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Morphological Aspects of Rat Ovaries When Exposed to Caffeine Containing Drink

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***Annotation*:** *Caffeine consumption increases in early adulthood, which has an adverse effect on the reproductive system. This study was aimed at assessing the effect of the embryonic effects of caffeine on the ovaries of rats in adulthood.*

Key words: *caffeine; follicle; ovary; Stereology.*

Caffeine is a methylated xanthine that acts as a mild central nervous system stimulant. It is present in many drinks, including coffee, tea and cola, as well as chocolate. Caffeine accounts for 1-2% of roasted coffee beans, 3.5% of fresh tea leaves and approximately 2% of mate leaves.Many over-the-counter medications, such as cold and allergy pills, headache medications, diuretics, and stimulants, also contain caffeine, although they result in relatively minimal consumption. Epidemiological studies suggest that one cup of coffee contains <or = 100 mg of caffeine, and soft drinks such as cola contain 10-50 mg of caffeine per 12-ounce serving. Caffeine consumption per capita from all sources is estimated at about 3-7 mg/kg per day or about 200 mg/day. Consumption of caffeinated beverages during pregnancy is quite common and is estimated at about 144 mg/day, or 2.4 mg/kg for a person weighing 60 kg. However, pregnant women seem to consume slightly less than other adults, approximately 1 mg/kg per day. This decrease may be related to taste aversion. The medical literature contains many different references that seem to indicate that caffeine causes adverse reproductive/developmental effects in humans. If caffeine does cause such effects, the reproductive consequences can be very serious, since caffeinated foods and beverages are consumed by the majority of the world's population, and consumption in the United States is estimated at 4.5 kg/person/year. Therefore, the medical literature concerning the developmental and reproductive risks of caffeine was reviewed, and the biological reliability of epidemiological and animal results, as well as methods and conclusions of previous researchers were evaluated. Epidemiological studies describe women's exposure to caffeine during pregnancy, as well as the occurrence of congenital malformations, fetal growth retardation, young children, miscarriages (spontaneous abortions), behavioral effects and maternal fertility problems that are presumably the result of caffeine consumption. Several

epidemiological studies have been devoted to the genetic effects of the preconceptional effects of caffeine. Animal studies conducted mainly on pregnant rats and mice were designed to produce malformations. The objectives of this review are to summarize the results of various clinical and animal studies, to objectively discuss the advantages and/or disadvantages inherent in research, and to create a global assessment of the reproductive risk of human caffeine consumption during pregnancy. It should be noted that the assessment of the risks of caffeine development, based solely on epidemiological studies, is difficult because the results are contradictory. Even more important is the fact that caffeine users are subject to a variety of confounding factors that make analysis difficult and prevent researchers from reaching final conclusions. For example, the caffeine content in foods and beverages can vary significantly, which can prevent reliable interpretations from being obtained from many human studies. Isolated epidemiological studies concerning the risk of abortion, without assessing other developmental and reproductive effects, are the most difficult to interpret because they present special problems that are sometimes ignored in epidemiological studies. The results of animal studies are probably most useful for solving some of the dilemmas created by epidemiological studies. An animal study published in 1960 first drew our attention to the potential developmental effects of caffeine. However, the exposure reported by Nishimura and Nakai was an intraperitoneal dosage of 250 mg/kg in a mouse, an extremely high dosage that would result in a blood plasma level that could never be obtained from consuming caffeinated foods. More recent animal studies have shown that, depending on the method of administration and the species, developing NOEL in rodents is approximately 30 mg / kg per day, teratogenic NOEL is 8,100 mg/ kg per day, and reproductive NOEL is approximately 80-120 mg / kg per day. The lack of biological plausibility to support the concept that caffeine is responsible for human malformations is another important part of this analysis. For example, no one has described the caffeine "teratogenic syndrome", a cluster of malformations associated with caffeine intake. Proven human teratogens have an identifiable syndrome. The malformations described in animal studies at very high doses correspond to the description of vascular destructive types of malformations.

