Effect of caffeine and retinoic acid on skeleton of mice embryos

Fakhr El-Din M. Lashein*, Amin A. Seleem, Abeer A. Ahmed

Department of Zoology, Faculty of Science, Sohag University, Egypt

Received 14 April 2016; accepted 22 June 2016

Abstract The present study was conducted to evaluate the effect of caffeine and retinoic acid either separately or in combination on the skeleton of the developing embryos of mice. Pregnant females were treated with either caffeine or retinoic acid at the onset of organogenesis (7th day of gestation). At morphological level no abnormalities in either caffeine or retinoic acid in the developing embryos at 14th day of gestation whose mothers’ were administered caffeine (2 mg/100 g b.w.) or those of the mothers’ treated with retinoic acid up to 4 mg/kg b.w. during the onset of the second trimester of pregnancy were observed. However, dose-dependent retinoic acid treatment initiates chondrocyte vacuolation, depression of PAS+ve intracellular inclusions and depression of nuclear fluorescence that were concomitant with downregulation of TGFβ2 expression in the perichondrium of the developing vertebrae. Co-administration of caffeine was found to ameliorate the effects of 2 mg/kg b.w. rather than 4 mg/kg b.w. of retinoic acid treatment. At the 18th day of gestation the uterine horns appeared normal without any signs of fetoresorption in all treatments. However, the effect of both caffeine (2 mg/100 g b.w) and retinoic acid at both doses (2, 4 mg/kg b.w) in Alizarin Red stain of wholemount revealed minor phalange deformation of the developing limbs either separately or in combined treatments.

© 2016 The Egyptian German Society for Zoology. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Introduction

The increasing popularity of caffeine-containing energy drinks (Mintel International, 2008), along with a variety of newly marketed caffeinated products contribute in higher caffeine intake during pregnancy. One of the prenatal exposures examined for association with preterm birth has been caffeine consumption. Caffeine (1,3,7-trimethylxanthine), a plant alkaloid found in coffee, tea, cocoa, and cola soft drinks, is one of the most frequently consumed substances (Nawrot et al., 2003). 1994–1996 survey found that 68% of pregnant women consumed caffeine in US, with an average intake of 125 mg (approximately equivalent to 1.25 cups of coffee) per day (Frary et al., 2005). A study suggests an increased risk of growth restriction, cardiovascular abnormalities, and skeletal abnormalities in children of women with high caffeine intake during pregnancy (Golding, 1995). Caffeine easily crosses the placenta and is known to decrease placental blood flow, fetal heart rate and has been detected in uterine secretions and amniotic fluid (Kirkinen et al., 1983; Mose et al., 2008). During pregnancy, the rate of caffeine metabolism decreases progressively from the first to third trimester, with a doubling of the half-life of caffeine. Delayed clearance...
leading to higher concentrations in the fetus and a higher half-life of caffeine in neonates than in adults was known (Aldridge et al., 1979; Soyka, 1981). Whether maternal caffeine intake during pregnancy is associated with preterm birth has been examined during the past 30 years with inconsistent results (Nawrot et al., 2003; Pacheco et al., 2007). Results of epidemiologic studies investigating caffeine teratogenicity have been mixed; however, relatively few have examined exposure from all major sources of caffeine in association with specific types of birth defects. No statistically significant associations with maternal dietary caffeine intake were observed for congenital heart defects (Browne et al., 2007), orofacial clefts (Collier et al., 2009), or bilateral renal agenesis or hypoplasia (Slickers et al., 2008). Moreover, caffeine has the potential to interact with many other exposures. For example, smoking is known to increase the rate of caffeine metabolism in humans (Landi et al., 1999). In rodents and chicken embryos, caffeine enhanced teratogenicity of substances such as nicotine, alcohol, bronchodilators, and anti-seizure medications (Nehlig and Deby, 1994).

