Pathogenesis of Pregabalin-Induced Limb Defects in Mouse Embryos

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ABSTRACT - Purpose. It is known that antiepileptic drugs might adversely affect neuronal function and thus influence brain development. However, we have reported that limb deformities are one of the most prominent disturbances caused by pregabalin (PGB) in the developing embryo. The aim of this work is to gain a better understanding of possible molecular mechanisms behind the musculoskeletal injuries and limb deformities associated with PGB. **Methods**: Pregnant mice divided into four groups. Each mouse received an intraperitoneal injection (IP) of 0, 20 (group I), 40 (group II) or 80 (group III) mg/kg/day of PGB during the organogenesis period. On gestational day 18, embryos were separated and their limbs were dissected. Levels of apoptotic proteins were analyzed by Western blotting. To establish whether apoptosis is present in the limbs, the specimens were examined by TUNEL. Pathological findings were also reported as a score ranging from 1 to 3 based on the level of differentiation. **Results**: Western blot analysis demonstrated that PGB in all PGB-treated groups significantly upregulated the levels of cleaved caspase-3, 8 and 9. Also, the results showed that PGB exposure increased the percentage of TUNEL positive cells in different limb tissues especially the mesenchymal tissue. The histopathological findings revealed that PGB administration to pregnant mice inhibited limb tissue differentiation, albeit to varying degrees. **Conclusions**: The result of our study revealed that apoptosis and inhibition of limb tissue differentiation play an important role in the pathogenesis of PGB-induced limb malformations. Both intrinsic and extrinsic caspase-dependent pathways of cell death are important in mediating the abnormal limb development triggered by insult with the PGB. Evaluating the effect of PGB on molecules involved in the cross-talk between intrinsic and extrinsic apoptotic pathways and cell adhesion, migration, proliferation, and differentiation during embryonic development can further help to identify and clarify the involved mechanisms.

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INTRODUCTION

Pregabalin (PGB) is a structural derivative of gamma- aminobutyric acid (GABA) that is widely used in the treatment of epilepsy and anxiety disorder (1). It is also FDA approved for the treatment of neuropathic pain associated with diabetic peripheral neuropathy and postherpetic neuralgia. PGB and gabapentin are chemically related compounds. Compared with gabapentin, PGB has more rapid absorption and greater efficacy. The most common side effects of PGB include dizziness, somnolence, headache, weight gain, and blurred vision (2). The manufacturer-issued PGB prescribing information describes creatine kinase elevation and rhabdomyolysis in treated patients as another potential side effect (Lyrica®, Pfizer, New York City, USA). Considering the broad therapeutic indications of PGB, its use during pregnancy has been reported. Little information is available concerning PGB side effects on fetal development. According to the manufacturer's published data, PGB can induce structural fetal abnormalities, growth retardation, lethality and nervous system dysfunctional and neural tube defect at high doses (3). In our previous

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investigation, PGB injection to pregnant mice during organogenesis at low doses caused fetus malformations including limb, vertebral column and craniofacial abnormalities. The most common abnormality was limb deformity. Also, reduced degree of ossification of fetal skeleton bone at doses higher than the maximum recommended dose in humans has been reported (1).

The exact mechanism of PGB action is still unclear. Recent gene targeting technology studies have revealed that PGB decreases the release of central neurotransmitters by binding to the α2δ-1 subunit of the voltage-dependent calcium channel. It is claimed that PGB is virtually inactive at GABA receptors. To explain the teratogenicity of anti-epileptic drugs (AEDs), several mechanisms have been proposed (4). There is some evidence that several AEDs such as valproate sodium, phenobarbital, clonazepam, vigabatrin and diazepam induce embryonic malformations mediated by apoptosis (5). Due to the effect of AEDs on neuronal function, they especially influence brain development. However, in the case of PGB, limb deformities were the most common observed disorders (6). The aim of this work is to gain a better understanding of possible molecular mechanisms behind the musculoskeletal injuries and limb deformities associated with PGB.

