Prenatal developmental toxicity study of the pyridoindole antioxidant SMe1EC2 in rats

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Abstract

OBJECTIVE: The 2-ethoxycarbonyl-8-methoxy-2,3,4,4a,5,9b-hexahydro-1H-pyrido-[4,3b] indolinium chloride (SMe1EC2) is a prospective antioxidant and neuroprotectant drug. The aim of the study was to evaluate the effect of SMe1EC2 on embryofetal development of rats.

METHODS: The substance tested was administered orally to Wistar/DV rats from day 6 to day 15 of gestation at the doses 5, 50 and 250 mg/kg/day. The animals were killed on day 20 of gestation and uterine content was inspected. Live fetuses were examined for gross, skeletal and visceral anomalies.

RESULTS: Administration of SMe1EC2 did not induce any signs of maternal toxicity. No adverse effect of the substance tested was found on reproductive variables. Morphological examination of fetuses revealed no evidence of teratogenesis.

CONCLUSION: The prenatal toxicity study showed that the substance SMe1EC2 tested did not have embryotoxic and teratogenic effects on developing rats. Neither were any signs of maternal toxicity found.

ORIGINAL ARTICLE

INTRODUCTION

Oxidative stress can interact and/or interfere with developmental processes during prenatal and perinatal period. Due to the poorely prepared developing organism to protect itself against oxidative stress, many chemical substances, drugs as well as pathophysiological processes can modify redox status and in turn may disrupt embryofetal development (Hansen, 2006). Therefore, administration of antioxidants *via* maternal organism may be a rational approach to protect the embryo and fetus during periods of increased risk of oxidative stress (e.g. pre-eclampsia, iron imbalances, infections, gestational diabetes) as well as in prevention and treatment of diseases associated with oxidative stress, such as bronchopulmonary dysplasia, idiopathic respiratory distress syndrome, asphyxia, etc. (Saugstad, 2005)

The use of natural and synthetic antioxidants during periods of increased free radical production is not a new concept. They have been used to advantage also in peri- and neonatology. Substances such as allopurinol, α-tocopherol, flavonoids, superoxide dismutase, melatonin were reported to be protective in preterm and term animals as well as in infants (Saugstad, 1990; Cohen-Keren and Koren, 2003; Chaudhari and McGuire, 2008). However, in case of developmental administration of any substance/drug, a serious precaution should be taken into account. Vitamin E (α-tocopherol) is generally considered safe in pregnancy and lactation. However, until recently there were no consistent teratological data on vitamin E (Martin and Hurley, 1977). Our previous studies on rats showed that prenatal vitamin E treatment resulted in persistent body weight gain depression, manifested from the fetal period up to adulthood (Mach et al., 2005; 2006). Intrauterine growth retardation/restriction, in turn, represents a risk factor for development of chronic diseases in adulthood. This concept has been known as "fetal/developmental origin of adult diseases" (Barker, 1998; Bezek et al., 2008). Thorough evaluation of chemical substances/drugs which are considered to be used in peri- and neonatology, has to be conducted in sense of their relative safety assessment in pregnancy. Therefore, teratological studies need to be conducted also by using laboratory animals.

The 2 - ethoxycarbonyl - 8 - methoxy - 2, 3, 4, 4a, 5, 9b - hexahydro - 1H - pyrido - [4,3b] indolinium chloride (SMe1EC2) is a prospective antioxidant and neuroprotectant drug designed and synthesized at the Institute of Experimental Pharmacology SAS, Bratislava, Slovakia. SMe1EC2 is a derivative of stobadine (STO), a pyridoindole drug derived from gamma-carboline antidepressant and neuroleptic drug Carbidine® (Barkov, 1973) as its active (–)-enantiomer. SMe1EC2 was found to have a better antioxidant capability than the parent molecule (Štolc *et al.*, 2008) and virtually full elimination of the undesired alpha1-adrenolytic activity of STO has been attained by appropriate modifications of its molecule (Štolc et al., 2006). We assume that this new molecule might represent the future in development of more effective and safer drugs with high antioxidant capacity. In our ongoing research, we intend to investigate potential protective effects of this drug on experimental chronic intrauterine hypoxia and neonatal/perinatal anoxia induced structural and functional injuries on rats. From this point of view it is important to know the potential developmental toxicity of SMe1EC2. The aim of the present study was to evaluate effects of SMe1EC2 on embryofetal development of rats.

