Effects of Maternal Caffeine Consumption on Ovarian Follicle Development in Wistar Rats Offspring

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Abstract

Background: In recent years concerns have been raised about human reproductive disorders, specially the effects of environmental factors on human fertility and pregnancy outcome. Therefore, the present study was designed to assess the effects of maternal caffeine consumption on ovarian follicles development in rat offspring.

Methods: 60 pregnant female rats were randomly divided into a control and two experimental groups. The rats in the two experimental groups received caffeine via drinking water during gestation (26 and 45 *mg/kg*) and lactation (25 and 35 *mg/kg*). The ovaries of the offspring were removed at 7, 14, 28, 60, 90 and 120 days after birth, and fixed in Bouin's solution. By preparing serial tissue sections, structural changes in ovarian follicles and corpora lutea were studied during postnatal development.

Results: The weight of ovaries decreased significantly ($p<0.05$) in the high dose caffeine-treated group at all stages of postnatal development. Significant ($p<0.05$) decreases were seen in the number of primordial follicles from day 7 to 120 after birth in the high dose caffeine-treated group. Moreover, the number of primary and secondary follicles decreased significantly on days 7, 14 and 28 as did the number of antral follicles on days 14 and 28 after birth ($p<0.05$) in the high dose caffeine-treated group. The diameter of secondary and antral follicles decreased significantly ($p<0.05$) in high dose caffeine-treated group on the early days of postnatal development. No statistically significant differences were seen in the number of corpora lutea between the groups.

Conclusion: The present study shows that caffeine consumption during gestation and lactation affects the early stages of ovarian follicle development and reduces reproductive efficiency in the offspring of Wistar rats.

Keywords: Caffeine, Fertility, Ovarian follicles, Postnatal development.
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Background

In recent years, concerns have been raised regarding the increases in human reproductive disorders. Exposure to environmental and occupational toxicants and progressive changes in many aspects of lifestyle, including dietary habits, has been shown to deteriorate reproductive health, thus, affecting the ability of couples to conceive and maintain a healthy pregnancy (1).

Among dietary factors, caffeine consumption has been found to adversely affect reproduction (2).

Caffeine is a naturally occurring alkaloid found in the seeds, leaves and fruits of more than 60 plants such as in coffee and cocoa beans, kola nuts, and tea leaves. Caffeine is present in a wide variety of foods and beverages including coffee, tea, cocoa, chocolate, colas and energy drinks, and also in a number of prescription drugs including cold and flu remedies, headache treatments, diet pills, diuretics and stimulants (3). The amount of caffeine in foods and beverages varies widely depending on

the type of product and the preparation methods. In general, brewed coffee contains the highest amount of caffeine, with an average of 137 *mg* per an-8-ounce cup and chocolate contains the lowest amount of caffeine (4). The total daily intake of caffeine from all sources is estimated to be about 3-7 *mg/kg* per day or 200 *mg/day* (5). Moreover, it has been shown that caffeine in shampoos is absorbed through the skin, mostly through hair follicles, and reaches the blood stream (6).

Peak plasma caffeine concentration is reached between 15-120 *min* after oral ingestion in humans. Caffeine half-life is about three hours but it increases to 5.6 hours in the first trimester and to 18 hours in the 35th week of pregnancy (7). Caffeine half-life increases to 80 hours during neonatal period due to the decreased activity of cytochrome P-450 (8). Caffeine crosses the placenta easily and is found in both the fetus and in newborn infants (9).