MATERIALS AND METHODS

Animals and Treatment

Female BALB/c mice, 10-12 weeks of age, body weight 20-30 g were obtained from the Animal Center, School of Pharmacy, Mashhad University of Medical Sciences. Animals were maintained at 18- 22 °C with a 12-h light/12-h dark cycle and had free access to food and water. All experiments were approved by the Animal Care Committee of Mashhad University of Medical Sciences.

Study Protocol

Two females were pair-housed with one male mouse of the same strain in a cage. Pregnant mice were separated by checking the vaginal plug the day after mating had taken place and was considered as day zero of pregnancy (Gestation Day 0: GD 0). The animals were divided to four groups. Three treated groups received a daily intraperitoneal injection (IP) of 20 (group I), 40 (group II) and 80 (group III) mg/kg/day PGB, during gestation days

6–15 (organogenesis period). The fourth group as control received equivalent volume of normal saline. On D18, pregnant mice were anesthetized and embryos were dissected out of the uterus. The fetuses were cleared of their membranes and their limbs were dissected. Six embryos were used from each litter, and each litter was considered as one statistical unit. Limb samples were frozen in liquid nitrogen or fixed by immersion in 4% paraformaldehyde until further use.

Western Blot Analysis

Western blot assays of caspase 3, 8 and 9 were carried out on protein extracts from the limb tissues. Tissue samples were homogenized in the homogenization buffer (Tris 50 mM pH 7.4, 2 mM EDTA, 10 mM NaF, 1 mM Na3VO4, 10 mM b-glycerol-phosphate, 0.2% W/V sodium deoxycholate, 1 mM phenyl methyl sulfonyl fluoride and complete protease inhibitor cocktail) in ice and then were centrifuged at 10,000 g for 15 min at 4 ^{0}C. The total protein content was measured out from supernatant using the Bradford protein assay kit (Bio-Rad). The supernatant was then mixed with SDS-buffer and boiled for 5 min. Limb proteins (50 µg) were separated by SDS–PAGE gel and were transferred to PVDF membrane. After blocking with 5% non-fat milk for three hours, blots were incubated with antibodies: caspase-3 (Cell Signaling, #9665), caspase-8 (Cell Signaling, #4790) and caspase-9 (Abcam, # 32539, E23) at a dilution of 1:1000. After washing three times with TBST, the secondary antibody (Horseradish-peroxidase conjugated anti-rabbit antibody, Cell Signaling, #7074) was applied for one hour at room temperature. Enhanced chemiluminescence (Pierce, IL, and USA) and Alliance gel doc (Alliance 4.7 Gel doc, UK) were used for detection of the antigen-antibody complexes. Pixel intensity of bands was analyzed using UVtec software (UK) and normalized against beta actin intensity.

Detection of in situ Nuclear DNA Fragmentation

Cells containing fragmented DNA were evaluated in the paraffin sections using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL), with a commercial kit (Roche/ Germany). Briefly, after deparaffinising, tissue sections were pre-treated with proteinase K (20 µg/ml in 10 mM Tris/Hcl, 30 min) and H$_2$O$_2$

(3% in methanol, 10 min), for protein digestion and endogenous peroxidase inactivation, respectively. Then, sections were incubated under a coverslip with the TUNEL reaction mixture (mixture of terminal deoxynucleotidyl transferase and nucleotides mixture in a reaction buffer) for 1 h at 37 ^{0}C. After immersing the slides in 3% BSA for 1 h at room temperature, sections were treated with a peroxidase-labeled digoxigenin sheep Fab antibody in the presence of a diaminobenzidine substrate. Sections treated with 0.003 U/ml DNase I (Fermentas, USA) were used as the positive control. Negative control was performed using only label solution y instead of the TUNEL reaction mixture during the labeling process. Semi-quantitative analysis was done by counting the total number of apoptotic cells on ten fields in a section.

