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Full Length Research Paper

The effects of caffeine and carvedilol on skeletal system of rat embryos in prenatal period

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Caffeine at high doses is a known rodent teratogen and induces limb malformations along with cleft palate in various strains of rats and mice. The teratogenic effects of some drugs can be prevented by the application of antioxidant drugs and stimulation of the maternal immune system. Also, there are some evidence that carvedilol is antioxidant. Therefore, in this study, the prophylactic effect of carvedilol on teratogenic effects of caffeine was evaluated. This study was performed on 24 pregnant rats that were divided into four groups. Control group received normal saline and test groups received caffeine (80 mg/kg), caffeine (80 mg/kg) plus carvedilol (5 mg/kg) and carvedilol (5 mg/kg), intraperitoneally at 9 to 11th 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. Cleft palate incidence was 33.33% in fetuses of rats that received only caffeine, while it was 2.85% in group which received caffeine plus carvedilol (5 mg/kg). The means of weight and length of fetuses from rats that received carvedilol were significantly greater than those that received only caffeine. It is concluded that carvedilol decreased cleft palate induced by caffeine, but this subject needs more detailed evaluation.

Key words: Caffeine, carvedilol, gestation, cleft palate, teratogenicity, fetus, rat.

INTRODUCTION

Caffeine, or 1, 3, 7-trimethylxanthine, is a widely used substance present in habitual beverages and chocolate-based foods (Olcina et al., 2006). Caffeine represents one of the most common pharmacologically active substances used by pregnant women. Exposure of the conceptus to this drug occurs primarily as a result of maternal consumption of caffeine containing beverages, especially coffee (Nash and Persaud, 1988).

Caffeine at high doses is a known rodent teratogen and induces limb malformations along with cleft palate in various strains of rats and mice (Moriguchi and Scott, 1986). Several studies have demonstrated the teratogenicity of caffeine in laboratory animals (Fujii and Nishimura, 1969; Fujii et al., 1969; Palm et al., 1978), but the experimental results cannot be applied to humans due to the variability of caffeine dose, exposure time and species differences.

The sensitivity of different animal species is variable. Malformations have been demonstrated in mice at 50 to 75 mg/kg of caffeine (Nehlig and Debry, 1994), whereas the lowest dose usually needed to induce malformations is 80 mg/kg in rats (Nehlig and Debry, 1994). However, when caffeine is administered in fractioned amounts during the day, 330 mg/kg/day are necessary to reach teratogenicity in rats (Nehlig and Debry, 1994). In rodents, the most frequently observed malformations are those of the limbs and digits, ectrodactyly, craniofacial malformations (labial and palatal clefts) and delays in ossification of limbs, jaw and sternum (Nehlig and Debry, 1994).

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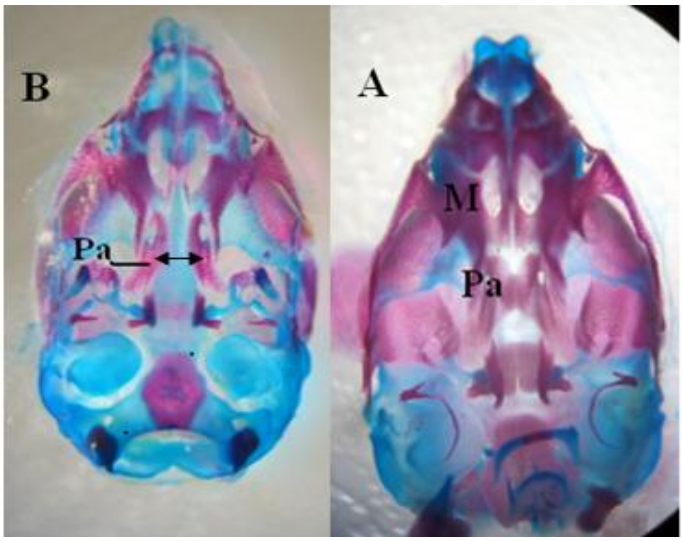

Figure 1. Ventral view of skull of rat fetuses of GD 20, stained with alizarin red S- alcian blue. A, Normal palatine bone; B, cleft palate induced by caffeine (arrow). M, maxilla; Pa, palatine.