Caffeine consumption has been found to be associated with low birth weight, small head, cleft palate, retardation, miscarriage or spontaneous abortion, increased incidences of digital defects and delayed ossification (9, 10). Wilcox et al. found a 50% reduction in the probability of conception per cycle with tintake of one cup of coffee per day (11). Jagilo et al. reported that subcutaneous administration of 25 *mg/kg* per day of caffeine prevented hormonally induced superovulation in adult mice (12). Similarly, it was found that oral gavage of 136 *mg/kg* per day of caffeine, 5 days per week for a total of 100 days, blocks normal oogenesis in adult rats (13). Furthermore, it has been shown that caffeine prolongs the meiotic arrest of porcine oocytes at the germinal vesicle stage (14). However, epidemiological studies in Danish women showed no association between delayed conception and consumption of caffeinated beverages (15, 16). Likewise, Caan et al. found that women who drank more than half a cup of tea per day had a significant increase in fertility (17). Furthermore, caffeine administeration during pregnancy did not affect the rate of early mitotic proliferation of germ cells at day 20 of pregnancy in rats, nor did it prevent their development or decrease their meiosis (18). Caffeine treatment can prevent the decline in the developmental potential of ovine oocytes matured and aged in vitro (19).

Regarding the conflicting results of epidemicological studies on caffeine and its effects on reproductive outcomes (20) and caffeine-containing foods and beverages are so widely consumed by most human populations of the world, its health effects have been and are still being studied extensively. The present study was designed to investigate the effects of maternal caffeine consumption during gestation and lactation on postnatal development of ovaries in the offspring of Wistar rats.

Methods

Animals: Wistar rats were obtained from animal house of Jondishapour Medical Sciences University of Ahwaz and kept under specific conditions on a constant 12-hour light/dark cycle and at a controlled temperature of $22 \pm 2° C$. Standard pellet food and distilled water were available ad libitum. Female Wistar rats (100 ± 10 days old) were mated overnight at a 3:1 females:male ratio. Vaginal smears were daily prepared and examined for the presence of sperm. The day of sperm detection in vaginal smears was considered day 0 of pregnancy. 60 Pregnant female Wistar rats were randomly divided into three equal groups; a control group and two treatment groups. The two treatment groups, respectively received caffeine via drinking water during gestation (26 and 45 *mg/kg*) and lactation (25 and 35 *mg/kg*) (23, 24) i.e. until day 21 after birth. The doses were established from related reproductive and developmental studies. On days 7, 14, 28, 60, 90 and 120 five pups were randomly selected, weighed and their ovaries were removed under chloroform inhalation anesthesia. The ovaries were trimmed of fat and extraneous tissue, weighed and fixed by immersion in Bouin's solution for 24 hours.

Microscopic Study: Following tissue processing, 5 *μm* serial paraffin sections were prepared and stained by haematoxylin-eosin. For microscopic examination, sections were selected using a non-random 10% sampling method and ovarian follicles and corpora lutea were counted in every 10th section of the ovary (25), in a fashion that each counted section was separated by a distance of approximately 50-60 *μm* from the next 10th section.

Later, the mean number of ovarian follicles and corpora lutea of each ovary were calculated in the

selected sections. Differential follicle counting and categorizing were performed blindedly. Ovarian follicles were classified on the basis of ovarian follicle morphology. Follicles containing a single layer of squamous follicular cells were considered as primordial. A primary follicle contains an oocyte surrounded by a single layer of cuboidal follicular cells; the secondary follicle contains more than one layer of follicular cells around the oocyte. Follicles containing scattered spaces or a distinct antrum were considered as antral (26). All the follicles were classified as either healthy or atretic, respectively, according to the absence or presence of signs of oocyte and/or granular degeneration such as pyknosis of the nucleus, infolding of the cell wall in oocyte, ingression of granulosa cells within the antral cavity, pulling away of granulosa cells from the basement membrane, infolding or thickening of base membrane and uneven layers of granulosa cells (27).

For measuring the diameter of ovarian follicle in each developmental stage, 45 microscopic fields were randomly chosen in each rat. Then, using the ocular micrometer of a light microscope (Olympus EH, America Inc.), at a magnification of 10×, the diameter of each ovarian follicle were measured. To avoid recounting the same follicle, only individual follicles, containing an oocyte with a nucleus, were evaluated.

