VIVIANE DE FÁTIMA MESTRE

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

Londrina
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Orientadora: Profa. Dra. Maria José Sparça Salles

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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 gabapentinoides neuromodelador com ação antiepilética 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 α2-δ 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 5° dia de prenhez ao dia antecessor ao parto (20° 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 α2-δ 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 spermatozoids (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

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LISTA DE ABREVIATURAS E SIGLAS

2D	Bidimensional
3D	Tridimensional
α2-δ	Alfa-2-delta
AED	*Antiepileptic drugs*
AGA	*Adequate for gestational age*
AMBN	Ameloblastina
AMEL	Amelogenina
cAMP	*Cyclic adenosine monophosphate*
BV	Volume do osso (*Bone volume*)
BS	Superfície óssea (*Bone surface*)
BS/BV	Superfície/volume (*Bone surface-to-volume ratio*)
BV/TV	Volume ósseo/volume tecidual (*Bone volume-to-tissue ratio*)
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 (*Dentin surface*)
DS/DV	Superfície/volume da dentina (*Dentin surface/volume ratio*)
DV	Volume da dentina (*Dentin volume*)
ENAM	Esmalteina
EV	Volume do esmalte (*Enamel volume*)
ES	Superfície do esmalte (*Enamel surface*)
ES/EV	Superfície/volume do esmalte (*Enamel surface/volume ratio*)
FDA	*Food and Drug Administration*
FGR	*Fetal growth restriction*

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

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

SUMÁRIO

INTRODUÇÃO

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

Estruturalmente, a PGB (Fig. 1-a) é semelhante ao transmissor inibitório do ácido gama aminobutírico (GABA) (Fig. 1-b); porém, seu mecanismo de ação não está relacionado à ligação ou degradação do GABA (EVOY *et al*., 2021). Acredita-se que o mecanismo de ação da PGB se dá pela modulação de neurotransmissores por meio da ação pré-sináptica mediada pela subunidade alfa-2-delta (α2-δ) dos canais de cálcio dependentes de voltagem atenuando o influxo de cálcio neuronal (FIELD *et al*., 2006; LOTARSKI *et al*., 2014; SILLS; ROGAWSKI, 2020), diminuindo a liberação de glutamato, noradrenalina e substância P, contribuindo para suas ações anticonvulsivante, analgésica e ansiolítica (MATHIESON *et al*., 2020).

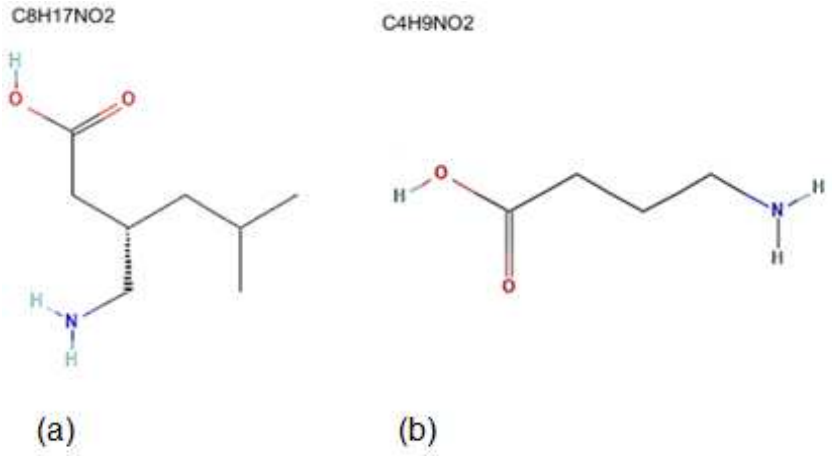

Figura 1- Estrutura química da PGB (a) e do GABA (b)
Fonte: Adaptado de PubChem [Internet]

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

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

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

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	77.086.569
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.

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

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

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

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

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

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

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

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

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

2 OBJETIVOS

2.1 Objetivo Geral

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

2.2 Objetivos Específicos

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

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

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

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

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

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

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

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

3 MATERIAIS E MÉTODOS

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

3.1 Fármaco

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

3.2 Artigo 1

3.2.1 Animais

Para a avaliação da exposição da PGB durante a espermatogênese, foram utilizados 20 camundongos machos e 28 fêmeas adultos da linhagem Swiss (*Mus musculus*), com aproximadamente 35 g de massa corporal provenientes do Biotério Central do Centro de Ciências Biológicas da UEL (Londrina, Paraná, Brasil). Os animais foram mantidos em gaiolas de polipropileno forradas com maravalhas e sob condições de iluminação controlada em ciclo de 12 h claro-escuro a temperatura entre 22 ± 2ºC com água e ração livres.

3.2.2 Delineamento experimental

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

3.2.3 Toxicidade

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

3.2.4 Eutanásia

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

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

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

3.2.5 Análise da espermatogênese

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

3.2.6 Concentração sérica de testosterona

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

3.2.7 Análise do desenvolvimento intrauterino e embriofetotoxicidade

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

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

Para a análise visceral, os fetos foram examinados pela combinação

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

3.3 Artigo 2

3.3.1 Animais

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

3.3.2 Delineamento experimental

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

3.3.3 Toxicidade

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

3.3.4 Eutanásia

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

3.3.5 Análise do desenvolvimento intrauterino e embriofetotoxicidade

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

3.3.6 Avaliação das malformações congênitas

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

3.4 Artigo 3

3.4.1 Animais

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

3.4.2 Delineamento experimental

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

3.4.3 Pós-natal

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

3.4.4 Eutanásia

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

hemimandíbulas direitas foram dissecadas e acondicionadas em formalina neutra tamponada a 10% por 24 h e mantidas em álcool 70% até o momento da análise por microtomografia computadorizada (μCT).

