# Prophylactic Effects of Melatonin on Sodium Valproate-Induced Neural Tube Defects and Skeletal Malformations in Rat Embryos

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**Abstract: Problem statement:** Some reports showed the teratogenic effects of sodium valproate can be prevented by application of antioxidant drugs and stimulation of the maternal immune system. Therefore, in this study, the prophylactic effect of melatonin on teratogenic effects of sodium valproate was compared. **Approach:** This study was performed on 31 pregnant rats that were divided into five groups. Control group received normal saline and test groups received sodium valproate (300 mg $kg^{-1}$), sodium valproate (300 mg $kg^{-1}$) plus melatonin (5 mg $kg^{-1}$) and sodium valproate (300 mg $kg^{-1}$) plus melatonin (10 mg $kg^{-1}$) and melatonin (10 mg $kg^{-1}$), intraperitonealy at 8-9th days of gestation, respectively. Fetuses were collected at 20th day of gestation and after determination of weight and length; they were stained by Alizarin red-Alcian blue method. **Results:** Cleft palate, spina bifida and exencephaly incidence were 17.70, 20 and 20% in fetuses of rats that received only sodium valproate. Cleft palate, spina bifida and exencephaly incidence were 4.16, 8.33 and 8.33% range in group which received sodium valproate plus melatonin (5 mg $kg^{-1}$), respectively. However, Cleft palate, spina bifida and excencaphaly incidence were 4/76, 0 and 0% in group which received sodium valproate plus melatonin (10 mg $kg^{-1}$), respectively. The mean of weight and length of animals' fetuses that received melatonin were significantly greater than those received only sodium valproate. **Conclusion:** It is concluded that melatonin with dose of 10 mg $kg^{-1}$ had significantly more prophylactic effect than melatonin with dose of 5 mg $kg^{-1}$ on incidence of sodium valproate-induced skeletal malformations.

**Key words:** Melatonin, cleft palate, valproic acid, Reactive Oxygen Species (ROS), skeletal malformations, antioxidant activity, oxidative stress

## INTRODUCTION

Valproate is often prescribed as a long-term therapeutic mood-stabilizing agent for individuals with bipolar disorder (Wang *et al*., 2003). Valproate (Valproic acid-VAP) is a major anti-epileptic drug with a broad spectrum of anti-epileptic activity. It has been the drug of choice in the treatment of most forms of primary generalized epilepsies and is also efficient against partial seizures (Rowan *et al*., 1997). Valproic acid, a commonly used antiepileptic agent, is associated with a 1-2% incidence of neural tube defects when taken during pregnancy; however, the molecular mechanism by which this occurs has not been elucidated (Defoort and Winn, 2006). As many as 10% of the 12000 infants that are exposed to anti-epileptic drugs during pregnancy every year show malformations (Finnell, 1991;

Lindhout and Omtzigt, 1992). Use of VPA during the first trimester of pregnancy significantly increases the risk for spina bifida as well as other malformations such as heart defects, limb abnormalities, cleft palate and craniofacial abnormalities (Finnell, 1991; Lammer *et al*., 1987). Together these abnormalities constitute the fetal valproate syndrome (Clayton-Smith and Donnai, 1995). Many other structurally unrelated anti-epileptic and anti-manic drugs, such as lithium, barbiturates and carbamazepine, are also teratogenic when used during pregnancy (Finnell, 1991, Koch *et al*., 1992).

Several studies suggest that valproic acid exposure leads to an increase in Reactive Oxygen Species (ROS). Long-term use of antiepileptic drugs has been shown to increase free radical formation and cause oxidative damage within neuronal cells (Maertens *et al*., 1995). The metabolism of valproate may trigger oxygen

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dependent tissue injury and elevate the free radicals in the body (Cengiz *et al*., 2000).