Some of these caffeine-derived effects could favour the production of free radicals and a subsequent increase of oxidative stress such as the metabolic inactivation of catecholamines Jewett et al., 1989) and the increase of oxidative metabolism (Shigenaga et al., 1994) including its own hepatic metabolism (Vistisen et al., 1992). There are also reports suggesting that caffeine is capable of including certain forms of oxidative damage by increasing lipid peroxidation (Dianzani et al., 1991).

One of the free radical scavengers currency used in clinical setting is carvedilol which is a blocker of α1- and β- adrenoreceptors (Yue et al., 1992). Because of its unique ability to interact with free radicals, carvedilol has been proposed to be a useful adrenergic antagonist in the treatment of hypertention and cardiac diseases in which oxidant stress prevails (Feuertein et al., 1998). In physicochemical, biochemical and cellular assays, carvedilol inhibited the formation of reactive oxygen radicals and lipid peroxidation, scavenged oxygen free radicals, and prevented the depletion of endogenous anti-oxidants. It has been suggested that carvedilol may provide greater benefit than traditional β-blockers in chronic heart failure because of its antioxidant actions that synergize with its nonspecific β- and α-blocking effects (Noguchi et al., 2000).

In the present study, the preventive effect of carvedilol on caffeine-induced cleft palate in rats was evaluated.

MATERIALS AND METHODS

Caffeine powder (Merck, Germany) and carvedilol (Abidi Co, Iran) were purchased**.** Male and female healthy rats of Wistar strain, 3 to 4 months old, weighing 200 to 250 g were purchased (Joundishapour Jaboratory Animal Center, Ahvaz, Iran) and housed individually (males) or at 10 per polycarbonate cage (female) for a 2-week acclimation period. Rats were fed *ad libitum* by standard laboratory pellet (Pars khurakdam, Tehran, Iran.) and tap water. Rats were maintained in animal rooms controlled for temperature (23 ± 2°C), relative humidity of 45 to 55% and light (12/12 h light/ dark cycle).

Females were mated overnight with males. Pregnancy was ascertained the next morning by presence of a vaginal plug, and this time was designated as gestational day (GD) 0. Pregnant rats (n = 24) were randomly divided into four groups (16 pregnant rats in treatment groups, 8 pregnant rats in control group) and treated as follows;

Group 1 (Control group): Normal saline in equal volume of caffeine was injected to pregnant rats for inducing similar condition (injection and handling) to other groups.

Group 2 (Caffeine group): Caffeine (80 mg/kg) was administrated intraperitoneally at 9 to 11th day of gestation.

Group 3 (Carvedilol group): Carvedilol (5 mg/kg) was administrated intraperitoneally at 9 to 11th day of gestation.

Group 4 (Caffeine + carvedilol group): Caffeine (80 mg/kg) plus carvedilol (5 mg/kg) was administrated intraperitoneally at 9 to 11th day of gestation.

The animals were sacrificed by cervical dislocation at 20th day of gestation. Following laparotomy, the uterus was exteriorized and the number and location of fetuses and resorption were noted, then their weight and length (crown- rump length) were measured. Individual fetuses were examined carefully for external anomalies then were stained in a mixture of 0.14% Alcian blue and 0.12% alizarin red S in ethanol and glacial acetic acid. Fetuses are then macerated in 2% KOH, cleared and hardened in 1:1 glycerin and distilled water, and stored in pure glycerin (Kimmel and Trammekl, 1981) and investigated by stereomicroscope (Nikon, SMZ200, Japan) for skeletal malformations. The incidence of skeletal malformations was determined and was compared in the groups.