Female Wistar rats (240-270 g) were divided into 5 groups (n = 6): experimental groups were exposed to 26, 45, 100 and 150 mg/kg of caffeine through drinking water during pregnancy, and the control group received only drinking water. The ovaries of the offspring were taken out by the day 7, 14, 28, 60, 90, and 120 postnatal development, and then they were fixed in a 10% formaldehyde solution. Ovarian follicles were studied by stereological methods, and the data were analyzed using a one-sided ANOVA followed by a Tukey test in SPSS software. The value of $p < 0.05$ was considered significant. Body weight, ovarian mass, ovarian volume and the number of primordial follicles significantly decreased ($p < 0.05$) in the 45 and 100 mg/kg groups and ($p < 0.001$) in the 150 mg/kg groups treated with caffeine at all stages of postnatal development. There was a significant decrease in the number of primary and secondary follicles at 45 and 100 mg/kg ($p < 0.05$) and ($p < 0.001$) in the groups receiving caffeine at a dose of 150 mg/kg on the 7th, 14th, 28th and 60th days compared with the control group. The number of Graafia follicles also decreased significantly ($p < 0.001$) in the groups receiving caffeine at doses of 45, 100 and 150 mg/kg on days 14 and 28. In addition, the average volume of oocytes in Graaf follicles decreased significantly in the groups receiving caffeine at doses of 45, 100 and 150 mg/kg compared to other groups ($p < 0.05$). The thickness of zona pellucida (ZP) in secondary follicles ($p < 0.02$) and Graaf follicles ($p < 0.05$) showed a significant decrease in the groups receiving caffeine at doses of 100 and 150 mg/kg on the 14th, 28th and 60th days. In conclusion, the consumption of caffeine in high doses during pregnancy affects all stages of the development of ovarian follicles in the offspring of rats. Some chemical and biological agents had irreversible effects on the reproductive system and fertility. Caffeine is one of the available nutrients widely used by

teenagers, and is found in coffee and tea, as well as in chocolate, cola, energy drinks and some medications. The total daily intake of caffeine from any source is estimated at about 3-7 mg/kg/day to 200 mg/day. The peak level of caffeine in blood plasma is reached between 15 and 120 minutes after oral administration in humans. Concerns have been raised about the deterioration of reproductive health, as some studies have pointed to the adverse effects of caffeine on the female and male reproductive systems.

The half-life of caffeine increases during pregnancy and during the newborn period due to increased estrogen levels and reduces the activity of cytochrome P450. Caffeine easily penetrates the placenta due to its high lipid solubility and low molecular weight.

Caffeine consumption causes low birth weight, cleft palate, growth retardation, spontaneous abortion, reduction in the number of implantation sites and sacral length (CRL). Treatment of female rats with caffeine during pregnancy (26 and 45 mg/kg) and lactation (25 and 35 mg/kg) affects the early stages of ovarian follicle development and reduces reproductive efficiency in rat offspring. They also reported that ovarian weight decreased significantly in the group receiving caffeine at a dose of 45 mg/kg at all stages of postpartum development. Jagiello et al . caffeine administration was reported to prevent hormone-induced superovulation in adult mice.

Another study reported that caffeine blocked normal oogenesis in adult rats. Caffeine has been shown to prolong the meiotic arrest of porcine oocytes at the germinal vesicle stage, possibly by increasing cAMP levels and suppressing Cdc2 kinase and MAP kinase activity in oocytes. On the other hand, the results of some studies have not shown any connection between delayed conception and caffeine consumption.

Regarding the conflicting results of studies conducted on caffeine causing reproductive outcomes, the exact effect of caffeine on pregnancy has been less characterized. The present study is conducted to study the effect of caffeine consumption by the mother during pregnancy on postpartum ovarian development in the offspring of Wistar rats.

Materials and methods

In this experimental study, 30 female Wistar rats with an average weight of 230-280 g were kept under controlled conditions (at 22 ± 2 °C and a 12:12 h light cycle:darkness) with free access to food and water. Pregnant female rats were divided into 5 groups (n = 6), namely experimental groups that were exposed to doses of caffeine at doses of 26, 45, 100 and 150 mg/kg through drinking water, and a control group that received only drinking water during pregnancy. All experiments were conducted in accordance with the relevant national guidelines and animal use, which were approved by the Ethics Committee of Shahid Sadughi University of Medical Sciences, Yazd, Iran (IR.SSU.MEDICINE.REC.1394.540).

After routine histological treatment, the paraffin blocks of the ovaries were collected and separated according to a system random sample. To estimate the average value of the total volume of the ovary, a counting probe was randomly superimposed on the images. The physical dissector method was used to estimate the number of follicles in the ovary. The slices were then selected using a non-random 10% sampling method. Ovarian follicles were counted in each 15th section of the ovary in such a way that each counted section was separated by a distance of 50-60 microns from the next 15th section. The average number of ovarian follicles of each ovary was calculated in the selected sections.

