

652 genes, interfering with the development of the placenta and fetus.

653           In the intrauterine development of the offspring of male mice treated with PGB  
654 during the period of spermatogenesis, an increase in the rate of resorption and post-  
655 implantation loss and a decrease in the rate of fetal viability were observed. These results  
656 suggest a toxic effect of PGB on male fertility. GBP has a similar mechanism of action to  
657 PGB and both are analogues of gamma-aminobutyric acid (GABA) (Bockbrader et al.,  
658 2010). In a study performed with anticonvulsants (GBP, lamotrigine, and vigabatrin) in  
659 male rats, Daoud et al. (2004) showed that there is a significant reduction in the fertility  
660 rate when follicle stimulating hormone (FSH) levels are reduced in animals treated with  
661 anticonvulsants. The authors' findings could explain the reduction in fertility rates in our  
662 study. FSH and luteinizing hormones (LH) are the main endocrine hormones that  
663 regulate testicular functions. Testosterone, stimulated by LH, plays a key role in  
664 spermatogenesis, including Sertoli cell differentiation (Huhtaniemi, 2015). FSH, through  
665 the signaling cascade, promotes cell growth and survival by regulating gametogenesis  
666 and reproduction (Casarini and Crépieux, 2019; Recchia et al., 2021).

667           Pre-clinical animal studies associate PGB exposure with increased spermatozoid  
668 morphological abnormalities and decreased gamete number and motility (Etemad et al.  
669 2013; Ding et al. 2017; Rayburn et al. 2018). The data presented in the current study  
670 showed significant alterations in spermatozoid morphology, in both the head and tail  
671 regions, which interfered with embryonic losses and motility, respectively. Although these  
672 alterations did not induce infertility, considering that females mated with males exposed  
673 to PGB became pregnant, in view of the alterations found in the fetuses, there was a  
674 decrease in the rate of fetal viability.

675           For the establishment and maintenance of male reproduction and fertility to occur,  
676 complete spermatogenesis is essential. The differentiation of germ cells into functional  
677 spermatozooids depends on factors involving somatic cells, such as Leydig cells, myoid  
678 cells, and Sertoli cells. In addition to testosterone, growth factors, transcription factors,

679 and receptors associated with Leydig cells and myoid cells mediate spermatogenesis  
680 (Hai et al. 2014).

681 In the histological evaluation of the testes, the Johnsen score showed that the  
682 PGB group presented cellular alterations in spermatogenesis. The factors that influenced  
683 the score were the disorganization of the cells of the germinal epithelium, few  
684 spermatozoa in the lumen, and the increase in Leydig cells.

685 LH induces testosterone secretion in Leydig cells, directly impacting Sertoli  
686 cells. Factors such as cytokines, xenobiotics, and pharmacological agents are involved  
687 in developmental processes in Sertoli cells (Shah et al., 2021). Androgen hormones  
688 control the signaling pathways of male germ cells that bind to androgen receptors on  
689 Sertoli cells and peritubular myoid cells participating in spermatogenesis, under  
690 regulation of the hypothalamic-pituitary-gonadal axis (De Gendt et al. 2004; Welsh et al.  
691 2009; O'Shaughnessy et al. 2010; Willems et al. 2015; Ni et al. 2020). It is important to  
692 highlight the fundamental role of Leydig cells in spermatogenesis, influencing growth  
693 factors and steroidogenesis (Haider 2004; Svechnikov et al. 2010). In this way,  
694 alterations in the functions of these cells can culminate in the abnormal production of  
695 androgens, which affects not only spermatogenesis, but also gonadal development and  
696 sex differentiation (Zhou et al. 2019).

697 Pharmacological treatments that interfere with the endocrine function of the  
698 testes may affect spermatogenesis by producing alterations in Sertoli cells, Leydig cells,  
699 and testosterone levels (Semet et al. 2017; Ray et al. 2017). Analysis of the seminiferous  
700 tubules in the PGB group revealed a significant increase in Leydig cells and a decrease  
701 in the Johnsen Score. Salem Hareedy et al. (2020) reported that the administration of  
702 PGB in rats caused degeneration in the seminiferous tubules and an increase in Leydig  
703 cells. The authors state that Leydig cells are mainly controlled by LH which aids  
704 testosterone synthesis mediated by a steroidogenesis dependent on cyclic adenosine  
705 monophosphate (cAMP) and phosphokinase.

706           The serum concentration of testosterone was significantly reduced in the PGB  
707 group, corroborating previous studies which revealed that the administration of PGB  
708 alters testicular morphometry and causes a drop in testosterone, affecting reproduction  
709 and inhibiting spermatogenesis (Shokry et al. 2020; Taha et al. 2020; Salem Hareedy et  
710 al. 2020). The present study showed that although PGB increased the number of Leydig  
711 cells, there was a reduction in serum testosterone concentration, suggesting that the  
712 drug alters the synthesis of testosterone produced by Leydig cells. The effect of PGB on  
713 Leydig cells is not yet known, however, it is possible that the drug interferes with  
714 intracellular enzymatic and signaling pathways (effects on cAMP and phosphokinase)  
715 and/or modulates calcium transport in Leydig cells (Salem Hareedy et al. 2020).

716           The presence of vacuolization in the PGB group may indicate Sertoli cell  
717 involvement (Tripathi et al. 2009), impairing the spermiogenesis phase that occurs in  
718 spermatids. PGB showed cytotoxicity to the seminiferous epithelium, altering  
719 morphometric and histological parameters of the testes.

720           Alterations in spermatozoid head morphology increase the risk of congenital  
721 anomalies associated with aneuploidies (Van Assche et al. 1996). In the consulted  
722 literature, it was not possible to find any studies on teratozoospermia and PGB. However,  
723 Siklenka et al (2015), state that alterations in histone methylation during  
724 spermatogenesis can reduce survival and cause developmental abnormalities for up to  
725 three subsequent generations. Sperm defects may be the product of functional variations  
726 that occur during spermatogenesis and in the period of spermatozoid maturation in the  
727 epididymis (Barth and Oko 1989; Kleshchev et al. 2021).

728           The results obtained regarding the percentages of spermatozoid abnormalities  
729 enable the establishment of a correlation with alterations in the seminiferous epithelium,  
730 although the abnormalities did not interfere with the fertility rate. However, the survival  
731 of the offspring was influenced, with a reduction in the number of live fetuses, which  
732 resulted in a decrease in fetal viability, an increase in resorptions, and post-implantation

733 losses, causing a greater number of spontaneous abortions. One of the causes of  
734 spontaneous abortions is morphological alterations in the spermatozoid heads correlated  
735 with the levels of DNA damage in the spermatozoid (Robinson et al. 2012). These  
736 alterations are associated with chromosomal aneuploidies, that is, the genetic  
737 information necessary for the offspring is compromised. Some aneuploid embryos  
738 implant and evolve with chromosomal abnormalities and others are aborted (Van Assche  
739 et al. 1996).

740         Statistically significant skeletal and visceral malformations observed in the  
741 offspring of males exposed to PGB were: alteration in kidney size, absence of  
742 metacarpals and phalanges, alteration in shape and size of the sternum, and  
743 supernumerary thoracic vertebrae. These results suggest a relationship between drug  
744 administration in male mice and birth defects in their offspring since the females used for  
745 mating were not exposed to the drug. The occurrence of congenital malformations can  
746 be attributed to paternal exposure to PGB. Evgeni et al. 2014, showed that alterations in  
747 the head of spermatozoids caused congenital abnormalities in the offspring of these  
748 individuals, due to changes in the paternal genetic material (Aitken and Koppers 2011).  
749 According to the findings of this work, it is believed that PGB has genotoxic potential in  
750 spermatozoids, promoting an increase in the spontaneous abortion rate and skeletal and  
751 visceral malformations observed in the offspring.

## 752         **Conclusion**

753         This study appears to be the first to provide evidence that spermatozoid  
754 alterations in male mice exposed to PGB mated with untreated females increase the risk  
755 of congenital malformations in the offspring. The drop in testosterone level and the  
756 increase in the number of Leydig cells suggest that PGB altered spermatogenesis and  
757 testosterone synthesis. The data obtained in this work could help future investigations  
758 and debates on the use of PGB and possible alterations in male reproductive  
759 performance, as well as congenital malformations in their offspring.

760 **Declaration of conflicts of interest**

761 The authors declare no conflicts of interest.

762 **Data Availability Statement**

763 The authors did not publish the research data.

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999 **Artigo 2 – Submetido à Semina: Ciências Biológicas e da Saúde**

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1001 **Pregabalin promotes embryotoxicity and congenital malformation in mouse fetuses**

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1009 **Abstract:** Pregabalin is an anticonvulsant indicated for the treatment of fibromyalgia, diabetic  
1010 neuropathic pain, postherpetic neuralgia, and epilepsy. The aim of the current study was to  
1011 evaluate the effects of pregabalin on embryo-fetal development. Pregnant mice were divided into  
1012 two groups. The treated group received 200 mg/kg of pregabalin via gavage during the gestational  
1013 period and the control group received distilled water under the same design. No clinical signs of  
1014 maternal toxicity were observed. The analysis of the intrauterine development showed significant  
1015 decrease in placental weight ( $P<0.0001$ ), weight ( $P=0.0484$ ) and length of the fetuses ( $P=0.0249$ ),  
1016 an increase in the rate of reabsorption ( $P=0.0485$ ), and decreases in the number of live fetuses  
1017 ( $P=0.0493$ ) and fetal viability ( $P=0.0038$ ), in the treated group. More than 67% of fetuses in the  
1018 treated group were small for gestational age. Malformations observed were significantly higher  
1019 in the treated group, including ventriculomegaly ( $P<0.0001$ ) and incomplete ossification of the  
1020 supraoccipital bone ( $P<0.0001$ ). The findings of this study show that pregabalin was embryotoxic  
1021 and teratogenic in mice fetuses.

1022 **Key-words:** pregabalin, embryotoxicity, ventriculomegaly, incomplete ossification

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## 1030 **Introduction**

1031 Pregabalin (PGB) is an anticonvulsant drug belonging to the gabapentinoids class, with  
1032 modulating action on voltage-dependent calcium channels and with therapeutic indication for  
1033 neuropathic pain, fibromyalgia, generalized anxiety disorder, and as an adjuvant for epilepsy.<sup>(1)</sup>  
1034 PGB was the first medication approved by the United States Food and Drug Administration  
1035 (FDA) for the treatment of fibromyalgia and was previously approved as an anticonvulsant and  
1036 for the control of chronic pain associated with diabetic neuropathy and postherpetic neuralgia.<sup>(2,3)</sup>

1037 According to Kennedy (2011)<sup>(4)</sup>, 85% of pregnant women use some type of medication,  
1038 with analgesics being the most common, second only to vitamins and supplements. A study by  
1039 Daugaard et al. (2019)<sup>(5)</sup>, reported an increase in the use of antiepileptic drugs (AEDs) by women  
1040 of childbearing age and pregnant women between 2001 and 2016, with PGB being one of the  
1041 three most widely prescribed AEDs. The authors suggest that this increase was likely due to  
1042 indications other than epilepsy, such as for the treatment of bipolar mood disorders, migraine, and  
1043 neuropathic pain syndrome.