Histopathological Examination

The limbs were fixed in 10% neutral buffered formalin and processed for paraffin embedding. All paraffin blocks were cut at a thickness of 5 μm tissue sections and stained with hematoxylin and eosin. Each tissue type includes bone, cartilage, muscle, skin and nerve were evaluated separately in different groups. All sections were examined in 10 random fields on each slide (40X magnification). Pathological finding were reported as a score ranging from 1 to 3. A score of 1, 2 and 3 means undifferentiated, partial differentiated and differentiated status, respectively.

Statistical Analysis

All results are reported as mean ± SEM. Two-way repeated-measures ANOVA was used to evaluate differences in continuous numerical variables of different groups followed by Tukey test. Kruskal-Wallis ANOVA test was used if necessary. Dunn test was set as post test only if the results were positive. Data analysis was done using SPSS version 16.0 software. Significance levels were established at p-values<0.05.

RESULTS

Apoptosis Proteins (caspases 3, 8 and 9)

By performing Western blot we initially compared the PGB-induced enzymatic activation of 32 kDa procaspase 3 to produce the 17 kDa large subunit (cleaved form) in limb tissues. Caspase-3 plays a key role in the apoptotic pathway. Results in

Figure 1 show that PGB exposure at doses of 20, 40 and 80 mg/kg/day during organogenesis significantly upregulates the expression of cleaved caspase3/pro-caspase 3 (p<0.01 and p<0.001). The activations of the caspase 8 and 9 as crucial mediators of the death receptor and mitochondria apoptosis pathway were also measured. PGB in all treated groups significantly increased the ratio of cleaved caspase 8/pro-caspase 8 (p<0.01 and p<0.001) (Figure 2). Also, protein expressions of the cleaved caspase 9/pro-caspase 9 were upregulated by PGB at all doses (p<0.05 and p<0.01) (Figure 3).
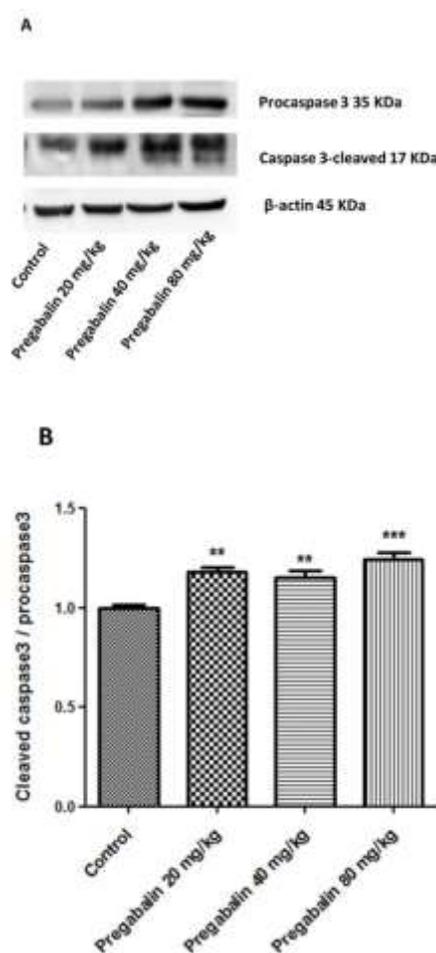
Figure 1. Effect of PGB on the protein level of caspase-3 in the mouse embryonic limb. The animal groups received 0, 20, 40 and 80 mg /kg/day of PGB. (A) Representative photograph of the Western blot analysis. (B) Densitometric data of protein analysis. Data are expressed as mean±SEM. **p<0.01 and ***p<0.001 compared to the control group.