3.4.5 Microtomografia computadorizada

As medidas de μCT foram realizadas utilizando o equipamento *SkyScan-Bruker*, modelo 1173 *(Bruker BioSpin Corporation, Kontich,* Bélgica). O escaneamento das amostras foi padronizado usando a tensão de 50 kV e 120 μA de corrente. A resolução das imagens obtidas foi de 6 μm. A reconstrução foi realizada pelo programa NRecon (*Bruker BioSpin Corporation, Kontich,* Bélgica, versão 1.7.4.2) e as análises quantitativas foram realizadas no programa CTan (*Bruker BioSpin Corporation, Kontich,* Bélgica, versão 1.20.8). As imagens 2D ou 3D foram obtidas pelos *softwares DataViewer (Bruker BioSpin Corporation, Kontich,* Bélgica*,* versão 1.6.0.0*)* e CTVox (*Bruker BioSpin Corporation, Kontich,* Bélgica, versão 3.3.1), respectivamente. Os parâmetros avaliados no PMID foram o volume do esmalte (EV, mm^3) e dentina (DV, mm^3), superfície do esmalte (ES, mm^2) e dentina (DS, mm3) e a relação entre superfície/volume do esmalte (ES/EV, mm^{-1}) e a superfície/volume da dentina (DS/DV, mm^{-1}). Para a análise do osso trabecular adjacente, foram considerados os parâmetros: volume do osso (BV, mm^3), a superfície óssea (BS, mm^2), razão entre a superfície e o volume (BS/BV, mm^{-1}), a relação entre o volume ósseo e o volume tecidual (BV/TV, %), número trabecular (Tb.N, mm-1), distância entre as trabéculas, (Tb.Sp, mm) e média da espessura trabecular (Tb.Th, mm), volume de poros fechados (Po.V(cl), mm^3), porosidade fechada (Po(cl), %), volume do espaço poroso aberto (Po(op), mm^3), porosidade aberta (Po(op), %) volume total do espaço poroso (Po.V(tot), mm^3) e porosidade total (Po(tot), %).

3.4.6 Análise histológica

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

3.5 Análise estatística

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

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

4 RESULTADOS E DISCUSSÃO

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

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

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

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

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

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

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Abstract:

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

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

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

Introduction

Pregabalin (PGB) is an anticonvulsant drug belonging to the gabapentinoids class, with modulating action on voltage-dependent calcium channels, and therapeutic indication for neuropathic pain, fibromyalgia, generalized anxiety disorder, and as an adjuvant for epilepsy (Lyrica®, 2020). Its analgesic effect is due to its ability to bind to the alpha 2 delta protein subunit (α2-δ) of voltage-gated calcium channels in the central nervous system (Di Guilmi et al., 2011). It has also been administered in the prophylaxis of chronic pain after acute events, decreasing intraoperative opioid consumption (Hennemann-Krause and Sredni, 2016). PGB is often prescribed for off-label use for insomnia, social phobia, migraines, panic disorder, bipolar disorder, and alcohol withdrawal (Evoy et al., 2017; Alles et al., 2020; Mathieson et al., 2020).

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

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

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

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

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

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

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

Materials and methods

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

Animals

To evaluate the effects of exposure to PGB during spermatogenesis, 20 male 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 ± 2°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

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

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

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

Spermatogenesis analysis

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

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

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

Serum testosterone concentration

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

Statistical analysis

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

Results

Paternal toxicity

No clinical signs of parental toxicity, such as piloerection, diarrhea, weight change, motor coordination abnormalities, and death were observed during the treatment period. Regarding the organs evaluated, there was a statistically significant 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[a] (g)	0.18 ± 0.02	0.18 ± 0.033	0.737
Weight of the lungs[b] (g)	0.25 ± 0.056	0.22 ± 0.049	0.094
Weight of the liver[a] (g)	1.79 ± 0.26	1.87 ± 0.21	0.406
Weight of the kidneys[a] (g)	0.52 ± 0.074	0.54 ± 0.062	0.519
Weight of the epididymis[a] (g)	0.041 ± 0.0047	0.04 ± 0.012	0.796
Seminal vesicle volume[a] (g)	0.074 ± 0.035	0.075 ± 0.043	0.952
Weight of the testicle[a] (g)	0.099 ± 0.009	0.095 ± 0.012	0.498
Size of the testicle[a] (mm)	6.28 ± 0.35	6.82 ± 0.51*	0.009*

Data expressed as mean ± standard deviation. Student's t test[a]. Mann-Whitney U test[b]
* 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[a] (g)	20.2 ± 3.07	19.33 ± 4.06	0.528
Weight of the placenta[a] (g)	0.091 ± 0.013	0.069 ± 0.013*	0.0001*
Weight of the fetuses[a] (g)	1.44 ± 0.054	1.314 ± 0.139*	0.0035*
Size of the fetuses[a] (cm)	2.32 ± 0.16	2.121 ± 0.161*	0.003*
Live fetuses[a]	10.14 ± 2.14	10.79 ± 2.91	0.5119
Dead fetuses[b]	0	0.21 ± 0.58	0.5491
Resorptions[b]	0.43 ± 0.65	3.36 ± 4.05*	0.003*
Implantations[b]	10.57 ± 2.57	14.36 ± 3.74	0.5837
Resorption rate[b] (%)	3.28 ± 4.99	20.63 ± 24.05*	0.003*
Post-implantation loss[b] (%)	3.28 ± 4.99	22.33 ± 23.75*	0.0458*

Placental index[b] (%)	6.33 ± 0.93	4.94 ± 1.19*	0.0016*
Fetal viability rate[b] (%)	96.72 ± 4.99	77.67 ± 23.75*	0.0458*

Data expressed as mean ± standard deviation. Student's t test[a]. Mann-Whitney U test[b]
* 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

Visceral malformations	**C (n=75) (%)**	**PGB (n=72) (%)**	**P value**
Kidneys (change in size)	0	7 (9.72)*	0.0058*
Heart (reduced auricles)	0	1 (1.39)	0.4898
Skeletal malformations	**C (n=75) (%)**	**PGB (n=74) (%)**	**P value**
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).

599

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.