1044 According to Mcugh and Delanty (2008)<sup>(6)</sup>, there is a high prevalence of women of  
1045 childbearing age with neuropathic pain and epilepsy and, therefore, the possible teratogenic  
1046 effects of the drugs are of concern. AEDs such as topiramate are associated with microcephaly  
1047 and low birth weight, while valproate increases the risks of heart defects.<sup>(7)</sup> In 2016, Winterfeld  
1048 et al.<sup>(8)</sup> collected data from 164 pregnant women exposed to PGB and observed a significantly  
1049 higher rate of birth defects than in women not exposed to the drug. However, the authors report  
1050 limitations of the study, such as the small sample size, different maternal conditions between  
1051 groups, and concomitant exposure to other medications.

1052 Few clinical studies have evaluated the safety of PGB in pregnant women. A survey  
1053 carried out by Andrade in 2018<sup>(9)</sup> pointed to only four studies on the effects of PGB exposure  
1054 during pregnancy. The author concluded that, although the available data did not identify adverse  
1055 effects associated with exposure to PGB, the studies presented biases such as a small sample size,  
1056 dosage, and unspecified time of exposure. On the other hand, studies in animals that received  
1057 PGB during pregnancy showed morphological and developmental alterations in fetuses.<sup>(10-14)</sup>

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1059 Due to the increasing prescription of PGB for women of childbearing age, the present  
1060 study aimed to investigate the effects of PGB during pregnancy in mice and its teratogenic  
1061 potential.

## 1062 **Materials and methods**

1063 *Animals*

1064 This study was carried out in accordance with the Ethical Principles of Animal  
1065 Experimentation, adopted by the National Council for the Control of Animal Experimentation –  
1066 CONCEA, under Law No. 11,794, of Oct/08/2008, and approved by the Animal Experimentation  
1067 Ethics Committee of the State University of Londrina, (UEL), Londrina, Paraná, Brazil (CEUA-  
1068 UEL n. 11174.2018.36).

1069 For the evaluation of PGB exposure during pregnancy, 15 male and 30 adult female mice  
1070 of the Swiss strain (*Mus musculus*) were used, weighing approximately 35 g, from the Central  
1071 Animal Facility of the Biological Sciences Center of UEL, Londrina, Parana Brazil. The animals  
1072 were kept in polypropylene cages lined with wood shavings, under controlled lighting conditions  
1073 in a 12 h light-dark cycle, at a temperature between  $22 \pm 2^\circ\text{C}$ , and with free access to water and  
1074 food.

1075 *Experimental design*

1076 The females were divided into two experimental groups: a control group (C) and a treated  
1077 group (PGB) and mating took place in the proportion of two females for each untreated male. In  
1078 the following days, with an interval of 12 h, the vaginas of the females were examined to verify  
1079 the occurrence of the “vaginal plug”, which determined day zero of pregnancy. From the 5th to  
1080 the 17th day of pregnancy, females in the PGB group received a daily concentration of 200 mg/kg  
1081 of PGB<sup>(1,15)</sup>, diluted in distilled water, via gavage, and with the dose readjusted every three days  
1082 according to the weight of the animal. The C Group received distilled water under the same  
1083 experimental design. At the end of the experiment, at 18 days of pregnancy, all females were  
1084 euthanized by overdose of the combination of 100 mg/kg of Dopalen® (Ketamine) and 10 mg/kg  
1085 of Anasedan® (Xylazine) intramuscularly, followed by cervical dislocation in order to ensure  
1086 rapid cerebral anoxia for the fetuses. Laparotomy and hysterectomy were performed to assess the  
1087 intrauterine development and analysis to detect possible external malformations in the fetuses.  
1088 Subsequently, half of the fetuses were immersed in Bodian's fixative solution for visceral analysis  
1089 and the other half in acetone, followed by staining in potassium hydroxide (KOH 1%) and  
1090 Alizarin (Synth, São Paulo, Brazil) for skeletal analysis.

1091 *Toxicity*

1092 For toxicity analysis, during the treatment period each female was monitored regarding  
1093 body mass and the presence of clinical signs, such as: piloerection, reddened eyes, diarrhea,  
1094 change in motor coordination, and death. After euthanasia and laparotomy, the heart, lungs, liver,  
1095 and kidneys were removed, analyzed externally and weighed on an analytical scale (Gehaka AG  
1096 200, São Paulo, Brazil).

1097 *Analysis of intrauterine development and embryotoxicity*

1098 The uterine content of females was analyzed for the number of implantation sites,  
1099 presence of resorptions, number of live and dead fetuses, and fetal and placental weight according  
1100 to the Salewski method (1964).<sup>(16)</sup> The following parameters were analyzed and calculated: fetal  
1101 viability rates, post-implantation losses rate, embryonic resorption rate, and placental index.

1102 *Evaluation of congenital malformations*

1103 In order to detect possible external structural, visceral, and skeletal malformations, the  
1104 fetuses were evaluated under a stereoscopic microscope (Motic SMZ 140, Motic Co, Xiamen,  
1105 China) with a magnification of 10x.

1106 Visceral analysis was performed using the combination of cuts/microdissection proposed  
1107 by Barrow and Taylor (1969)<sup>(17)</sup> to study the thorax and abdomen, and the strategic cuts proposed  
1108 by Wilson (1965).<sup>(18)</sup> Fetuses submitted to skeletal analysis were evaluated to detect anomalies in  
1109 the bones of the skull, thorax, spine, and upper and lower limbs.

1110 *Statistical analysis*

1111 The data were analyzed in the program GraphPad Prism 5® (GraphPad Software, Inc.,  
1112 La Jolla, CA, USA) using the Student's t test for parametric data and the Mann-Whitney test for  
1113 non-parametric data. The normality test adopted was the Shapiro-Wilk test. For frequency data,  
1114 Fisher's exact test or the chi-square test was used. Categorical variables are expressed as absolute  
1115 number (n) and percentage (%) and continuous variables as mean and standard deviation (SD).  
1116 Differences were considered significant when  $P < 0.05$ .

1117 **Results**

1118 *Toxicity*

1119 No clinical signs of maternal toxicity, such as piloerection, diarrhea, weight change, motor  
1120 coordination abnormalities, and death were observed during the PGB treatment period. The  
1121 evaluated organs also did not show significant alterations (Table 1).

Table 1 – Effects of treatment with PGB (200 mg/Kg) in female mice from the 5th to the 17th day of pregnancy

Parameters of the females	C (n=15)	PGB (n=15)	P value
Weight gain (g) <sup>a</sup>	22.97 ± 5.29	22.88 ± 7.92	0.4442

Weight of the heart (g) <sup>a</sup>	0.18 ± 0.03	0.17 ± 0.03	0.4804
Weight of the lungs (g) <sup>a</sup>	0.22 ± 0.03	0.19 ± 0.02	0.0518
Weight of the liver (g) <sup>a</sup>	2.6 ± 0.31	2.82 ± 0.29	0.0577
Weight of the kidneys (g) <sup>a</sup>	0.43 ± 0.05	0.4 ± 0.05	0.0646

Data expressed as means ± standard deviation. Student's t test<sup>(a)</sup>. Mann-Whitney U test<sup>(b)</sup>.

\*P<0.05

Legend: Control Group (C); Treated group (PGB); Pregabalin (PGB).

Source: own authors

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### *Effect of pregabalin during pregnancy*

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The analysis of the uterine content of the females showed developmental alterations in the PGB group. There were significant decreases in fetal and placenta weights, and fetal length. The number of live fetuses, fetal viability, and placental index were significantly lower in the PGB group. The number of resorptions showed a significant increase in the group treated with PGB when compared to the control group (Table 2).

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Figure 1 shows the adequacy of fetal weight to gestational age in the PGB group. It was verified that 67.5% of the fetuses were small for the gestational age (SGA) and 32.5% had adequate weight for the gestational age (AGA). No fetuses were large for gestational age (LGA).

Table 2 – Effects of PGB (200 mg/kg) treatment on intrauterine development in offspring of female mice

<b>Parameters of the females</b>	<b>C (n=15)</b>	<b>PGB (n=15)</b>	<b>P value</b>
Weight of the uterus (g) <sup>a</sup>	15.9 ± 5.9	19.21 ± 5.49	0.1223
Weight of the placenta (g) <sup>a</sup>	0.11 ± 0.16	0.07 ± 0.02*	<0.0001
Live fetuses <sup>a</sup>	11.6 ± 3.42	9.13 ± 3.72*	0.0493
Weight of the fetuses (g) <sup>a</sup>	1.41 ± 0.15	1.11 ± 0.14*	0.0484
Measurement of the fetuses (cm) <sup>a</sup>	2.3 ± 0.27	2.12 ± 0.11*	0.0249
Resorptions <sup>b</sup>	1.2 ± 2.21	1.87 ± 2.64*	0.041
Resorption rate (%) <sup>b</sup>	11.38 ± 15.82	13.71 ± 20.16*	0.0485
Fetal viability (%) <sup>b</sup>	88.62 ± 15.82	84.74 ± 20.44*	0.0038
Placental index (%) <sup>a</sup>	7.542 ± 1.29	5.79 ± 1.63*	0.0029

Data expressed as means ± standard deviation. Student's t test<sup>(a)</sup>. Mann-Whitney U test<sup>(b)</sup>.

\*P<0.05

Legend: Control Group (C); Treated group (PGB); Pregabalin (PGB).

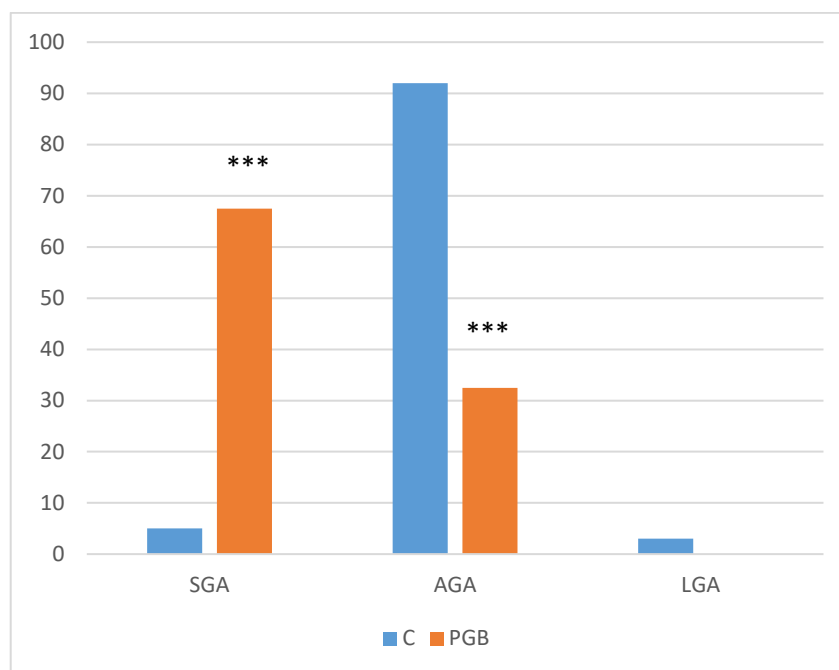
Source: own authors

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Figure 1 - Correlation of fetal weight with pregnancy age of offspring exposed to PGB and offspring not exposed during pregnancy.