Statistical Analysis: The data were analyzed using SPSS, version 10.0 for Windows. Body and ovarian weights, the number and diameter of follicles and corpora lutea in the control and caffeine-treated groups were compared by one-way analysis of variance (ANOVA) and Tukey's post hoc test. Comparisons between different groups were made in each developmental stage, independent from other days of postnatal development. The differences were considered to be significant when $p < 0.05$.

Results

Mean body weight showed significant ($p < 0.05$) decreases in high dose caffeine-treated group in all stages of postnatal development in comparison with the control group. There were significant ($p < 0.05$) differences between the mean ovary weight in high dose caffeine-treated and control groups in all stages of postnatal development. No significant differences were seen between the mean body and ovary weight in low dose caffeine-treated and the control groups in all stages of postnatal development (Table 1).

The mean number of primordial follicles decreased significantly ($p < 0.05$) in high dose caffeine-treated group from the 7th to 120th day in comparison with the control group (Table 2).

Table1. Distribution of body weight (*gr*) and ovary weight (*mg*) in the controls and caffeine-treated offspring of Wistar rats during different stages of postnatal development (Mean±SEM)

Parameters	Age of Pups (day)	Groups		
		Control	Caffeine-treated	
			Low dose	High dose
Body weight (*gr*)				
	7	8.91±0.17[a]	7.84±0.41[a,b]	7.02±0.30[b]
	14	12.14±0.76[a]	11.35±0.28[a,b]	9.86±0.38[b]
	28	25.00±1.18[a]	22.75±1.21[a,b]	20.93±1.25[b]
	60	58.17±3.62[a]	52.96±2.89[a,b]	50.96±2.78[b]
	90	82.61±4.18[a]	77.14±3.62[a,b]	73.79±3.59[b]
	120	95.12±4.26[a]	91.65±4.01[a,b]	88.05±4.11[b]
Ovary weight (*mg*)				
	7	2.20±0.15[a]	1.80±0.18[a,b]	1.20±0.20[b]
	14	6.30±0.20[a]	5.80±0.31[a,b]	5.20±0.41[b]
	28	18.40±1.33[a]	17.50±1.24[a,b]	15.80±1.03[b]
	60	35.00±2.32[a]	32.70±2.22[a,b]	30.60±2.17[b]
	90	45.61±3.54[a]	40.90±3.44[a,b]	38.70±3.28[b]
	120	48.33±5.01[a]	46.60±4.41[a,b]	42.21±4.12[b]

a,b Different superscripts in the same row indicate significant difference between groups (P<0.05)

Table 2. The number of ovarian follicles in the control and caffeine-treated offspring of Wistar rats during different stages of postnatal development (Mean±SEM)

Follicular stage	Age of pups (days)	Groups		
			Caffeine-treated	
		Control	Low dose	High dose
Primordial F.				
	7	48.57±5.29[a]	44.57±4.27[a,b]	39.57±5.27[b]
	14	38.70±4.72[a]	36.40±4.27[a,b]	32.10±3.27[b]
	28	25.50±3.14[a]	21.20±2.25[a,b]	17.10±1.36[b]
	60	19.00±1.67[a]	16.33±1.21[a,b]	11.41±1.28[b]
	90	17.66±1.46[a]	12.73±1.18[a,b]	10.33±1.16[b]
	120	15.32±1.25[a]	13.87±1.20[a,b]	10.65±1.04[b]
Primary F.				
	7	35.90±4.97[a]	28.10±3.21[b]*	25.90±2.39[b]**
	14	30.40±3.27[a]	22.90±2.27[b]*	20.40±2.13[b]**
	28	18.08±2.25[a]	9.16±1.18[b]*	8.75±1.11[b]**
	60	8.26±1.21	6.20±0.27	5.80±0.22
	90	8.40±1.38	5.13±0.24	6.53±0.62
	120	6.43±0.03	6.30±0.25	5.05±0.23
Secondary F.				
	7	3.00±0.15[a]	1.90±0.11[b]*	1.50±0.11[b]**
	14	16.80±1.45[a]	10.70±1.28[b]*	9.00±1.15[b]**
	28	11.75±1.41[a]	5.75±0.22[b]*	4.00±0.22[b]**
	60	8.10±1.20	6.30±0.28	5.90±0.19
	90	3.93±0.15	3.50±0.14	3.44±0.14
	120	4.64±0.17	3.55±0.18	3.84±0.16
Antral F.				
	7	N	N	N
	14	3.62±0.15[a]	1.25±0.10[b]*	1.04±0.10[b]**
	28	4.83±0.18[a]	2.04±0.12[b]*	2.00±0.12[b]**
	60	4.80±0.17	3.30±0.15	3.20±0.16
	90	5.07±0.28	4.41±0.21	4.23±0.18
	120	7.65±0.41	6.71±0.30	6.46±0.28

