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PHENYTOIN INDUCED OXIDATIVE STRESS IN PRE- AND POSTNATAL RAT DEVELOPMENT – EFFECT OF VITAMIN E ON SELECTIVE BIOCHEMICAL VARIABLES

Jana Navarová, Eduard Ujházy, Michal Dubovický, Mojmír Mach

Institute of Experimental Pharmacology, Slovak Academy of Sciences, Bratislava, Slovakia, e-mail: Jana.Navarova@savba.sk

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A pre- and postnatal study was carried out to investigate the effect of high dose (500 mg/kg) of the natural antioxidant vitamin E (VIT E) on biochemical variables in the model of chronic intrauterine hypoxia. Chronic hypoxia was induced by administration of the anticonvulsant phenytoin (PHT) during pregnancy. Rats were orally treated with PHT (150 mg/kg) from day 7 to 18 of gestation and VIT E prior to PHT orally on the same days. The activity of the lysosomal enzyme *N*-acetyl-ß-D-glucosaminidase (NAGA) and the level of glutathione (GSH) were used as markers of tissue damage. In the prenatal study PHT-induced embryofoetal toxicity was associated with an increase in NAGA activity and decrease of GSH level in maternal serum and heart and with an increase in NAGA activity in the placenta. Administration of VIT E did not inhibit the above given changes. PHT increased the activity of NAGA and decreased the level of GSH in foetal organs (liver, lungs, brain). VIT E did not reverse these changes. In the postnatal study, we did not find any significant differences in NAGA activity in the organs of 1-day-old pups. An increase of liver GSH level was found in PHT and VIT E+PHT groups of pups and in the group VIT E+PHT in the lungs. In conclusion, supplementation with a high-dose of VIT E failed to protect maternal, foetal and new-born rat organs from PHT induced changes of selective biochemical variables.

INTRODUCTION

It has been suggested that PHT teratogenicity is induced by embryonic hypoxia with vascular disruption and tissue necrosis as a result of ischaemic damage and/or reactive oxygen species generation at reoxygenation[1]. Phenytoin (PHT) is thought to cause chronic intrauterine hypoxia/ischaemia and embryo- foetal toxicity via reactive oxygen intermediates. Reactive oxygen species can oxidise molecular targets such as DNA, protein and lipid, in a process referred to as oxidative stress resulting in in utero death or teratogenicity. The pathology of oxidative stress can be prevented by antioxidants known to be effective in treating conditions associated with oxidative damage[2–4]. Vitamin E (VIT E), a natural antioxidant, is believed to help prevent diseases associated with oxidative stress. Toxicity of VIT E is very low and adverse effects were rarely observed with dosages up to 2000 mg VIT E/day in human subjects. At much higher dosages, side effects and intolerance were increasingly noted. The only consistent side effect of VIT E treatment was coagulation abnormalities in individuals with previous vitamin K deficiency[5]. VIT E is considered safe in pregnancy[6], although experiments evaluating the safety of high-dose VIT E treatment in pregnancy have not been reported. The fact that premature infants have poorly developed antioxidant systems[7, 8] lends special relevance to the study of antioxidant protection in preterm rats.

In our previous study, we compared the effect of the natural antioxidant melatonin and the synthetic antioxidant stobadine in the PHT model of intrauterine hypoxia. We showed that in pregnancy and early postnatal development stobadine inhibited and melatonin partially prevented the biochemical changes induced by PHT (ref.[9]). The objective of this study was to test the hypothesis that VIT E could prevent the biochemical changes in maternal, foetal and newborn organs induced by PHT administration to mothers.

MATERIAL AND METHODS

Animals: Wistar/DV pregnant rats (n= 50, initial body weight 200-220 g, 3-4 months old) from the Breeding Facility IEP SASc Dobrá Voda, Slovakia, were used. The animals were kept under controlled conditions at 22 ± 2 °C and 55 ± 5% relative humidity. Food pellets and tap water were available *ad libitum.* Animals were exposed to a 12/12 hr light/dark cycle. Experiment was approved by the respective Ethical Committee.

Treatment: PHT dissolved in tap water and adjusted to pH 11 with NaOH was administered orally from day 7 until day 18 of gestation in the dose of 150 mg/kg. Control groups received water with pH 11 over the same period. Sodium salt of PHT, batch No. 0080499, was a kind gift from Slovakofarma, J. S. Co. Hlohovec, Slovakia.

Dosage volume was 0.5 ml/100 g body weight. Vitamin E (tocoferolum aceticum, Slovakofarma J.S.Co., Hlohovec, Slovakia; batch No. 0070597, 99% purity) was administered 2 hours prior to PHT orally (0.5 ml/100 g) from day 7 to day 18 of gestation.

