Diphenylhydantoin Promotes Proliferation in The Subventricular Zone and Dentate Gyrus

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Abstract: Problem statement: Diphenylhydantoin (phenytoin) is an antiepileptic drug that generates hyperplasia in some tissue by stimulating Epidermal Growth Factor (EGFR) and Platelet-Derived Growth Factor beta (PDGFR-β) receptors and by increasing serum levels of basic fibroblast growth factor (bFGF, FGF2 or FGF-β). Neural stem cells in the adult brain have been isolated from three regions: the Subventricular Zone (SVZ) lining the lateral wall of the lateral ventricles, the Subgranular Zone (SGZ) in the dentate gyrus at the hippocampus and the Subgranular Zone (SZC) lining between the hippocampus and the corpus callosum. Neural stem cells actively respond to bFGF, PDGFR-β or EGF by increasing their proliferation, survival and differentiation. The aim of this study was to evaluate the effect of phenytoin on proliferation and apoptosis in the three neurogenic niches in the adult brain.

Approach: We orally administrated phenytoin with an oropharyngeal cannula for 30 days: 0 mg kg\(^{-1}\) (controls), 1, 5, 10, 50 and 100 mg kg\(^{-1}\). To label proliferative cells, three injections of 100 mg kg\(^{-1}\) of BrdU was administrated every 12 h. Immunohistochemistry against BrdU or Caspase-3 active were performed to determine the number of proliferative or apoptotic cells.

Results: Our results showed that phenytoin induces proliferation in the SVZ and the SGZ in a dose-dependent manner. No statistically significant effects on cell proliferation in the SCZ neither in the apoptosis rate at the SVZ, SGZ and SCZ were found.

Conclusion: These data indicate that phenytoin promotes a dose-dependent proliferation in the SVZ and SGZ of the adult brain. The clinical relevance of these findings remain to be elucidated.

Key words: Epidermal Growth Factor (EGFR), Subventricular zone (SVZ), Subgranular Zone (SGZ), neural stem cells, Platelet-Derived Growth Factor beta (PDGFR-β)

INTRODUCTION

Diphenylhydantoin (5-Ethyl-3-Methyl-5-Phenylhydantoin) also known as phenytoin is an effective anticonvulsant in tonic-clonic epilepsy (Cornacchio et al., 2011). Voltaged-gated channels are involved in the epilepsy pathophysiology (Abuhamed et al., 2008). The primary target of phenytoin in depolarizing neurons is voltage-dependent sodium channels, where phenytoin blocks sodium influx, reducing neuronal excitability and limiting the spread of electrical activity of seizures (Shaw et al., 2007). Other mechanisms possibly contributing to the antiepileptic activity of phenytoin include a suppression of sodium action potentials by stimulating the sodium pump, inhibition of calcium influx in neurons, blockage of ionotropic receptors for glutamate and enhancement of GABA neurotransmission (Escueta and Appel, 1971; Kaindl et al., 2006; Yang et al., 2007). Thus, its safety profile and ease of use make phenytoin an attractive drug for the seizure prophylaxis and the control of status epilepticus. Some of side effects of phenytoin include gingival hyperplasia (Eyer et al., 2008), hypertrichosis (hirsutism) (Vivard et al., 1989), acne (Jenkins and Ratner, 1972), cerebellar atrophy (Ohmori et al., 1999), hyperglycemia (Yang et al., 2007) and others.

Recent evidence indicates that phenytoin promotes proliferation of primary progenitors in several tissues,
such as: skin (Swamp et al., 2004), heart (Zhou et al., 2006), bone (Lau et al., 1995) and oral mucosa (Arya and Gulati, 2012; Sano et al., 2004). Phenytoin-induced tissue hyperplasia seems to be mediated by increasing levels of connective tissue growth factor (CCN2/CTGF), transforming growth factor β1 (TGF-β1) (Kantarci et al., 2007; Kura et al., 2004), mRNA of Platelet-Derived Growth Factor (PDGF-β) (Dill et al., 1993; Iacopino et al., 1997), Fibroblast Growth Factor type-2 (FGF-2) (Saito et al., 1996; Sasaki and Maita, 1998; Turan et al., 2004) and Epidermal Growth Factor Receptors (EGFR) (Modeer and Andersson, 1990; Soory and Kasasa, 1997).