\*\*\* P<0.0001

Legend: Small for Gestational Age (SGA); Adequate for Gestational Age (AGA); Large for Gestational Age (LGA). Control group (C); Treated group (PGB); Pregabalin (PGB).

Source: own authors

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### *Analysis of malformations*

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Visceral and skeletal malformations observed in the offspring of the PGB group are shown in Table 3. There was a significant increase in fetuses with ventriculomegaly (Fig. 2) and incomplete ossification of the supraoccipital bone.

Table 3 - Malformations observed in fetuses after exposure to PGB (200 mg/kg) from the 5th to the 17th day of pregnancy

Visceral malformations	C (n=79) (%)	PGB (n=61) (%)	P value
Microphthalmia	0	3 (4.92)	0.0683
1st, 2nd, and 3rd cerebral ventricles (ventriculomegaly)	0	18 (29.51)*	<0.0001
Change in the size of the kidneys	0	3 (4.92)	0.0804

Decreased testicles	0	1 (1.64)	0.4357
<b>Skeletal malformations</b>	<b>C (n=71) (%)</b>	<b>PGB (n=62) (%)</b>	<b>P</b>
Parietal and interparietal (incomplete ossification)	3 (4.23)	8 (12.90)	0.2833
Supraoccipital (incomplete ossification)	0	16 (25.81)*	<0.0001
Palate (cleft)	0	3 (4.84)	0.0987
Ribs (incomplete/reduced ossification)	0	3 (4.84)	0.0987
Phalanges (front and back feet, distal and proximal)	2 (2.82)	3 (4.84)	0.6637

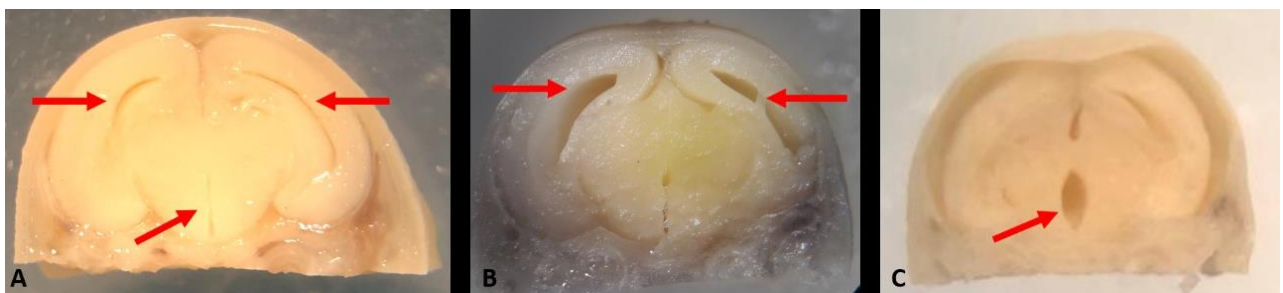
Data presented as absolute number and percentage of affected fetuses. Fisher's Exact test. \*P<0.05

Legend: Control Group (C); Treated group (PGB); Pregabalin (PGB).

Source: own authors

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1148 Figure 2 - Frontal section of the cerebral hemisphere in fetus exposed to PGB



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1150 Arrows indicate normal ventricles in the C group (A), arrows indicate dilated ventricles in the PGB

1151 group (B and C).

1152 Legend: Control group (C); Treated group (PGB); Pregabalin (PGB).

1153 Source: own authors

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### Discussion

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The present study showed that PGB did not induce maternal toxic effects during pregnancy, but altered embryonic development. Placental weight, fetal viability, and the placental index were significantly lower in the PGB group. Similar results were reported by Etemad et al. (2013)<sup>(19)</sup> and Singh and Gupta (2018).<sup>(13)</sup>

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The use of AEDs during pregnancy presents potential risks for fetuses, and correlations were reported between congenital malformations and anticonvulsants such as phenobarbital, phenytoin, carbamazepine, and valproate.<sup>(20,21)</sup> However, the teratogenic potential of PGB has not yet been established.<sup>(9)</sup>

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Embryonic resorptions in mice are analogous to spontaneous abortions in humans.<sup>(22)</sup> The PGB group presented a statistically higher resorption rate than the C group, which explains the

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1166 decrease in the fetal viability rate. The study by Hirama et al. (2008)<sup>(23)</sup> showed that women with  
1167 epilepsy tend to present decreased blood levels of AEDs during pregnancy, which return to pre-  
1168 gestational levels in the first or second month after delivery. This decrease seems to result mainly  
1169 from the lower amount of AED binding to plasma proteins and increased metabolism and  
1170 elimination; the decrease in protein binding leads to an increase in the free blood fraction, which  
1171 may lead to toxic effects in the pregnant woman and also in the fetus or embryo. These findings  
1172 are in agreement with the results obtained in the current study, regarding the increase in resorption  
1173 rates and decrease in fetal viability.<sup>(23)</sup>

1174 According to the Society for Maternal-Fetal Medicine, a variety of maternal, fetal, and  
1175 placental conditions can lead to fetal growth restriction (FGR), which is a leading cause of  
1176 perinatal morbidity and mortality.<sup>(24)</sup> The present study showed that the length and body weight  
1177 of the fetuses were significantly lower in the group exposed to PGB. Approximately 20% of FGR  
1178 cases are due to congenital malformations and chromosomal abnormalities.<sup>(25)</sup> When structural or  
1179 genetic defects of the fetus are excluded, the primary cause of FGR is placental insufficiency.<sup>(26)</sup>  
1180 The study carried out by Margulis (2019)<sup>(27)</sup> on the association of AEDs and birth size, showed  
1181 that babies exposed to PGB during pregnancy had lower weight and length when compared to the  
1182 group exposed to lamotrigine. The data obtained in this study suggest that PGB interfered with  
1183 intrauterine development.

1184 Pregnant women who use AEDs, whether for epilepsy or other neuropsychiatric  
1185 indications, are at an increased risk of preterm delivery and SGA newborns.<sup>(28,29)</sup> The analysis of  
1186 fetal weight to gestational age in the current study showed that 67.5% of fetuses in the PGB group  
1187 were SGA. Although the long-term consequences of the relationship between AEDs and SGA  
1188 remain unclear, being born with a low weight for gestational age has been associated with  
1189 unfavorable outcomes not only in the perinatal period (stillbirths, impaired thermoregulation and  
1190 hypoglycemia) but also in adulthood (cardiovascular diseases and diabetes).<sup>(29,30)</sup> A study  
1191 involving pregnant women who were users of different AEDs showed an increased risk of SGA  
1192 newborns after exposure to carbamazepine or valproate monotherapy.<sup>(30)</sup> GBP, an AED with  
1193 similar action to PGB, has also been associated with an increased risk of SGA newborns.<sup>(31)</sup>

1194 The UK Medicines and Healthcare Products Regulatory Agency, based on an  
1195 observational study involving over 2,700 pregnancies, states that PGB is associated with a slightly  
1196 increased risk of birth defects when compared to pregnancies without exposure to any AEDs or  
1197 lamotrigine or duloxetine monotherapy.<sup>(32)</sup> The present study showed that PGB caused incomplete  
1198 ossification of the supraoccipital bone in fetuses. Similar evidence in rabbits exposed to higher  
1199 doses (1250 mg/kg) was reported by Morse (2016).<sup>(11)</sup> The incomplete ossification of the  
1200 supraoccipital bone can be explained by the embryonic development deficit evidenced by the

1201 shorter length and lower body weight of fetuses in the PGB group. In contrast, administration of  
1202 PGB at different doses in pregnant rats induced skeletal variations in the offspring, especially  
1203 advanced ossification from the jugal bone to the maxilla and fusion of the nasal sutures.<sup>(12)</sup>

1204 Ventriculomegaly can be defined as abnormal enlargement of the cerebral ventricles in  
1205 utero, related or not to increased intracranial pressure (hydrocephaly).<sup>(33,34)</sup> Data obtained in the  
1206 current work suggest that PGB caused ventriculomegaly in fetuses in the PGB group. In a  
1207 multicenter study involving 116 babies exposed to PGB, 4 cases of cerebral ventricle enlargement  
1208 were observed. The authors, however, reported that, due to the limited data, it was not possible to  
1209 establish an association between the drug and the anomaly found.<sup>(8)</sup>

1210 Some research has been carried out to explain the teratogenic effects of AEDs. Prenatal  
1211 exposure to, for example, oxcarbazepine or valproic acid induces apoptosis-mediated  
1212 neurodegeneration.<sup>(35)</sup> According to Etemad et al. (2015),<sup>(36)</sup> PGB causes caspase-dependent  
1213 apoptosis (cysteine protease) and inhibits the differentiation of limb tissues during fetal  
1214 development in mice. Similar data were found by Sayin and Simsek (2018),<sup>(37)</sup> suggesting that  
1215 PGB induced neural tube defects and malformations in chicken embryos by increasing apoptosis  
1216 involving activation of the caspase-3 protease pathway. Programmed cell death plays an important  
1217 role in organogenesis and tissue remodeling, which, when out of balance, is associated with  
1218 developmental abnormalities.<sup>(38)</sup>

1219 Based on the findings of the current work, it is believed that PGB presents embryotoxic  
1220 potential on the intrauterine development of fetuses, promoting a decrease in placental weight, an  
1221 increase in abortion rate, and a decrease in fetal viability. PGB also had a teratogenic effect,  
1222 significantly increasing the number of fetuses with skeletal and visceral malformations, such as  
1223 ventriculomegaly and incomplete ossification of the supraoccipital bone.

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## 1225 **Conclusion**

1226 The findings of the current study showed that PGB administered to pregnant mice was  
1227 embryotoxic. In addition, the drug showed teratogenic potential, with a significant increase in  
1228 ventriculomegaly and incomplete ossification of the supraoccipital bone. Considering the high  
1229 percentage of unplanned pregnancies, caution should be employed when prescribing PGB for  
1230 women of childbearing age.

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1235 Personnel), Brazil.

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1419 **Artigo 3 – Submetido à Birth Defects Research Part B: Developmental and**  
1420 **Reproductive Toxicology**

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1422 **Evaluation of the effects of prenatal exposure to pregabalin and postnatal analysis**  
1423 **of dental and mandibular bone tissue development in rat offspring**

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1437 **Abstract**

1438 **Background:** Pregabalin is a gabapentinoid indicated for the treatment of peripheral  
1439 neuropathic pain, fibromyalgia, and as an adjuvant therapy for epilepsy. The aim of this  
1440 study was to evaluate the effects of pregabalin on postnatal development,  
1441 odontogenesis, and mandibular trabecular tissue using computed microtomography.

1442 **Methods:** Pregnant rats (n=24) were divided into two groups: control group (C), and  
1443 treated group (PGB). The PGB group received 200 mg/kg of pregabalin via gavage  
1444 during the embryonic period and the C group received distilled water under the same  
1445 design. On the 21st day of pregnancy, delivery occurred naturally. On the first postnatal  
1446 day, the litters were reduced to four pups (2 males and 2 females) and followed up for  
1447 30 days. On postnatal day 30, eight animals from each group were randomly selected  
1448 for analysis of the lower right first molar and adjacent trabecular bone.