On day 20 of pregnancy one group of animals were sacrificed by cervical dislocation. The peritoneal cavity and uterus were opened and live foetuses were dissected. Placentas, maternal heart and foetal organs (brain, liver and lungs) were removed. Pups of the other group were sacrificed on postnatal day 1.

Determination of biochemical variables: Tissue samples of 50–60 mg were put in ice-cold phosphate buffer pH 7.4, containing Triton X-100 (0.1%), and homogenised in a hand glass homogeniser. Homogenates were centrifuged at 15 000 × g for 20 min. The activity of NAGA, the level of GSH and proteins were assayed according to standard methods[10–12] used in our previous studies in supernatants and in serum[13, 14]. All chemicals and enzyme substrates (Sigma, USA) were of analytical grade.

Statistical evaluation: ANOVA, one way analysis of variance, with Bonferroni multiple comparison test was used for statistical analysis, $p<0.05$ was considered significant.

RESULTS

The results of the experiments are divided into three sets, biochemical changes (1) in maternal organs and placenta, (2) in foetal organs and (3) in organs of pups. In the prenatal study PHT-induced embryofoetal toxicity was associated with an increase in NAGA activity and decrease of GSH level in maternal serum and heart and with an increase in NAGA activity in the placenta.. Administration of VIT E did not inhibit the above given changes (Table 1). PHT increased the activity of NAGA and decreased the level of GSH in foetal organs (liver, lungs, brain). VIT E did not reverse the biochemical changes. The values of NAGA and GSH in the VIT E+PHT treated groups were comparable to the values of the groups treated with PHT alone (Table 2). In the postnatal study, we did not find any significant differences in NAGA activity in the organs of 1-day-old pups. An increase of liver GSH level was found in PHT and VIT E+PHT groups of pups and in the lungs of the group VIT E+PHT (Table 3).

DISCUSSION

The present pre- and postnatal study was carried out to investigate the effect of VIT E in the *in vivo* model of intrauterine hypoxia by assessing biochemical variables, i.e. NAGA and GSH, measured in our previous studies[13, 14]. Results of these studies confirmed a high teratogenic potential of PHT (ref.[15]), neuro-behavioural alterations[16], accompanied with a broad spectrum of biochemical changes in NAGA activity and GSH level[14]. The findings of Juránek and coworkers (personal communication) support the concept of intrauterine hypoxia. On day 20 of gestation of the rat, they observed declined oxygen saturation of the blood (about 20%) in the common iliac artery after single intravenous administration of 150 mg/kg PHT.

In vivo and *in vitro* studies indicated that PHT-initiated teratogenesis may involve, at least in part, peroxidase-catalysed bioactivation of PHT to a reactive free radical intermediate. If not detoxified, it may initiate oxidative stress leading to oxidation of embryonic lipids, proteins, and DNA (ref.[17]). The authors hypothesised that highly reactive oxygen species, such as hydroxyl radicals, could be generated by the PHT free radical. Potentially, ·OH could be generated indirectly by the PHT free radical via the Fenton reaction. *In vivo* catalytic iron may be found loosely bound to membrane lipids, DNA, and phosphate complexes. While ·OH is generally thought to be a primary intermediate of *in vivo* damage, there is some controversy which reactive oxygen species may be ultimately responsible for cellular damage. However, it is known that if these reactive oxygen species are not detoxified by cytoprotective enzymes or antioxidants, they can cause lipid peroxidation and protein oxidation and degradation. These changes can lead to structural and functional changes and also to injuries on biochemical level[18]. Understanding of the biochemical and molecular changes associated with oxidative stress may promote establishment of experimental models for testing drugs protecting tissues from injury.

A number of observations suggest that detoxification of a xenobiotic free radical intermediate with antioxidants may provide important embryoprotection[19]. GSH may be involved in the detoxification of a teratogenic reactive intermediate of PHT and/or in cytoprotection against oxidative stress. GSH depletors or inhibitors of GSH synthesis potentiate PHT teratogenicity in mice[20]. Winn and Wells demonstrated the teratologic importance of antioxidant balance: maternal administration of the antioxidative enzyme catalase enhanced embryonic activity and inhibited PHT teratogenicity. Yet on the other hand, maternal pretreatment with another antioxidative enzyme, superoxide dismutase, increased the teratogenicity of PHT. In the current study we found that administration of the natural antioxidant VIT E during pregnancy did not inhibit the biochemical changes induced by PHT (ref.[21]).

Administration of VIT E to pregnant diabetic animals decreased the rate of embryonal malformations and increased their weight and enhanced their maturation[22]. However, high doses (500 mg/kg) exerted a negative effect on the conceptus, as shown by increased rate of reabsorptions[23]. Boskovic et al. reported that consumption of high doses of VIT E during the first trimester of pregnancy did not appear to be associated with an increased risk for major malformations, but may be associated with decrease in birth weight[24]. On the other hand, supranutritional vitamin E supplementation of the ewe resulted in a significant increase in lamb birth weight[25].

