Journal of Applied Sciences Research, 9(4): 3219-3227, 2013
ISSN 1819-544X
This is a refereed journal and all articles are professionally screened and reviewed

ORIGINAL ARTICLES

Adverse prenatal neurotoxicity induced by administration of caffeine to pregnant rats.

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ABSTRACT

The effects of caffeine on the developing brain remain controversial; therefore, the present study examined the developmental profiles of nucleic acids, proteins, and monoaminergic metabolism in immature rat brain and their impact on brain development in fetuses exposed to caffeine throughout pregnancy. Thirty pregnant rats were divided into three groups ten rats each, treated orally *via gavage tube* with caffeine at a doses of 0, 75 or 120 mg/kg b.wt./day, from the 1st to the 19th day of gestation. At the end of the experimental period, rats were sacrificed; blood samples and fetal amniotic fluids were taken. The maternal serum and amniotic fluid concentrations of calcium, phosphorus and the total protein content were estimated. The uteri were removed, weighed and examined. Caffeine levels in the maternal serum and fetal brain were evaluated. Fetal brain samples were obtained for different biochemical analyses and histopathological examination. The results indicated that dams received caffeine at either75 or 120 mg/kg bwt did not produce any adverse effect on the number of implantation, live fetuses or pre- or post-implantation loss. However, a significant reduction of fetal body weight was apparent in both doses. Caffeine levels of maternal serum and fetal brain were elevated significantly with the increase of caffeine dosage. High dose treated dams exhibited significant reduction of serum total protein content and their fetuses exhibited significant decrease of nucleic acids, total protein contents and neuronal density of fetal brain. Caffeine ingestion by pregnant rats at two doses induced a significant decrease of norepeniphrine in fetal brain but there was a significant increase in dopamine level. Therefore, caffeine received by pregnant rats can induce alterations in the steady-state concentrations of some neurotransmitters. Also, it can interfere with neuronal proliferation in the fetal brain especially at the high dose resulting in abnormal cerebral development that could lead to postnatal neurobehavioral disturbances.

Key words: Caffeine; Developing brain; Fetuses; Monoamines; Nucleic acids; Protein.

Introduction

Caffeine and theophylline are the most widely consumed psychoactive drugs in the world. Men and women of all ages, including children and pregnant women, intake daily doses of these stimulants which are present in many beverages and foods (e.g. coffee, tea, chocolate bars and cola drinks) (Gilbert and Scott, 2000). Caffeine enhances urine production and gastric secretion. Also it is perhaps best known as a stimulant of the central nervous system; certain aspects of this action include the abatement of drowsiness and fatigue, and changes in appetite (Curatolo and Robertson, 1983).

Recently there is a widespread interest in the potential harmful effects of caffeine on fetal development when ingested by pregnant women. Caffeine metabolism becomes slower in pregnancy, and ingested caffeine easily crosses the placenta (Cook *et al.*, 1996). Although it has been suggested that the risk of fetal toxicity from caffeine in humans is low, several studies have shown that, moderate to heavy caffeine consumption increases the risk of spontaneous abortion or low fetal birth weight (Hey, 2007).

In this regard, animal studies have shown caffeine intake is a risk factor due to adverse reproductive effects (Jacombs *et al*., 1999 and Li *et al*., 2012). Caffeine is readily absorbed in the gastrointestinal system, and passes into blood and fetal tissues including the central nervous system, when it is administered to the mother (Matijasevich *et al*., 2005). The highest blood caffeine levels are reached 3 -120 minutes after ingestion, during which time it is rapidly distributed throughout body tissues, achieving equilibrium between blood and tissue levels (Golding, 1995). It has been suggested that the immature brain is very susceptible to caffeine. It can pass through biological barriers and acts on the immature brain, altering several biochemical and behavioral parameters (Da Silva *et al*., 2005) that seems to have some effects on the expression levels and distribution of adenosine receptors (Montandon *et al*., 2006), cause transient motor impairments (Tchekalarova *et al*., 2005)

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and be neurotoxic to the newborn rat (Kang *et al*., 2002). The majority of earlier developmental neurotoxicity studies on caffeine focused on postnatal subjects rather than on the fetus. In the present work neurotoxicolgical studies were conducted in fetuses, focusing on the developmental profiles of nucleic acids, proteins, and monoaminergic metabolism in immature rats brain and their impact on brain development.