MATERIAL AND METHODS

Animals

Monitored conventional breeding of virgin female Wistar/DV rats (weight 200–220 g, age 3–4 months, n=70) obtained from the breeding station Dobrá Voda (Slovak Republic, reg. No. SK CH 4004) had free access to water and food pellets and was kept on a 12/12 hr light-dark cycle. After 7 days of adaptation, the females were mated with males in the ratio 1 male : 4 females (presence of spermatozoa in vaginal smear indicated day 0 of gestation). The experiments were performed in compliance with the Principles of Laboratory Animals Care issued by the Ethical Committee of the Institute of Experimental Pharmacology, Slovak Academy of Sciences and the experimental design was approved by the State Veterinary and Food Administration of the Slovak Republic.

Drugs

2 - ethoxycarbonyl - 8 - methoxy - 2, 3, 4, 4a, 5, 9b - hexahydro - 1H - pyrido - [4,3b] indolinium chloride (m.w. 312.79 Da, chemical purity <99 %) (SMe1EC2) was prepared at the Institute of Experimental Pharmacology, Slovak Academy of Sciences. The substance tested was dissolved in saline at a constant dosage volume 0.5 ml/100 g body weight. The dams were treated by oral gavage with SMe1EC2 at doses of 5, 50 and 250 mg/kg/day from day 6 to 15 of gestation. The doses were determined according to LD_{50} of SMe1EC2 (p.o. LD_{50} < 2 400 mg/kg), with the highest dose representing approximately 10% from LD_{50}. Controls received vehicle over the same period.

Teratological examination

The group of 51 pregnant rats (controls = 12; 5 mg/kg = 11; 50 mg/kg = 16; 250 mg/kg = 12 animals) was followed up concerning body weight and clinical signs of toxicity till day 20 of gestation, when they were killed by cervical dislocation. The peritoneal cavity and uterus were opened and live fetuses and placentas were removed from the uterus. Fetal and placental wet weight, number of *corpora lutea*, implantations, resorptions, live and dead fetuses were recorded. All fetuses

were then inspected for external malformations. Two-thirds of fetuses from each litter were exsanguinated, eviscerated, stripped of most subcutaneous tissues, fixed in 96% ethanol, then cleared by 1 % KOH solution and stained in a dilute alkaline solution of Alizarin red S for examination of the skeleton (Lorke, 1977). The remaining fetuses were fixed in Bouin´s solution for soft tissue examination by Wilson's free hand razor slicing method (Wilson, 1965).

Statistical evaluation

The data were analyzed by means of ANOVA and Fisher´s exact test (skeletal and visceral abnormalities). The data are expressed as mean ± S.E.M. The significance limit of $p<0.05$ was considered statistically significant.

RESULTS

Throughout the course of the experiment all dams were in good physical condition. No maternal death or abortion occurred in the controls or the dose groups. The maternal weight gains were not affected by the treatment with SMe1EC2 at any dose used (Figure 1). There was no significant effect of SMe1EC2 treatment on uterine content variables, namely implantations, early and late resorptions, live and dead fetuses, pre- and postimplantation loss and sex ratio (Table 1). We found significant changes in the number of *corpora lutea* [$F(3, 47)=3.99$; $p<0.05$] among experimental groups. One-way ANOVA revealed significant increase of weight of fetuses [$F(3, 447)=7.45$; $p<0.001$] and placentas [$F(3, 447)=6.81$; $p<0.001$] compared to controls. Concerning the evaluation of possible structural anomalies, no macroscopically visible abnormalities were observed in any of the groups inspected. Skeletal and visceral examination of the fetuses revealed no significant effect of developmental administration of the substance tested (Tables 2 and 3).

DISCUSSION

Pregnancy and development are delicate states of balance between mother, fetus and environment. Disturbances during development can have long lasting effects and determine the future quality of life. Oxygen is one of the most important elements in our life and could be life giving as well as life disturbing. Its excess can cause generation of free radicals which in turn could shift the balances into oxidative stress. Oxidative stress is a silent life threatening situation which can be eliminated by endogenous antioxidative systems or exogenous