606

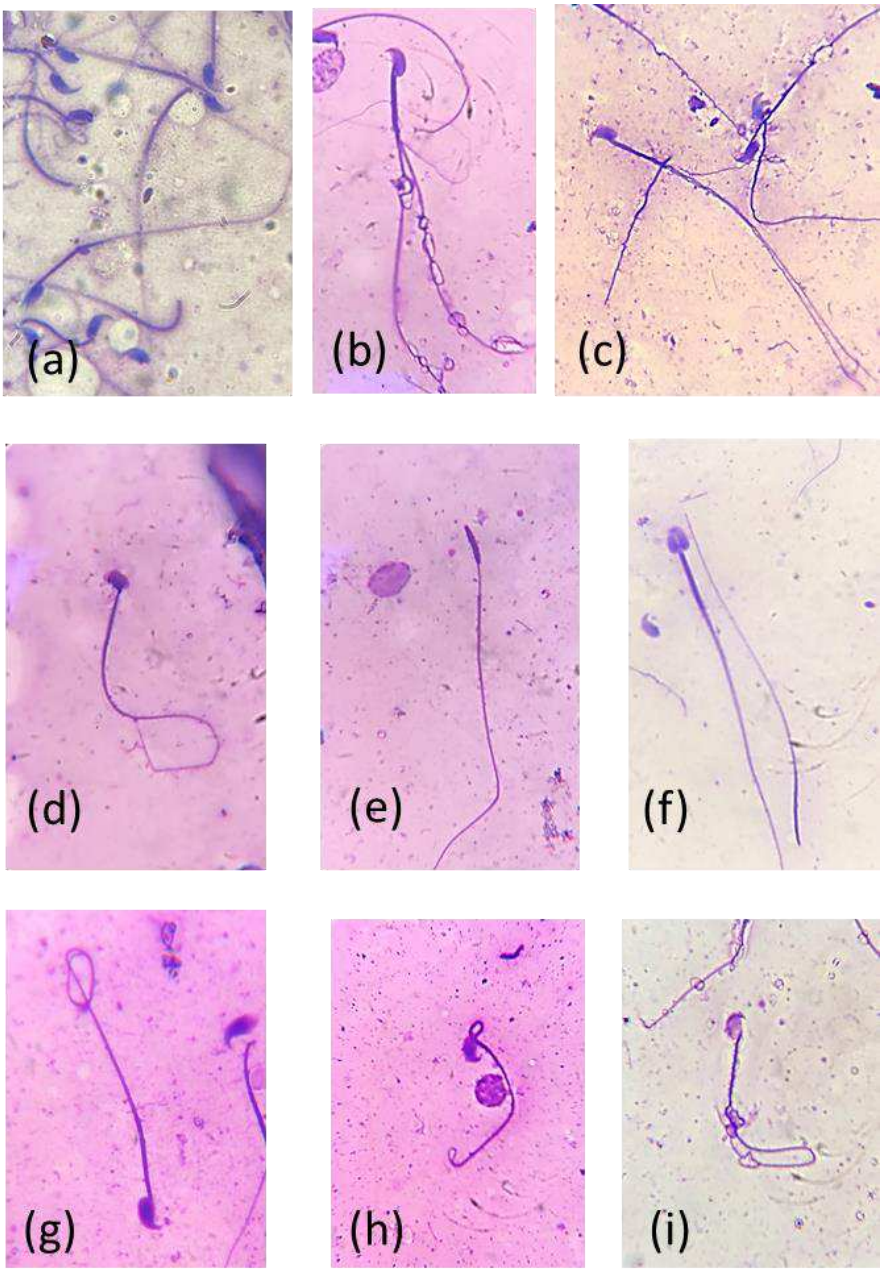


Figure 1. Morphological alterations found in spermatozoids from mice treated with PGB (200 mg/kg) during the period of spermatogenesis. Normal spermatozoids (a). Note two-tailed spermatozoid (b, c). Amorphous head (d, e). Biencephalic spermatozoid (f). Curled tail (g,h). Vacuolated head (i). Legend: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

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

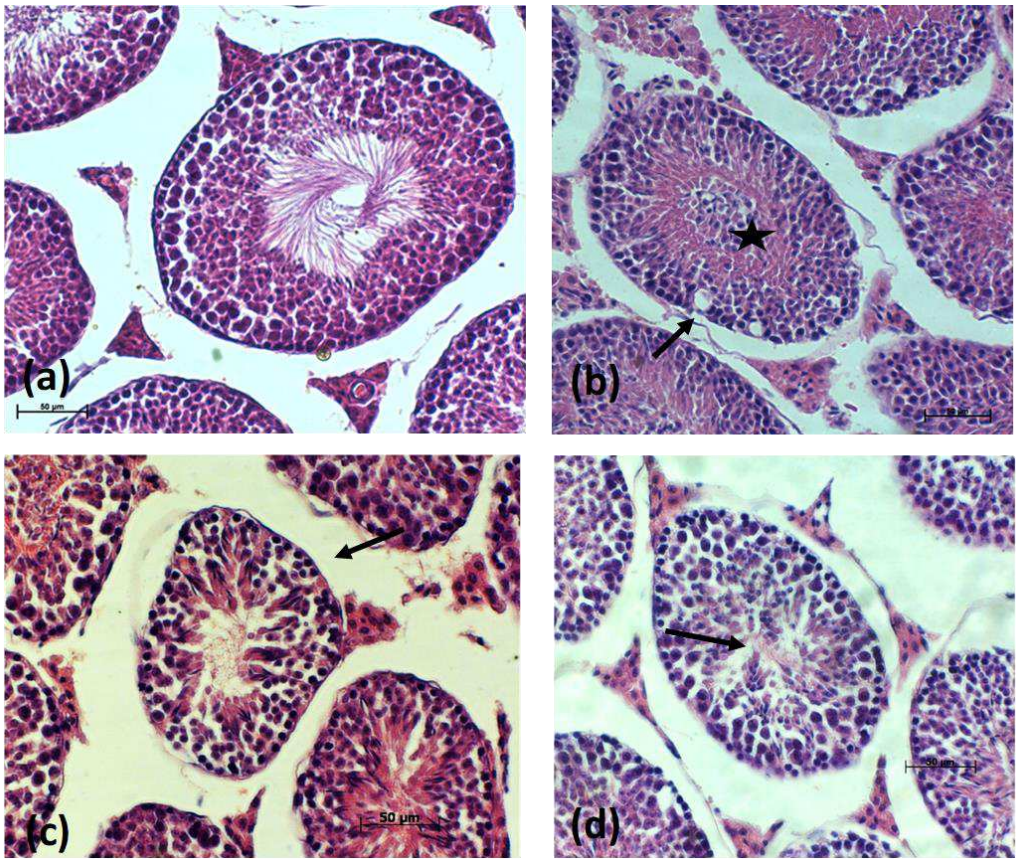
Alterations	C (n=4000) (%)	PGB (n=4000) (%)	P value
Tail	828 (20.7)	1270 (31.75)*	<0.0001
Head	24 (0.6)	425 (10.62)*	<0.0001