1449 **Results:** In the maternal parameters, the data revealed a significant decrease in body  
1450 weight gain in addition to a smaller number of live pups. In the postnatal analysis, the  
1451 exposed pups showed a significant decrease in weight and length and a delay in the

1452 eruption of the incisor teeth. Microtomography analysis revealed a significant reduction  
1453 in enamel volume, a lower volume and percentage of open pores, lower total porosity,  
1454 and a higher percentage of bone volume.

1455 **Conclusions:** The findings of this study showed that pregabalin altered the eruption  
1456 chronology of the incisors, decreased enamel volume, affected the microarchitecture of  
1457 mandibular trabecular bone, and impaired postnatal development.

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1459 **Key-words:** pregabalin, congenital malformation, tooth enamel, mandibular bone tissue,  
1460 computed microtomography

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## 1480 Introduction

1481 Pregabalin (PGB) is a gabapentinoid indicated for the treatment of neuropathic  
1482 pain, fibromyalgia, generalized anxiety disorder, and as an adjuvant for epilepsy (Cross,  
1483 Viswanath & Sherman, 2022; Lyrica®, 2020). PGB is often prescribed for off-label use  
1484 for migraine, insomnia, social phobia, bipolar disorder, alcohol withdrawal, and in an  
1485 acute dose for postoperative pain (Alles, Cain & Snutch, 2020; Cross et al., 2022; Evoy,  
1486 Morrison & Saklad, 2017; Mathieson, Lin, Underwood, & Eldabe, 2020). It is believed  
1487 that the effect of the drug occurs through the inhibition of voltage-gated calcium channels  
1488 binding to the  $\alpha 2\text{-}\delta$  subunit and, even though it is analogous to gamma-aminobutyric acid  
1489 (GABA), it does not bind to GABA receptors (Chincholkar, 2018).

1490 Animal studies have shown that PGB is up to five times more potent than  
1491 gabapentin (GBP) for treating visceral pain, acute pain in spinal cord injury, irritable  
1492 bowel syndrome, and fibromyalgia (Łuszczki, 2010; Shamsi Meymandi & Keyhanfar,  
1493 2013; Tanabe, Ono, Honda, & Ono, 2009).

1494 Between 2002 and 2015, the use of gabapentinoids (PGB and GBP) more than  
1495 tripled among adults in the United States (Johansen, 2018). The increase in the  
1496 prescription of PGB may be related to the low dependence potential compared to opioids  
1497 for the treatment of chronic pain (Goins, Patel & Alles, 2021).

1498 The guidelines from the Centers for Disease Control and Prevention indicate the  
1499 prescription of gabapentinoids as the first choice for neuropathic pain, with PGB being  
1500 indicated for the treatment of post-herpetic neuralgia and neuropathic pain associated  
1501 with diabetes and spinal cord injuries (Dowell, Haegerich & Chou, 2016).

1502 Spoenclin et al. (2021) studied the prescription of anticonvulsants during  
1503 pregnancy and concluded that PGB was the third most commonly used drug during  
1504 pregnancy and the first for women of childbearing age. In another study conducted by  
1505 Blotière et al. (2019) investigating antiepileptic drugs prescribed for pregnant women,  
1506 PGB ranked second, only behind lamotrigine.

1507 However, research suggests that the use of PGB during pregnancy induces fetal  
1508 toxicity and an increased risk of teratogenicity, such as skeletal alterations and reduced  
1509 ossification rate (Etemad, Mohammad, Mohammadpour, Vahdati Mashhadi & Moallem,  
1510 2013; Singh & Gupta, 2018; Winterfeld et al., 2016).

1511 Computed microtomography ( $\mu$ CT) is an X-ray imaging exam considered the gold  
1512 standard for evaluating 3D bone morphology and morphometry in small animals by

1513 visualizing the bone at a microstructural scale (1–100  $\mu\text{m}$  resolution) (Kim, Brodt, Tang  
1514 & Silva, 2021), allowing quantitative and qualitative analyses of bone tissue (Rovaris et  
1515 al., 2018).

1516 With applications in different areas of medical research,  $\mu\text{CT}$  has been used in  
1517 dentistry as an alternative to conventional histological analysis. The technique is used  
1518 for the characterization of carious lesions, evaluation of dental structure (enamel and  
1519 dentin), alterations in bone metabolism, and the effects of drug use that impair bone  
1520 remodeling (Free et al., 2017; Sinibaldi et al., 2018).

1521 No studies regarding the possible effects of PGB on tooth development and  
1522 mandibular bone tissue, were found in the consulted literature. Thus, the aim of the  
1523 current study was to evaluate the effects of prenatal PGB exposure in rat offspring on  
1524 odontogenesis and mandibular trabecular tissue using  $\mu\text{CT}$  and histomorphometry.

## 1525 **Materials and methods**

### 1526 *Animals*

1527 This study was carried out in accordance with the Ethical Principles of Animal  
1528 Experimentation adopted by the National Council for the Control of Animal  
1529 Experimentation - CONCEA under Law No. State University of Londrina, (UEL),  
1530 Londrina, Paraná, Brazil (Opinion n. CEUA-UEL n. 11174.2018.36).

1531 In total, 24 adult female, and 12 adult male Wistar rats weighing approximately  
1532 250 g - 350 g were kept in polypropylene cages lined with wood shavings and under  
1533 controlled lighting conditions in a 12 h light-dark cycle at a temperature of  $22 \pm 2^\circ\text{C}$  with  
1534 water and feed ad libitum.

1535 Rats identified as in estrus were mated at the ratio of two females to one male.  
1536 Pregnancy was confirmed by the presence of spermatozoa in the vaginal smear and  
1537 considered day zero of pregnancy. The pregnant rats were identified, weighed, and  
1538 randomly distributed into a control group (C) and a treated group (PGB).

### 1539 *Drug*

1540 PGB (LYRICA®, Pfizer) was administered at a dose of 200 mg/kg, based on a  
1541 previous study (Hasanein & Shakeri, 2014).

### 1542 *Design*

1543 Rats in the PGB group received daily, via gavage, 200 mg/kg of PGB diluted in  
1544 distilled water from the 5th day of pregnancy to the day before parturition (20th day). The  
1545 dose was adjusted every 3 days according to the animal's body weight. Group C received  
1546 distilled water under the same experimental design. On the 21st day of pregnancy,  
1547 delivery occurred naturally and newborn animals were identified as aged postnatal day  
1548 zero (PND0).

#### 1549 *Assessment of postnatal development*

1550 On the first postnatal day (PND1), litters were reduced to four pups, 2 males and  
1551 2 females. The four animals in each litter were weighed and measured on days PND1,  
1552 7, 14, and 21 according to Alder and Zbien (1977, modified).

1553 Eruption of incisor teeth was followed from PND1 to postnatal day 30 (PND30).  
1554 PND30 was chosen because it corresponds to the final process of root development and  
1555 formation of cellular cementum (Kaneko, Hashimoto, Enokiya, Ogiuchi & Shimono,  
1556 1999). Eight animals from each group were randomly selected for analysis of the right  
1557 mandibular first molar (RMFM) and adjacent trabecular bone. The animals were  
1558 euthanized through an overdose of the association of 100 mg/kg of Dopalen® (Ketamine)  
1559 and 10 mg/kg of Anasedan® (Xylazine) intramuscularly, and the right hemimandibles  
1560 were dissected and placed in 10% neutral buffered formalin for 24 h and kept in 70%  
1561 alcohol until analysis by  $\mu$ CT.

#### 1562 *Evaluation of Computerized Microtomography*

1563 The  $\mu$ CT measurements were performed using tSkyScan-Bruker equipment,  
1564 model 1173 (Bruker BioSpin Corporation, Kontich, Belgium). Sample scanning was  
1565 standardized using a 50 kV voltage and 120  $\mu$ A current. The resolution of the images  
1566 obtained was 6  $\mu$ m. Reconstruction was performed using the NRecon program (Bruker  
1567 BioSpin Corporation, Kontich, Belgium, version 1.7.4.2) and quantitative analyses were  
1568 performed using the CTan program (Bruker BioSpin Corporation, Kontich, Belgium,  
1569 version 1.20.8). The 2D or 3D images were obtained using the software DataViewer  
1570 (Bruker BioSpin Corporation, Kontich, Belgium, version 1.6.0.0) and CTVox (Bruker  
1571 BioSpin Corporation, Kontich, Belgium, version 3.3.1), respectively. The parameters  
1572 evaluated in the RMFM were the enamel (EV,  $\text{mm}^3$ ) and dentin volume (DV,  $\text{mm}^3$ ),  
1573 enamel (ES,  $\text{mm}^2$ ) and dentin surface (DS,  $\text{mm}^3$ ), and enamel surface/volume ratio  
1574 (ES/EV,  $\text{mm}^{-1}$ ), and dentin surface/volume ratio (DS/DV,  $\text{mm}^{-1}$ ). For the analysis of  
1575 adjacent trabecular bone, the following parameters were considered: bone volume (BV,  
1576  $\text{mm}^3$ ), bone surface (BS,  $\text{mm}^2$ ), bone surface-to-volume ratio (BS/BV,  $\text{mm}^{-1}$ ), bone

1577 volume-to-tissue ratio (BV/TV, %), trabecular number (Tb.N, mm<sup>-1</sup>), distance between  
 1578 trabeculae (Tb.Sp, mm), mean trabecular thickness (Tb.Th, mm), closed pore space  
 1579 volume (Po.V(cl), mm<sup>3</sup>), closed porosity (Po(cl), %), open pore space volume (Po(op),  
 1580 mm<sup>3</sup>), open porosity (Po(op), %) total pore space volume (Po.V(tot), mm<sup>3</sup>), and total  
 1581 porosity (Po(tot), %).

1582 After obtaining the microtomographic images, the samples were sent for  
 1583 histological analysis following the preparation protocol, with staining in hematoxylin-  
 1584 eosin (HE) and mounting of the slides. The cuts were made with a thickness of 7µm and  
 1585 a cutting plane parallel to the long axis of the RMFM. The slides were analyzed under an  
 1586 optical microscope (Motic BA210, Motic Co, Xiamen, China) with a magnification of 40X.

### 1587 *Statistics*

1588 The mean value of the parameters was calculated and used for statistical  
 1589 analysis. Data referring to each analysis were tabulated and subsequently analyzed in  
 1590 the GraphPad Prism 5 program (GraphPad Software, Inc., La Jolla, CA, USA). For  
 1591 variables with normal distribution and homogeneity of variance, the significance of  
 1592 differences was assessed using two-way analysis of variance (ANOVA; factors: weight  
 1593 and length) and the post hoc Bonferroni test or Student's t test. Data that did not have  
 1594 normal distribution and/or did not show homogeneity of variance were analyzed using  
 1595 the non-parametric Mann-Whitney or Kruskal-Wallis tests, followed by the post hoc Dunn  
 1596 test. The normality test adopted was the Shapiro-Wilk test. Categorical variables were  
 1597 expressed as absolute number (n) and percentage (%). Continuous variables were  
 1598 expressed as mean and standard deviation (SD). The significance level adopted was  
 1599 5%.

## 1600 **Results**

### 1601 *Maternal toxicity*

1602 During the administration of PGB (200 mg/kg) in pregnant rats, no clinical signs  
 1603 of toxicity, such as piloerection, diarrhea, motor coordination abnormalities, and death  
 1604 were observed. However, there was a significant decrease in maternal body weight gain  
 1605 and number of live pups per litter (Table 1).