In the adult mammalian brain, there are neural stem cells that produce new neurons and oligodendrocytes (Mackay-Sim, 2010). These multipotent progenitor cells are located in restricted regions: the Subventricular Zone (SVZ), lining the lateral wall of the lateral ventricle located in restricted regions: the Subventricular Zone (Mackay-Sim, 2010). These multipotent progenitors are cells that produce new neurons and oligodendrocytes (Aguirre et al., 2007; 2010; Ayuso-Sacido et al., 2010; Balu and Lucki, 2009; Ming and Song, 2005). Thus, neural progenitor cells may be a pharmacological target for phenytoin effects. The aim of this study was to analyze whether phenytoin promoted proliferation or apoptosis in the SVZ, the SGZ and the SCZ. Our findings indicate that phenytoin induces a dose-dependent proliferative effect in the SVZ and SGZ. No changes were observed in apoptosis. These results may be of clinical relevance because neural stem cells have been successfully isolated in the adult human brain (Sanai et al., 2004) and phenytoin is a drug commonly used in epileptic patients.

MATERIALS AND METHODS
Animal care and tissue processing: All animal procedures followed the Committee on Animal Research guidelines in the University of Colima. Adult (P60) Balb/C mice were sacrificed by an overdose of pentobarbital (100 mg kg$^{-1}$ body weight) before transcardial perfusion. For light microscopy analysis, mice (n = 5 per group) were perfused with 4% Paraformaldehyde (PFA) dissolved in 0.1M phosphate buffer and the brains were post-fixed overnight at 4°C in the same fixative. Then, 40-µm thick coronal sections were cut with a vibratome.

Phenytoin administration: 5. 5-Diphenylhydantoin (Sigma, Cat. No. D4505) re-suspended in distilled water (vehicle) or vehicle alone were orally administered for 30 days with an oropharyngeal cannula. We used the following doses (n = 5 animals per dose): 0, 1, 5, 10, 50 and 100 mg kg$^{-1}$.

Bromodeoxyuridine (BrdU) administration: BrdU is a synthetic thymidine that incorporates into DNA during the S-phase of the cell cycle (Cameron and McKay, 2001; Falconer and Galea, 2003; Taupin, 2007). To label all progeny derived from the SVZ, SGZ and SCZ precursors, we injected 3 doses of 100 mg kg$^{-1}$ i.p. BrdU every 12h (Cameron and McKay, 2001; Gonzalez-Perez et al., 2011) before animals’ sacrifice.

Immunohistochemistry (IHC): Sections were then incubated in pre-warmed (at 37°C) 2 N HCl for 30 min. Then, a single wash with 0.1 M borate buffer (pH = 8.5) for 10 min was utilized to neutralize HCl. Then, samples were rinsed (10 min×3) in 0.1 M buffer Phosphate Buffer Saline (PBS). After peroxidase inactivation with 30% H$_2$O$_2$ for 30 min, sections were blocked in 0.1M PBS containing 0.1% Triton-X and 10% of normal goat serum for 1 h at room temperature, sections were incubated overnight at 4°C in primary antibodies diluted in blocking solution. The following primary antibodies were used: rat monoclonal to BrdU (1:500; Accurate Chemical OBT0030) or mouse IgG anti-Caspase-3 active (Casp3; Imgenex IMG-144A) dilution 1:800. Sections were washed in 0.1M PBS, incubated in blocking solution with the appropriate biotinylated secondary antibody (1:200; Vector Laboratories, Burlingame, CA) for 1 h at room temperature, incubated in ABC peroxidase kit (Vector Laboratories, Burlingame, CA) for 1 h and revealed with 0.03% diaminobenzidine and 0.01% H$_2$O$_2$. Controls in which primary antibodies were omitted showed no detectable staining.

Quantification: To quantify the number of BrdU-positive or Casp3-positive cells, we analyzed at least ten 40-µm sections randomly selected, 160-µm apart (n = 5 animals per group). For SVZ quantifications, the number of BrdU+ or Casp3+ cells was quantified within 100 µm from the ependymal layer along the ventricle per every section. For SGZ quantifications, the number of immuno-positive cells found along the dentate gyrus was quantified in every section. The SCZ is a caudal extension of the SVZ that is no longer

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RESULTS

Phenytoin administration was well-tolerated and no side effects were observed at any of doses used throughout the study. We recorded the weight gain during phenytoin administration (Fig. 1) and did not find significant differences between the control-vehicle group vs. the phenytoin groups (ANOVA–Tukey, P = 0.63). This suggested that the potential sedative effects of phenytoin did not change the body development of animals.