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).

621

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

626



627
628 Figure 2. Photomicrographs of the testes of adult mice. Seminiferous tubule of the
629 control group without alterations (a); Seminiferous tubules of the group treated with
630 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[b]	9.912 ± 0.1018	9.04 ± 0.416*	0.0002*
Sertoli cells[a] (unid./mm^2)	19.74 ± 2.508	17.94 ± 2.738	0.1317
Leydig cells[a] (unid./mm^2)	37.92 ± 3.806	61.43 ± 12.33*	0.0002*
Testosterone[b] (ng/ml)	996.7 ± 551.6	355.6 ± 296.0*	0.0152*

Data expressed as means ± standard deviation. Student's t test[a]. Mann-Whitney U test[b]
*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

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

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

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

For the establishment and maintenance of male reproduction and fertility to occur, complete spermatogenesis is essential. The differentiation of germ cells into functional spermatozoids depends on factors involving somatic cells, such as Leydig cells, myoid 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.

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

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

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

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

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

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

Conclusion

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

Declaration of conflicts of interest

The authors declare no conflicts of interest.

Data Availability Statement

The authors did not publish the research data.

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

Pregabalin promotes embryotoxicity and congenital malformation in mouse fetuses

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

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

Introduction

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

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

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

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

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

Materials and methods

Animals

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

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

Experimental design

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

Toxicity

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

Analysis of intrauterine development and embryotoxicity

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

Evaluation of congenital malformations

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

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

Statistical analysis

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

Results

Toxicity

No clinical signs of maternal toxicity, such as piloerection, diarrhea, weight change, motor coordination abnormalities, and death were observed during the PGB treatment period. The 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)[a]	22.97 ± 5.29	22.88 ± 7.92	0.4442

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

Data expressed as means ± standard deviation. Student's t test[(a)]. Mann-Whitney U test[(b)].
*P<0.05
Legend: Control Group (C); Treated group (PGB); Pregabalin (PGB).
Source: own authors

Effect of pregabalin during pregnancy

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).

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).

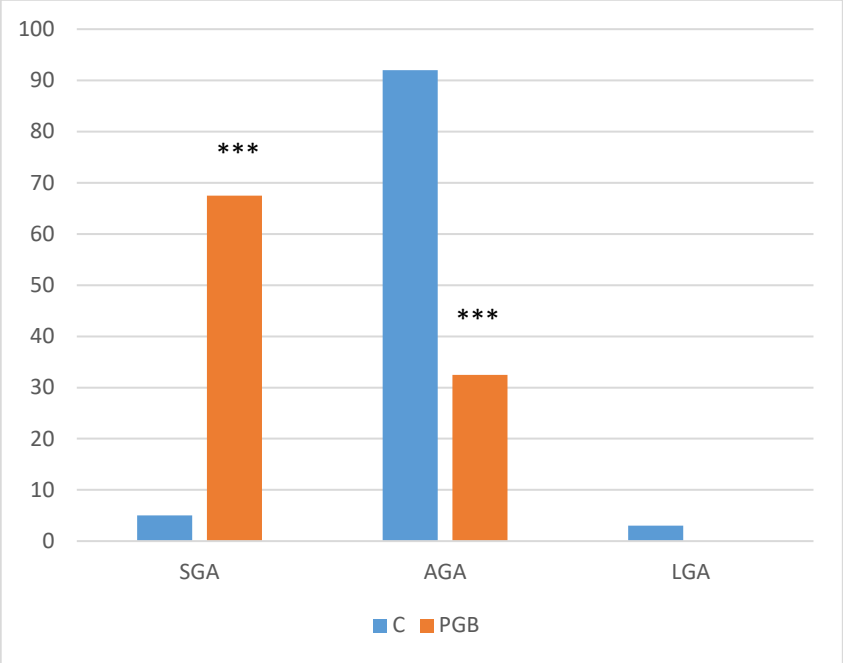
Table 2 – Effects of PGB (200 mg/kg) treatment on intrauterine development in offspring of female mice

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

Data expressed as means ± standard deviation. Student's t test[(a)]. Mann-Whitney U test[(b)].
*P<0.05
Legend: Control Group (C); Treated group (PGB); Pregabalin (PGB).

Source: own authors

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

Analysis of malformations

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.