A

B
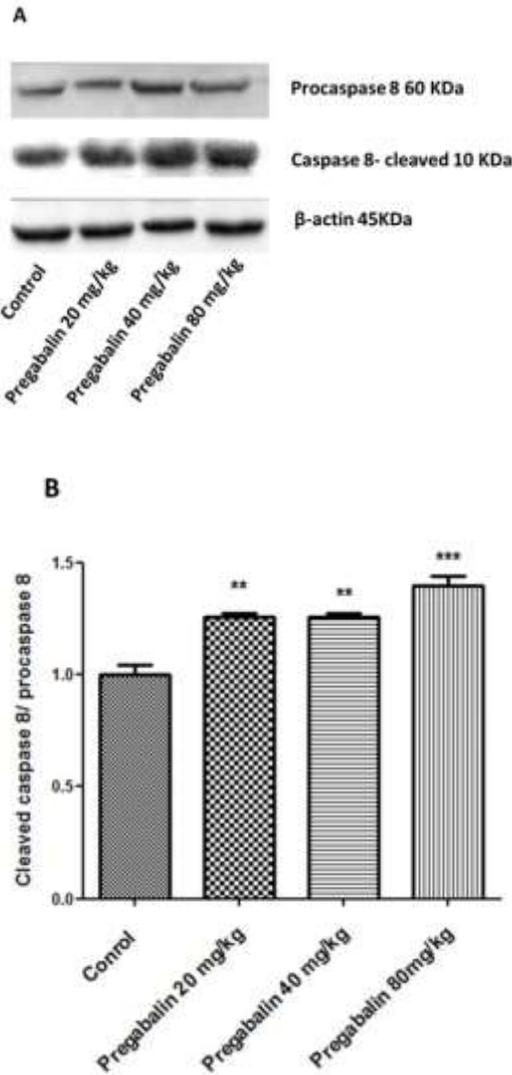
Figure 2. Effect of PGB on the protein level of caspase-8 in the mouse embryonic limb. The animal groups received 0, 20, 40 and 80 mg /kg/day of PGB. (A) Representative photograph of the Western blot analysis. (B) Densitometric data of protein analysis. Data are expressed as mean±SEM. **$p<0.01$ and ***$p<0.001$ compared to the control group.

TUNEL Assay
The TUNEL assays revealed an induction of apoptosis in limb tissues (Figure 8). In comparison with the control group, the percentages of TUNEL positive cells in the mesenchymal tissues were significantly increased in groups that received 20,40 and 80 mg/kg/day PGB (11.6±0.79%, 18.8±2.28% and 17.5±1.52%, respectively vs 2.62±0.26% in the control group) ($p<0.01$, $p<0.01$ and $p<0.001$,

respectively) (Figure 4). PGB-treated groups also showed a significant increase in the mean apoptotic index in cartilage tissues, ranging from 6.6% to 15.5% (Figure 5). A significant difference in the apoptotic index was detected between the treated groups and the control group in muscle and skin tissues (Figures 6 and 7). The apoptotic indices were ranged from 7.6% to 14.5% and 7.8% to 14.8%, respectively compared with their control groups (1.75±0.49% and 2.2 ±0.54%, respectively).

