© 2022 Journal of Pharmacy & Pharmacognosy Research, 10 (3), 397-405, 2022
ISSN 0719-4250
https://jppres.com

DOI: https://doi.org/10.56499/jppres21.1312_10.3.397

Original Article

The ovulation assessment of regular cyclic rats following subacute oral administration of monosodium glutamate: An *in vivo* study

[Evaluación de la ovulación en ratas con ciclos regulares después de la administración oral subaguda de glutamato monosódico: Un estudio *in vivo*]

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Abstract

Context: The possible toxic effects of monosodium glutamate (MSG) on ovarian functions have not been thoroughly evaluated in contrast to testicular functions. Few studies documented that MSG showed histological alterations.

Aims: To investigate the subacute effects of oral MSG (2 g/kg) on estrogen level and numbers of ovulated oocytes and histological changes in ovary of Sprague-Dawley (SD) rats.

Methods: Virgin young adult SD female rats with a regular estrous cycle were randomly assigned to either MSG-treated group or control group, and the duration of treatment was 14-16 days for oral administration MSG or vehicle (distilled water), respectively.

Results: Oral MSG treatment with doses of 2 g/kg/day showed significantly ($p<0.01$) reduced numbers of ovulated oocytes in the oviduct, newly formed corpora lutea, large follicles in histology of ovarian sections and attenuated serum estrogen levels.

Conclusions: Subacute oral administration of MSG may negatively influence the ovarian function of young female rats via reduction of ovulated oocytes and the attenuation of estrogen level.

Keywords: corpora lutea; estrogen level; follicles ovarian; monosodium glutamate; oocytes; ovulation; toxicity.

Resumen

Contexto: Los posibles efectos tóxicos del glutamato monosódico (MSG) en las funciones ováricas no se han evaluado a fondo en contraste con las funciones testiculares. Pocos estudios documentaron que el GMS mostrara alteraciones histológicas.

Objetivos: Investigar los efectos subagudos del glutamato monosódico oral (2 g/kg) sobre el nivel de estrógeno y el número de ovocitos ovulados y los cambios histológicos en el ovario de ratas Sprague-Dawley (SD).

Métodos: Se asignaron al azar ratas SD hembra adultas jóvenes vírgenes con un ciclo estral regular al grupo tratado con MSG o al grupo de control, y la duración del tratamiento fue de 14 a 16 días para la administración oral de MSG o vehículo (agua destilada), respectivamente.

Resultados: El tratamiento con MSG oral con dosis de 2 g/kg/día mostró un número significativamente reducido ($p<0,01$) de ovocitos ovulados en el oviducto, cuerpos lúteos recién formados, folículos grandes en la histología de las secciones ováricas y niveles atenuados de estrógeno sérico.

Conclusiones: La administración oral subaguda de MSG puede influir negativamente en la función ovárica de ratas hembra jóvenes a través de la reducción de los ovocitos ovulados y la atenuación del nivel de estrógeno.

Palabras Clave: cuerpos lúteos; folículos ováricos; glutamato monosódico; nivel de estrógeno; ovocitos; ovulación; toxicidad.

ARTICLE INFO
Received: November 19, 2021.
Received in revised form: January 8, 2022.
Accepted: January 14, 2022.
Available Online: January 19, 2022.

INTRODUCTION

Disrupted ovarian cyclicity and modified follicle maturation can directly impact female fertility and are considered adverse health outcomes. Clinical and preclinical studies revealed that disruption in ovarian development or ovarians' function is one of the common causatives of many female reproductive disorders, including infertility, polycystic ovary syndrome (PCOS), premature ovarian insufficiency (POI), and early menopause (Johansson et al., 2017). Endocrine disturbances caused by unintended chemical exposures during adulthood can disturb ovarian function or worsen the effects of chemical exposure during developmental processes. These chemicals can disrupt the ovary's folliculogenesis, in which the activated primordial follicles grow and mature to follicles that contain oocytes (pre-ovulatory follicles). The growth of primordial follicles into pre-antral is considered the first phase and independent of the hypothalamic-pituitary-ovary axis (Gougeon, 1986).

