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Effect of caffeine and retinoic acid on skeleton of mice embryos

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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.

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

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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 y 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 Debry, 1994).

In addition, retinoic acid (RA) is known to plays 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 (Lofberg 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, $C_5H_{10}N_4O_2$, 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 ($C_{20}H_{28}O_2$, 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

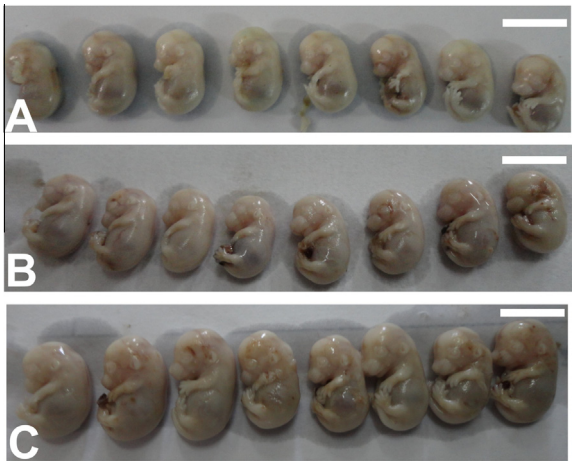

Plate 1 Photographs of embryos at E14 of gestation representing the growth morphology of control (A), caffeine (B) and 4 mg/kg b.w. retinoic acid (C) treatments. No malformations were recorded. Scale bar 1 cm.