Non–specific stimulation of the maternal immune system in mice during the peri-conception period appears to have a broad spectrum efficacy for reducing teratogen induced birth defects from a variety of sources including chemical agents, x-rays and diabetes mellitus (Nomura *et al*., 1990; Holladay *et al*., 2000; Punareewattana and Holladay, 2004). Maternal immune stimulation reduced or blocked digit and limb defects (Prater *et al*., 2004), tail malformation, cleft palate (Sharova *et al*., 2002) cranial defects (Hrubec *et al*., 2006) and neural tube defects (Turchinsky *et al*., 1997; Punareewattana and Holladay, 2004). The operating mechanisms by which such immune stimulation reduces fetal dysmorphogenesis are unknown; however, the collective literature suggests that immunoregulary cytokines of maternal or placental origin may be effector molecules that normalize dysregulated apoptosis or timing of cell proliferation in the fetus (Punareewattana and Holladay, 2004; Sharova *et al*., 2000). Stimulation of maternal immune system or antioxidant drugs can decrease or prevent drug- induced embryonic abnormalities (Holladay *et al*., 2002; Prater *et al*., 2004). For example, macrophage activation decreases incidence of cleft palate and digital and tail anomalies in fetuses of mice that received urethane and methylnitrous urea (Holladay *et al*., 2000). Interferon gamma reduces urethane-induced cleft palate and granulocyte-colony stimulating factor decreases cyclophosphamide -induced distal limb abnormalities in mice (Syska *et al*., 2004).

Melatonin or N-Acetyl-5-methoxytryptamine, the main secretary product of pineal gland, participates in many physiological functions due to its efficacy as a free radical scavenger and indirect antioxidant (Tan *et al*., 2002; Reiter and Tan, 2003). Because of its small size and lipophilicity, melatonin crosses biological membrane easily, thus, reaching all compartments of the cell. Melatonin has also been shown to be an efficient protector of DNA, protein and lipids in cellular membrane (Cuzzocrea and Reiter, 2002) as well as antagonist of a number of endogenous and exogenous free radicals attach or during cellular processes (Zhang *et al*., 1998).

Melatonin has been shown to have antiepileptic activity in animal studies using different seizure models (Mevissen and Ebert, 1998; Srivastava *et al*., 2002). A few mechanisms for anticonvulsant activity of melatonin have been suggested. It exerts neuroprotection due to its antioxidant, antiexcitotoxic and free scavenging properties within the central nervous system (Espinar *et al*., 2000). It has also been demonstrated to be safe in humans even in high pharmacological doses (Reiter *et al*., 1994).

In present study, the preventive effect of melatonin on VAP-induced neural tube defects and skeletal malformations in rats was compared.

## MATERIALS AND METHODS

**Drugs:** Sodium valproate (Sigma, USA) and melatonin (Sigma, USA) purchased from commercial sources.

**Animals:** Male and female healthy rat of Wistar strain, 3-4 month old, weighing 200-250g were purchased (Razi Institute, Karadje, Iran) and housed individually (males) or at 10 per polycarbonate cage (female) for a 2-week acclimatization period. Rats were fed *ad libitum* standard laboratory pellet (Pars khurakdam, Shushtar, Iran.) and tap water. A 12-h light: 12-h dark cycle was maintained. Room temperature was at $23 \pm 2°$ C with a relative humidity of 45-55%.

Female rats were mated overnight with males. The vaginal plug was assumed as first day of gestation (GD1). Pregnant rats (n=31) were randomly divided into five groups (25 pregnant rats in treatment groups, 6 pregnant rats in control group).

**Exprimental protocol:** Control group received normal saline, the test groups received sodium valproate (300 mg $kg^{-1}$) (Menegola *et al*., 1998), sodium valproate (300 mg $kg^{-1}$) plus melatonin (5 mg $kg^{-1}$) (Konar *et al*., 2007), sodium valproate (300 mg $kg^{-1}$) plus melatonin (10 mg $kg^{-1}$) (Konar *et al*., 2007) and melatonin (10 mg $kg^{-1}$) (Konar *et al*., 2007) intraperitonealy, respectively.

The animals were sacrificed by cervical dislocation 20th day of gestation and fetuses were collected and numbered, then their weight and length (crown- rump length) were measured. Fetuses were stained by Alizarin red-Alcian blue method (Kimmel and Trammekl, 1981) and examined by stereomicroscope for neural tube defects and skeletal malformations. The incidence of neural tube defects and skeletal malformations were determined.

**Statistical analysis:** Statistical significance between groups was determined using SPSS program and comparisons were made by one way Analysis Of Variance (ANOVA) and Chi-square test. The minimum level of significance was p<0.05.