Statistical significance between groups was determined using SPSS program and compared by one way analysis of variance (ANOVA) and Post hoc least significant difference (LSD). The minimum level of significance was $p< 0.05$.

RESULTS

Sixty-two (62) fetuses were obtained from 8 rats of control group. There were not observed macroscopic anomalies in the control animals. In the control group, palatal closures of fetuses were normal at GD 20 (that is, palatal shelves had grown vertically on the sides of the tongue, then horizontally to meet and fuse) (Figure 1A). No maternal death or abortion occurred in any experimental groups. There were not any aborted fetuses from total groups but percentage of resorbed fetuses were 0, 3.38, 2.77 and 0% in groups that received normal saline, caffeine, caffeine plus carvedilol and carvedilol, respectively; so carvedilol decreased resorption rate (Table 1).

Caffeine induced cleft palate (Figure 1B) at 33.33% incidence. Caffeine plus carvedilol (5 mg/kg) significantly reduced incidence of cleft palate to range of 2.85%.

The mean of weight of animals' fetuses that received caffeine (80 mg/kg) in 9 to 11th days was significantly

Table 1. Incidence of anomalies in rat fetuses of groups.

Group	No. of litters	Implantations	Resorbed fetuses	Live fetuses	Fetal length (mm): (mean ± SEM	Fetal weight (g): (mean ± SEM)	%fetuses with cleft palate
Control	8	62	0 (0)	61	38.01 ± 0.26*	4.93 ± 0.08*	0 (0)
Caffeine	6	59	2 (3.38)	57	28.92 ± 0.81**	3.03 ± 0.17**	19 (33.33)#
Caffeine + carvedilol	5	36	1 (2.77)	35	35.68 ± 0.57	4.30 ± 0.13	1 (2.85)
Carvedilol	5	38	0 (0)	38	36.31 ± 0.40	4.49 ± 0.11	0 (0)

Numerals in parentheses are percentages; *, Significant difference when compared with other groups ($p < 0.05$); **, significant difference when compared with other groups; #, significant difference when compared with other groups ($p < 0.05$), Incidence of cleft palate was significantly difference at groups which received caffeine with control and carvedilol group ($p = 0.0001$).

decreased in comparison with other groups (Table 1). The mean of weight and length of animals' fetuses that received normal saline in 9 to 11th days was significantly increased in comparison with other groups (Table 1).

DISCUSSION

Since data are not available on the effect of carvedilol on the teratogenicity of caffeine in rat embryos this study was initiated. Several studies have reported that the maternal immune stimulation can reduce teratogenic anomalies (Ivnitsky et al., 2001). Mechanisms of this effect remain unclear, but it is thought that the fetal gene expression has been modulated (Holladay et al., 2002).

The enhancing antioxidative effects can protect fetuses against 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).

In the present study for first time, the effect of carvedilol on teratogenicity of caffeine in rat embryos was evaluated.

According to a recent report by Lelo et al. (1986), the average daily human caffeine intake of moderate to heavy consumers ranges from approximately 300 to 600 mg/kg/day, or from 3 to 6 cups of coffee (assuming 100 mg/cup). The dosage level therefore in a person weighing 70 kg ranges from approximately 4.3 to 8.6 mg/kg/day. In comparison, caffeine doses administrated to laboratory animals ranged from 30 mg/kg (Palm et al., 1978) to 250 mg/kg (Fujii and Nishimura, 1969). Even when species variation is taken into account, the practical application of the results obtained from many of these animal experiments to the human condition is unrealistic due to the excessive dose levels administered (Nash and Persaud, 1988).

A moderate dosage level of 80 mg/kg caffeine was administered as a three intraperitoneal injection on GDs 9 to 11. Fujii et al. (1969) demonstrated that in mice, whereas embryo-lethality is related to the duration of caffeine exposure, teratogenic effects are more dependent on a sufficiently high concentration of the drug. Though intraperitoneal injections dose not stimulate human caffeine consumption the method of caffeine administration in present study was the most expedient and in accordance with that utilized by others.