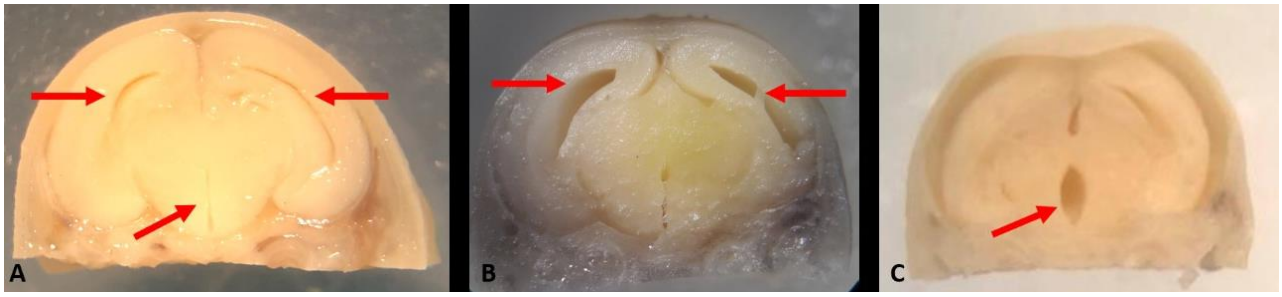
Table 3 - Malformations observed in fetuses after exposure to PGB (200 mg/kg) from the 5th to the 17th day of pregnancy

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
Skeletal malformations	**C (n=71) (%)**	**PGB (n=62) (%)**	**P**
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

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



Arrows indicate normal ventricles in the C group (A), arrows indicate dilated ventricles in the PBG group (B and C).
Legend: Control group (C); Treated group (PGB); Pregabalin (PGB).
Source: own authors

Discussion

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)[19] and Singh and Gupta (2018).[13]

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.[20,21] However, the teratogenic potential of PGB has not yet been established.[9]

Embryonic resorptions in mice are analogous to spontaneous abortions in humans.[22] The PGB group presented a statistically higher resorption rate than the C group, which explains the

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

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

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

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

shorter length and lower body weight of fetuses in the PGB group. In contrast, administration of PGB at different doses in pregnant rats induced skeletal variations in the offspring, especially advanced ossification from the jugal bone to the maxilla and fusion of the nasal sutures.[12]

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

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

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

Conclusion

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

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

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

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Abstract

Background: 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 microtomography.

Methods: Pregnant rats (n=24) were divided into two groups: control group (C), and treated group (PGB). The PGB 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 up for 30 days. On postnatal day 30, eight animals from each group were randomly selected for analysis of the lower right first molar and adjacent trabecular bone.

Results: In the maternal parameters, the data revealed a significant decrease in body weight gain in addition 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.

Conclusions: 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 impaired postnatal development.

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

Introduction

Pregabalin (PGB) is a gabapentinoid indicated for the treatment of neuropathic pain, fibromyalgia, generalized anxiety disorder, and as an adjuvant for epilepsy (Cross, Viswanath & Sherman, 2022; Lyrica®, 2020). PGB is often prescribed for off-label use for migraine, insomnia, social phobia, bipolar disorder, alcohol withdrawal, and in an acute dose for postoperative pain (Alles, Cain & Snutch, 2020; Cross et al., 2022; Evoy, Morrison & Saklad, 2017; Mathieson, Lin, Underwood, & Eldabe, 2020). It is believed that the effect of the drug occurs through the inhibition of voltage-gated calcium channels binding to the α2-δ subunit and, even though it is analogous to gamma-aminobutyric acid (GABA), it does not bind to GABA receptors (Chincholkar, 2018).

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

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

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

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

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

Computed microtomography (μCT) is an X-ray imaging exam considered the gold standard for evaluating 3D bone morphology and morphometry in small animals by

visualizing the bone at a microstructural scale (1–100 μm resolution) (Kim, Brodt, Tang & Silva, 2021), allowing quantitative and qualitative analyses of bone tissue (Rovaris et al., 2018).

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

No studies regarding the possible effects of PGB on tooth development and mandibular bone tissue, were found in the consulted literature. Thus, the aim of the current study was to evaluate the effects of prenatal PGB exposure in rat offspring on odontogenesis and mandibular trabecular tissue using μCT and histomorphometry.

Materials and methods

Animals

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

In total, 24 adult female, and 12 adult male Wistar rats weighing approximately 250 g - 350 g were kept in polypropylene cages lined with wood shavings and under controlled lighting conditions in a 12 h light-dark cycle at a temperature of 22 ± 2˚C with water and feed ad libitum.

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

Drug

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

Design

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

Assessment of postnatal development

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

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

Evaluation of Computerized Microtomography

The µCT measurements were performed using tSkyScan-Bruker equipment, model 1173 (Bruker BioSpin Corporation, Kontich, Belgium). Sample scanning was standardized using a 50 kV voltage and 120 µA current. The resolution of the images obtained was 6 um. Reconstruction was performed using the NRecon program (Bruker BioSpin Corporation, Kontich, Belgium, version 1.7.4.2) and quantitative analyses were performed using the CTan program (Bruker BioSpin Corporation, Kontich, Belgium, version 1.20.8). The 2D or 3D images were obtained using the software DataViewer (Bruker BioSpin Corporation, Kontich, Belgium, version 1.6.0.0) and CTVox (Bruker BioSpin Corporation, Kontich, Belgium, version 3.3.1), respectively. The parameters evaluated in the RMFM were the enamel (EV, mm^3) and dentin volume (DV, mm^3), enamel (ES, mm^2) and dentin surface (DS, mm3), and enamel surface/volume ratio (ES/EV, mm^{-1}), and dentin surface/volume ratio (DS/DV, mm^{-1}). For the analysis of adjacent trabecular bone, the following parameters were considered: bone volume (BV, mm^3), bone surface (BS, mm^2), bone surface-to-volume ratio (BS/BV, mm^{-1}), bone

volume-to-tissue ratio (BV/TV, %), trabecular number (Tb.N, mm^{-1}), distance between trabeculae (Tb.Sp, mm), mean trabecular thickness (Tb.Th, mm), closed pore space volume (Po.V(cl), mm^3), closed porosity (Po(cl), %), open pore space volume (Po(op), mm^3), open porosity (Po(op), %) total pore space volume (Po.V(tot), mm^3), and total porosity (Po(tot), %).

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

Statistics

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

Results

Maternal toxicity

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

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

Female	**C (n=12)**	**PGB (n=12)**	**P value**
Weight gain[b] (g)	116.3 ± 21.58	103.08 ± 35.53*	0.0139*

[1]Live pups (unit/litter)[a]	12.67 ± 1.5	10.75 ± 2.22*	0.0213*

Data expressed as means ± standard deviation. Student's t test [a]. Mann-Whitney test [b]
* P<0.05
Legend: [1]number of live pups per litter; Control group (C); Treated group (PGB); Pregabalin (PGB).

