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Full Length Research Paper

Protection of rat fetuses by quercetin against caffeine-induced cleft palate

Mahmood Khaksary Mahabady[1]* and Hossein Najafzadeh Varzi[2]

[1]Department of Anatomy and Embryology, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran.
[2]Department of Pharmacology, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran.

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Caffeine at high doses is a known rodent teratogen and it 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 quercetin is an antioxidant. Therefore, in this study, the prophylactic effect of quercetin on teratogenic effects of caffeine was evaluated. This study was performed on 26 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 quercetin (75 mg/kg), and quercetin (10 mg/kg), intraperitoneally at 9 to 11th days of gestation, respectively. Fetuses were collected at the 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 and incidence was 1.49% in the group that received caffeine plus quercetin (75 mg/kg). The means of weight and length of fetuses from rat that received quercetin were significantly greater than those that received only caffeine. It is concluded that quercetin decreased cleft palate induced by caffeine, but this subject needs more detailed evaluation.

Key words: Caffeine, quercetin, pregnancy, 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 (Oclina 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, because of 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, whereas the lowest dose usually needed to induce malformations is 80 mg/kg in rats. 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).

Caffeine potentiates the teratogenic effect of other substances, such as tobacco, alcohol, and acts synergistically with ergotamine and propranolol to induce

*Corresponding author. E-mail: mkhaksarymahabady@yahoo.com or mkhaksary@scu.ac.ir. Tel: +986113330073 or +989131619252. Fax: +986113360807.

materno-fetal vasoconstrictions leading to malformations induced by ischemia. Therefore, even though caffeine does not seem to be harmful to the human fetus when intake is moderate and spread out over the day, some associations, especially with alcohol, tobacco, and vasoconstrictive or anti-migraine medications should be avoided. Maternal exposure to caffeine induces long-term consequences on sleep, locomotion, learning abilities, emotivity, and anxiety in rat offspring, whereas in humans, more studies are needed to ascertain long-term behavioral effects of caffeine ingestion by pregnant mothers (Nehlig and Debry, 1994).

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).

Flavonoids are polyphenolic compounds widely distributed in dietary fruits and vegetables. The average daily intake in the occidental diet is about 23 mg, of which quercetin (3,3,4,5,7-pentahydroxyflavone) is one of the most abundant representing the 60 to 75% of the average polyphenol ingestion (Goldberg et al., 1995). Quercetin directly scavenges the superoxide anion (Robak and Gryglewski, 1988) and inhibits several superoxide-generating enzymes such as xanthine oxidase (XO) (Sanhueza et al., 1992; Chan et al., 1993) or the neutrophil membrane nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex (Tauber et al., 1984).

Quercetin has been reported to have biological, pharmacological, and medicinal activities (Perez-Vizcaino et al., 2009) that are believed to arise from its antioxidant properties. It is found in frequently consumed foods, including apples, berries, onion, tea, nuts, seed and vegetables that represent an integral part of the human diet (Bhutada et al., 2010). Quercetin was reported to have many beneficial effects on human health, including cardiovascular protection, anticancer activity, antiulcer activity, anti-allergic activity, cataract prevention, anti-inflammatory and antiviral activity (Yao et al., 2004). In addition, quercetin could prevent oxidant injury and cell death by several mechanisms, such as scavenging oxygen radicals, protecting against lipid peroxidation, and chelating metal ions (Boots et al., 2008).

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

MATERIALS AND METHODS

Male and female healthy rats of Wistar strain, 3 to 4 months old, weighing 200 to 250 g were purchased (Joundishapour Laboratory Animal Center, Ahvaz, Iran) and housed individually (males) or at10 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. A 12 h light: 12 h dark was mentioned. Room temperature was at 23±2°C with a relative humidity of 45 to 55%.