Fujii and Nishimura (1974) postulated that caffeine was teratogenic by virtue of catecholamine release from maternal or embryonic tissue. They reported that administering 175 mg/kg of caffeine intraperitoneally on days 11 and 12 of pregnancy in mice induced malformation that is initiated by release of catecholamines from the maternal adrenal gland.

Ross and Persaud (1989) reported neural tube defects in early rat embryos following maternal treatment with caffeine. Kimmel et al. (1984) reported a significant in resorptions following oral administration of caffeine to pregnant rats at a dose level of 120 mg/kg on the day 12 of gestation. Even though epidemiological studies have found no real association between coffee consumption during pregnancy and adverse fetal outcome (Linn et al., 1982), the United State Food and Drug Administration still advised pregnant women to avoid caffeine-containing foods and methylxanthine which resembles the purines found in genetic material. Thus, caffeine possesses the potential to derange the processes involved in cell proliferation. Because it has been known for some time that caffeine readily crosses

the placenta and reaches the fetus (Goldestein and Warren, 1962), the warning of the Food and Drug Administration merits serious consideration. In the present study, embryo from mothers treated with caffeine revealed a significant reduction in crown-rump length. It is believed that maternal treatment with caffeine alters utero-placental circulation to such an extent that normal embryonic development is impaired (Adamson et al., 1971).

Burdan (2003) reported that the mixture of paracetamol and caffeine decreased fetal length and body weight, and placental weight. Nishimura and Nakai (1960) reported increased cleft palate and digital defects in mice offspring exposed to caffeine at a dose of 250 mg/kg.

In one study, Colomina et al. (2001) reported a single oral dosage of caffeine or aspirin (ASA) on p.c.d 9 was given to mice orally exposed to toxic levels of caffeine (30 mg/kg/day), ASA (250 mg/kg), or a combination of caffeine and ASA (30 and 250 mg/kg, respectively). Three additional groups were given the same doses and restrained for 14 h. The pregnant mice were restrained 2 h/day on p.c.ds 0 to 18 by placing them in methacrylate cylindrical holders and keeping them in a prone position with the paws immobilized with elastic adhesive tape, a procedure the authors previously reported to produce stress in pregnant mice (Colomina et al., 1995; 1999). Other mice were given toxic dosages of caffeine by gavage at 30, 60, and 120 mg/kg/day on GDs 0 to 18, and another group was administered the same dosages of caffeine immediately followed by restraint stress for 2 h/day on the same days (Colomina et al., 1999). No caffeine levels were recorded. Although the authors do not identify maternal toxicity, it is noteworthy that the weekly intervals measured for body weights are inappropriate (drug treatments and restraint occurred on one day; the intervals are evaluated for 3 or 4 days). Maternal toxicity was evident, with reductions or frank weight losses in body weight and feed consumption measurements. Regarding caffeine, these effects were most severe for the three groups of interest (restraint, 30 mg/kg caffeine and combined 30 mg/kg of caffeine and 14 h of restraint), on p.c.ds 9 to 11. Of these three groups, the effects were most severe for the combined caffeine and stress group. The 30 mg/kg plus restraint group also had an increase in post-implantation loss, including dead fetuses and late resorptions. An increase in early resorptions was seen in the restraint alone group, but the group with both restraint and 30 mg/kg of caffeine were increased compared with the restraint alone group. As would be expected, there was an increase in reduced ossification in the restraint group alone, the 30 mg/kg caffeine alone, and the combined caffeine and stress group. There was no increase in malformations in any group. The authors considered there to be some clinical relevance for the data because real life involves multiple simultaneous exposure to many chemicals. However, the duration of oral exposure to ASA and caffeine on GD 9 in this study is not analogous to the type of stress experienced by pregnant women who drink coffee and take ASA. Interspecies differences and pharmacokinetics and bioavailability are both important considerations (Brent et al., 2011).