The number of colored oocyte nuclei visible in the original structure determined the number of cells. The sections were analyzed using a light microscope with a magnification of 100 times. To quantify

the number of follicles, slides from each ovary were classified according to the following classification: primary follicle (one layer of flat granulosa cells), primary follicle (monolayer of cuboid granulosa cells), secondary follicle (oocyte surrounded by two or more granulosa cells). the cell layer of the ulose) and follicles containing scattered spaces or a distinct antrum, which were considered Graafian. All follicles were classified as either healthy or atretic. These classifications correspond respectively to the absence or presence of signs of oocytic and/or granular degeneration, such as pyknosis of the nucleus, proliferation of the cell wall in the oocyte, penetration of granulosa cells into the antral cavity, separation of granulosa cells from the main membrane, proliferation or thickening of the main membrane and uneven layers of granulosa cells.

On average, 80-100 microscopic fields were collected in each ovary by systematic sampling. Then the number of different types of follicles was evaluated. Then the result of the equation was multiplied by the total volume of the ovary to get the total number of follicles.

Diameter of ovarian follicles

To measure the diameter of ovarian follicles at each stage of development, 60 microscopic fields were randomly selected in each rat. Subsequently, the diameter of each ovarian follicle was measured using an ocular micrometer light microscope (Olympus America) at magnification × 100. Egg volume (mm3)

The nucleator method was used to estimate the volume of the egg. An average of 15 slices were randomly selected from slices with a thickness of 20 microns and studied using an Olympus microscope with a magnification of × 100.To approximate the volume of the oocyte in the selected cells using an unbiased counting system, the distance from the center of the nucleolus to the oocyte membrane was measured.

To calculate the average thickness of the transparent zone (ZP), an average of 40 slices were randomly selected from slices with a thickness of 5 microns and studied using a light microscope with a magnification of × 100.A certain linear grid (3 parallel lines) was randomly superimposed on the selected fields. The orthogonal intercept method was used to calculate the average thickness of ZP. The length of the extended line was measured vertically from the inner membrane to the outer surface of the ZP at each intersection of the grid line with the zona membrane, and this was considered an orthogonal intersection. The average value of 100-200 measurements was estimated, and the average harmonic thickness was calculated using the following formula.

The data was analyzed using SPSS software. The normal distribution of the data was verified using the Kolmogorov-Smirnov normality criterion. Normally distributed data were analyzed using a one-way ANOVA followed by a post-special Tukey test.

Results

Studies conducted on rats have shown that the formation of primary follicles is completed by the 3rd-4th postpartum day, and most primary follicles remain dormant when some grow and move into the primary follicle stage. Several preantral and antral follicles will be present from day 9 to day 20. In this study, caffeine treatment at a dose of 45, 100 and especially 150 mg/kg reduced the population of primary follicles in the resting pool from the 7th to the 120th day of the experiment. Consequently, the decrease in the number of ovarian follicles in the current study may be due to the effect of caffeine on ovarian differentiation or folliculogenesis. Hoyer and Sipes showed that the stage of development of ovarian follicles, at which the follicles are destroyed, determines the effect of exposure to chemical or toxic substances on reproduction. Chemicals or toxicants that intensively destroy the primordial

follicles lead to permanent infertility, since the primordial follicle is destroyed and cannot be replaced. Some studies have reported that caffeine can interfere with cell division, reduce the number of cells and cause cell death and suppression of meiosis.

The reducing effects of caffeine in the present study may be related to the effects of caffeine on reducing the number of cells and inducing cell death and suppression of meiosis. In this study, ZP thickness, ovarian follicle diameter, and egg volume were examined. At a dose of 150 mg/kg, these changes were more noticeable and stable, since changes in follicular parameters were detected even on the 60th day after birth. Apparently, exposure to high doses of caffeine had a permanent effect on the development of follicles. Toxicants that damage growing or antral follicles only temporarily interrupt reproductive function, since these follicles can be replaced by recruiting from a larger pool of primary follicles. Thus, these agents cause an easily reversible form of infertility, which manifests itself relatively soon after the exposure period.

Caffeine does not accumulate in the body over time, and it is usually eliminated from the body within a few hours after consumption. However, exposure to high doses of caffeine had a permanent effect on follicle development and ovulation in rat offspring during postnatal development.

Conclusion

In conclusion, the present study showed that the consumption of caffeine in low doses in the mother has no effect on the development of follicles, and the consumption of caffeine in high doses in the mother during pregnancy affects the early stages of follicle development and has a permanent effect on folliculogenesis, especially at doses of 100 and 150 mg/kg.

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