a,b Different superscripts in the same row indicate significant difference between groups (P<0.05)
* p<0.01, ** p<0.01
F: Follicle, N: Not seen

Significant ($p<0.05$) decreases were observed in the mean number of primary and secondary follicles on days 7, 14 and 28 in both treatment groups in comparison with the controls. There were no significant differences between the mean number of primary and secondary follicles in the controls and both treatment groups on days 60, 90 and 120 (Table 2).

Antral follicles were not seen on day 7 in any of the experiment groups. The mean number of antral follicles decreased significantly ($p<0.05$) in both treatment groups on days 14 and 28 in comparison with the control group (Table 2). No significant differences were seen between the mean number of antral follicles in the control and both treatment groups on days 60, 90 and 120 (Table 2).

There were no significant differences between the means of primordial and primary follicles in diameter in the control and treatment groups in all stages of postnatal development. The mean for secondary follicles diameter decreased significantly ($p<0.05$) in high dose caffeine-treated group on days 7, 14, 28 and 60 in comparison

Table 3. The diameter of ovarian follicles (μm) in control and caffeine-treated offspring of Wistar rats during different stages of postnatal development (Mean±SEM)

Follicular stage	Age of Pups (day)	Groups		
		Control	Caffeine-treated	
			Low dose	High dose
Primordial F.				
	7	19.50±1.80	19.30±2.41	18.90±1.08
	14	20.25±2.85	19.59±1.82	19.30±1.32
	28	21.60±3.10	20.92±2.73	20.25±2.36
	60	21.35±3.83	20.74±2.55	20.79±1.47
	90	20.90±2.90	20.25±1.18	20.43±2.16
	120	20.74±2.45	20.32±2.20	20.45±2.24
Primary F.				
	7	45.29±2.28	44.74±2.20	44.30±2.10
	14	50.24±3.26	49.73±2.67	48.55±2.30
	28	50.30±3.31	50.25±3.28	49.58±2.03
	60	51.55±2.01	50.67±3.39	50.38±2.33
	90	51.80±3.84	51.35±2.53	50.61±3.36
	120	51.84±2.86	51.40±2.57	50.80±3.41
Secondary F.				
	7	70.10±3.27[a]	68.20±4.10[a,b]	61.30±3.87[b]
	14	88.72±4.70[a]	83.59±3.47[a,b]	78.55±4.31[b]
	28	109.89±4.41[a]	100.81±4.05[a,b]	94.65±3.34[b]
	60	112.93±5.47[a]	106.88±5.49[a,b]	99.37±4.83[b]
	90	116.39±5.56	112.32±4.61	110.33±3.26
	120	117.65±4.60	114.71±5.10	111.46±5.39
Antral F.				
	7	N	N	N
	14	200.00±6.12[a]	196.62±4.47[a,b]	190.15±4.67[b]
	28	201.90±5.60[a]	199.05±5.25[a,b]	192.74±5.10[b]
	60	205.09±6.03[a]	201.31±6.42[a,b]	195.17±4.80[b]
	90	220.80±5.22	218.55±5.11	215.23±6.21
	120	222.65±5.80	220.71±6.16	217.42±5.33