Assessment of Postnatal Development

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

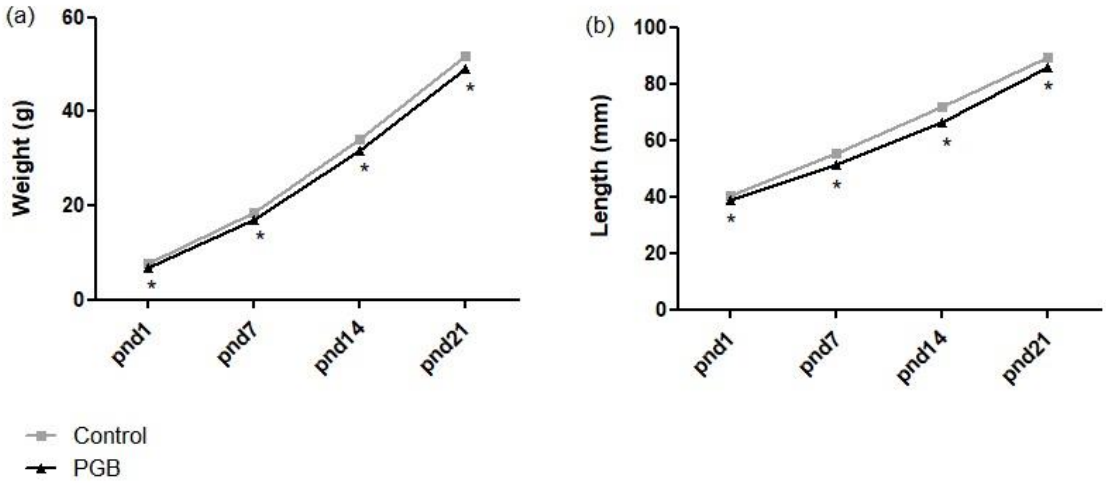

Figure 1. Weight gain (a) and body length (b) of the offspring of female rats from PND1 to PND 21 of group C and group PGB. Data are mean ± s.e.m. of 48 pups from each group. The two-way ANOVA analysis of repeated measures indicated that the weight and length of the offspring of the PBG group in the postnatal period were significantly lower when compared to the C group.
*P<0.05
Legend: postnatal day (pnd); Control group (C); Treated group (PGB), Pregabalin (PGB).

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

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**

Eruption of incisors (day)[b]	7.13 ± 0.33	7.55 ± 0.68*	0.001

Data expressed as means ± standard deviation. Mann-Whitney U test[b]
* P<0.05
Legend: Control group (C); Treated group (PGB); Pregabalin (PGB).

Computerized Microtomography

Enamel, dentin and mandibular trabecular bone

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

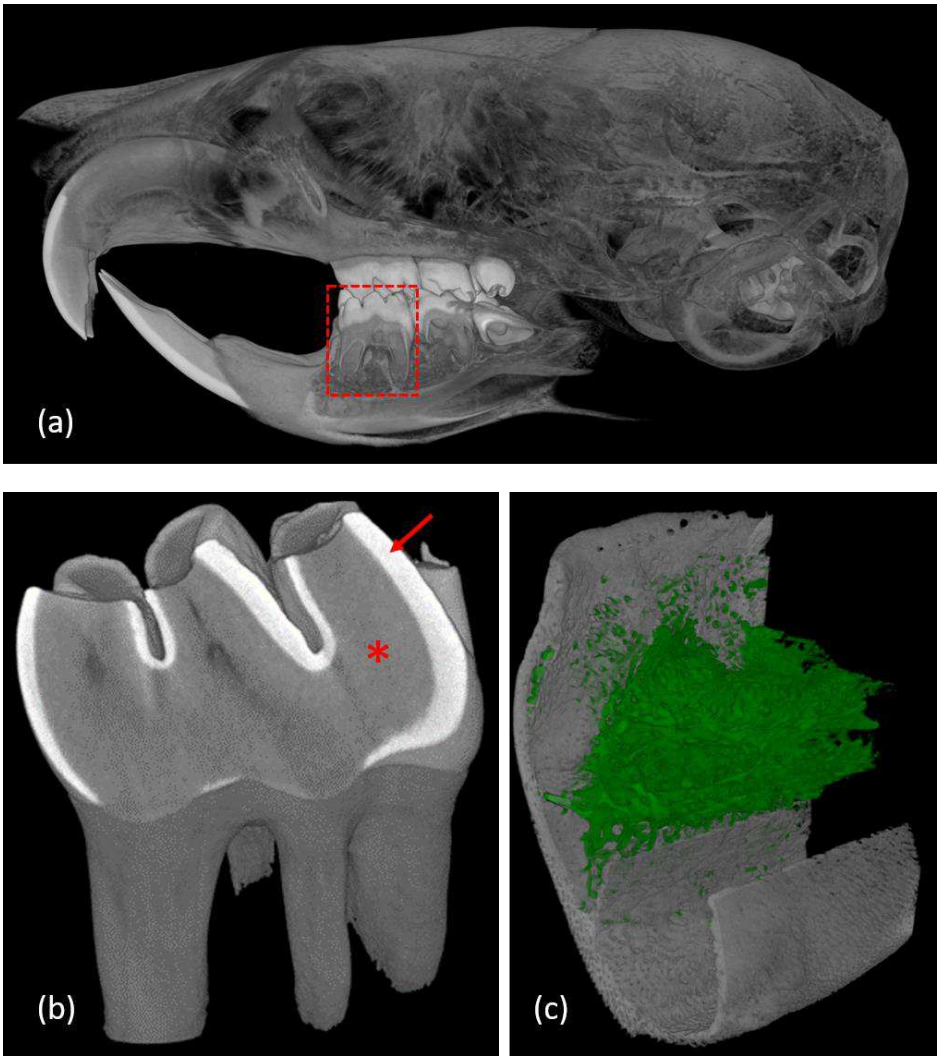

Figure 2. Reconstructed µCT images. RMFM Region of Interest (ROI) indicated by the dotted rectangle (a). Enamel area indicated by arrow and dentin area indicated by asterisk (b). Volume of interest (VOI) of trabecular bone represented in green (c).
Legend: Computed microtomography (µCT); Right mandibular 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^3)[a]	1.24 ± 0.077	1.09 ± 0.10*	0.0043*
ES (mm^2)[a]	33.07 ± 1.74	30.98 ± 2.67	0.0850
ES/EV (mm^{-1})[b]	26.71 ± 0.94	28.53 ± 0.88*	0.0045*
Dentin	**C (n=8)**	**PGB (n=8)**	**P**
DV (mm^3)[a]	3.72 ± 0.20	3.60 ± 0.19	0.2410
DS (mm^2)[a]	53.59 ± 2.40	52.54 ± 3.24	0.4743