## RESULTS

Fourty-seven fetuses were obtained from six rats of control group. In control group, palatal closures of

fetuses were normal on gestational day 20 (i.e. palatal shelves had grown vertically on the sides of the tongue, then horizontally to meet and fuse) (Fig. 1, 2A) and no macroscopic anomalies were observe in them. VAP induced cleft palate (Fig. 1, 2B), spina bifida (Fig. 3 B) and exencephaly (Fig. 4) at 17.70, 20 and 20% incidence, respectively. Sodium valproate plus melatonin (5 mg kg$^{-1}$) significantly reduced incidence of cleft palate, spina bifida and exencephaly to 4.16%, 8.33 and 8.33% range but sodium valproate plus melatonin (10 mg kg$^{-1}$) significantly reduced incidence of cleft palate, spina bifida and exencaphaly to 4.76%, 0 and 0% range, respectively. Incidence of omphalocele and fusion of two or more sternebrae, absence of sternebrae and malpositioning of two halves of the sternebrae (Fig. 5) in group which received sodium valproate were 11.1 and 15.5%, respectively.
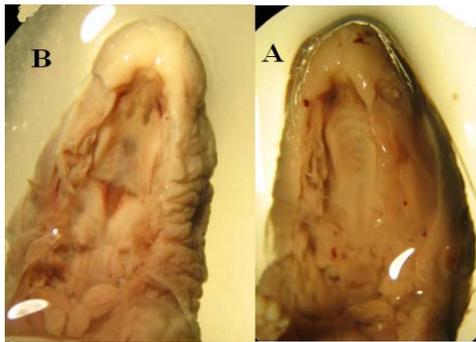


Fig. 1: Razor blade sections of rat fetuses of GD 20. (A) Control skeleton. Note the cleft palate due to palatal shelf hypoplasia (B) in the treated case (300 mg kg$^{-1}$ of VAP, treated on GD 8,9)
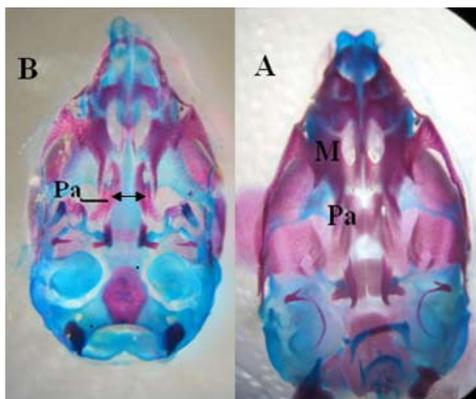


Fig. 2: Ventral view of skull of rat fetuses of GD 20, stained with alizarin red S- alcian blue. (A) Normal palatine bone (B) Cleft palate induced by VAP (arrow). M: maxilla; Pa: palatine
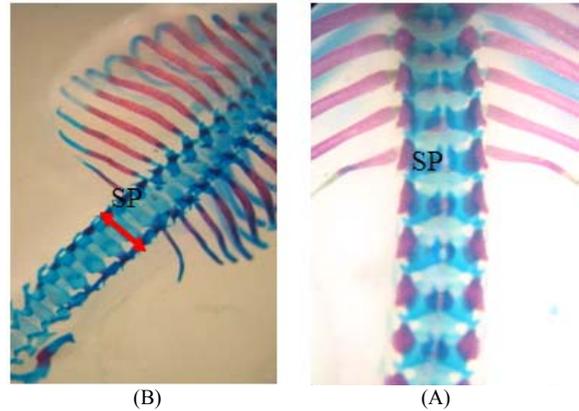


Fig. 3: Dorsal view of vertebral column of rat fetuses of GD 20, stained with alizarin red S- alcian blue. (A) Normal (B) Spina bifida (arrow) induced by VAP. SP; spinous process



Fig. 4: Exencephaly (blue arrow) and omphalocele (yellow arrow) in experimental group treated with 300 mg kg$^{-1}$ of VAP on GD 8-9
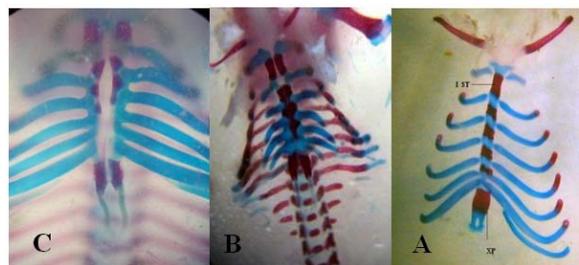


Fig. 5: Sterna of fetuses of GD 20, stained with alizarin red S--alcian blue. (A) Control. The chest wall together with the sternum and costal elements are dissected out and shown here. (B and C) Experimental group treated with 300 mg kg$^{-1}$ of VAP on GD 8-9. Observe fusion of sternebra (B, C), hemisternebra (C) and unusal shape of xiphoid process