In addition, retinoic acid (RA) is known to play a key role in pattern formation during vertebrate development. It has long been known that retinol (vitamin A) is essential for normal growth, vision, reproduction, maintenance of numerous tissues, and overall survival of embryos (Lohnes et al., 1994; Niederreither et al., 1996). Retinoic acid (RA), which exists in both cis and trans isomeric forms, is the most biologically active metabolite of vitamin A and is also essential for normal development (Abu-Abed et al., 2002). Several studies indicated that in utero exposure to excessive retinoic acid during pregnancy generates different congenital malformations (Loberg et al., 1990; Niederreither et al., 1996; Mulder et al., 2000; Amini et al., 2005; Quemelo et al., 2007). These studies used isomeric forms of retinoic acid at high dose at early stages during embryonic development. Hence, the present study was conducted to evaluate the effect of caffeine, retinoic acid and their interaction during co-exposure at lowest doses on the skeleton of the developing embryos of mice, Mus musculus, for their wide uses of therapeutic purposes.

Materials and methods

Caffeine

Caffeine, anhydrous pure crystals (Merck, C_{8}H_{10}N_{4}O_{2}, 194.2 g/mol) was obtained commercially. Stock solution in saline was prepared and renewed as required during the experimental period.

Retinoic acid

Retinoic acid (C_{15}H_{24}O_{2}, 300.4 g/mol) in the form of 13-cis form (Isotretinoin) product of Sigma 500 mg/package was obtained. In olive oil, stock solution was prepared and renewed as required during the experimental period.

Animals and experimental design

Immature mice, Mus musculus, after weaning were obtained from animal house of Assiut University. Animals were acclimatized in laboratory under normal light and temperature conditions with free access of food and water. Males and females were kept separately till maturity in cages. For the prenatal study at days 14, 18 of pregnancy, one male was mixed with two females in several cages. After the insurance of vaginal plug following copulation in one round of gestation, the pregnant females were classified into six groups from the 7th day of gestation (the first day of the organogenesis period in mice) (Strömland et al., 1991). G1 a control, G2 administered orally with caffeine at dose (2 mg/100 g b.w.), G3, G4 intraperitonally injected with retinoic acid at doses 2, 4 mg/kg b.w., respectively. G5, G6 a combined groups caffeine-administered at morning (2 mg/100 g b.w.) and at evening intraperitonally injected with retinoic acid (2, 4 mg/kg b.w., respectively). Caffeine dosing of pregnant females was carried out according to Reis et al. (2014) for prenatal exposure of rat embryos. Meanwhile, Vitamin A (Isotretinoin) dosing was carried out at 2, 4 mg/kg b.w., a range of low doses that induce different abnormalities on injected presomite of mouse embryos (Sulik et al., 1995). Treatment of pregnant females with caffeine was conducted daily, while retinoic acid treatment was conducted at day after the other day for either the separate or combined treatments from the 7th to the 18th day of gestation. Pregnant females at day 14 and at day 18 of pregnancy for different groups were dissected. The uterine horns and the embryos of repeated patches of the different experimental groups were examined morphologically. Embryos of 14 days old were fixed in Carnoy’s fixatives for histological, histochemical and immunohistochemical investigations of the developing vertebrae, while the embryos at day 18 of gestation were stored in 95% ethanol for skeleton staining.

Histological and histochemical study

Embryos at E14 of gestation were fixed in Carnoy’s fixative, dehydrated in ethyl alcohol, cleared in methyl benzoate and processed for sectioning. Serial sections of embryos 7μ thick in paraffin were mounted on glass slides and dried at 40°C
in an oven for 3 days. Sections were stained with Hematoxylin and Eosin for general histological picture, Periodic acid Schiff’s (PAS) reaction for polysaccharide detection (Drury and Wallington, 1976), and Acidine orange/Ethidium bromide stain for nuclear fluorescence (Kasibhatla et al., 2006). In addition, alcohol-fixed 18-day whole embryos of different groups were stained with Alizarin Red S for skeleton visualization. Sections were dehydrated in ascending grades of ethanol, cleared in xylene and mounted with DPX mounting media. Selected sections of the developing vertebrae at 14th day of gestation and 18 days of whole Alizarin Red S-stained embryos were photographed and processed as required.