Table 1. Reproductive variables in pregnant rats

Variables	Control n=12	DOSE I n=11	DOSE II n=16	DOSE III n=12
Corpora lutea	12.42±0.34	13.00±0.49	12.00±0.35	13.83±0.46
Implantations	10.17±1.01	12.18±0.75	10.94±0.52	12.50±0.36
Live fetuses	9.42±0.96	11.09±1.01	10.31±0.55	10.92±0.68
Sex ratio M/F	62/51	66/56	90/75	71/60
Dead fetuses	0	0	0	0
Early resorptions	0.17±0.17	0.91±0.53	0.31±0.18	1.25±0.73
Late resorptions	0.58±0.19	0.09±0.09	0.31±0.12	0.25±0.13
Total resorptions	0.75±0.28	1.00±0.52	0.63±0.26	1.50±0.72
Preimplantation loss[1]	2.25±1.05	0.91±0.58	1.06±0.34	1.42±0.53
Postimplantation loss[2]	0.67±0.28	1.00±0.52	0.63±0.26	1.50±0.72
Weight of fetuses [g]	3.38±0.03	3.49±0.03	3.54±0.03***	3.40±0.03
Weight of placenta [g]	0.52±0.01	0.56±0.01**	0.56±0.01***	0.55±0.01*

n = number of dams, M - males, F - females; Dose I, II, III - 5, 50, 250 mg/kg SMe1EC2, [1]*corpora lutea* – implantation sites / *corpora lutea* (× 100); [2]implantation sites – viable fetuses / implantation sites (×100); *$p<0.05$; **$p<0.01$; ***$p<0.001$ compared to control (ANOVA) (data are presented as Means ± S.E.M.)

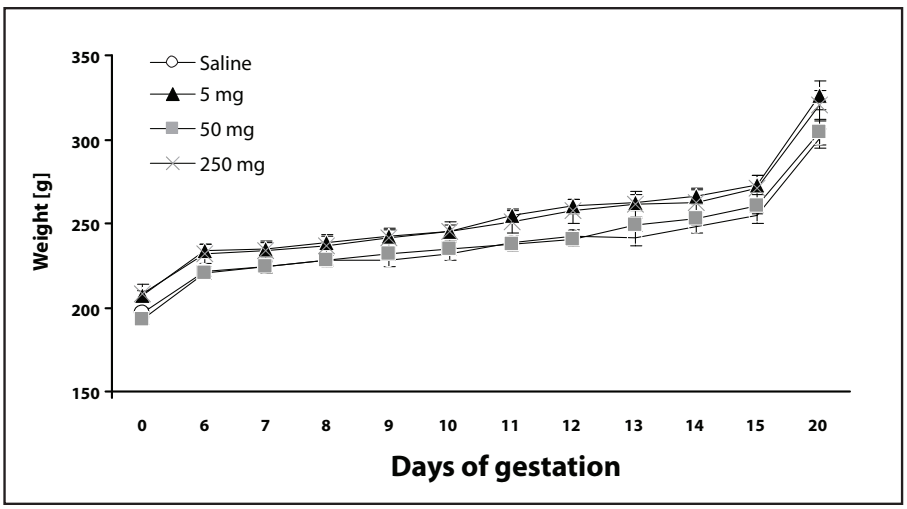

Figure 1. Maternal body weight gain. DOSE I, II, III – 5, 50, 250 mg/kg SMe1EC2. Vertical bars represent S.E.M. (Repeated two-way ANOVA)

Table 2. Effect of SMe1EC2 on skeletal anomalies

Variables	Control (n=76)	DOSE I (n=85)	DOSE II (n=116)	DOSE III (n=92)
Skull[a]	4 (5.26%)	0	9 (7.76%)	3 (3.26%)
Sternebrae[b]	20 (26.32%)	14 (16.47%)	22 (18.97%)	20 (21.74%)
Ribs[c]	6 (7.90%)	6 (7.06%)	10 (8.62%)	14 (17.39%)
Vertebrae[d]	1 (1.32%)	0	0	0
Pelvic bones[e]	0	2 (2.35%)	1 (0.86%)	0

n - number of fetuses inspected; DOSE I, II, III – 5, 50, 250 mg/kg SMe1EC2, Fisher's exact test
a - delayed ossification of interparietal and supraoccipital bone
b - delayed or unossified sternebrae
c - 13th and 14th accessory rudimentary ribs
d - delayed ossification
e - delayed ossification of ilium and ischium