Table 1 - Effect of treatment with PGB (200 mg/kg) in pregnant rats

<b>Female</b>	<b>C (n=12)</b>	<b>PGB (n=12)</b>	<b>P value</b>
Weight gain <sup>b</sup> (g)	116.3 ± 21.58	103.08 ± 35.53*	0.0139*

<sup>1</sup>Live pups (unit/litter)<sup>a</sup>                      12.67 ± 1.5                      10.75 ± 2.22\*                      0.0213\*

Data expressed as means ± standard deviation. Student's t test <sup>a</sup>. Mann-Whitney test <sup>b</sup>

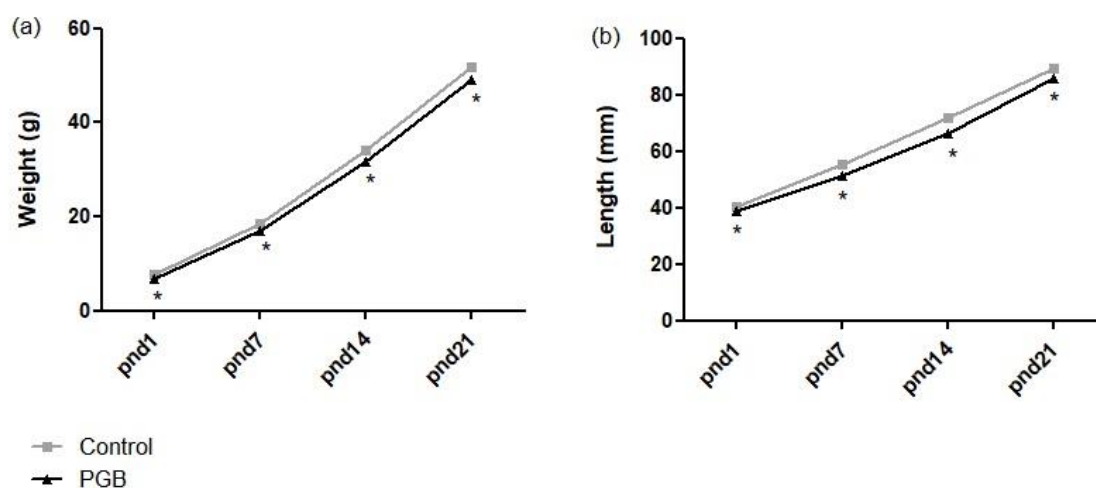
\* P<0.05

Legend: <sup>1</sup>number of live pups per litter; Control group (C); Treated group (PGB); Pregabalin (PGB).

## 1606                      *Assessment of Postnatal Development*

1607                      In the postnatal analysis of the offspring, the evolution of the weight, the length  
1608 of the animals (Fig.1), and the eruption of the incisor teeth were evaluated.

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1611 Figure 1. Weight gain (a) and body length (b) of the offspring of female rats from PND1 to PND  
1612 21 of group C and group PGB. Data are mean ± s.e.m. of 48 pups from each group. The two-way  
1613 ANOVA analysis of repeated measures indicated that the weight and length of the offspring of the  
1614 PGB group in the postnatal period were significantly lower when compared to the C group.

1615 \*P<0.05

1616 Legend: postnatal day (pnd); Control group (C); Treated group (PGB), Pregabalin (PGB).

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1619                      The two-way ANOVA analysis indicated the effect of PBG exposure on weight  
1620 gain (F=2.67; P=0.048) and length (F=9.49; P<0.0001) of the offspring exposed to PGB  
1621 when compared to the C group. In addition, the data revealed a delay in the eruption of  
1622 incisor teeth in the PGB-exposed group (Table 2).

1623

Table 2- Monitoring the eruption of incisor teeth in the offspring of animals exposed to PGB (200 mg/kg) during gestational development

Offspring	C (n=48)	PGB (n=48)	P value
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Eruption of incisors (day) <sup>b</sup>	7.13 ± 0.33	7.55 ± 0.68*	0.001
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Data expressed as means ± standard deviation. Mann-Whitney U test<sup>b</sup>

\* P<0.05

Legend: Control group (C); Treated group (PGB); Pregabalin (PGB).

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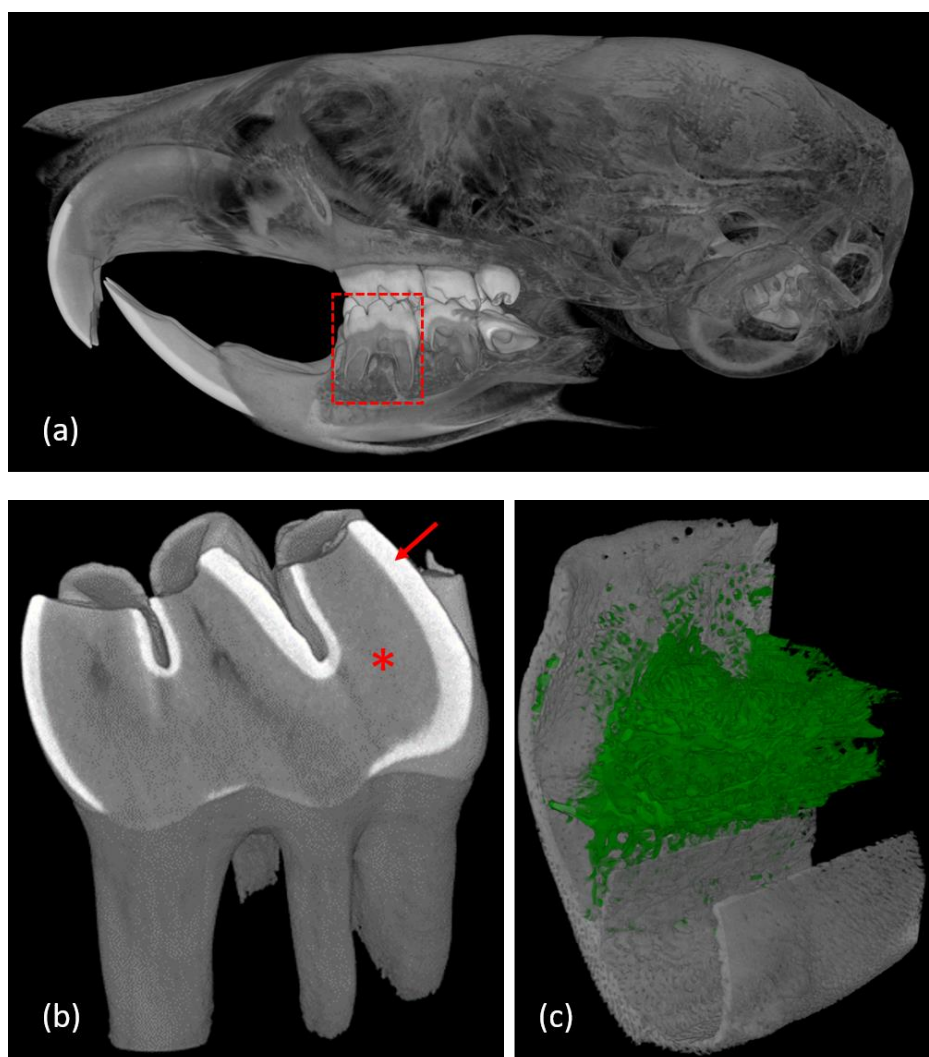
1626            *Computerized Microtomography*

1627            *Enamel, dentin and mandibular trabecular bone*

1628            The regions of interest of enamel, dentin, and trabecular bone for  $\mu$ CT analysis  
1629 are shown in Figure 2. The parameters analyzed in the RMFM showed a significant  
1630 reduction in EV in the PGB group when compared to the C group (Table 3). No significant  
1631 alterations were observed in dentin.

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Figure 2. Reconstructed  $\mu$ CT images. RMFM Region of Interest (ROI) indicated by the dotted rectangle (a). Enamel area indicated by arrow and dentin area indicated by asterisk (b). Volume of interest (VOI) of trabecular bone represented in green (c).  
Legend: Computed microtomography ( $\mu$ CT); Right mandibular first molar (RMFM).

Table 3 - Evaluation of RMFM enamel and dentin in the offspring of rats treated with PGB (200 mg/kg) during the gestational period

Enamel	C (n=8)	PGB (n=8)	P value
EV (mm <sup>3</sup> ) <sup>a</sup>	1.24 ± 0.077	1.09 ± 0.10*	0.0043*
ES (mm <sup>2</sup> ) <sup>a</sup>	33.07 ± 1.74	30.98 ± 2.67	0.0850
ES/EV (mm <sup>-1</sup> ) <sup>b</sup>	26.71 ± 0.94	28.53 ± 0.88*	0.0045*
Dentin	C (n=8)	PGB (n=8)	P
DV (mm <sup>3</sup> ) <sup>a</sup>	3.72 ± 0.20	3.60 ± 0.19	0.2410
DS (mm <sup>2</sup> ) <sup>a</sup>	53.59 ± 2.40	52.54 ± 3.24	0.4743

DS/DV (mm<sup>-1</sup>)<sup>a</sup>                      14.42 ± 0.35                      14.60 ± 0.51                      0.4098

Data expressed as means ± standard deviation. Student's t test<sup>a</sup>. Mann-Whitney U test<sup>b</sup>

\* P<0.05

Legend: EV: enamel volume; ES: enamel surface; ES/EV: enamel surface/volume ratio; DV: dentin volume; DS: dentin surface; DS/DV: dentin surface/volume ratio; Control group (C); Treated group (PGB); Pregabalin (PGB).

1642                      The evaluation of the trabecular bone adjacent to the RMFM showed that PGB  
1643 was able to significantly increase the percentage of bone volume (BV/TV). Regarding  
1644 bone microarchitecture, the PGB group showed a lower percentage of open pores  
1645 (Po(op)) and a lower total pore volume (Po.V(tot)) and, therefore, a lower percentage of  
1646 total porosity (Po(tot)) (Table 4).