Material and Methods

Chemicals:

Caffeine of 99% purity (caffeine monohydrate, Sigma Chemical Company St. Louis, MO 63103, USA.) was used in the experiment. The molecular formula is $C_8H_{10}N_4O_2.H_2O$ and the molecular weight is 212.2.The caffeine was dissolved in distilled water administered intragastrically *via gavage tube* at a dose of 75 or 120 mg/kg b.wt.

Animals:

The experiment was carried out on adult female albino Wister rats strain (175-190 g) and adult males of the same strain (200-220 g) were obtained from Animal House of EL-Salam Farm, Giza, Egypt. The animals' room was maintained at approximately 25 ± 2 °C with a 12-hour light/12-hour dark cycle and relative humidity (50 ± 10). Tap water and standard laboratory pellets rat chow (NRC, 1995) were provided ad libitum. After fifteen days of acclimatization, pregnancy was established by housing females in the pro-estrous stage with sexually potent males overnight. The next morning, females with positive vaginal smears were considered pregnant, and the day of detection was defined as the zero day of pregnancy. Experiment was carried out according to the internationally valid guidelines and the institutional animal ethics committee.

Experimental design:

Thirty pregnant females were randomly divided into three groups 10 rats each, treated orally *via gavage* tube with caffeine dissolved in 1 ml distilled water at doses of 0 (control group), 75 (C-75 group) or 120 (C- 120 group) mg/kg bwt at 8:30- 9:00 am daily from the 1st to the 19th day of gestation. The doses were based on earlier studies in the rat (Christian and Brent, 2001). Animals were observed daily for clinical signs of toxicity. Maternal body weights were recorded on gestational day (GD) 0, 6, 14 and 20. On GD 20 the rats were sacrificed under ether anesthesia.

Serum and amniotic fluid biochemical parameters:

Blood samples were taken from heart of pregnant females. The samples were left at 37 °C for 15 min. and then centrifuged at 3000 rpm for 20 min. Serum was separated and kept at −20 °C in plastic vials until analysis. Amniotic fluid from each litter was collected from the fetal sacs by syringe and pooled before freezing at −20 °C until analysis. The serum and amniotic fluid concentrations of calcium, phosphorus and the total protein content were estimated by using colorimetric method kits (Biggs and Moorehead, 1974; EL-Merzabani *et al.*, 1977 and Doumas, 1975), respectively.

Pregnancy outcome:

The uteri were removed and weighed. The uterine contents were examined to determine the number of the implantation sites, resorptions, the number and location of viable and nonviable fetuses. The number of corpora lutea in each ovary was recorded. The pre implantation and post implantation loss was calculated as follows: Preimplantation loss= [(number of corpora lutea –number of implants) / (number of corpora lutea) x 100], and post implantation loss= [(number of implants –number of viable fetuses) / (number of implants) x 100]. Corrected maternal body weight gain (final body weight minus uterus weight minus body weight at GD 0) was calculated. Live fetuses were weighed and examined for external malformations including those of the oral cavity. Fetal brain was dissected on ice, weighed and pooled before freezing at −20 °C for estimation of caffeine, monoamines, DNA, RNA and total protein content.

Caffeine levels in the maternal serum and fetal brain:

The extraction of caffeine from serum and brain was performed as previously described by Kaplan *et al.* (1989) using high performance thin layer chromatography (HPTLC) system Camag Software Wincats, Camag TLC scanner 3. Fetuses' brains were weighed and homogenized. The homogenate was extracted in 5 ml of

chloroform, agitated in a vortex mixer and then centrifuged. The lower organic phase was transferred by pipette to conical centrifuge tubes. The same extraction procedure was performed an additional two times and the contents of the three organic phases were combined and evaporated at room temperature in the conical tubes. The residue for HPTLC analysis was reconstituted in the mobile phase. Serum samples were extracted in 4 ml of ethyl acetate:isoamyl alcohol (98:2) by agitation in a vortex mixer. The solution was centrifuged at room temperature and the upper organic layer was transferred to a conical centrifuge tube and evaporated to dryness. The residue for HPTLC analysis was reconstituted with 300 µl of mobile phase.