A

B
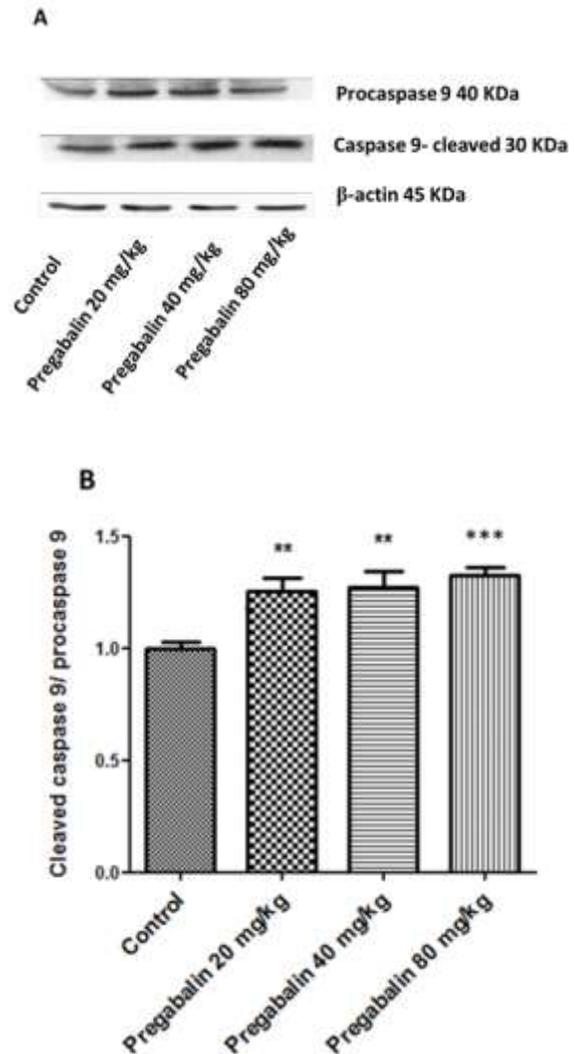
Figure 3. Effect of PGB on the protein level of caspase-9 in the mouse embryonic limb. The animal groups received 0, 20, 40 and 80 mg /kg/day of PGB. (A) Representative photograph of the Western blot analysis. (B) Densitometric data of protein analysis. Data are expressed as mean±SEM. **$p<0.01$ and ***$p<0.001$ compared to the control group.
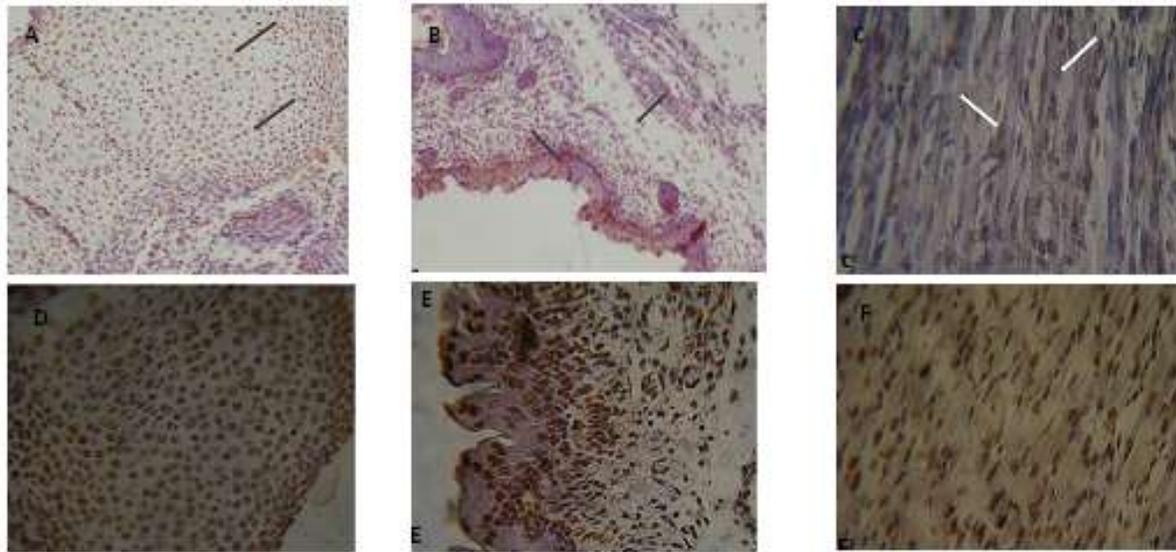
Figure 8. Apoptotic profile of PGB (40 mg/kg/day) in the embryonic limb. A) Cell death in the cartilage tissue, B) Cell death in the dermal and mesenchymal tissues, C) Cell death in the muscle tissue, D, E and F) tissue sections of cartilage, dermal, mesenchymal and muscle tissues treated with DNase as the positive controls. Apoptotic cells are shown by arrows.
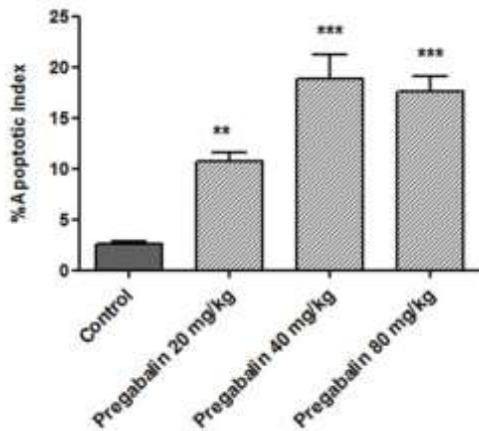
Figure 4. Effect of PGB on embryonic limb mesenchymal cells apoptosis as revealed by TUNEL assay. The animal groups received 0, 20, 40 and 80 mg /kg/day of PGB, through 6-15 gestational days, respectively. Values are presented as mean±SEM. ** $p < 0.01$ and ***$p < 0.001$ compared to control group.