Table 4- Evaluation of the adjacent trabecular bone tissue by RMFM of the offspring of rats treated with PGB (200 mg/kg) during the gestational period

Parameters	C (n=8)	PGB (n=8)	P value
BV, (mm <sup>3</sup> ) <sup>a</sup>	2.66 ± 0.28	2.5 ± 0.28	0.2857
BS (mm <sup>2</sup> ) <sup>a</sup>	147.8 ± 11.94	140.9 ± 14.34	0.3115
BS/BV (mm <sup>-1</sup> ) <sup>a</sup>	55.74 ± 3.1	56.47 ± 4.16	0.6962
BV/TV (%) <sup>a</sup>	92.2 ± 1.5	93.78 ± 0.86*	0.0208*
Tb.Th (mm) <sup>a</sup>	0.07 ± 0.003	0.07 ± 0.004	0.722
Tb,N (mm <sup>-1</sup> ) <sup>a</sup>	13.31 ± 0.76	13.7 ± 0.9	0.37
Tb.Sp (mm) <sup>b</sup>	0.03 ± 0.003	0.03 ± 0.002	0.2902
Po.V(cl) (mm <sup>3</sup> ) <sup>a</sup>	0.0005 ± 0.0001	0.0005 ± 0.0003	0.9857
Po(cl) (%) <sup>a</sup>	0.018 ± 0.005	0.018 ± 0.0105	0.9337
Po(op) (mm <sup>3</sup> ) <sup>a</sup>	0.228 ± 0.066	0.165 ± 0.028*	0.0266*
Po(op) (%) <sup>a</sup>	7.786 ± 1.497	6.198 ± 0.856*	0.0208*
Po.V(tot) (mm <sup>3</sup> ) <sup>a</sup>	0.229 ± 0.066	0.166 ± 0.028*	0.0267*
Po(tot) (%) <sup>a</sup>	7.802 ± 1.496	6.216 ± 0.855*	0.0208*

Data expressed as means ± standard deviation. Student's t test<sup>a</sup>. Mann-Whitney U test<sup>b</sup>

\* P<0.05

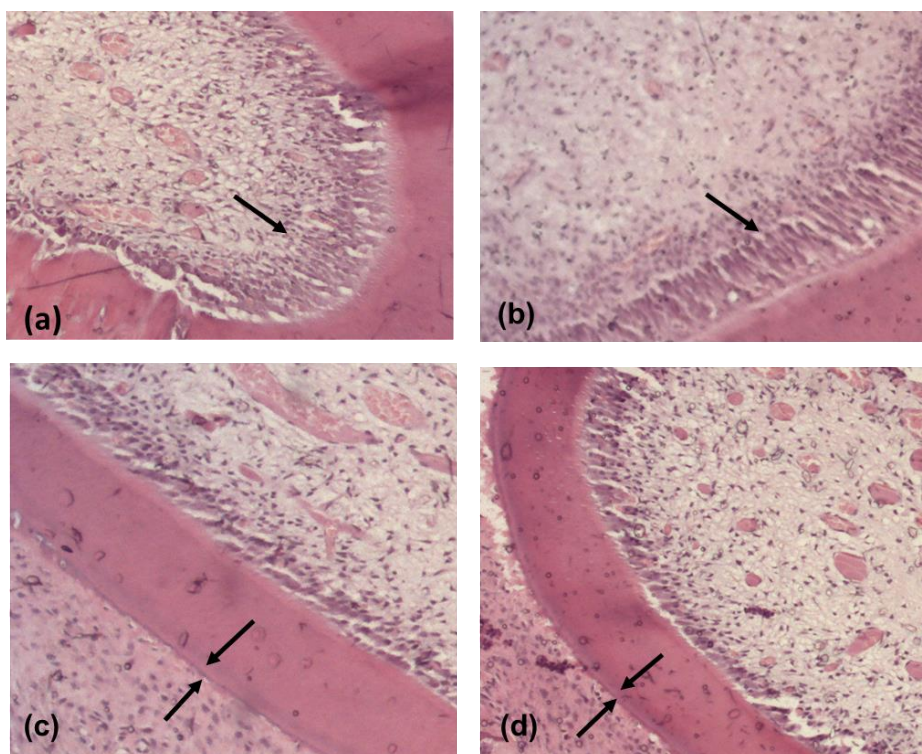
Legend: BV: bone volume, BS: bone surface, BS/BV: bone surface-to-volume ratio, BV/TV bone volume-to-tissue ratio, Tb.N: trabecular number, Tb.Sp: distance between trabeculae, Tb.Th: mean trabecular thickness, Po.V(cl): closed pore space volume, Po(cl): closed porosity, Po(op): open pore space volume, Po(op): open porosity, Po.V(tot): total pore space volume, and Po(tot): total porosity. bone volume (BV, mm<sup>3</sup>), bone surface (BS, mm<sup>2</sup>), surface-to-volume ratio (BS/BV, mm<sup>-1</sup>), bone volume-to-tissue ratio (BV/TV, %), trabecular number (Tb.N, mm<sup>-1</sup>), distance between trabeculae (Tb.Sp, mm), mean trabecular thickness (Tb.Th, mm), closed pore space volume (Po.V(cl), mm<sup>3</sup>), closed porosity (Po(cl), %), open pore space

volume (Po(op), mm<sup>3</sup>), open porosity (Po(op), %) total pore space volume (Po.V(tot), mm<sup>3</sup>), and total porosity (Po(tot), %); Control group (C); Treated group (PGB); Right mandibular first molar (RMFM); Pregabalin (PGB).

1647

1648 *Histological analysis*

1649 In the histological analysis, no alteration was observed in cellular organization.  
 1650 The evaluated parameters were: organization of the odontoblast layer, cementum layer,  
 1651 and pulp organization (Figure 3).



1652

1653 Figure 3- HE-stained histological sections of the RMFM region of the offspring  
 1654 of rats after  $\mu$ CT analysis. C Group (a,c) and PGB Group (b,d). Arrows indicate  
 1655 odontoblast layer (a, b) and cementum layer (c, d). Legend: Hematoxylin-eosin  
 1656 (HE); Right mandibular first molar (RMFM); Computed microtomography ( $\mu$ CT);  
 1657 Pregabalin (PGB).

## 1658 Discussion

1659 The administration of PGB (200 mg/kg) in pregnant rats showed clinical signs of  
 1660 maternal toxicity with a significant decrease in the body weight gain of animals in the  
 1661 PBG group when compared to the C group. Similar data in mice were found in the study  
 1662 by Etemad et al. (2013). However, the authors report that, when analyzing maternal body

1663 weight gain minus gravid uterine weight, the means were not significantly different  
1664 between the control and treated groups. In the present study, it was not possible to  
1665 subtract the gravid uterine weight from the maternal body weight, as the delivery  
1666 occurred naturally, however, according to the European Medicines Agency, among the  
1667 uncommon adverse reactions of PGB is anorexia and weight loss (EMA, 2020).

1668           Insufficient maternal weight gain is associated with impaired intrauterine growth  
1669 and low birth weight (Kleinman et al., 2007). Qi and He (2021) state that newborns with  
1670 low birth weight are at a greater risk of motor difficulties and delay in the development of  
1671 coordination, while Othman (2021) reports delay in speech and language.

1672           Maternal exposure to drugs, chromosomal alterations in the fetus, and  
1673 abnormalities in the placenta can lead to fetal death. In the current study, there was a  
1674 reduction in the number of live pups in the PGB group, characterizing alterations in  
1675 embryo-fetal development, suggesting that PGB may increase the risk of spontaneous  
1676 abortion. Similar findings were reported by Etemad et al. (2013). In addition, the  
1677 newborns in the PGB group had lower weight when compared to the C group,  
1678 demonstrating an intrauterine development deficit. Similar data were found in the study  
1679 developed by Morse, Henck and Bailley (2016). Another study with mice carried out in  
1680 our laboratory in 2022 (unpublished data), showed that 67.5% of fetuses exposed to  
1681 PGB were small for pregnancy age. The weight of the offspring in the present study  
1682 continued to be lower in the PGB group on PND7, PND14, and PND 21, showing that  
1683 there was no weight recovery during postnatal development. It is suggested that the use  
1684 of PGB during pregnancy, compromised the weight gain of the offspring.

1685           Margulis et al. (2019) observed that maternal exposure to PGB in humans  
1686 generated newborns with lower weight and length when compared to exposure to other  
1687 anticonvulsants used during pregnancy, such as lamotrigine. Veiby, Daltveit, Engelsen  
1688 & Gilhus (2014) evaluated fetal growth in children exposed to antiepileptic drugs and  
1689 concluded that the mean birth weight, length, and head circumference were lower in the  
1690 group exposed to topiramate, when compared to other drugs, such as PGB,  
1691 carbamazepine, lamotrigine, and levetiracetam.

1692           Regarding the eruption of incisor teeth, no studies were found regarding the use  
1693 of PGB during pregnancy and eruption chronology. The data presented show a delay in  
1694 the eruption of incisors in the group exposed to PGB. Systemic disturbances during  
1695 odontogenesis can affect not only tooth structure and morphology, but also alter the  
1696 eruption process (Seminaro & Ivancaková, 2004). The mechanism of tooth eruption is

1697 still unclear and several theories have been proposed. The theory of alveolar bone  
1698 growth is one of the most accepted and assumes that bone deposition and resorption  
1699 are responsible for tooth eruption (Kjær, 2014).

1700           According to research carried out by Liu, Yao, Pan & Wise (2005) and He et al.  
1701 (2021), the follicle around the unerupted tooth regulates a sequence of events, directing  
1702 the osteoclastogenesis necessary for tooth eruption, which, in turn, is dependent on bone  
1703 resorption in the coronal region and neoformation in the apical region. In addition,  
1704 according to the authors, osteoclastogenesis depends on the balance of expression of  
1705 genes encoding proteins activating the receptor activator of ligand Kappa-B (RANKL)  
1706 and osteoprotegerin (OPG) present in the dental follicle. It is possible that PGB may have  
1707 altered this balance of events between the RANKL, responsible for inducing osteoclast  
1708 formation, and OPG, which, when bound to RANKL, inhibits osteoclast differentiation  
1709 and activity (Brodetska et al., 2020; Ponzetti & Rucci, 2019). Future research is needed  
1710 to clarify this event.

1711           The literature shows that other anticonvulsants, such as phenytoin, are  
1712 associated with delayed tooth eruption, gingival hyperplasia, and short tooth roots  
1713 (Appleton & Leach, 2008; Doufexi, Mina & Ioannidou, 2005). Jacobsen, Henriksen,  
1714 Haubek & Østergaard (2014) suggest that valproate administered during prenatal care  
1715 is related to tooth agenesis. In this way, generations of different anticonvulsants can also  
1716 alter tooth formation and eruption.

1717           Analysis of enamel and dentin in RMFM, performed by  $\mu$ CT, suggests that PGB  
1718 did not alter dentin development; however, there was a significant reduction in EV.  
1719 According to the Federacion Dentarie Internacional (DDE Index, 1992), hypoplasia is a  
1720 quantitative defect of the dental enamel caused by deficient formation of the organic  
1721 matrix. Hypoplastic teeth are more susceptible to the development of dental caries and  
1722 acid secretions from cariogenic bacteria (Alotaibi et al., 2022).

1723           Amelogenesis (enamel formation) is a complex biological process regulated by  
1724 ameloblasts involving two phases: secretion and maturation. Secreted enamel proteins  
1725 are encoded by genes belonging to the calcium-binding secretory phosphoprotein family  
1726 that are expressed by secretory ameloblasts: amelogenin (*AMEL*), enamelin (*ENAM*),  
1727 and ameloblastin (*AMBN*) (Simmer et al., 2021). It is possible that PGB may have  
1728 interfered with the expression of enamel secretory genes, since any failure in the  
1729 secretion stage results in enamel hypoplasia (Lacruz, Habelitz, Wright & Paine, 2017;  
1730 Simmer et al., 2021).