The subventricular zone: To characterize the effect of different concentrations of phenytoin on SVZ precursors, we delivered different doses of phenytoin (0, 1, 5, 10, 50 and 100 mg kg\(^{-1}\)) and administrated 3 injections of 100 mg kg\(^{-1}\) of BrdU before sacrifice. After 30 days of phenytoin administration, we found no statistically significant differences in the number of BrdU+ cells in the group of 1 mg kg\(^{-1}\) of phenytoin (22.04±0.55 cells per field) as compared to the control group (16.62±2.07 cells per field) (Fig. 2). However statistically significant differences were found with the doses of 5 mg kg\(^{-1}\) (27.58±1.56 cells per field), 10 mg kg\(^{-1}\) (28.42±2.09 cells per field), 50 mg kg\(^{-1}\) (27.42±2.11 cells per field) and 100 mg kg\(^{-1}\) of phenytoin (24.90±1.11 cells per field; p<0.05, ANOVA–Tukey’s test) as compared to controls. Interestingly, upon 10 mg kg\(^{-1}\) of phenytoin no further changes in proliferation were observed.

To analyze the apoptosis rate in the SVZ, we quantified the number of CASP3+ cells in this region. In all cases, we did not find statistically significant differences among groups: the control group (5.27±1.36 cells per field), 1 mg kg\(^{-1}\) (5.06±0.96 cells per field), 5 mg kg\(^{-1}\) (5.37±0.82 cells per field), 10 mg kg\(^{-1}\) (5.30±1.22 cells per field), 50 mg kg\(^{-1}\) (6.83±1.85 cells per field) and 100 mg kg\(^{-1}\) of phenytoin (13.14±3.45 cells per field; ANOVA–Tukey). Taken together these data suggests that phenytoin promotes proliferation of SVZ neural progenitors in a dose-dependent manner without changing the apoptosis rate in this region.

The subgranular zone: To characterize the effect of different concentrations of phenytoin on SGZ precursors, we orally delivered 0, 1, 5, 10, 50 and 100 mg kg\(^{-1}\) of phenytoin per day and administrated BrdU before sacrifice. At day 30th, we quantified the number of BrdU+ cells in the dentate gyrus in the hippocampus (Fig. 3). We found an increase in the number of BrdU+ cells in the SGZ with the dose of 10 mg kg\(^{-1}\) of phenytoin (5.87±0.34 cells per field) as compared to controls (4.01±0.26 cells per field; p<0.05, ANOVA–Tukey). Interestingly, no significant differences were observed with the doses of 1 mg (4.62±0.39 cells per field), 5 mg kg\(^{-1}\) (4.46±0.32 cells per field), 50 mg kg\(^{-1}\) (4.04±0.24 cells per field) and 100 mg kg\(^{-1}\) (4.39±0.20 cells per field) of phenytoin. We then quantify the number of CASP3+ cells in the SGZ. Our findings indicate that there are not statistical significant differences among groups: the control group (0.04±0.01 cells per field), 1 mg kg\(^{-1}\) (0.08±0.02 cells per field), 5 mg kg\(^{-1}\) (0.07±0.02 cells per field), 10 mg kg\(^{-1}\) (0.05±0.02 cells per field), 50 mg kg\(^{-1}\) (0.05±0.01 cells per field) and 100 mg kg\(^{-1}\) of phenytoin (0.09±0.02 cells per field; ANOVA–Tukey). These results suggest that only the dose of 10 mg kg\(^{-1}\) of phenytoin promotes proliferation of SGZ progenitors and that this drug did not change the apoptosis rate in this region.