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 = 26) were randomly divided into four groups (18 pregnant rats in treatment groups, 8 pregnant rats in control group), and were treated as follow: 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 administered intraperitoneally at 9 to 11th days of gestation; group 3 (quercetin group): quercetin (75 mg/kg) was administered intraperitoneally at 9 to 11th days of gestation; group 4 (caffeine + quercetin group): caffeine (80 mg/kg) plus quercetin (75 mg/kg) was administered intraperitoneally at 9 to 11th days of gestation.

The animals were sacrificed by euthanized and cervical dislocation at the 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 stereo-microscope (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 was 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 fetuses were obtained from eight rats of the control group. There were no observed macroscopic anomalies in the control animals. In the control group, palatal closures of fetuses were normal at gestational day 20 (that is, palatal shelves had grown vertically on the sides of the tongue, then horizontally to meet and fuse) (Figures 1, 2 A). There were no any aborted fetuses from total groups.

Caffeine induced cleft palate (Figures 1 and 2B) at 33.33% incidence. Caffeine plus quercetin (75 mg/kg) significantly reduced incidence of cleft palate to 1.49% range.

The mean weight of animals' fetuses that received caffeine (80 mg/kg) in 9 to 11th days was significantly decreased in comparison with normal saline group (Table 1). The mean weight and length of animals' fetuses that received quercetin (75 mg/kg) in 9 to 11th days was significantly increased in comparison with other groups (Table 1).

DISCUSSION

Since there are no data available on quercetin, on the

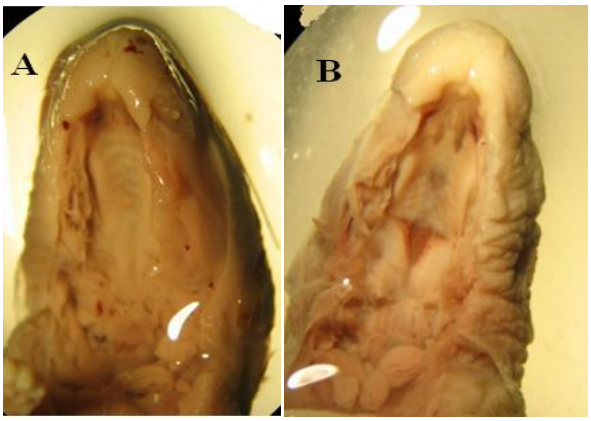

Figure 1. Razor blade sections of rat fetuses of GD 20: (A) control skeleton and cleft palate due to palatal shelf hypoplasia (B) in the treated case (80 mg/kg of caffeine, treated on gestation days 8, 9, and 11).

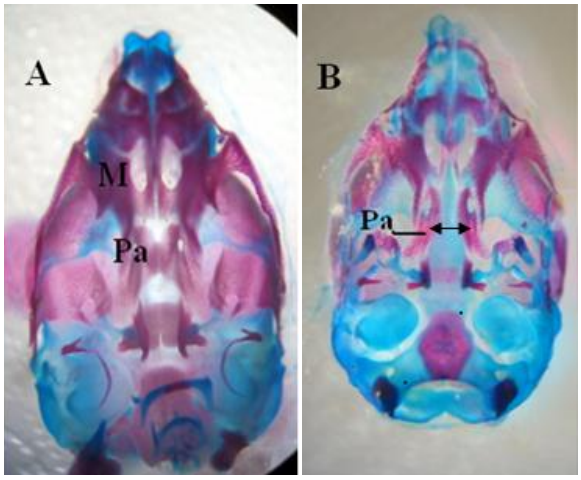

Figure 2. Ventral view of skull of rat fetuses of gestation day 20, stained with alizarin red S-alcian blue: (A) normal palatine bone and (B) cleft palate induced by caffeine (arrow). M: maxilla; Pa: palatine.

teratogenicity of caffeine in rat embryos, the present study for first time, evaluates the effect of quercetin on teratogenicity of caffeine in rat embryos.

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 administered to laboratory animals ranged from 30 (Palm et al., 1987) to 250 mg/kg (Fujii and Nishimurta, 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 gestational days 9 to 11th. Fujii et al. (1969) demonstrated that in mice, whereas embryolethality 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 this 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. We reported that administering 175 mg/kg of caffeine intraperitoneally at 16:00 h in day 11 and 9:00 h in day 12 in mice induced malformation that is initiated by release of catecholamines from the maternal adrenal gland.