Table 1: Incidence of anomalies in rat fetuses of groups

| Groups | No. of litters | Implantations | Resorbed fetuses | Live fetuses | Fetal length (mea n± SEM) | Fetal weight (g): (mean ± SEM) | No . of fetuses with malformations | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Omphalocele | Cleft palate | Spina bifida | Stennal malformations | Exencephaly |
| Control | 6 | 49 | 2(4.08) | 47 | 37.48±0.38 | 4.73±0.09 | 0 | 0 | 0 | 0 | 0 |
| VAP | 6 | 51 | 6(11.76) | 45 | 26.38±0.48* | 2.10±0.09* | 5(11.1) | 8(17.7) | 9(20) | 7(15.5) | 9(20) |
| VAP+Mel (5 mg kg$^{-1}$) | 7 | 52 | 4(7.69) | 48 | 31.84±0.88** | 3.44±0.21** | 4(8.3) | 2(4.1) | 4(8.3) | 4(8.3) | 4(8.3) |
| VAP+Mel (10 mg kg$^{-1}$) | 6 | 44 | 2(4.54) | 42 | 35.39±0.3# | 4.06±0.09# | 0 | 2(4.7) | 0 | 3(7.1) | 0 |
| Melatonin | 6 | 43 | 2(4.65) | 41 | 35.59±0.85 | 4.39±0.16 | 0 | 0 | 0 | 0 | 0 |

Numerals in parantheses are percentages,*: Significant difference when compared with other groups (p<0.05),**: Significant difference when compared with control,VAP and melatonin groups (P<0.05),#: Significant difference when compared with VAP, melatonin and VAP+ melatonin (5 mg kg$^{-1}$) groups (p<0.05),Incidence of anomalies was significantly difference at groups which received VAP with control and melatonin group (p = 0.0001), Also this incidence was difference between groups received VAP (p<0.05)

Incidence of omphalocele (Fig. 4) and fusion of two or more sternebrae, absence of sternebrae and malpositioning of two halves of the sternebrae in group which received sodium valproate plus melatonin(5 mg kg$^{-1}$) were 8.3 and 8.3%, respectively. Incidence of omphalocele and fusion of two or more sternebrae, absence of sternebrae and malpositioning of two halves of the sternebrae in group which received sodium valproate plus melatonin (10 mg kg$^{-1}$) were 0 and 7.1%, respectively. Mean weight and Crown Rump Length (CRL) were significantly (P<0.001) decreased in group received only VAP. The means weight and length in groups that received melatonin were greater than the group received only VAP (Table 1). There were not any aborted fetuses from total groups but percentages of resorbed fetuses were 4.08, 11.76, 7.69, 4.54 and 4.65% in groups of 1-5, respectively (Table 1).

## DISCUSSION

VPA is a widely used antiepileptic drug since the 1970s., leading to skeleton defects, fetal growth retardation, neural tube defects and in-uterus death (Elmazara and Nau, 1995). It is routinely used to treat epilepsy (Dalessio, 1985). However, valproic acid is also known to cross the placenta and to cause a wide spectrum of congenital anomalies, including craniofacial and skeletal defects (Lammer et al., 1987). The teratogenic effect mechanism of VAP has not been precisely clarified. Although VAP induces exencephaly in experimental animal embryos, its pathogenic mechanism is not known reported that, in the 8th day of gestation, VAP application cause growth retardation in fetuses (AL Deeb et al., 2000). In our study, growth retardation that exhibits similar findings to pregnant rat fetuses was macroscopically observed.

Numerous studies point towards the role of oxygen derived free radicals in the pathogenesis of neuaral tube defects (AL Deeb et al., 2000). Exposure of rat embryos to high concentration of oxygen during early neurulation significantly increases the incidence of neural tube defects aqnd is dependent on the capacity of the antioxidant system to combat oxygen derived free radicals (Ishibashi et al., 1997).

Several studies have reported that the maternal immune stimulation or antioxidant drugs can reduce teratogenic anomalies. Mechanisms of this effect have remained unclear (Holladay et al., 2002); however, there are indications that immunoregulatory cytokines of maternal or placental origin normalize dysregulated apoptosis or timing of cell proliferation in the fetus (Sharova et al., 2000; Hrubec et al., 2006). Vitamin E reduces fetal malformation in diabetic animals (Simon and Eriksson, 1997).

In the present study, melatonin reduced the frequency of incidence of valproate induced malformations.