Determination of brain fetuses' monoamines:

Determination of brain 5-Hydroxytryptamine (serotonin), noradrenaline, and dopamine was carried out using high performance liquid chromatography (HPLC) system, Agilent technologies 1100 series, equipped with a quaternary pump (Quat pump, G131A model). Separation was achieved on ODS-reversed phase column. The mobile phase consisted of potassium phosphate buffer/methanol 97/3 (v/v) and was delivered at a flow rate of 1 ml/min. UV detection was performed at 270 nm, and the injection volume was 20 µl. The concentration of both catecholamines and serotonin were determined by external standard method by using peak areas. Serial dilutions of standards were injected, and their peak areas were determined. A linear standard curve was constructed by plotting peak areas versus the corresponding concentrations. The concentration in samples was obtained from the curve.

Determination of brain fetuses DNA, RNA and Total protein content:

Extraction of DNA and RNA were performed by using a QIAamp DNA Mini kit (QIAGEN, Merck Euro lab, Stockholm, Sweden), which isolates nucleic acids by a spin column elution method (Sambrook and Russell, 2001) using a UV spectrophotometer at wavelength of 260 nm. Protein content was determined by the method of Lowry *et al.* (1951).

Histopathological study:

For histopatholgical examination, whole fetal rat brains were fixed in 10% buffered formalin, dehydrated with ethanol series, paraffin embedded, and then fetal rat brains were coronally sectioned at 5 micrometer thickness through the frontal cerebral cortex. The sections were stained with hematoxylin and eosin (H & E) then checked for histological alterations by using a light microscope (Humason, 1979).

Statistical analysis:

Data were expressed as the mean ± standard error (SE). Statistical analysis was performed by using the statistical package for social science (SPSS) version 17 statistical software. Data were analyzed by one-way analysis of variance followed by post hoc-least significant difference analysis. The values were considered statistically significant at levels $p < 0.05$, $p < 0.01$ and $p < 0.001$. Percentage of difference representing the percent of variation with respect to the control was also calculated.

Results:

Evaluation of maternal and fetal toxicity:

There was no maternal death or abnormal behavior observed in any group throughout the whole experimental period. Gestational administration of caffeine at low dose (75 mg/kg b.wt.) induced a significant decrease in maternal body weight gain only in the first week of gestation. On the other hand, caffeine significantly reduced the weight gain of mothers treated with the higher dose (120 mg/kg b.wt.) along the experimental period. The corrected weight gain was significantly decreased ($P<0.01$) in C-120 group only (Table 1). The number of corpora lutea, implantation sites, live fetuses, resorptions, preimplantation and postimplantation loss in the treated groups did not show any significant difference compared to the control group (Table 2). No differences in the number of dead fetuses or external malformations were observed between the treated groups and the control group, except for an insignificant number of subcutaneous hematomas detectable in the 120 mg/kg bwt dosage group. The gravid uterus weight was significantly decreased ($P < 0.01$) only in the C- 120 group. Fetal body weights were significantly reduced in rats of both treated groups compared to the control group (Table 2).

Table 1: Gestation body weight gain (g) of control dams and dams treated with caffeine at doses of 75 and 120 mg/kg bwt. (Values are mean ± SE.)

Gestation interval	Control	C-75	C-120
0-6 GD	16.13 ± 0.85	6.25 ± 1.54[c]	6.50 ± 1.53 [c]
6- 14 GD	23.5± 1.19	24.5 ± 0.30	13.7 ± 2.81 [a]
14-20 GD	52.4 ± 3.77	49.6 ± 1.52	39.3 ± 4.79 [a]
Corrected wt gain	43.8 ± 2.11	41. 7 ± 3.28	31.8 ± 1.70 [b]
Corrected weight gain = Final body weight-uterus weight – gestation day 0 body weight (g).			
a, b & c : compared with the control group at P<0.05, P<0.01 & P<0.001, respectively.			

Table 2: Pregnancy outcome of control dams and dams treated with caffeine at doses of 75 and 120 mg/kg bwt.