Immunohistochemistry of transforming growth factor β2

In immunohistochemical study, deparaffinized Superfrost/Plus slide-mounted sections of embryos at 14th day of gestation of the different experimental groups were retrieved for re-antigenicity using 10 mM citrate buffer at pH6 in 100 °C for an hour (Buchlowalow and Bocker, 2010). After cooling at room temperature, sections were treated for 10 min with 0.3% hydrogen peroxide block and then with protein block (phosphate buffer solution, pH 7.6, with 0.5% BSA, 0.5% casein and less than 0.1% sodium azide) for 10 min to block nonspecific background staining, then sections were incubated with primary antibody (Rabbit Anti-human TGF β2 polyclonal antibody, Spring Bioscience, USA) washed using phosphate buffer and incubated with secondary antibody, Biotinylated Goat Anti-polyvalent (Anti-polyvalent HRP DAB detection system, Spring Bioscience, USA) according to the manufacture protocol. Reactions and color were visualized by using chromogene mixed with 3,3′-diaminobenzidin (1:10) (DAB substrate, chromogen, Spring Bioscience). In all cases, negative control sections in which the primary antibody was not applied to tissue sections were carried out. Sections were dehydrated in ascending grades of ethanol, cleared in xylene and mounted with DPX mounting media. Sections were examined microscopically to evaluate the effect of caffeine, retinoic acid and the co-administration of both in experimental groups compared to the control. Selected sections of the developing vertebrae and whole Alizarin Red S-stained embryos were photographed and processed as required.

Results

14 days-old embryos

Morphological examination of the developing embryos at day 14 of gestation did not reveal any abnormalities in either the embryos whose mothers were administered caffeine or those of the mother’s treated with retinoic acid even at the higher dose of 4 mg/kg b.w.. The growth morphology of the

Plate 2 Photomicrographs of histological sections through the developing vertebral centrae at E 14 of control (A); caffeine (B); retinoic acid 2, 4 mg/kg b.w. (C, D) and the combined treatments with caffeine and retinoic acid (E, F). H & E stain. Scale bar 20 μm.
developing embryos looks similar to the control (Plate 1). Head, trunk, limbs and tail are well developed in caffeine treatment (Plate 1B). In retinoic acid treatments the characteristic malformations, omphalocele, gastrochisis, lower limb defects, imperforated anus and tail agenesis of retinoic acid treatment are not detected (Plate 1C). Histological examination of the developing cartilaginous skeleton of the vertebral centrae of the control, caffeine, retinoic acid at doses of 2, 4 mg and the combined groups that were treated with caffeine and the two doses of retinoic acid revealed the dose-dependent effect of retinoic acid on the chondrocyte vacuolation and their abundance (Plate 2C and D, respectively) in the background of the homogenous matrix as compared to both the control and caffeine treated embryos (Plate 2A and B) in which the chondrocytes are dense and uniformly distributed in its background matrix. In combined treatments with caffeine, severely vacuolated condrocytes were noted in 4 mg dose (Plate 2F) compared to 2 mg dose (Plate 2E) and those treated with retinoic acid at 2 mg dose only (Plate 2C). PAS-stained sections of cartilaginous substance revealed positive intracellular inclusions of the developing chondrocytes of control (Plate 3A) and those of caffeine treated (Plate 3B). In retinoic acid treatments, either with 2 mg/kg b.w (Plate 3C) or with 4 mg/kg b.w (Plate 3D), a dose-dependent decrease in which faintly-stained PAS-positive intracellular inclusions were observed was compared with either the control or caffeine treatment. Caffeine was also found to exert a counter effect on retinoic acid by upregulating the intensity of the intracellular inclusions in combined treatment at 2 mg/kg dose (Plate 3E) rather than those treated with 4 mg (Plate 3F). In acridin orange/ethedium bromide stained sections, chondrocytes with their inclusions and a network of extracellular matrix are sharply acridinophilic that well sharply fluorescent in control (Plate 4A). Slight inhibition of fluorescence among the extracellular network was observed in caffeine treatment (Plate 4B), while retinoic acid at low dose (2 mg/kg b.w) results in darkness of the central regions of the nuclei due to the incorporation of ethedium bromide (Plate 4C) as compared to both the control and caffeine treatment. In addition to the inhibited fluorescence of the nuclei of the developing cartilage, the extracellular network that represents the limits of the differentiated lacunae was not observed in retinoic acid treatment. Recovered lacunar limits with slight inhibition of the nuclear fluorescence reflect the mitigating effect of caffeine on the alterations induced by retinoic acid (Plate 4D). Immunostained sections of the developing cartilage revealed a slight downregulating effect of caffeine (Plate 5B), dose-dependent in retinoic acid treatments (Plate 5C and D) downregulation in the combined treatment at 2 mg/kg (Plate 5E) that inhibited completely at 4 mg/kg of retinoic acid treatment with caffeine.