Ross et al. (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 this 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 that a single oral dosage of caffeine or aspirin on gestation day 9 was given to mice orally exposed to toxic levels of caffeine (30 mg/kg/day), aspirin (250 mg/kg), or a combination of caffeine and aspirin (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 gestation days 0 to 18 by placing them in methacrylate cylindrical holders and keeping them in a prone position with the paws

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

Group	No. of litter	Implantation	Resorbed foetus	Live foetus	Fetal length (mm) (mean ± SEM	Fetal weight (g) (mean ± SEM)	Fetuses with cleft palate (%)
Control	8	62	1 (1.58)	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+ Quercetin	6	69	2 (2.89)	67	31.50 ± 0.38	4.54 ± 0.07	1 (1.49)
Quercetin	6	47	1 (2.12)	46	38.60 ± 0.28	4.08 ± 0.09	0 (0)

Numerals in parentheses are percentages. *Significant difference when compared with other groups (P<0.05); **Significant difference when compared with other groups except caffeine + quercetin group (P<0.05); #Significant difference when compared with other groups (P<0.05). Incidence of cleft palate was significantly different at groups that received caffeine with control and quercetin group (P=0.0001).

immobilized with elastic adhesive tape, a procedure the authors previously reported to produce stress in pregnant mice (Colomina et al., 1995; Colomina et al., 1999). Other mice were given toxic dosages of caffeine by gavage at 30, 60, and 120 mg/kg/day on gestation days 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 three or four 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 gestation days 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 group alone, but the group with both restraint and 30 mg/kg of caffeine were increased when 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 aspirin and caffeine on gestational day 9 in this study is not analogous to the type of stress experienced by pregnant women who drink coffee and take aspirin. Interspecies differences and pharmacokinetics and bioavailability are both important consideration (Brent et al., 2011).

Colomina et al. (2001) exposed mice to caffeine (30 mg/kg) and aspirin (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. These 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. Lack of embryo or fetotoxicity or teratogenicity was observed when caffeine was administered 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 ton 62 mg/kg per day. In contrast, Nolen (1981, 1982) 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 significance 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).

Prater et al. (2008) reported that low-dose quercetin (66 mg/kg supplemented in rodent chow throughout gestation; approximately 70% of human dose) and high-dose quercetin (333 mg/kg

supplemented in rodent chow throughout gestation; approximately 3.5 times daily human dose), impair placental oxidative stress and fetal skeletal malformation induced by methylnitrosourea.

In one study, quercetin reduced abnormal development of mouse embryos produced by hydroxyurea (Pérez-Pastén et al., 2010). Liang et al. (2009) reported that quercetin (66 mg/kg supplemented diet) significantly improves high fatty saturated induced fetal skeletal mal-development, perhaps in part due to antioxidant effects of quercetin in placenta. This speculation is supported by previous reports that demonstrate quercetin prevention of oxidant injury and cell death by reactive oxygen species (ROS) scavenging and protection against lipid peroxidation (Boots et al., 2008).

In several studies that reported quercetin, a flavonoid antioxidant, prevents and protects against ethanol-induced oxidative stress in mouse (Molina et al., 2003) and rat (Liu et al., 2010) liver. Abdelmoaty et al. (2010) reported that quercetin could prevent hyperglycemia induced by streptozotocin in rats. In another study, quercetin with 50 mg/kg oral dose was the most effective in preventing arsenic poisoning by reducing oxidative stress (Dwivedi and Flora, 2011).

In conclusion, the present study showed the effects of quercetin for the first time on cleft palate induced caffeine in rat fetuses. The present results indicate that 80 mg/kg of caffeine exposure in 9 to 11th days of gestation of rat decreases weight and length of embryos and did have influence on skeletal system. The protective effect of quercetin in 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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