a,b Different superscripts in the same row indicate significant difference between groups (P<0.05)
F: Follicle, N: Not seen

with the controls. Moreover, significant ($p<0.05$) decreases were seen between the mean of antral follicle diameter in the control and high dose caffeine-treated group on the 14th, 28th and 60th days (Table 3).

There were significant ($p<0.05$) increases in the mean number of atretic follicles on days 7, 14 and 28 in both treatment groups in comparison with the controls. No statistically significant differences were seen between the mean number of corpora lutea in the controls and both treatment groups on the 60th, 90th and 120th days.

Discussion

The aim of present study was to establish whether in utero and lactational exposure of Wistar rats to caffeine affects ovarian development in their offspring during pre- and post pubertal development. The results clearly showed that high dose maternal caffeine consumption during gestation and lactation significantly reduced ovarian weight and primordial follicle population and diminished fertility and reproductive efficiency in the offspring of Wistar rats. These alterations in the ovary were associated with a significant growth retardation of the female off-

spring. It seems that reductions in the number of primordial follicles, the number and diameter of secondary and antral follicles and the increase in the number of atretic follicles caused a significant decrease in ovarian weight in different stages of postnatal development.

In this study, reduction in number of primordial, primary and secondary follicles increased from birth to 28 days on wards but decreased then after, up to the end of the study (120th day) in both treatment groups. However, the highest ovarian weight decrease was seen on day 7 after birth which contiued until the 120th day in both treatment groups.

Female fertility depends on the supply and maturation of the ovarian germ cells, i.e., the oocytes and the differentiation and proliferation of ovarian somatic cells, or granulosa and thecal cells (28). During folliculogenesis, oocytes grow and they are surrounded by an increasing number of granulosa cell layers. From the preantral stage onward, theca cells differentiate outside the follicles (29). Improper ovarian differentiation or folliculogenesis, due to intra- or extra-ovarian regulation defects, often results in premature ovarian failure, leading to infertility (30). In rats, the formation of primordial follicles is completed by postnatal day three or four (31). Most primordial follicles remain quiescent, but some grow and undergo transition into the primary follicle stage. High numbers of preantral and antral follicles would be present from day 9 to 20 (32), and the first estrus and ovulation will occur between days 35-42 after birth (33).

The present study showed that caffeine treatment altered the number of primordial follicles in the resting pool from day 7 until day 120 in the high dose caffeine group.

For reproductive toxicants that cause direct damage to ovarian follicles, the stage of development at which follicles are destroyed determines the impact that exposure to the chemical will have on reproduction (34). Chemicals that extensively destroy primordial follicles cause permanent infertility and premature ovarian failure, since once a primordial follicle is destroyed, it cannot be replaced. Destruction of primordial follicles will have an irreversible effect on reproduction until such a time that recruitment for the number of growing and antral follicles can no longer be

supported (35, 36). It seems that caffeine could interfere with cell division resulting in decreased number of cells and cell death. Axelrod and Reichenthal described this hypothesis that caffeine acts by genetic damage to the cells replicating DNA (37). Comet assay studies indicate that caffeine-mediated increases in radiation risk of embryos is due to the inhibition of DNA repair (38). Furthermore, Jagiello et al. (1972) reported meiosis suppression by caffeine in female mice (9).