Colomina et al. (2001) exposed mice to caffeine (30 mg/kg) and ASA (250 mg/kg by gavage on the 9th post conception day.) There was no significant maternal or developmental toxicity in this group of animals and offspring. The studies also included stressful restraint. However, the exposure and the stress in the mouse studies cannot be utilized to determine human developmental risks, especially since the developmental results were minimal and the exposure equivalency in the human is unknown.

Differences in outcome after intrauterine caffeine exposure dependent on dose and rout of administration were also seen in rats. A lack of embryo or fetotoxicity or teratogenicity was observed when caffeine was administrated for whole gestational period at doses 16 to 17 and 25 to 33 per day (Aeschbacher et al., 1980). A reduction in fetal weight was found after maternal pregnancy exposure to 62 mg/kg per day. In contrast, Nolen (1981) reported that daily, long-term caffeine exposure at doses up to 80 mg/kg per day in drinking water did not affect fetal development. They also showed that such administration caused no differences in body weight gain or feed consumption. Aeschbacher et al. (1980) reported that caffeine dietary concentration of 0.25 and 0.5 g/kg throughout gestation and lactation had no significant effect on birth weight, litter size or development. At 1 g/kg, there was a slight reduction of birth weight. In animal studies, fetal loss, decreased fetal weight and size, and major skeletal defects have been reported when dosages of more than 80 mg/kg of caffeine were used (McKim, 1991).

A number of observations suggest that detoxification of a xenobiotic free radical intermediate with antioxidants may provide important embryo protection (Wells et al., 1997).

In one study, carvedilol protected oxidative stress induced by okadaic acid in N1E-115 cells. It seems that protective effect of carvedilol, as well as its ability to modify cell response to okadaic acid, involving like cytoprotective mechanism is its antioxidative properties (Tunez et al., 2006). Huang et al. (2006) reported carvedilol treatment increased activities of antioxidant enzymes and expression of Bcl-2 in healthy rats as well as diabetic rats. These results indicated that carvedilol partly improves cardiac function via its antioxidant properties in diabetic rats (Haung et al., 2006).

Prakash and Kumar (2009) reported the effectiveness of carvedilol in preventing cognitive deficits as well as the oxidative stress caused by intracerbroventicular administration of streptozotocin in rats (Prakash and Kumar, 2009). Carvedilol has been shown to preserve the endogenous antioxidant system, that is, vitamin E

and reduced glutathione (GSH), which are normally consumed when tissues or cells are exposed to oxidative stress (Feuerstein et al., 1997).

Antelava et al. (2009) reported that antioxidant and positive treatment effects of carvedilol could be explained by its wide range of pharmacological ability: as non-selective beta-adrenergic blocker (via inhibition of adenylatecyclase and decreasing cyclic adenosinemonophosphate), alpha 1-adrenoblocker [decreasing activation of phospholipase C and concentration of inositoltriphosphate, diacylglicerole and Ca (++)] and antioxidant (Antelava et al., 2009). Tasset et al. (2009) reported that carvedilol and melatonin prevented the increases in lipid peroxidation and total lactate dehydrogenase (LDH) activity, as well as the depletion of reduced GSH and the reduction of antioxidative enzymes activities in N1E-115 cells incubated with 100 mM 3NP (Tasset et al., 2009).

In conclusion, the present study showed the effects of carvedilol for the first time on cleft palate induced caffeine in rat fetuses. The present results indicate that exposure 80 mg/kg of caffeine in 9 to 11th days of gestation of rat decreases weight and length of embryos and did influence on skeletal system. It is probable that caffeine influences antioxidant system that produces teratogenic effects including cleft palate. Effects of caffeine immune suppression are mediated indirectly by inducing oxidative stress. The protective effect of carvedilol against caffeine-induced cleft palate in rat may, at least in part, be due to its antioxidant activity, which we believe deserves further investigation.

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