The results of the study (Mach et al.[26]) showed that supplementation with a high-dose of VIT E did not reduce the maternal and foetal toxicity of PHT and failed to protect the rat foetuses. Due to the occurrence of slight

Table 1. Effect of PHT and VIT E on NAGA activity and GSH level in rat maternal serum, heart and in placenta

		CONTROL	VIT E	PHT	VIT E + PHT
Serum	NAGA.102	10.90 ± 0.41	11.86 ± 1.06	14.93 ± 0.62 **	11.75 ± 0.72
	GSH	2.23 ± 0.32	2.17 ± 0.14	1.69 ± 0.09 ***	1.89 ± 0.18 **
Heart	NAGA	1.39 ± 0.53	1.89 ± 0.08***	1.89 ± 0.07 ***	1.71 ± 0.08*
	GSH	1.67 ± 0.14	1.58 ± 0.08	1.20 ± 0.14 *	1.32 ± 0.08
Placenta	NAGA	6.07 ± 0.37	8.11 ± 0.41 **	7.88 ± 0.42 *	8.25 ± 0.43 **
	GSH	1.38 ± 0.10	1.36 ± 0.15	1.43 ± 0.08	1.00 ± 0.08

Values are given as means ± S.E.M., (n = 10). Activity of N-acetyl-ß-D-glucosaminidase (NAGA) is expressed in µg 4-nitrophenol / min / mg protein. Glutathione (GSH) is expressed in µg / mg protein. VIT E – vitamin E; PHT – phenytoin; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus control

Table 2. Effect of PHT and VIT E on NAGA activity and GSH level in foetal organs

		CONTROL	VIT E	PHT	VIT E+PHT
Liver	NAGA	3.63 ± 0.32	5.08 ± 0.27 *	6.03 ± 0.45 ***	6.27 ± 0.23 ***
	GSH	3.78 ± 0.24	4.27 ± 0.24*	3.61 ± 0.18	3.10 ± 0.19
Lungs	NAGA	2.44 ± 0.13	3.83 ± 0.09 ***	4.53 ± 0.31***	3.93 ± 0.19 ***
	GSH	3.26 ± 0.17	2.64 ± 0.16	1.99 ± 0.19 ***	1.87 ± 0.13 ***
Brain	NAGA	2.14 ± 0.12	3.06 ± 0.08 ***	3.47 ± 0.16***	3.21 ± 0.19 ***
	GSH	2.58 ± 0.25	2.78 ± 0.15 ^{o}	2.20 ± 0.11	2.09 ± 0.14

Values are given as means ± S.E.M., (n = 10). Activity of N-acetyl-ß-D-glucosaminidase (NAGA) is expressed in µg 4-nitrophenol / min / mg protein. Glutathione (GSH) is expressed in µg / mg protein. VIT E – vitamin E; PHT – phenytoin, * $p < 0.05$, *** $p < 0.001$ versus control, ^{o} $p < 0.05$, versus PHT

Table 3. Effect of PHT and VIT E on NAGA activity and GSH level in organs of pups

		CONTROL	VIT E	PHT	VIT E+PHT
Liver	NAGA	7.99 ± 0.41	7.91 ± 0.36	9.09 ± 0.61	9.13 ± 0.40
	GSH	3.99 ± 0.12	3.38 ± 0.31 ^{oo}	4.72 ± 0.21 *	5.19 ± 0.32 * $^{+++}$
Lungs	NAGA	4.12 ± 0.11	4.11 ± 0.14	3.66 ± 0.20	4.26 ± 0.08 ^{o}
	GSH	2.49 ± 0.21	1.91 ± 0.13	1.70 ± 0.15	2.91 ± 0.20 ^{oo} $^{++}$
Brain	NAGA	3.43 ± 0.19	3.91 ± 0.21	4.00 ± 0.25	4.09 ± 0.10
	GSH	2.91 ± 0.14	3.11 ± 0.26	3.04 ± 0.18	3.74 ± 0.17 *

Values are given as means ± S.E.M., (n = 6–10). Activity of N-acetyl-ß-D-glucosaminidase (NAGA) is expressed in µg 4-nitrophenol / min / mg protein. Glutathione (GSH) is expressed in µg / mg protein. VIT E – vitamin E; PHT – phenytoin; * $p < 0.05$, versus control, ^{o} $p < 0.05$, ^{oo} $p < 0.01$ versus PHT, $^{++}$ $p < 0.01$, $^{+++}$ $p < 0.001$ versus VIT E

skeletal anomalies in the group supplemented with VIT E, high doses of VIT E in pregnancy appear to involve a risk to the foetus[26].

In conclusion, supplementation with a high-dose of VIT E failed to protect maternal, foetal and new-born rat organs from PHT induced changes of selective biochemical variables.

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