DS/DV (mm^{-1})[a]	14.42 ± 0.35	14.60 ± 0.51	0.4098

Data expressed as means ± standard deviation. Student's t test[a]. Mann-Whitney U test[b]
* 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).

The evaluation of the trabecular bone adjacent to the RMFM showed that PGB was able to significantly increase the percentage of bone volume (BV/TV). Regarding bone microarchitecture, the PGB group showed a lower percentage of open pores (Po(op)) and a lower total pore volume (Po.V(tot)) and, therefore, a lower percentage of 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^3)[a]	2.66 ± 0.28	2.5 ± 0.28	0.2857
BS (mm^2)[a]	147.8 ± 11.94	140.9 ± 14.34	0.3115
BS/BV (mm^{-1})[a]	55.74 ± 3.1	56.47 ± 4.16	0.6962
BV/TV (%)[a]	92.2 ± 1.5	93.78 ± 0.86*	0.0208*
Tb.Th (mm)[a]	0.07 ± 0.003	0.07 ± 0.004	0.722
Tb,N (mm^{-1})[a]	13.31 ± 0.76	13.7 ± 0.9	0.37
Tb.Sp (mm)[b]	0.03 ± 0.003	0.03 ± 0.002	0.2902
Po.V(cl) (mm^3)[a]	0.0005 ± 0.0001	0.0005 ± 0.0003	0.9857
Po(cl) (%)[a]	0.018 ± 0.005	0.018 ± 0.0105	0.9337
Po(op) (mm^3)[a]	0.228 ± 0.066	0.165 ± 0.028*	0.0266*
Po(op) (%)[a]	7.786 ± 1.497	6.198 ± 0.856*	0.0208*
Po.V(tot) (mm^3)[a]	0.229 ± 0.066	0.166 ± 0.028*	0.0267*
Po(tot) (%)[a]	7.802 ± 1.496	6.216 ± 0.855*	0.0208*

Data expressed as means ± standard deviation. Student's t test[a]. Mann-Whitney U test[b]
* 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^3), bone surface (BS, mm^2), surface-to-volume ratio (BS/BV, mm^{-1}), bone volume-to-tissue ratio (BV/TV, %), trabecular number (Tb.N, mm^{-1}), distance between trabeculae (Tb.Sp, mm), mean trabecular thickness (Tb.Th, mm), closed pore space volume (Po.V(cl), mm^3), closed porosity (Po(cl), %), open pore space

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

Histological analysis

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

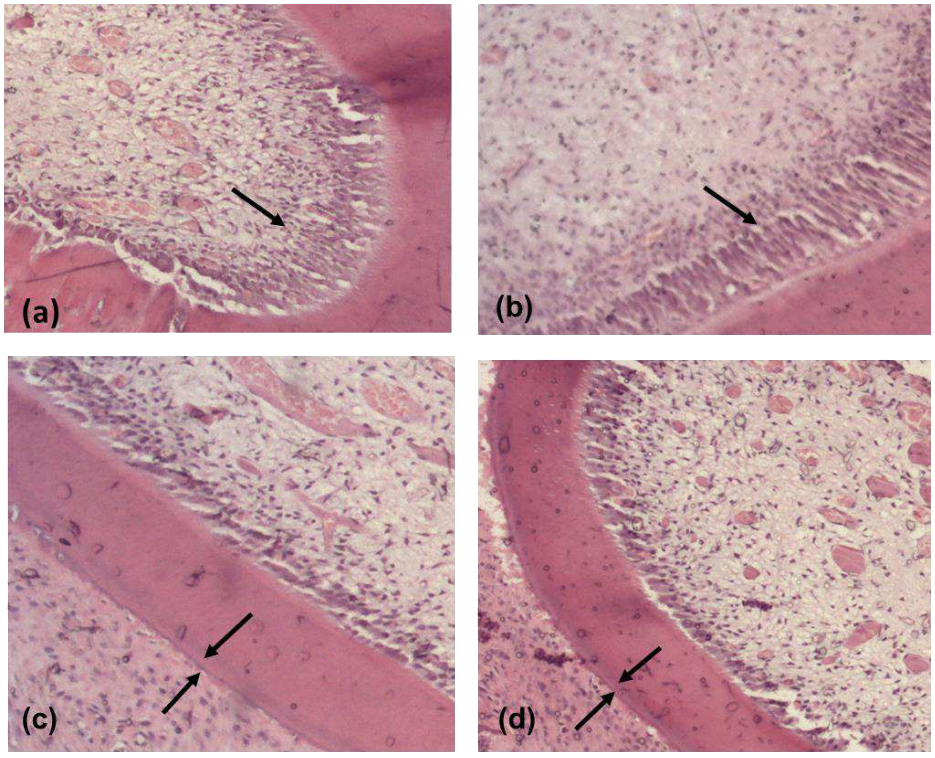

Figure 3- HE-stained histological sections of the RMFM region of the offspring of rats after µCT analysis. C Group (a,c) and PGB Group (b,d). Arrows indicate odontoblast layer (a, b) and cementum layer (c, d). Legend: Hematoxylin-eosin (HE); Right mandibular first molar (RMFM); Computed microtomography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

Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Conclusion

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

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

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5 CONCLUSÕES

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

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

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

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

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

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

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

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

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

significativo das reabsorções quando comparado ao grupo C;