Plate 3 Photomicrographs of PAS-stained sections through the developing vertebral centrae at E 14 showing the dose-dependent decrease in PAS-positive inclusions of chondrocytes in retinoic acid 2, 4 mg/kg b.w. (C, D) compared to control (A), caffeine (B) that upregulated in combined treatments with caffeine (E, F). PAS stain Scale bar 20 μm, 10 μm for the inserts.
as compared to control (Plate 5A). In all treatments and in control the expression of TGFβ2 was restricted to the perichondrial connective tissue rather than the developing chondrocytes.

18 days-old embryos

The growth morphology of the uterine horns containing the developing foeti was not affected in either caffeine or retinoic acid treatments. Embryo resorption was not observed in either caffeine, retinoic acid or in combined treatments with both (Plate 6). Alizarin Red-S staining of whole embryos revealed a well developed skeleton of the major components (skull, limbs, ... etc) (Plate 7A–F), on magnifying the skeleton of the most distal parts of the limbs (hand and foot). It was obvious that the effect of both caffeine and retinoic acid in both doses is best manifested in the skeleton of the foot rather than in the skeleton of the hand. Metacarpals and the skeleton of phalanges are well developed in caffeine treated embryos (Plate 8B) as compared to control (Plate 8A). Dose-dependent effect of retinoic acid was noted. Missing of the second row of the skeleton of phalanges was noted in 4 mg/kg b.w-treated embryos (Plate 8C) which looks like those of control and caffeine-treated embryos. In combined treatment with caffeine and retinoic acid doses, missing of the skeleton of the second row of phalanges was noted (Plate 8E and F) similar to that observed in 4 mg/kg b.w of retinoic acid-treated embryos. In the skeleton of the foot, the effect of caffeine and retinoic acid doses was much obvious. In caffeine-treated embryos and those treated with retinoic acid, rudimentary phalanges (Plate 9B–D) as compared to the control (Plate 9A) were noted. Also, in combined treatments enhancement of the phalange malformations was found to be dose-dependent of retinoic acid (Plate 9E and F) in the presence of caffeine.

Discussion

The present study did not reveal morphological abnormalities in either caffeine or retinoic acid in the developing embryos at 14th day of gestation whose mothers’ were administered caffeine (2 mg/100 g b.w) or those of the mother’s treated with retinoic acid even at 4 mg/kg b.w dose during the onset of the second trimester of pregnancy. However, dose-dependent retinoic acid treatment initiates chondrocyte vacuolation, depression of PAS+ve intracellular inclusions and depression of nuclear fluorescence that were concomitant with downregulation of TGFβ2 expression in the perichondrium of the developing vertebrae. Co-administration of caffeine was found to ameliorate the effects of low (2 mg/kg b.w) rather than the high dose (4 mg/kg b.w) of retinoic acid. At 18th day of gestation the uterine horns appeared normal without any signs of fetoresorption in all treatments. However, the effect of both caffeine (2 mg/100 g b.w) and retinoic acid at both doses (2, 4 mg/kg b.w) in Alizarin Red S stain of wholemount revealed malformed phalanges of the developing limbs either in separate or in combined treatments. Caffeine is a methylxanthine alkaloid present in coffee, tea, and soft drinks and some drugs excite the central nervous system and have shown positive actions on the cardiovascular system and in reducing fatigue. In contrast to our findings, studies indicate that caffeine intake may have adverse effects on reproduction and fetal development including an increase in the risk of premature delivery, spontaneous abortion and intrauterine growth retardation (Kuczkowski, 2009). Treatment of female mice with caffeine (60, 120 and 240 mg/kg per day) before and during pregnancy was found to initiate a series of adverse reproductive and developmental effects, including delay in the timing of conception, lower maternal weight gain, placental weight, lower fetal/placental weight ratio and changes in indices of fetal growth and development. Similar results, particularly in terms of placental weight, body weight and intrauterine growth retardation were seen in rats after caffeine treatment (20, 60 and 180 mg/kg per day) from gestational day 11 to gestational day 20 (Huang et al., 2012).