	Control	C-75	C-120
No. pregnant rats	10	10	10
Corpora Lutea number/litter	10.13 ± 0.35	10.75 ± 0.75	9.87 ± 0.58
Implants/litter	9.63 ± 0.32	10.25 ± 0.82	9.00 ± 0.65
No. desorbed & dead /litter	0.13 ± 0.13	0.25 ± 0.16	0.75 ± 0.49
Preimplantaion loss index-%	4.78 ± 1.83	4.85 ± 2.65	8.77 ± 4.01
Postimplantaion loss index-%	1.25 ± 1.25	2.29 ± 1.51	6.81 ± 4.15
No. litters with viable fetuses	10	10	10
No. litter with resorption	1	2	3
No. viable fetuses/litter	9.50 ± 0.33	10.00 ± 0.80	8.25 ± 0.45
Gravid uterus weight (g)	51.25 ± 2.30	48.54 ± 2.49	38.99 ± 1.53[b]
Body weight of fetuses (g)	3.96 ± 0.07	3.21 ± 0.11[b]	2.95± 0.08[c]
Values are mean ± SE. b &c : compared with the control group at P<0.01 & P<0.001, respectively			

Caffeine levels in the maternal serum and fetal brain:

Caffeine concentrations of dams serum and fetal brain were elevated significantly with the increase of caffeine dosage (P<0.001) compared to the control group (Table 3).

Table 3: Maternal serum and fetal brain caffeine levels of control dams and dams treated with caffeine at doses of 75 and 120 mg/kg bwt.

	Control	C-75	C-120
Maternal serum (µg/ml)	0.19 ± 0.06	6.86 ± 0.10[c]	8.20 ± 0.11[c]
Fetal brain (µg/g tissue)	0.14 ± 0.06	4.65 ± 0.22[c]	6.66 ± 0.48[c]
Values are mean ± SE. c: compared with the control group at *P*<0.001.			

Calcium, phosphorus and total protein content in amniotic fluid and maternal serum:

Caffeine ingestion by pregnant rats at a dose of 75 or 120 mg/kg b.wt. did not significantly alter calcium or phosphorus level in maternal serum and amniotic fluid. On the other hand, total protein content was significantly decreased in maternal serum in C-120 group by -16.3% when compared to control group. Caffeine treatment at the low dose (75 mg/kg b.wt.) did not affect the total protein contents of maternal serum (Table 4).

Table 4: Calcium (Ca), phosphorus (P) and total protein (T.P.) content in amniotic fluid and serum of control dams and dams treated with caffeine at doses of 75 and 120 mg/kg bwt.

	Amniotic fluid			Serum		
	Ca (mg/dl)	P(mg/dl)	T. P. (g/dl)	Ca (mg/dl)	P(mg/dl)	T. P. (g/dl)
Control	5.31± 0.58	3.93 ± 0.26	0.471 ± 0.03	10.96 ± 0.40	6.35 ± 0.41	7.35 ± 0.17
C-75	6.25± 0.52	4.08 ± 0.24	0.466 ± 0.02	11.03 ± 0.37	5.82 ± 0.48	6.90 ± 0.27
C-120	6.13± 0.48	4.03 ± 0.29	0.450 ± 0.04	11.85 ± 0.41	6.37 ± 0.47	6.15 ± 0.18[b]
Values are mean ± SE. b: compared with the control group at P<0.01.						

Nucleic Acids and Total protein in fetal brain:

Administration of caffeine to pregnant rats produced a significant decrease (P< 0.01) in brain nucleic acids content in C-120 group (DNA: - 31.7% and RNA: - 31.3%) compared to control. The exposure produced also a significant decrease (- 33.6%) in the content of total protein (P<0.001) (Table 5). Caffeine ingestion at low dose (75 mg/kg b.wt.) did not affect the nucleic acids or protein contents in fetal brain (Table 5).

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Table 5: Cerebral DNA, RNA and total protein (T.P.) contents of control fetuses and fetuses maternally treated with caffeine at doses of 75 and 120 mg/kg bwt.