Table 3. Effect of SMe1EC2 on visceral anomalies

Variables	Control (n=35)	DOSE I (n=37)	DOSE II (n=49)	DOSE III (n=39)
ULCV[a]	5 (14.29%)	5 (13.51%)	13 (26.53%)	12 (30.77%)
DPP[b]	1 (2.86%)	0	0	0
HP[c]	3 (8.57%)	0	4 (8.16%)	1 (2.56%)
RM[d]	0	6 (16.22%)	6 (12.24%)	2 (5.13%)

n - number of fetuses inspected; DOSE I, II, III – 5, 50, 250 mg/kg SMe1EC2, Fisher's exact test
a - undilatation of lateral cerebral ventricles
b - diminished pulmonary parenchyma
c - hepatal petechiae
d - renal malposition

antioxidants. A delicate balance must be established between oxidants and antioxidants (Dennery, 2007).

Prenatal developmental toxicity studies are necessary to predict risk or safety of chemical compounds and drugs administered during embryofetal development. The thalidomide tragedy in the early 1960s alarmed the medical community about the dangers of drugs to the unborn child *in utero*. However, supplements, food additives and vitamins are widely used during pregnancy. One example is the use of vitamin E in pregnancy, but scarcely reported in the literature. Martin and Hurley (1977) showed that high doses of vitamin E (from 22.5 up to 2 252 mg/kg/day) did not have a negative effect on the course of pregnancy and lactation in rats. Nevertheless, in our previous studies, we found that vitamin E at the dose of 500 mg/kg administered orally from day 7 to day 18 of gestation caused slight skeletal anomalies and persistent growth retardation apparent up to adulthood (Mach et al., 2005; 2006). The results are suggestive of necessary caution in using any vitamin, food additive and herbal product during pregnancy.

Within the present study, we performed teratological evaluation of the new pyridoindole antioxidant SMe1EC2 in compliance with OECD guidelines (OECD, 1981). No adverse signs of maternal toxicity were found in pregnant rats. Prenatal administration of SMe1EC2 did not have teratogenic effect on developing rat embryos. Although we found significantly increased number of *corpora lutea*, post-hoc comparison did not reveal any significant changes in SMe1EC2 treated groups compared to controls. We consider this significant alteration as a consequence of interindividual variation which could not be caused by the SMe1EC2 treatment since the tested substance was administered from day 6 to 15 of gestation when *corpora lutea* were already formed. Our study showed increased weight of fetuses in the 50 mg/kg group and of placentas in all SMe1EC2 groups. In our experience, this change is within the normal range of biological variability because this increase represents less than 5% of control values, and on balance, this increase may be rather beneficial than unfavorable for development. Many pathological conditions are associated with decreased fetal weight (hypoxia, malnutrition, pre-eclampsia, stress, alcohol and drug abuse) and thus our compound tested might favorably affect the detrimental effect of the risk conditions mentioned.

Concerning skeletal and visceral alterations, we found spontaneous incidence in controls as well as in SMe1EC2

groups. There were no significant differences among the groups. These changes are common and represent only transient stages of development (Palmer, 1977).

The substance SMe1EC2 tested is a derivative of stobadine (STO), substituted with methoxy-group in the aromatic cycle and ethoxycarbonyl-group substituted in the gamma position of pyrimidine nitrogen. The parent drug STO has high antioxidant properties and was found promising for long-term administration during diseases accompanied with excessive oxidative stress and free radical formation (Horáková and Štolc, 1998). Therefore it was subjected to extensive toxicological and teratological studies in different animal species (Balonová et al., 1991; Miháliková et al., 1993; Ujházy et al., 1994; Gajdošíková et al., 1995; Ujházy et al., 1999; Dubovický et al., 1999; Navarová et al., 2006; Ujházy et al., 2006). These experimenatal studies showed low toxicity of STO in adult animals and no teratogenic effect on the developing organism.

As mentioned above, SMe1EC2 is even more effective and with less undesired alpha1-adrenolytic activity, exhibiting more favorable properties than the parent pyridoindole STO (Štolc et al., 2008). Due to these properties and its low toxicity, this substance might find use as a protective agent in situations in which oxidative stress can be presumed, including hypoxia/ischemia insults during development. In conclusion, the present study showed that the substance SMe1EC2 tested did not have embryotoxic and teratogenic effects on developing rats. We did not find any signs of maternal toxicity. The results indicate that this substance would be appropriate as a model drug in the study of its potential effects on injuries evoked by hypoxia/ischemia in the developing organism.

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