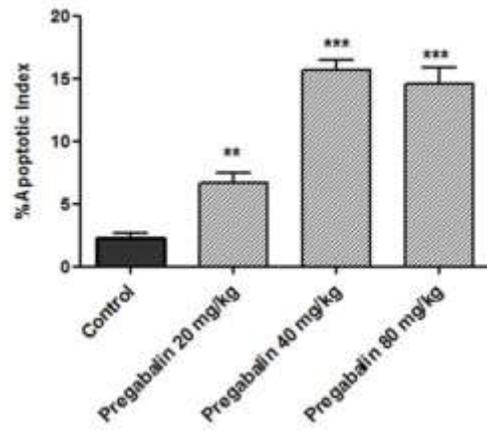
Figure 5. Effect of PGB on embryonic limb cartilage cells apoptosis as revealed by TUNEL assay. The animal groups received 0, 20, 40 and 80 mg /kg/day of PGB, through 6-15 gestational days, respectively. Values are presented as mean±SEM. ** $p < 0.01$ and *** $p < 0.001$ compared to control group.
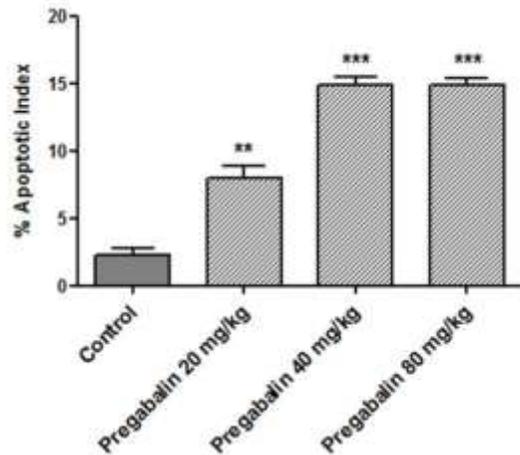
Figure 6. Effect of PGB on embryonic limb muscle cells apoptosis as revealed by TUNEL assay. The animal groups received 0, 20, 40 and 80 mg /kg/day of PGB, through 6-15 gestational days, respectively. Values are presented as mean±SEM. **p<0.01 and ***p<0.001 compared to control group.
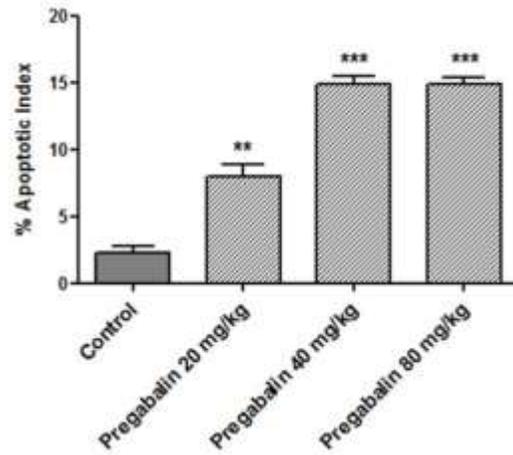
Figure 7. Effect of PGB on embryonic limb skin cells apoptosis as revealed by TUNEL assay. The animal groups received 0, 20, 40 and 80 mg /kg/day of PGB, through 6-15 gestational days, respectively. Values are presented as mean±SEM. **p<0.01 and ***p<0.001 compared to control group.