	Control	C-75	C-120
DNA (mg/g tissue)	2.93± 0.15	2.73± 0.19	2. 00± 0.11[b]
RNA(mg/g tissue)	2.33 ± 0.12	2.20 ± 0.15	1.60 ± 0.10[b]
T. P. (mg/g tissue)	74.23 ± 4.29	70.45 ± 1.60	49.24 ± 4.82[c]
Values are mean ± SE , b &c : compared with the control group at P<0.01 & P<0.001, respectively.			

Fetal Brain Monoamines:

Caffeine ingestion by pregnant rats at a dose of 75 or 120 mg/kg bwt resulted in a significant decrease of norepinephrine (P<0.001) in the brains of developing fetuses by -40.9% and -52.8%, respectively. On the other hand, a significant increase in dopamine level was observed in C-75 and C-120 groups by 19.8% and 33.7%, respectively; whereas 5-Hydroxytryptamine level was not affected by the caffeine exposure compared to control group (Table 6).

Table 6: Norepinephrine (NE), Dopamine (DOPA) and 5-Hydroxytryptamine (5-HT) of Brain control fetuses and fetuses maternally treated with caffeine at doses of 75 and 120 mg/kg bwt.

	Control	C-75	C-120
NE (ng/g tissue)	10.34± 0.46	6.11 ± 0.67[c]	4.88 ± 0.68[c]
DOPA (ng/g tissue)	17.82± 0.60	21.36 ± 0.79[a]	23.83 ± 1.79[b]
5-HT(µg/g tissue)	0.34± 0.049	0.30 ± 0.063	0.22 ± 0.020
Values are mean ± SE, a, b& c: compared with the control group at *P*<0.05, *P*<0.01 & *P*<0.001, respectively			

Histopathological analysis:

Histopathological examination of fetal cerebrum revealed reduction in the thickness of cerebral cortex layers, the cortical plate (CP), intermediate (migratory) zone (IZ) and ventricular (proliferative) zone (VZ), with the massive dilation of the ventricles (V) in caffeine –treated groups. Morphological evidence of neuronal or glial cell death via apoptosis was suggested by hyperchromatic, condensed nuclei within cortex in fetuses exposed to the higher dose of caffeine (120 mg/kg bwt). Also, caffeine induced severe vascular congestion, reduction in cell population, shrunken cells and devastation zones. The severity of those histological alterations was dose dependent (Fig. 1).