The subcallosal zone: We finally characterize the effect of different concentrations of phenytoin on SCZ neural precursors. At day 30th, we quantified the number of BrdU+ cells in the SCZ (Fig. 4).
Fig. 2: BrdU+ cells in the adult SVZ in mice after 30 days of phenytoin or vehicle administration (A). The quantification of BrdU+ cells is summarized in the graph (B). The bars represent the mean ± standard deviation. Arrows indicate some BrdU+ cells. V: ventricle; (*) p = 0.05 ANOVA-Tukey; (**) p = 0.01 ANOVA-Tukey. Scale bar = 200 µm

Fig. 3: BrdU+ cells in the adult SGZ after 30 days of phenytoin or vehicle administration (A). The quantification of BrdU+ cells is summarized in the graph (B). The bars represent the mean ± standard deviation. Arrows indicate some BrdU+ cells. (*) p = 0.05 ANOVA-Tukey. Scale bar = 100 µm
We did not find significant differences in the number of BrdU+ cells among groups: the control group (8.4±0.76 cells per field), 1 mg kg⁻¹ (7.8±0.82 cells per field), 5 mg kg⁻¹ (7.37±0.31 cells per field), 10 mg kg⁻¹ (7.45±1.50 cells per field), 50 mg kg⁻¹ (6.04±0.47 cells per field) and 100 mg kg⁻¹ of phenytoin (7.88±0.48 cells per field). The analysis of CASP3+ cells in this region did not show statistical significant differences: the control group (0.04±0.02 cells per field), 1 mg kg⁻¹ (0.03±0.01 cells per field), 5 mg kg⁻¹ (0.04±0.02 cells per field), 10 mg kg⁻¹ (0.03±0.01 cells per field), 50 mg kg⁻¹ (0.04±0.03 cells per field) and 100 mg kg⁻¹ of phenytoin (0.01±0.01 cells per field; ANOVA-Tukey). Taken together, our data suggest that phenytoin cannot induce changes in proliferation and apoptosis of the SCZ neural progenitors.

**DISCUSSION**

In this study, we show that: (1) Different doses of phenytoin did not alter weight gain in adult mice; (2) Phenytoin induces proliferation in the SVZ and the SGZ in a dose-dependent manner; (3) Phenytoin has no significant effect on the proliferation rate in the SCZ; and (4) No statistically significant changes on the apoptosis rate in any of the analyzed regions are induced by phenytoin. Taken together, these data indicate that phenytoin promotes proliferation in the main neurogenic niches of the adult brain in vivo without changing the apoptosis rate.

As described above, our findings indicated that different doses of phenytoin did not alter weight gain. Similar findings have been reported in Sprague Dawley® rats (Mowery et al., 2008). However, another report indicated that phenytoin administration at early-postnatal stages reduced food intake and weight gain (Mowery et al., 2008). These changes were reversible when phenytoin supplementation was suspended or when administrated at older development stages (Mowery et al., 2008; Okada et al., 1997; 2001). Hence, this evidence suggests that the metabolism of phenytoin or its cellular receptors vary according to the age stages (Ogura et al., 2002).

In our study, we quantified the number of BrdU+ cells along the SVZ. BrdU is a reliable proliferation marker that incorporates DNA during S phase and can be detected by immunohistochemistry (Kee et al., 2002; Taupin, 2007). The protocol and dose of BrdU administration used in this study has shown to reduce the false positive and lacks significant side effects (Cameron and McKay, 2001). Our findings indicated that the phenytoin-induced proliferative effect is observed from the dose of 5mg kg⁻¹, but it reaches a plateau at 10 mg kg⁻¹ of phenytoin in the SVZ. Interestingly, in the SGZ this drug shows proliferative effects only with 10 mg kg⁻¹ of phenytoin. Proliferative effects of phenytoin have been described in several tissues, such as: Skin (Swamy et al., 2004), cardiomyocytes (Zhou et al., 2006), bone (Lau et al., 1995), bone marrow stem cells (Ohta et al., 1995) and oral mucosa (Sano et al., 2004). These effects seem to
be mediated by increasing c-jun levels and suppression of p44/42, which indicates that phenytoin can modify MAPK signaling pathway (Zhao et al., 2003).

In addition, proliferative effects of phenytoin can be mediated by increasing levels of growth factors and cytokines (Okada et al., 2001), which modify the proliferation rate, apoptosis, migration and differentiation of neural stem cells (Alvarez-Palazuelos et al., 2011; Gonzalez-Perez et al., 2010). Oral administration of phenytoin in patients has shown to increase levels of osteocalcin, also known as Bone Gamma-Carboxylglutamic Acid-Containing Protein (BGLAP) (Koyama et al., 2000; Lau et al., 1995) and basic Fibroblast Growth Factor (FGF-2) (Saito et al., 1996; Sasaki and Maita, 1998; Turan et al., 2004). Other molecules associated with the phenytoin-induced proliferation are: bone morphogenetic protein 4 (BMP-4), endothelin 1 and Transforming Growth Factor β (TGF- β); (Koyama et al., 2000; Nakade et al., 1996; Sano et al., 2004).