1731           The evaluation of bone microarchitecture suggested a significant increase in the  
1732 relationship between bone volume and tissue volume (BV/TV). Simko et al. (2019)  
1733 evaluated the effect of PGB and GBP on bone remodeling in rats and concluded that  
1734 neither drug affected mechanical strength or bone mineral density. Morse et al. (2016)  
1735 evaluated the incidence of fusion of the jugal bone to the maxilla in newborn rats exposed  
1736 to PGB during pregnancy, and revealed a significant increase in the treated group. It has  
1737 been shown that some anticonvulsants are capable of producing deleterious effects on  
1738 bone metabolism and that GBP inhibits the process of osteoclastogenesis and  
1739 osteoblastogenesis (Rocha, Ferraz, Prudêncio, Fernandes & Costa-Rodrigues, 2019).  
1740 The bone modeling process involves coordinated actions of resorptions (osteoclasts)  
1741 and replacements (osteoblasts) by mechanisms of degradation of the extracellular bone  
1742 matrix and deposits of layers of collagen and hydroxyapatite (Rocha et al., 2019; Siddiqui  
1743 & Partridge, 2016; Soysa & Alles, 2016; Valenti, Dalle Carbonare & Mottes, 2016). Any  
1744 disorder in these actions can lead to alterations in bone composition and structure  
1745 (Rocha et al., 2019).

1746           The increase in BV/TV found in this study can be supported by the results of the  
1747 Po parameters. In the evaluation of pore space volume, the parameters open porosity,  
1748 total pore space volume, and total porosity were significantly smaller, that is, there was  
1749 a reduction in open spaces in the PGB group and, consequently, an increase in bone  
1750 volume in the region adjacent to the RMFM.

1751           Classical histological analysis with HE offers important information about  
1752 morphology, cell organization, and extracellular matrix elements (de Bournonville,  
1753 Vangrunderbeeck & Kerckhofs, 2019). However, this technique has limitations, such as  
1754 the destruction of the sample during the preparation process and 2D visualization (Liu,  
1755 Yan, Kang, Zhang & Li, 2015; Müller et al., 1998), in addition to making it impossible to  
1756 visualize the enamel layer due to the decalcification process. However, other techniques  
1757 for viewing the enamel of erupted teeth, such as grinding, prevent cell visualization.  
1758 Quantification of the volume of dentin and, especially, of enamel is only possible with  
1759  $\mu$ CT.

## 1760           **Conclusion**

1761           PGB negatively interfered with the intrauterine development of the offspring  
1762 exposed during pregnancy, with a decrease in weight and length at birth. This study  
1763 appears to be the first to provide evidence that PGB promotes delayed eruption of

1764 incisors, a significant reduction in the volume of dental enamel in the RMFM, and greater  
1765 trabecular bone volume, suggesting that the drug can alter several tissues. These  
1766 findings indicate that the prescription of PGB during pregnancy should be performed with  
1767 caution.

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**1969 5 CONCLUSÕES**

1970

1971 - Não foram observados sinais clínicos de toxicidade nos camundongos machos  
1972 durante o período de tratamento com PGB;

1973 - Observou-se aumento estatisticamente significativo da medida dos testículos  
1974 dos camundongos machos tratados com PGB durante a espermatogênese quando  
1975 comparados ao grupo C;

1976 - Os camundongos machos tratados com PGB durante a espermatogênese  
1977 apresentaram alterações morfológicas significativas de espermatozoides tanto em  
1978 cabeça como em cauda;

1979 - A avaliação da espermatogênese apontou um aumento significativo das células  
1980 de Leydig e diminuição significativa do Escore de Johnsen no grupo de camundongos  
1981 tratados com PGB;

1982 - A concentração sérica de testosterona após o tratamento com a PGB durante  
1983 a espermatogênese de camundongos apresentou-se significativamente diminuída  
1984 quando comparada ao grupo C;

1985 - O desenvolvimento intrauterino da prole de camundongos machos tratados  
1986 com PGB durante a espermatogênese e acasalados com camundongos fêmeas não  
1987 tratadas apontou uma diminuição significativa do peso da placenta, do peso e  
1988 comprimento dos fetos, do índice placentário e da taxa de viabilidade fetal; e um  
1989 aumento significativo do número de reabsorções, da taxa de reabsorção e da perda pós-  
1990 implantação quando comparado ao grupo C;

1991 - As malformações congênitas significativas observadas nos fetos de  
1992 camundongos machos tratados com PGB durante a espermatogênese e acasalados  
1993 com fêmeas não tratadas foram alterações de tamanho dos rins e da forma e tamanho  
1994 do esterno, metacarpos e falanges ausentes e vértebras torácicas supranumerárias;

1995 - Não foram observados sinais clínicos de toxicidade nos camundongos fêmeas  
1996 tratados com PGB durante a prenhez;

1997 - A análise do conteúdo uterino das fêmeas tratadas com PGB durante a prenhez  
1998 mostrou diminuição significativa do peso da placenta, do peso e do comprimento fetal,  
1999 do número de fetos vivos, da viabilidade fetal e do índice placentário; e um aumento

- 2000      significativo das reabsorções quando comparado ao grupo C;
- 2001              - As malformações congênitas significativas observadas nos fetos de  
2002 camundongos fêmeas expostos à PGB durante a gestação foram ventriculomegalia e  
2003 ossificação incompleta do osso supraoccipital;
- 2004              - O efeito do tratamento com PGB em ratas prenhes mostrou perda significativa  
2005 de ganho de peso e diminuição significativa do número de filhotes vivos por ninhadas  
2006 quando comparado ao grupo C;
- 2007              - O peso e o comprimento pós-natal da prole de ratas exposta à PGB foram  
2008 significativamente menores quando comparados ao grupo C;
- 2009              - A exposição da PGB na prole de ratas causou atraso na erupção dos dentes  
2010 incisivos;
- 2011              - A análise do esmalte do PMID sugeriu redução significativa de volume na prole  
2012 exposta à PGB; não houve alteração significativa da dentina;
- 2013              - A análise do osso trabecular adjacente ao PMID sugeriu aumento significativo  
2014 da relação entre o volume ósseo e o volume tecidual (BV/TV) na prole exposta à PGB  
2015 quando comparado ao grupo C.
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## 2030 **6 CONSIDERAÇÕES FINAIS**

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2032 Os dados obtidos neste estudo sugerem que a PGB promove toxicidade  
2033 reprodutiva em camundongos machos adultos, causando alterações morfológicas nos  
2034 espermatozoides dos animais.

2035 A PGB apresentou potencial teratogênico quando administrada em  
2036 camundongos prenhes. Também alterou a odontogênese e a formação do osso  
2037 trabecular mandibular da prole de ratas.

2038 Não estão claros na literatura os efeitos tóxicos da PGB nas células testiculares  
2039 e os efeitos teratogênicos do fármaco em humanos. Até que novos estudos sejam  
2040 realizados, é necessário cautela ao prescrever PGB para mulheres e homens em idade  
2041 fértil, bem como, orientação sobre a relação risco-benefício.

2042 O presente estudo apresentou fortalezas quanto a manipulação das amostras  
2043 sob ambiente controlado, uso de apenas um anticonvulsivante e propiciou informações  
2044 sobre o potencial tóxico e teratogênico da PGB em modelo animal e o uso da  $\mu$ CT para  
2045 avaliar a amelogênese, a dentinogênese e a microarquitetura trabecular da mandíbula.  
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2047 As limitações deste estudo foram a administração de apenas uma dose e a  
2048 ausência de dados sobre a concentração plasmática da PGB durante a  
2049 espermatogênese e prenhez dos animais. Além disso, há a incerteza associada à  
2050 extrapolação dos dados obtidos nos modelos animais para o ser humano e, também,  
2051 não foi possível avaliar a expressão do RANKL e da OPG na diferenciação e na  
2052 atividade dos osteoclastos nos animais expostos à PGB, que em equilíbrio, são  
2053 responsáveis pela osteoclastogênese necessária para a erupção dentária.

2054 Justifica-se a escolha de utilizar como modelo animal o rato para a técnica da  
2055  $\mu$ CT, em função das estruturas analisadas serem maiores em comparação aos  
2056 camundongos. Desta forma o modelo escolhido propicia melhor visualização e  
2057 acurácia dos resultados.

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**APÊNDICES**

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**APÊNDICE A**  
Ficha controle de tratamento camundongos machos

**Projeto** \_\_\_\_\_

**Grupo:** Macho:  
**Data da eutanásia**  
**Data de colocar para acasalar:**  
**Peso inicial (Dia 1):**  
**Início do tratamento:**  
**Dose fixa:**

**Tratamento:**

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
24	25	26	27	28	29	30	31	32	33	34	35	*	37	38	39	40	41	42	43	44	45	**

**Peso final (Dia 36 ou 46):**

Peso Órgão (g)	Fêmeas do machos					
	Fêmea 1			Fêmea 2		
	Peso Placenta	Peso Fetos	Medida fetos	Peso Placenta	Peso Fetos	Medida fetos
Coração:	1-			1-		
Pulmões:	2-			2-		
Fígado:	3-			3-		
Rins:	4-			4-		
Vesícula cheia:	5-			5-		
Vesícula vazia:	6-			6-		
Epidídimo esquerdo	7-			7-		
Epidídimo direito	8-			8-		
Testículo esquerdo:	9-			9-		
Testículo direito	10-			10-		
	11-			11-		
	12-			12-		
	13-			13-		
	14-			14-		
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**Observações:**

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## APÊNDICE B

Ficha controle de tratamento camundongos fêmeas e ratas

**Projeto** \_\_\_\_\_

**Grupo:** \_\_\_\_\_ **Fêmea:** \_\_\_\_\_  
**Data da rolha:** \_\_\_\_\_  
**Data da eutanásia:** \_\_\_\_\_  
**Data de tirar o macho:** \_\_\_\_\_  
**Início do tratamento:** \_\_\_\_\_  
**Fator multiplicador ou dose fixa:** \_\_\_\_\_

**Tratamento:**

5	6	7	8	9	10	11	12	13	14	15	16	17
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Dia de prenhes	Data	Dosagem (mL)	Peso (g)
0	___/___/___	-	
3	___/___/___		
6	___/___/___		
9	___/___/___		
12	___/___/___		
15	___/___/___		
18	___/___/___	-	

Peso de órgãos	Peso Placenta (g)	Peso fetos (g)	Medida fetos (cm)
Útero com fetos:	1 -		
Coração:	2 -		
Pulmões:	3 -		
Fígado:	4 -		
Rins:	5 -		
<b>Fetos vivos</b>	6 -		
Direito:	7 -		
Esquerdo:	8 -		
<b>Fetos mortos</b>	9 -		
Direito:	10 -		
Esquerdo:	11 -		
<b>Reabsorções precoces</b>	12 -		
Direito:	13 -		
Esquerdo:	14 -		
<b>Reabsorções tardias</b>	15 -		
Direito:	16 -		
Esquerdo:	17 -		
<b>Sítio de implantação</b>	18 -		
Direito:	19 -		
Esquerdo:	20 -		

Observações:

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**APÊNDICE C**  
Ficha de análise esquelética

**ANÁLISE ESQUELÉTICA**

Projeto:										
Grupo:	Fêmea:									
Data da eutanásia: ___/___/___	Data da análise: ___/___/___									
Número de fetos:										

ABREVIÇÕES	
OK: Normal	NA: Não Alinhado
A: Ausente	R: Reduzido
F: Fenda palatina	O: Ondulado
I: Forma irregular	D: Dividido

CRANIO	FETO 1	FETO 2	FETO 3	FETO 4	FETO 5	FETO 6	FETO 7	FETO 8	FETO 9	FETO 10
Frontal e nasal										
Zigomático										
Parietal										
Interparietal										
Supraoccipital e exoccip.										
Basoccipital										
Basoesfenóide										
Pterigóide										
Bulla timpanica										
corpo do hióide										
Palato										
Mandíbula e maxila										