Enhancing antioxidative effects can protect fetuses against anticonvulsant drugs teratogenicity (Winn and Wells, 1999). Sharova et al. (2002) showed that interferon-gamma and Freund's complete adjuvant reduced severity of the urethane - induced cleft palate in mice (Sharova et al., 2002). Torkinsky et al. (1997) reported that immune stimulation in diabetic mice, which show a high spontaneous rate of cleft palate, decreased in malformed fetuses, significantly (Torkinsky et al., 1997).

Delayed fetal development and reduced fetal sized are common sequelae of teratogenic exposures such as diabetes (Cederberg et al., 2003), ethyl carbamate (Sharova et al., 2000) and valproate (AL Deeb et al., 2000). Consistent with these previous studies, we observed decreased fetal size in the sodium valproate exposed fetuses. Maternal immune stimulation with melatonin did protect the fetus from the sodium valproate induced growth impairment, even though neural tube defects were prevented. Maternal immune stimulation reduced the incidence of ethyl carbamate

induced cleft palate (Sharova *et al*., 2000) and vitamin E reduced the incidence of valproate induced malformations, but had no positive effect on fetal survival or growth (Al Deeb *et al*., 2000).

This disparity between the reduction of fetal malformations by various interventions and lack of reductions in fetal growth impairment and mortality may be explained by differential genetic contributions by the dam and fetus to valproate teratogenicity.

Laboratory mice have been used to study the basis for VPA teratogenicity. Administration of VPA on days 8-9 of gestation results in failure of cranial neural tube closure and spina bifida, as well as limb abnormalities such as syndactyly and oligodactyly (Ehlers *et al*., 1992).

Menegola *et al*. (1998) reported that the administration of 150-300 mg $kg^{-1}$ sodium valproate subcutaneously to pregnant mice or rats every 8h during the first stages of somitogenesis is able to produce a very high incidence of malformations at the level of the axial skeleton. They observed fetal defects similar to our study including cleft palate. These anomalies were decreased by melatonin (5,10 mg $kg^{-1}$), but melatonin with dose of 10 mg $kg^{-1}$ significantly more prophylactic effect than melatonin with dose of 5 mg $kg^{-1}$ .

Melatonin or N-Acetyl-5-methoxytryptamine, the main secretary product of pineal gland, is an antioxidant (Reiter and Tan, 2003), scavenges the hydroxyl radical (Tan *et al*., 2002). The studies in laboratory rodents and other domestic species suggested that melatonin did not affect prenatal growth, survival, or morphology of the concepts once pregnancy had been stablished (Chan and Ng, 1994, 1995; Tigchelaar and Nalbandov, 1975). The present investigations (NTP, 1997) extend the dose range evaluated in pregnant animals and the results are consistent with earlier studies that falied to find adverse effects on prenatal growth, or gross morphological development. Jahnke *et al*. (1997) reported that melatonin with doses of 50 and 100 mg $kg^{-1}day^{-1}$ from gd 6 through 19 had no effect on prenatal survival, fetal body weight, or incidence of fetal malformations (Jahnke *et al*., 1997). The authors reported no significant differences in dam body weight or total numbers of fetuses, live fetuses, or abortions through Gd 18 (San Martin *et al*., 1995). In one study, melatonin with dose 5 or 10 mg $kg^{-1}$ in mice protects against lipopolysaccaride-induced intra-uterine fetal death and growth retardation via counteracting lipopolysaccaride-induced oxidative stress (Chen *et al*., 2006). Omurtag *et al*. (2008) reported melatonin with dose 10 mg $kg^{-1}$ $day^{-1}$ for 5 days protects against endosulfan-induced oxidative tissue damages in rats (Omurtag *et al*., 2008). In present study, the effect of melatonin is probably related to antioxidant activity.

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## CONCLUSION

In conclusion, probably VAP influences immune system that produces teratogenic effects including cleft palate and spina bifida. Effects of VAP immunosuppression are mediated indirectly by inducing oxidative stress. On the other hand, melatonin can be reduce the incidence of sodium valproate induced malformations such as cleft palate and spina bifida in fetuses of rat and had positive effect on fetal growth. Melatonin with dose of 10 mg $kg^{-1}$ had significantly more prophylactic effect than melatonin with dose of 5 mg $kg^{-1}$ on incidence of sodium valproate-induced skeletal malformations.This effect of melatonin may be due to its antioxidant property.

## ACKNOWLEDGEMENT

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