Caffeine crosses the placental barrier easily, where it can directly affect the fetus in several different aspects. The half-life of caffeine is greatly increased in pregnancy, as it cannot be metabolized by the fetus or the placenta (Aldridge et al., 1981). In the first trimester the half life is about ten hours, while it increases up to 18 h during the third trimester, as the enzymes in the human liver do not exist until the eight month of life (Grosso and Bracken, 2005). Several previous studies focused primarily on the associations between caffeine intake
during pregnancy and birth weight showed inconsistency (Linn et al., 1982; Martin and Bracken, 1987; Larroque et al., 1993; Shu et al., 1995; Bech et al., 2007). Based on the dose-conversion correlation between humans and mice (Wang, 2005), the low dose of caffeine used in the present study (2 mg/100 g per day for mice is 0.36-fold that of average caffeine intake during pregnancy in Europe or 0.2-fold that of average caffeine intake during pregnancy in the US (Barone and Roberts, 1996). According to a previous study conducted by Butt and Sultan (2011) a standard cup of coffee contains, on average, between 100 and 150 mg caffeine; thus, the aforementioned dose in the present study (2 mg/100 g daily) of caffeine exposure is equivalent to approximately a cup of coffee daily consumed in pregnant women. In this context, it has been reported that in women who consume more than 300 mg caffeine daily, the conception of a primigravida is reduced by 27% (Bolúmar et al., 1997). Furthermore, the risks of spontaneous abortion and low-weight fetuses increase significantly when the intake of caffeine during pregnancy is > 150 mg/day (Valsamakis et al., 2006). Moreover, as early as 1980, the US Food and Drug Administration (FDA) proposed that caffeine should be taken cautiously during pregnancy (Fernandes et al., 1998). We showed that caffeine intake at low dose (2 mg/100 g b.w) per day during pregnancy might be associated with skeletal related fetal growth characteristics that seemed to be most consistently affected from the second trimester onwards on the basis of TGFβ2 downregulation in the perichondrium as the site of chondrocyte proliferation. Further follow up studies are needed focused on the effects of fetal caffeine exposure on postnatal skeletal and bone measurements.

Furthermore, it is well known that retinoids are essential for adequate embryo development. They are important signaling molecules for the regulation of cell differentiation, proliferation and morphogenesis. Inadequate levels of these compounds (excess or deficiency) results in a set of defects denoted by retinoic acid embryopathy including neural crest, limbs and skeleton differentiation (Zile, 1998; Mulder et al., 2000; Ross et al., 2000; Ali-khan and Hales, 2006; Maden, 2006). Other types of anomalies such as omphalocele, gastrochisis, limb and rib alterations and tail regression were noted in pregnant mice in early developmental stages of conception at doses less than 60 mg/kg while doses of 80 mg/kg or higher provoked many reabsorptions (Quemelo et al., 2007). In the present study, however, a small dose of retinoic acid did not initiate such overmentioned teratogenicity. Dose-dependent alterations of chondrocytes were observed with concomitant downregulation of TGFβ2 expression in the perichondrium at the 14th day of gestation. These alterations were manifested at 18th day embryos in which a minor
defects of phalanges were observed. Specificity of exogenous trans-retinoic acid (RA) effects during embryogenesis, especially on developing and regenerating limbs in different vertebrates were recorded in either systemic or locally applied (Bryant and Gardiner, 1992; Mohanty-Hejmadi et al., 1992; Maden, 1993), unlike in chickens and amphibians, in which RA was applied locally to developing or regenerating limbs or to amputated tails. In mice, RA injections of pregnant females 4.5–5.5 days after mating were found to be effective in inducing the duplication effects on lower body including limbs at higher doses (Quemelo et al., 2007).