ESQUELETO AXIAL	FETO 1	FETO 2	FETO 3	FETO 4	FETO 5	FETO 6	FETO 7	FETO 8	FETO 9	FETO 10
Esterno										
Vértebras (arco e centro)										
Cauda (v. sacral + cauda)										
Costelas										

CINTURA ESCAPULAR	FETO 1		FETO 2		FETO 3		FETO 4		FETO 5		FETO 6		FETO 7		FETO 8		FETO 9		FETO 10		
	E	D	E	D	E	D	E	D	E	D	E	D	E	D	E	D	E	D	E	D	
Clavícula																					
Escápula																					
Úmero																					
Rádio																					
Ulna																					
Metacarpos																					
Falanges proximais																					
Falanges distais																					

CINTURA PÉLVICA	FETO 1		FETO 2		FETO 3		FETO 4		FETO 5		FETO 6		FETO 7		FETO 8		FETO 9		FETO 10		
	E	D	E	D	E	D	E	D	E	D	E	D	E	D	E	D	E	D	E	D	
Íleo																					
Ísquio																					
Púbis																					
Fêmur																					
Tíbia																					
Fíbula																					
Metatarsos																					
Falange <u>proximais</u>																					
Falanges distais																					

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**APÊNDICE D**  
Ficha de análise visceral

**ANÁLISE VISCERAL**

Projeto:	
Grupo:	Fêmea:
Data da eutanásia: ___/___/___	Data da análise: ___/___/___
Número de fetos:	

CORTES	
1~4: Cabeça	5: Pescoço
6: Pescoço	7: Abdômen

		FETO 1	FETO 2	FETO 3	FETO 4	FETO 5	FETO 6	FETO 7	FETO 8	FETO 9	FETO 10
1	Palato										
	Ouvido interno										
	Medula										
	Traguéia										
2	Cavidade nasal										
	Septo nasal										
	Palato										
3	Bulbo olfatório										
	Retina										
	Palato										
	Córnea										
4	Hemisfério cerebral										
	Ventrículos cerebrais										
	3º Ventrículo cerebral										
	Diencéfalo										
5	Glândula salivar										
	Tireóide										
	Medula espinhal										
	Esôfago										
6	Traguéia										
	Coração										
	Pulmões										
	Timo										
7	Traguéia										
	Diafragma										
	Bexiga										
	Rins										
	Pelve renal										
	Medula renal										
	Ureteres										
	Útero										
	Testículo										
	Epidídimo										

OBSERVAÇÕES:

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NÚMERO DE FETOS FEMININOS	NÚMERO DE FETOS MASCULINOS

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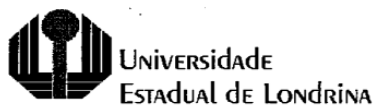
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**ANEXOS**

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**ANEXO A**  
Parecer do Comitê de Ética



**COMISSÃO DE ÉTICA NO USO DE ANIMAIS**

OF. CIRC. CEUA Nº 132/2018

Londrina, 21 de Agosto de 2018.

Prezado (a) professor (a)

Certificamos que o projeto intitulado: "Análise do efeito toxicológico do Pregabalina sobre as glândulas salivares de camundongos machos e fêmeas prenhes e avaliação das possíveis alterações na fertilidade masculina e teratogênese na prole" protocolo CEUA nº 11174.2018.36 sob a responsabilidade de Maria José Sparça Salles, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem) para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovado pela Comissão de Ética no Uso de Animais da Universidade Estadual de Londrina (CEUA/Uel), em 21/08/2018.

Este projeto tem por objetivo avaliar as possíveis alterações causadas pela Pregabalina, na concentração de 200mg/kg, quando administrada em camundongos machos e fêmeas prenhes sobre as glândulas salivares, a fertilidade masculina e as malformações congênitas na prole. Grau de invasividade=1

Finalidade	( ) Ensino ( x ) Pesquisa científica
Vigência da autorização	21/08/2018 a 31/08/2020
Espécie/ linhagem/ raça	Camundongo Heterogênico/ Swiss
Nº de animais	165
Peso/ Idade	35g/ Adulta
Sexo	Machos e Fêmeas
Origem	Biotério Central do Centro de Ciências Biológicas-Uel
Amostras a serem coletadas	Fetos, Órgãos: Coração, Pulmão, Fígado, Rins, Glândulas salivares, Testículos, Epidídimos e Sangue

Cumpra-se orientar que caso pretendam-se quaisquer alterações no protocolo experimental aprovado, deve-se submeter o novo protocolo à apreciação da CEUA/Uel anteriormente à execução das modificações.

Coloco-me à disposição, para quaisquer esclarecimentos que se fizerem necessários. Sem mais para o momento, subscrevo, cordialmente.

*Maria Fernanda R. Graciano*  
Prof.ª Dr.ª Maria Fernanda Rodrigues Graciano  
Coordenadora da CEUA/Uel

IImo.(a) Sr.(a)

Prof. (a) Dr (a). Maria José Sparça Salles

Responsável pelo projeto

Departamento de Biologia Geral/CCB

C/C para o Biotério Central/CCB

C/C para a Chefia do Depto de Biologia Geral/CCB

C/C para a Direção de Centro do CCB

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## ANEXO B

### Parecer da solicitação do uso da Pregabalina no Brasil (ANVISA)



Central de Atendimento ao Público - Anvisa <atendimento.central@anvisa.gov.br> sex., 9 de dez. de 2022 às 15:57  
Para: VIVIANEMESTRE@YAHOO.COM.BR

Prezado(a) Senhor(a),

Em atenção a sua solicitação, informamos que infelizmente não temos relatórios de medicamentos mais vendidos.

Por favor, avalie a resposta recebida acessando o link:

<https://pesquisa.anvisa.gov.br/index.php/241521?lang=pt-BR&encode=>

Atenciosamente,

Central de Atendimento  
Agência Nacional de Vigilância Sanitária  
0800 642 9782  
<https://www.gov.br/anvisa/pt-br>

Siga a Anvisa:

[www.twitter.com/anvisa\\_oficial](https://www.twitter.com/anvisa_oficial)  
[www.instagram.com/anvisaoficial](https://www.instagram.com/anvisaoficial)  
[www.facebook.com/AnvisaOficial](https://www.facebook.com/AnvisaOficial)

Este endereço eletrônico está habilitado apenas para enviar e-mails. Caso deseje entrar em contato com a Central, favor ligar no 0800 642 9782 ou acessar o "Fale Conosco", disponível no portal da ANVISA (link [https://www.gov.br/anvisa/pt-br/canais\\_atendimento/formulario-eletronico](https://www.gov.br/anvisa/pt-br/canais_atendimento/formulario-eletronico)). As ligações podem ser feitas de segunda a sexta-feira, das 7h30 às 19h30, exceto feriados.

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2585**ANEXO C**Submissão do Artigo 1 à *Reproduction, Fertility and Development*

- **Reproduction, Fertility and Development - Manuscript ID RD22287** Yahoo/Entrada ☆



• **Reproduction, Fertility and Development** qua, 7 de dez. de 2022 às 20:41 ☆

Para:

vivianemestre@yahoo.com.

, salmjs00@gmail.com,

k-yow@hotmail.com,

lvbrito\_@hotmail.com,

alinezeffa@hotmail.com

e 1 mais...

08-Dec-2022

Dear Dr Mestre

Your manuscript entitled 'Pregabalin alters reproductive performance in male mice and causes congenital anomalies in offspring' has been successfully submitted online and will be given full consideration for publication in *Reproduction, Fertility and Development*.

Your manuscript ID is RD22287. Please mention this manuscript ID in all future correspondence or when calling the office for questions.

If there are any changes in your street address or email address, please log in to ScholarOne Manuscripts at <https://mc.manuscriptcentral.com/csiro-rd> and edit your user information as appropriate. You can also view the status of your manuscript at any time by checking your Author Centre after logging in.

We encourage all co-authors to link their ORCID iDs to their author accounts in our submission system. To learn more about ORCID, please visit <http://orcid.org/content/initiative>.

Thank you for submitting your manuscript to *Reproduction, Fertility and Development*.

Sincerely,

Editorial Office, *Reproduction, Fertility and Development*

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## ANEXO D

Submissão do Artigo 2 à Semina: Ciências Biológicas e da Saúde

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● Fábio Henrique Kwasniewski via P



seg., 6 de fev. às 01:41 ☆

Para: Viviane de Fátima Mestre

Viviane de Fátima Mestre:

Obrigado por submeter o manuscrito, "Pregabalina promove embriotoxicidade e malformações congênitas em fetos de camundongos" ao periódico Semina: Ciências Biológicas e da Saúde. Com o sistema de gerenciamento de periódicos on-line que estamos usando, você poderá acompanhar seu progresso através do processo editorial efetuando login no site do periódico:

URL da Submissão:

<https://ojs.uel.br/revistas/uel/index.php/seminabio/authorDashboard/submission/47472>

Usuário: vivianemestre

Se você tiver alguma dúvida, entre em contato conosco. Agradecemos por considerar este periódico para publicar o seu trabalho.

Fábio Henrique Kwasniewski

*Semina: Ciências Biológicas e da Saúde*

Fábio H. Kwasniewski

Editor Chefe

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## Submission Overview

**Initial Submission** This manuscript has been submitted to the editorial office for review. Changes cannot be made during editorial review, but you can view information and files you submitted, below.

Article Type	Research Article		
Title	Evaluation of the effects of prenatal exposure to pregabalin and postnatal analysis of dental and maxillary bone tissue development in rat offspring		
Manuscript Files	Name	Type of File	Size
	<a href="#">Manuscript.docx</a>	Main Document - MS Word	11.3 MB
	<a href="#">Cover letter.docx</a>	Cover letter / Comments	14 KB
Abstract	<p><b>Background:</b> Pregabalin is a gabapentinoid indicated for the treatment of peripheral neuropathic pain, fibromyalgia, and as an adjuvant therapy for epilepsy. The aim of this study was to evaluate the effects of pregabalin on postnatal development, odontogenesis, and mandibular trabecular tissue using computed tomography.</p> <p><b>Methods:</b> Pregnant rats (n=24) were divided into two groups: control group (C), and treated group (PG). The PG group received 200 mg/kg of pregabalin via gavage during the embryonic period and the C group received distilled water under the same design. On the 21st day of pregnancy, delivery occurred naturally. On the first postnatal day, the litters were reduced to four pups (2 males and 2 females) and followed for 30 days. On postnatal day 30, 8 animals from each group were randomly selected for analysis of the left right first molar and adjacent trabecular bone.</p> <p><b>Results:</b> In the maternal parameters, the data revealed a significant decrease in body weight gain in the PG group compared to a smaller number of live pups. In the postnatal analysis, the exposed pups showed a significant decrease in weight and length and a delay in the eruption of the incisor teeth. Microtomography analysis revealed a significant reduction in enamel volume, a lower volume and percentage of open pores, lower total porosity, and a higher percentage of bone volume.</p> <p><b>Conclusions:</b> The findings of this study showed that pregabalin altered the eruption chronology of the incisors, decreased enamel volume, affected the microarchitecture of mandibular trabecular bone, and</p>		