However, our findings indicated that unlike days 7, 14 and 28, there were no significant differences in the number of growing follicles on days 60, 90 and 120 after birth in the treatment groups. Moreover, the diameter of secondary and antral follicles and the number of corpora lutea in the treatment groups did not differ with that of the control group on days 60, 90 and 120 after birth. Thus, it seems that caffeine exposure has transient effects on follicular development and ovulation in the offspring of Wistar rats during the postnatal development. This could be due to the fact that caffeine does not accumulate in the body over the course of time and it is normally excreted within several hours of consumption. Chemicals that selectively damage large growing or antral follicles only temporarily interrupt reproductive function because these follicles can be replaced by recruitment from the greater pool of primordial follicles. Thus, these chemicals produce a readily reversible form of infertility that is manifested relatively soon after exposure period (35, 36).

Conclusion

The present study showed that maternal caffeine consumption during gestation and lactation affects the early stages of follicular development and reduces reproductive efficiency in the offspring of Wistar rats.

Acknowledgement

References

1. Hruska KS, Furth PA, Seifer DB, Sharara FI, Flaws JA. Environmental factors in infertility. Clin Obstet Gynecol. 2000;43(4):821-9.

2. Kumar S, Kumari A, Murarka S. Lifestyle factors in deteriorating male reproductive health. Indian J Exp Biol. 2009;47(8):615-24.

3. Al-Shoshan AA. Predictors of caffeine consumption among young women. Pak J Nutr. 2007;6(6):597-602.

4. Monroe J. Caffeine's hook. Curr Health. 1998;2:5-12.

5. Barone JJ, Roberts HR. Caffeine consumption. Food Chem Toxicol. 1996;34(1):119-29.

6. Otberg N, Teichmann A, Rasuljev U, Sinkgraven R, Sterry W, Lademann J. Follicular penetration of topically applied caffeine via a shampoo formulation. Skin Pharmacol Physiol. 2007;20(4):195-8.

7. Golding J. Reproduction and caffeine consumption--a literature review. Early Hum Dev. 1995;43(1):1-14.

8. Aranda JV, Collinge JM, Zinman R, Watters G. Maturation of caffeine elimination in infancy. Arch Dis Child. 1979;54(12):946-9.

9. Ramlau-Hansen CH, Thulstrup AM, Bonde JP, Olsen J, Bech BH. Semen quality according to prenatal coffee and present caffeine exposure: two decades of follow-up of a pregnancy cohort. Hum Reprod. 2008;23(12):2799-805.

10. Nolen GA. The effect of brewed and instant coffee on reproduction and teratogenesis in the rat. Toxicol Appl Pharmacol. 1981;58(2):171-83.

11. Wilcox A, Weinberg C, Baird D. Caffeinated beverages and decreased fertility. Lancet. 1988;2(8626-8627):1453-6.

12. Jagiello G, Ducayen M, Lin JS. Meiosis suppression by caffeine in female mice. Mol Gen Genet. 1972;118(3):209-14.

13. Boyd EM, Dolman M, Knight LM, Sheppard EP. The chronic oral toxicity of caffeine. Can J Physiol Pharmacol. 1965;43(6):995-1007.

14. Kren R, Ogushi S, Miyano T. Effect of caffeine on meiotic maturation of porcine oocytes. Zygote. 2004;12(1):31-8.

15. Olsen J. Cigarette smoking, tea and coffee drinking, and subfecundity. Am J Epidemiol. 1991;133(7):734-9.

16. Alderete E, Eskenazi B, Sholtz R. Effect of cigarette smoking and coffee drinking on time to conception. Epidemiology. 1995;6(4):403-8.

17. Caan B, Quesenberry CP Jr, Coates AO. Differences in fertility associated with caffeinated beverage consumption. Am J Public Health. 1998;88(2):270-4.

18. Pollard I, Williamson S, Magre S. Influence of caffeine administered during pregnancy on the early differentiation of fetal rat ovaries and testes. J Dev Physiol. 1990;13(2):59-65.

19. Maalouf W, Lee JH, Campbell KH. Effects of caffeine on the developmental potential of in vitro matured, aged and denuded ovine oocytes. Hum Fertil. 2005;8(2):129-30.