- As malformações congênitas significativas observadas nos fetos de camundongos fêmeas expostos à PGB durante a gestação foram ventriculomegalia e ossificação incompleta do osso supraoccipital;

- O efeito do tratamento com PGB em ratas prenhes mostrou perda significativa de ganho de peso e diminuição significativa do número de filhotes vivos por ninhadas quando comparado ao grupo C;

- O peso e o comprimento pós-natal da prole de ratas exposta à PGB foram significativamente menores quando comparados ao grupo C;

- A exposição da PGB na prole de ratas causou atraso na erupção dos dentes incisivos;

- A análise do esmalte do PMID sugeriu redução significativa de volume na prole exposta à PGB; não houve alteração significativa da dentina;

- A análise do osso trabecular adjacente ao PMID sugeriu aumento significativo da relação entre o volume ósseo e o volume tecidual (BV/TV) na prole exposta à PGB quando comparado ao grupo C.

6 CONSIDERAÇÕES FINAIS

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

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

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

O presente estudo apresentou fortalezas quanto a manipulação das amostras sob ambiente controlado, uso de apenas um anticonvulsivante e propiciou informações sobre o potencial toxico e teratogênico da PGB em modelo animal e o uso da µCT para avaliar a amelogênese, a dentinogênese e a microarquitetura trabecular da mandíbula.

As limitações deste estudo foram a administração de apenas uma dose e a ausência de dados sobre a concentração plasmática da PGB durante a espermatogênese e prenhez dos animais. Além disso, há a incerteza associada à extrapolação dos dados obtidos nos modelos animais para o ser humano e, também, não foi possível avaliar a expressão do RANKL e da OPG na diferenciação e na atividade dos osteoclastos nos animais expostos à PGB, que em equilíbrio, são responsáveis pela osteoclastogênese necessária para a erupção dentária.

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

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APÊNDICES

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)
Coração:
Pulmões:
Fígado:
Rins:
Vesícula cheia:
Vesícula vazia:
Epidídimo esquerdo
Epidídimo direito
Testículo esquerdo:
Testículo direito

Medida órgãos (mm)
Testículo direito:
Testículo esquerdo:

Fêmeas do machos						
Fêmea 1			Fêmea 2			
	Peso Placenta	Peso Fetos	Medida fetos	Peso Placenta	Peso Fetos	Medida fetos
1 -						
2 -						
3 -						
4 -						
5 -						
6 -						
7 -						
8 -						
9 -						
10 -						
11 -						
12 -						
13 -						
14 -						
15 -						
16 -						
17 -						
18 -						
19 -						
20 -						

Observações:

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

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 -			
Fetos vivos	6 -			
Direito:	7 -			
Esquerdo:	8 -			
Fetos mortos	9 -			
Direito:	10 -			
Esquerdo:	11 -			
Reabsorções precoces	12 -			
Direito:	13 -			
Esquerdo:	14 -			
Reabsorções tardias	15 -			
Direito:	16 -			
Esquerdo:	17 -			
Sítio de implantação	18 -			
Direito:	19 -			
Esquerdo:	20 -			

Observações:

APÊNDICE C

Ficha de análise esquelética

ANÁLISE ESQUELÉTICA

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

ABREVIAÇÕES	
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 excoocip.										
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 próximais																				
Falanges distais																				

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										
	Traquéia										
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										
	Traquéia										
6	Coração										
	Pulmões										
	Timo										
	Traquéia										
	Diafragma										
7	Bexiga										
	Rins										
	Pelve renal										
	Medula renal										
	Ureteres										
	Útero										
	Testículo										
	Epidídimo										

OBSERVAÇÕES:

NÚMERO DE FETOS FEMININOS	NÚMERO DE FETOS MASCULINOS

ANEXOS

ANEXO A
Parecer do Comitê de Ética

UNIVERSIDADE ESTADUAL DE LONDRINA

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
N° 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

Cumpre 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
Profa. Dra. Maria Fernanda Rodrigues Graciano
Coordenadora da CEUA/UEL

Ilmo.(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

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
www.instagram.com/anvisaoficial
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). As ligações podem ser feitas de segunda a sexta-feira, das 7h30 às 19h30, exceto feriados.

ANEXO C

Submissão do Artigo 1 à *Reproduction, Fertility and Development*

Reproduction, Fertility and Development - Manuscript ID RD22287

Yahoo/Entrada

Reproduction, Fertility an

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

ANEXO D

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

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 embriotoxidade 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

ANEXO E

Submissão do Artigo 3 à *Birth Defects Research*

Wiley Authors | Submission

My Submissions Maria

Birth Defects Research

JOURNAL HOME AUTHOR GUIDELINES EDITORIAL CONTACT

Powered by Atypon ReX

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 vi 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 mand bone tissue development in rat offspring

Manuscript Files

Name	Type of File	Size
Manusorit .docx	Main Document - MS Word	11.3 MB
Cover letter.docx	Cover letter / Comments	14 KB

Abstract

Background: Pregabalin is a gabapentinoid indicated for the treatment of peripheral neuropathic pair fibromyalgia, and as an adjuvant therapy for epilepsy. The aim of this study was to evaluate the effects pregabalin on postnatal development, odontogenesis, and mandibular trabecular tissue using compu microtomography.
Methods: Pregnant rats (n=24) were divided into two groups: control group (C), and treated group (PG PGB 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 natur On the first postnatal day, the litters were reduced to four pups (2 males and 2 females) and followed 30 days. On postnatal day 30, 8 animals from each group were randomly selected for analysis of the k right first molar and adjacent trabecular bone.
Results: In the maternal parameters, the data revealed a significant decrease in body weight gain in ac to a smaller number of live pups. In the postnatal analysis, the exposed pups showed a significant dec in weight and length and a delay in the eruption of the incisor teeth. Microtomography analysis reveal significant reduction in enamel volume, a lower volume and percentage of open pores, lower total por and a higher percentage of bone volume.
Conclusions: 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, an