Still, however, low dose of retinoic acid treatment of pregnant mice, at the onset of organogenesis, is able to provoke developmental alterations of the skeleton during prenatal life. Regardless the downregulated expression of TGF\(\beta\)2 as an inducer of matrix deposition, caffeine co-treatment ameliorates the intracellular matrix biosynthesis as indicated in PAS-stained chondrocytes in 2 mg/kg b.w rather than 4 mg/kg b. w of retinoic acid treatments. In contrast with other reports for in vitro studies (Tassinari et al., 1991; Tsuang et al., 2006), caffeine at 50 mg/kg, increases the osteogenic potential of osteoblasts, as characterized by increased alkaline phosphatase activity, collagen synthesis, mineralization of nodules and expression of osteogenic genes including osteocalcin, osteopontin, sialoprotein, alkaline phosphatase and type I collagen. Here the most important aspect of our method of caffeine administration is that the drug was not added to the culture medium as in most studies; we administered caffeine to mothers during pregnancy, and the drug or its metabolites passed through the placenta to the fetus. The doses of caffeine used in this study were chosen based on previously observed effects on endochondral ossification in the offspring of rats treated with caffeine (Reis et al., 2014). When added directly to the culture medium, caffeine inactivates cell survival signaling and promotes programed cell death by a mitochondria-dependent cascade; cell death thus occurs by apoptosis and necrosis (Tsuang et al., 2006; Lu et al., 2008). In vitro assay is thus different from in vivo because the cellular microenvironment of the organism is difficult to reproduce in vitro due to numerous interdependent intrinsic and extrinsic factors. The control of proliferation, differentiation and cell maintenance is carried out by genes, cytokines, developmental and growth factors and cellular interactions. So, it is difficult to develop in vitro models that can simulate drug effects in the body because the drug is metabolized within the mother’s body.

Plate 6 Photographs of uterine horns at E 18 of pregnancy showing normal horns without embryo resorption in various treatments similar to control. A (Control), B (caffeine treated), C (2 mg/kg b.w. retinoic acid-treated), D (4 mg/kg b.w. retinoic acid-treated), E, F (combined treatment with caffeine and both doses of retinoic acid, respectively). Scale bar 1 cm.

Plate 7 Photographs of Alizarin Red S-stained foeti at E 18 of gestation of control (A); caffeine (B); retinoic acid at 2, 4 mg/kg b. w. (C, D) and combined with caffeine treatment (E, F) show a proper skeleton development. Alizarin Red S stain Scale bar 1 cm.
Plate 8  Photographs of Alizarin Red S-stained skeleton of hands at E 18 of gestation showing the effect of 4 mg/kg b.w retinoic acid on phalanges deformation (D) as compared to control (A), caffeine treatment (B) and 2 mg/kg b.w. retinoic acid (C) black arrows. Combined treatment leads to phalanges malformations in both doses of retinoic acid with caffeine (E, F) yellow arrows. Alizarin Red S stain Scale bar 0.5 cm.

Plate 9  Photographs of Alizarin Red S-stained skeleton of foot at E 18 of gestation showing malformed phalanges in caffeine (B) red arrow; retinoic acid 2, 4 mg/kg b.w. doses (C, D) white arrows. Dose-dependent malformation of retinoic acid (E, F) yellow arrows in presence of caffeine compared to control (A) black arrow. Alizarin Red S stain Scale bar 0.5 cm.
and a small amount may reach to the developing embryo. The aim of this study was to observe the effects of caffeine in embryos whose mothers were exposed to caffeine during pregnancy, taking into consideration the metabolism of the drug by the mother and its passage to the fetus through the placenta. In conclusion, the work presents relatively novel results using methodology of little use previously and demonstrates the need for further in vivo studies to better understand the significance of our results of caffeine and retinoic acid uses during pregnancy for their therapeutic importance.

References