Histopathological Studies

The histopathological findings showed that PGB administration to pregnant mice inhibits limb tissue differentiation, albeit to varying degrees (Figure 9). These results showed that PGB at different doses inhibit the differentiation of mesenchymal, cartilage, muscle, osteoblast and nervous tissues significantly (*p*<0.01 and p<0.001). No pathologic finding was observed in the skin tissue. PGB had greater suppressive effects on cartilage and muscle tissues than other limb tissues. PGB is able to suppress limb differentiation at higher doses (40 and 80 mg/kg/day) more than the lowest dose (20 mg/kg/day). There were no significant differences between the 40 and the 80 mg/kg/day doses (Table 1).

Table 1. Histopathological changes caused by PGB in embryonic limb. Treated and control groups received 0, 20, 40 and 80 mg/kg/day of PGB, through 6-15 gestational days, respectively. Values are presented as mean±SEM. **p<0.01 and ***p<0.001 compared to the control group.

Tissue	Degree of Differentiation			
	Control	PGB 20 mg/kg/day	PGB 40 mg/kg/day	PGB 80 mg/kg/day
Skin	2.83±0.17	2.83±0.16	2.5±0.22	2.5±0.22
Nervous	3±0	2±0.21**	1.71±0.18***	1.57±0.2***
Muscle	3±0	1.85±0.26**	1.57±0.2***	1.28±0.18***
Chondrocyte	2.85±0.14	1.86±0.26**	1.57±0.2***	1.28±0.18***
Osteoblast	3±0	2±0.21**	1.71±0.18***	1.28±0.18***
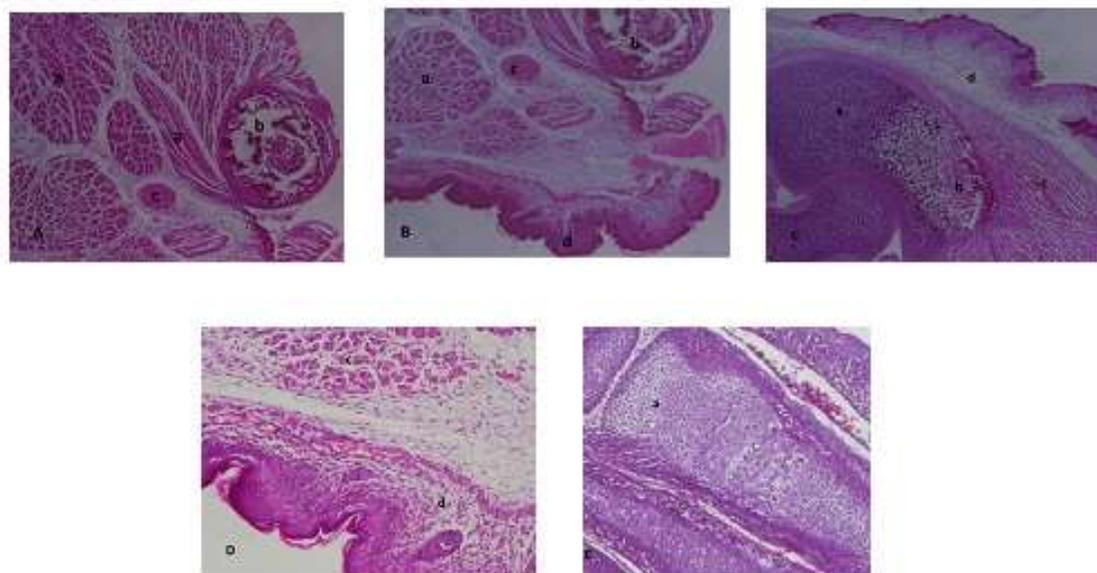
Figure 9. Light photomicrograph of hematoxylin and eosin–stained sections of embryonic limbs that were exposed to 40 mg /kg/day of PGB during the organogenesis period. A, B) Limb tissue differentiation in the control group (a: muscle tissue, b: osteoblast tissue, c: neuronal tissue and d: skin tissue). C, D, E) Limb tissue differentiation in the treated groups: partial differentiated cells of cartilage (a), osteoblast (b) and muscle (c) and immature mesenchymal tissue (d).