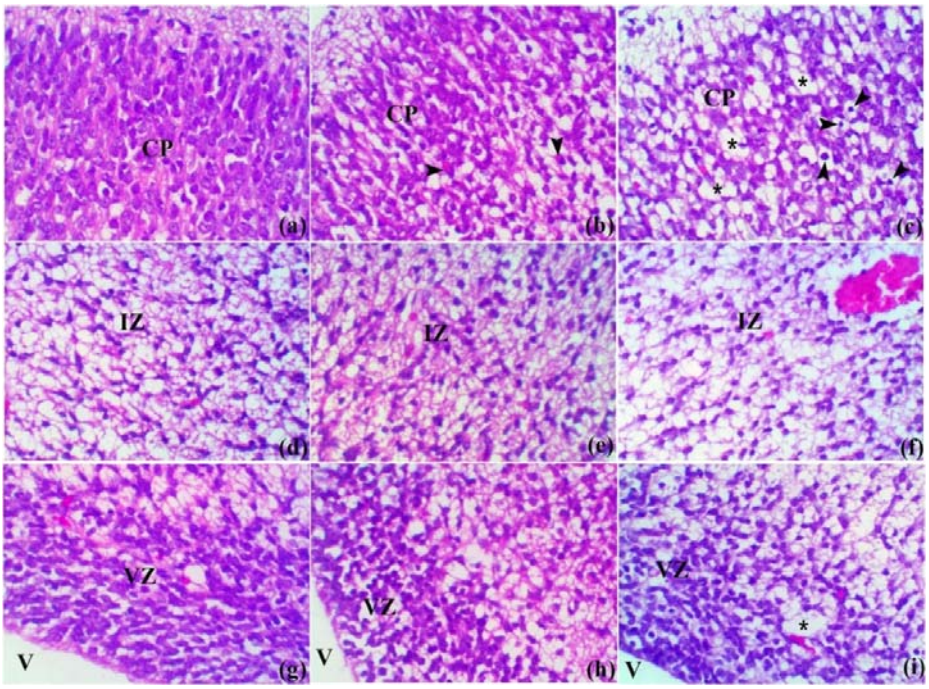

Fig. 1: Photomicrographs of coronal sections of cerebral cortex of 20-days-old fetuses. **(a)** cortical plate (CP) of control, **(b)** CP of fetus exposed to 75 mg/kg bwt caffeine showing shrunken and hyperchromatic cells (arrow head), and **(c)** represents the CP of 120 mg/kgbwt caffeine exposed fetus showing hyperchromatic and condensed nuclei(arrow head), devastation zones (asterisks) and reduced neuronal

density. **(d &e)** represents the intermediate (migratory) zone (IZ) of control and fetus exposed to 75 mg/kg bwt caffeine, respectively showing the normal appearance and distribution of the migratory cells in intermediate zone, and **(f)** represents IZ of fetus treated with 120 mg/kg bwt caffeine showing lower cellularity as well as congestion and dilatation of the blood vessel. **(g)**the ventricular or proliferative zone (VZ) and the lateral ventricle (V) of the control, **(h&i)** VZ of fetuses exposed to 75 and 120 mg/kg bwt caffeine, respectively showing dose dependant reduction in cell population, alteration of their distribution and devastation zone (asterisk). (HE – x 400 magnifications).

Discussion:

In the present study, daily maternal exposure to caffeine at the two studied doses (75 and 120 mg/kg b.wt.) throughout gestation did not produce significant alterations in the number of implantation, live fetuses or pre- or post- implantation loss in the treated dams. However, a significant reduction of fetal body weight was apparent in both doses that may be indicated that fetuses were more susceptible to caffeine at late stages of pregnancy than early stages. Jacombs *et al. (*1999) and Pollard *et al.* (1999) reported that much higher caffeine doses do not influence conception and preimplantation embryo development in the rat animal model. They did report that, caffeine affects postimplantation embryo development, which is consistent with the present results. It is possible that the interferences caused by caffeine in the fetal growth, could be due to toxicological alterations which can cause retardation in growth or development of specific organs and/or systems can create anatomic and biochemical alterations that generally occur in organisms in the late period of development (Bernardi, 1996).

On the other hand, the lack of any significant effect on the maternal weight gain during pregnancy of caffeine treated animals at dose 75 mg/kg bwt compared to the control indicated that the selected dose did not affect the dams in any overt manner. This suggested that any effects on the fetuses did not result from maternal under-nutrition and is not the result of obviously impaired dams. However, at the higher dose (120 mg/kg bwt) the observed fetal growth retardation may be a consequence of maternal toxicity as indicated by the significant decrease in maternal serum total protein content and the direct effect of caffeine on developing fetuses.

It is well established that caffeine is rapidly transferred from mother to fetus. The present results suggested that the concentration of caffeine was almost the same in maternal serum and fetal cerebrum. This could be explained by the fact that substantial quantities pass into the amniotic fluid and the umbilical cord blood (Fernandes *et al*., 1998). It could cross the blood–brain barrier, stored and accumulated in the brain without being metabolized (Li *et al*., 2012). Because caffeine rapidly crosses the placenta, fetal caffeine levels appear to rapidly equilibrate with maternal levels (Kimmel *et al*., 1984). Caffeine half-life was increased during early life due to lower activity of cytochrome P-450 (Aranda *et al*., 1979) and to the relative immaturity of some demethylation and acetylation pathways (Carrier *et al*., 1988).