20. Grosso LM, Bracken MB. Caffeine metabolism, genetics, and perinatal outcomes: a review of exposure assessment considerations during pregnancy. Ann Epidemiol. 2005;15(6):460-6.

21. Moore RW, Rudy TA, Lin TM, Ko K, Peterson RE. Abnormalities of sexual development in male rats with in utero and lactational exposure to the antiandrogenic plasticizer Di(2-ethylhexyl) phthalate. Environ Health Perspect. 2001;109(3):229-37.

22. Odum J, Lefevre PA, Tinwell H, Van Miller JP, Joiner RL, Chapin RE, et al. Comparison of the developmental and reproductive toxicity of diethylstilbestrol administered to rats in utero, lactationally, preweaning, or postweaning. Toxicol Sci. 2002;68(1):147-63.

23. Hughes RN, Beveridge IJ. Behavioral effects of exposure to caffeine during gestation, lactation or both. Neurotoxicol Teratol. 1991;13(6):641-7.

24. Hughes RN, Loader VG. Effects on elevated plus-maze behavior of exposure to caffeine during both gestation and lactation. Psychobiology. 1996;24(4):314-9.

25. Bolon B, Bucci TJ, Warbritton AR, Chen JJ, Mattison DR, Heindel JJ. Differential follicle counts as a screen for chemically induced ovarian toxicity in mice: results from continuous breeding bioassays. Fundam Appl Toxicol. 1997;39(1):1-10.

26. Britt KL, Drummond AE, Cox VA, Dyson M, Wreford NG, Jones ME, et al. An age-related ovarian phenotype in mice with targeted disruption of the Cyp 19 (aromatase) gene. Endocrinology. 2000;141(7):2614-23.

27. Myers M, Britt KL, Wreford NG, Ebling FJ, Kerr JB. Methods for quantifying follicular numbers within the mouse ovary. Reproduction. 2004;127(5):569-80.

28. McGee EA, Hsueh AJ. Initial and cyclic recruitment of ovarian follicles. Endocr Rev. 2000;21(2):200-14.

29. Hoyer PB. Damage to ovarian development and function. Cell Tissue Res. 2005;322(1):99-106.

30. Guigon CJ, Magre S. Contribution of germ cells to the differentiation and maturation of the ovary:

insights from models of germ cell depletion. Biol Reprod. 2006;74(3):450-8.

31. Kezele P, Skinner MK. Regulation of ovarian primordial follicle assembly and development by estrogen and progesterone: endocrine model of follicle assembly. Endocrinology. 2003;144(8): 3329-37.

32. Hirshfield AN. Development of follicles in the mammalian ovary. Int Rev Cytol. 1991;124:43-101.

33. Rennels EG. Influence of hormones on the histochemistry of ovarian interstitial tissue in the immature rat. Am J Anat. 1951;88(1):63-107.

34. Hoyer PB, Sipes IG. Assessment of follicle destruction in chemical-induced ovarian toxicity. Annu Rev Pharmacol Toxicol. 1996;36:307-31.

35. Hooser SB, Douds DP, DeMerell DG, Hoyer PB, Sipes IG. Long-term ovarian and gonadotropin changes in mice exposed to 4-vinylcyclohexene. Reprod Toxicol. 1994;8(4):315-23.

36. Generoso WM, Stout SK, Huff SW. Effects of alkylating chemicals on reproductive capacity of adult female mice. Mutat Res. 1971;13(2):172-84.

37. Axelrod J, Reichenthal J. The fate of caffeine in man and a method for its estimation in biological material. J Pharmacol Exp Ther. 1953;107(4):519-23.

38. Müller WU, Bauch T, Wojcik A, Böcker W, Streffer C. Comet assay studies indicate that caffeine-mediated increase in radiation risk of embryos is due to inhibition of DNA repair. Mutagenesis. 1996;11(1):57-60.