Phenytoin is also a competitive binding agonist of the Epidermal Growth Factor Receptor (EGFR) (Grenader et al., 2007) and increases the expression of EGFR (Modeer and Andersson, 1990). EGFR are highly expressed in the SVZ precursors (Doetsch et al., 2002) and control proliferation and migration of neural precursors in the adult SVZ (Gonzalez-Perez, 2010; Gonzalez-Perez et al., 2009). EGFR mitogenic effects are mediated through MAPK, Akt and IP3 downstream pathways (Gonzalez-Perez and Alvarez-Buylla, 2011; Jorissen et al., 2003). PI3K/AKT is involved in survivor and matures of oligodendrocytes in the early development of the central nervous system (Flores et al., 2008). Activation of EGFR activates PI3K/Akt signal transduction pathway that positively regulates Glycogen Synthase Kinase 3β (GSK-3β) (Zhang et al., 2002). In addition, EGFR stimulation of SVZ adult precursors promotes oligodendrogenesis and arrests neurogenesis (Gonzalez-Perez, 2010; Gonzalez-Perez and Alvarez-Buylla, 2011; Gonzalez-Perez et al., 2009). Since EGFR signaling has been related to brain tumor progression (Jorissen et al., 2003), the role of phenytoin in tumorigenesis remains to be elucidated.

This study indicates that cell proliferation in the dentate gyrus were only noticeable with the dose of 10 mg kg$^{-1}$ of phenytoin, whereas no significant differences were observed in the SCZ. Interestingly, in our study higher doses of phenytoin induced no proliferation in neuronal SGZ precursors. We hypothesize that regional differences may be due to different levels of EGFR expression in the neural precursor of the SGZ and SCZ as compared to the SVZ (Seri et al., 2004; 2006). Therefore, phenytoin may be exerting some differential proliferative effect on these regions. Dual effects of phenytoin have been previously described that, at certain doses, it reduce cerebral monoamines (Vazquez et al., 2003). On this regard, serotonin modulates the proliferation of SGZ precursors (Sahay and Hen, 2008; Warner-Schmidt and Duman, 2006). Therefore, high doses of phenytoin can reduce the levels of serotonin (Okada et al., 1997) that, in turn, decrease cell proliferation into the SGZ. Remarkably, another anticonvulsant drug, magnesium valproate, has shown either proliferation or apoptosis, depending on the dose used on microglial cells and neuronal precursors (Dragunow et al., 2006). Phenytoin also increases the levels of Adrenocorticotropic Hormone (ACTH) and corticosterone (Okada et al., 2001), probably mediated by the P450 cytochrome enzyme system (Putignano et al., 1998), which reduce proliferation of SGZ precursors (Gonzalez-Perez et al., 2011; Nichols et al., 2005). Therefore, high levels of glucocorticoids induced by phenytoin may also modify the proliferation rate of neural precursors in the dentate gyrus.

In addition, our data indicate that phenytoin did not induce changes in the number of CASP3+ cells in any of the analyzed regions, which suggest that the increase in the number of BrdU+ cells is not due to a reduction in apoptosis rate. Similar findings have been reported in epithelial cells from oral mucosa (Kantarci et al., 2007). However, other anticonvulsants have shown to induce apoptosis in microglia, which suggest that apoptosis induction is probably related to intrinsic drug metabolism (Dragunow et al., 2006).

There are several questions that remain to be elucidated, such as: (1) what kind of cell types proliferate in the SVZ and the SGZ; (2) Do these cells remain in the brain parenchyma; (3) Do they differentiate in the brain parenchyma; If so, (3) Do they play a functional role into the brain. In addition, it would be interesting to test the phenytoin in experimental models of disease (Anderson et al., 2008; Jqamadze et al., 2012). Therefore further studies are needed to address these questions.

CONCLUSION

Phenytoin induces cell proliferation of neural precursors in the SVZ in the forebrain and the SGZ in the dentate gyrus in a dose-dependent manner, without changing apoptosis rates of these neurogenic niches. Whether phenytoin may promote proliferation in the human brain remains to be elucidated.

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