Many animal experiments had studied the effects of caffeine during pregnancy. However, the exact consequence of caffeine on fetal neurodevelopment remains uncertain. During fetal maturation, monoamine neurotransmitters played an important role on brain development by transmitting maturation signals to organs and tissues (Lauder, 1993). But to some extent these maturation signals can be interrupted by exogenous substances, which in turn cause deficits of fetus (Li *et al*., 2012). The present study showed a significant increase in dopamine level in fetuses exposed to caffeine at both tested doses. The significant increase in the fetal cerebral level of dopamine is similar to that observed in the adult rat brain (Kirch *et al*., 1990). At doses achieved in normal human consumption, the only effect mediated by caffeine seems to be antagonism of adenosine. The biochemical mechanism that underlies the actions of caffeine in relevant concentrations for human consumption was blockade of adenosine A1 and A2A receptors (Fredholm *et al.,* 1999). Adenosine A1 receptors modulated synaptic activity by inhibiting the release of several neurotransmitters and were highly distributed in the central nervous system, with particularly high concentrations in the hippocampus, cortex, cerebellum, and thalamus (Dunwiddie and Masino, 2001). Thus, caffeine, by antagonizing the effects of endogenous adenosine, could facilitate dopaminergic neurotransmission by stimulating dopamine release and by potentiating the effects of dopamine receptor stimulation (Ferre´ *et al.,* 1997). On the other hand, Datta *et al.* (1996) found that chronic caffeine treatment increased expression of tyrosine hydroxylase mRNA in the ventral segmental area and substantia nigra pars compacta of rats given 20-80 mg/kg caffeine for 9 days.

In the present study caffeine consumption by pregnant rats at doses 75 and 120 mg/kg bwt resulted in a significant decrease of norepeniphrine and non significant decrease in 5-hydroxytryptamine in the brains of developing fetuses. In the central nervous system, 5-hydroxytryptamine and norepinephrine are interdependent, with the presence of one strengthening the effect of the other Jisheng (1993). The elevated dopamine level accompanied by a significant decrease in norepinephrine level in the brains of developing fetuses in the present study, suggesting the possible blockage of the pathway that transforms dopamine into norepinephrine or possibly due to suppression of the relevant metabolic enzymes. Daly and Fredholm (1998) and Kang *et al.* (2002) suggested that, caffeine at high doses can inhibit phosphodiesterases, block $GABA_A$ receptors or cause a release of intracellular calcium. In the present work, caffeine consumption by pregnant rats at doses 75 and 120

mg/kg bwt did not significantly alter calcium or phosphorus level in maternal serum and amniotic fluid. This was agreeing with Barone *et al.* (1993) who found no changes in serum calcium level in their study carried out in rats at 4 weeks of age.

The present study confirmed that caffeine given during gestation at both tested doses induced dose dependant histological alterations in the brain of developing fetuses such as reduction in cell population, severe vascular congestion and cell death. The influence of caffeine in fetal development period appears to be an inhibitor of DNA synthesis. From the present results DNA, RNA and protein content of fetal brain was significantly reduced in fetuses exposed to the high dose, which may suggest the inhibition of nucleic acids and protein synthesis and subsequent cell replication. It had been described that caffeine blocked DNA replication ultimately leading to double-strand breaks in *Escherichia coli* (Kang *et al.,* 2012). Furthermore, it could bind to DNA with higher affinity to damaged regions and thus interferes with the specific binding of repair enzymes (Selby and Sancar, 1990). Previous studies reported that caffeine not only inhibits DNA but also RNA and protein synthesis in yeast and *Escherichia coli* (Goth and Cleaver, 1976) and protein synthesis in fetal cerebrum in rats (Tanaka *et al.,* 1983). Zeidán-Chuliá, *et al.* (2013) reported that caffeine may exert neurotoxicological effects in part by disruption of redox homeostasis. It had been suggested that caffeine may affect cell cycle function, induce programmed cell death or apoptosis and may perturb key regulatory proteins, including the tumor suppressor protein, p53 (He *et al.,* 2003). Black *et al.* (2008) reported that, alteration in cell death was observed in the cerebral rat pups very shortly after exposure to caffeine. Thus, it is possible that prenatal caffeine exposure at high doses could induce mitotic disturbance of neurons at various stages of neuronal proliferation and differentiation. Accordingly, the development and functions of the brain could be adversely affected.

It could be concluded that caffeine administration to pregnant rats at both tested doses can result in alterations in the steady-state concentrations of some, but not all neurotransmitter substances in the developing fetuses brains. Also it can interfere with neuronal proliferation in the fetal brain reflecting in abnormal cerebral development that could lead to postnatal neurobehavioral disturbances.

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