DISCUSSION

In the present study, we have observed apoptosis, a form of programmed cell death as a potential mechanism of PGB-mediated developmental toxicity. The TUNEL results showed that PGB administration at 20, 40 and 80 mg/kg/day doses during the organogenesis period can increase the apoptosis index in limb tissues. Most cells undergoing apoptosis were detected within the mesenchyme. No significant differences were observed between groups that received 40 and 80 mg/kg/day. Activation of the caspase-3 protease revealed that PGB induces apoptosis through a caspase-mediated pathway in the embryonic limb tissues. The involvement of caspases in the process of limb malformation has been supported by studies with other teratogens. Single oral dose administration of retinoic acid (400 mg/kg) to pregnant mice leads to excessive apoptosis in the apical ectodermal ridge of malformed limbs (7). *In vitro* murine limb exposure to a preactivated analog of cyclophosphamide at 1- 10 μg/ml revealed that the apoptotic cell death play an important role in the pathogenesis of 4-hydroperoxycyclophosphamide-induced limb malformations (8). To determine the apoptotic pathway induced by PGB, we assessed activation of caspase-8, and -9 by Western blot. Increased activities of caspase-8 and -9 were observed due to an enhanced activation of both intrinsic (mitochondrial) and extrinsic (death receptors) apoptosis pathways. It is widely accepted that an intensive crosstalk can take place between the extrinsic and intrinsic pathways (9). Different mechanisms have been suggested for this crosstalk. Cleavage of Bid to truncated Bid by caspase-8 and ceramide generation as well as other plethora of molecular pathways are involved in the crossover activity (10). PGB has a high-affinity binding toward the alpha2-delta-1 subunit of the voltage-dependent calcium channels (11). This mechanism is frequently detected in both rodent- and human-based experiments. Wide distribution of calcium channel alpha 2 delta types 1 subunit in the human brain and skeletal muscles and also similar tissue distribution profiles between mouse and human tissues can explain why limb deformities are more prevalent (12). On the other hand, concentration of the α2/δ1 protein at the end of immature muscle cells revealed a critical role of this subtype in migration, attachment and spreading of myoblasts. It is postulated that the α2/δ1 subunit mediates

interaction of muscle cells and the extracellular matrix and is involved in extracellular signalings that are essential for muscle development and muscle repair (13). The presence of voltage-sensitive calcium channels (VSCC) has been well characterized in osteoblastic cells at various stages of differentiation. VSCCs are also responsible for most of the calcium influx into osteoblasts and it has been shown that specific calcium channel blockers affect bone formation bone and turnover (14). Indeed, the inhibition of limb tissue differentiation in varying degrees according to pathologic findings suggests a role for α2/δ subunit in contributing to differentiation of mesenchymal stem cells.

CONCLUSION

It is concluded that apoptotic cell death and inhibition of limb tissue differentiation play important roles in the pathogenesis of PGB-induced limb malformations. Both intrinsic and extrinsic caspase-dependent pathways of cell death are important in mediating the abnormal limb development triggered by insult with PGB. Studying the effect of PGB on molecules involved in the crosstalk between intrinsic and extrinsic apoptotic pathways and cell adhesion, migration, proliferation and differentiation during embryonic development can help to identify and clarify the involved mechanisms.

ACKNOWLEDGMENTS

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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