In contrast, the maturation of antral follicles to pre-ovulatory is referred to second phase and is dependent on the hypothalamic-pituitary-ovary axis (Gougeon, 1986). This disruption of the process at different stages is dependent on the duration of chemical exposure. The disruption of follicles in the first and second growth phases when exposed chemical for 4- 6 menstrual cycles (MCs) in humans (Gougeon, 1986) and 7 – 9 estrous cycles (ECs) in rodents (Zheng et al., 2014). This disruption in the growth phase follicles can be analyzed by measuring preantral and other follicles between primordial, preantral, and atretic follicles. The disruption follicles in the second phase (maturation phase) when exposed chemical for 1-3 MCs (≈ 35-90 days) in humans (Gougeon, 1986) and for 4-5 ECs in rodents (Zheng et al., 2014). This disruption of the second phase of folliculogenesis is imminent upon chemical exposure for 14-20 days in rodents (Zheng et al., 2014). This disruption in the second phase can be analyzed by counting the oocytes, estimating the estrogen level, and quantifying the maturing ovarian follicles and new corpus lutea using histological sections. Completion of folliculogenesis of ovulatory follicles in both phases takes several menstrual cycles (≈ 6-9 MCs) in humans (Gougeon, 1986) and several estrous cycles (≈ 9-12 ECs) in rodents (Zheng et al., 2014).

The scientific community differ in opinion on monosodium glutamate's (MSG) safety profile when used as a food additive. The amount of intake and its negative impact on reproductive function is highly troubling. Many regulatory authorities in different countries proposed an acceptable daily intake. The European Food Safety Authority (EFSA) suggested 30 mg/kg body weight (BW)/day of MSG as the acceptable daily intake (Roberts et al., 2018). The in-take of MSG in the United Kingdom ranged from 0.58 g/day to 4.685 g/day for average to extreme users (Rhodes et al., 1991). Many studies have reported the negative impact of MSG on body metabolism in adult rodents. MSG has been shown to cause type two diabetic mellitus in the experimental model (Boonnate et al., 2015) and increase body weight and fat mass in rodents (Hernández-Bautista et al., 2014; Calis et al., 2016; Pelantová et al., 2016). Evidence is growing that the influence of MSG on the body's metabolism may alter the biological process in the reproductive system (Gaspar et al., 2016). A recent review has addressed MSG-induced male reproductive system dysfunction (Kayode et al., 2020).

Previous studies on MSG's impact on the adult female rodent's reproductive systems have reported negatively the influence of MSG on histology of female reproductive organs and hormone levels (Obochi et al., 2009; Eweka et al., 2010; Koffuor et al., 2013; Mondal et al., 2017). MSG showed that cellular hypertrophy, degenerative and atrophic changes, and lysed red blood cells in the lumen in histological section of oviducts of rats (Eweka et al., 2010) and a significant increase in the number of follicles (primary and primordial), increase in the size of Graafian follicle, and decrease in the size of corpus luteum (Obochi et al., 2009). MSG showed in different studies that increase blood level of estrogen (Obochi et al., 2009; Koffuor et al., 2013; Mondal et al., 2017). A single study reported the MSG's effect on the estrous cycle in rodents (Mondal et al., 2017). In the mentioned studies above, the duration of MSG administration about 7-9 estrous cycles and addressed the MSG toxic effects for both phases of folliculogenesis lead to increasing the complexity and difficulty of identifying the possible pathway responsible for MSG toxic effects because different factors regulate each phase of folliculogenesis.

Although the previous studies (Obochi et al., 2009; Eweka et al., 2010; Koffuor et al., 2013) and the more recent experimental study published in 2017 (Mondal et al., 2017) have elucidated certain aspects of MSG's ovarian disruption in rodents. Most of the MSG toxic effects on reproductive organs studies have not fully provided an overview of the integrated effects of MSG on ovulation and follicular maturation. Therefore, this study aimed to investigate subacute oral MSG effects with the dose 2 g/kg/day on numbers of ovulated oocytes, estrogen level and histological morphometric changes in Sprague-Dawley (SD) rats' ovary at the estrus phase.

MATERIAL AND METHODS

Experimental animals

Virgin sexually matured Sprague Dawley young adult female rats with regular estrous cycles, 9-13 weeks old and weighing 180–250 g, were obtained from the Animal Research and Service Centre of Qassim University (QU), Al Qassim, Saudi Arabia. The animals were housed in a controlled temperature room (23-26°C) with a 12 h light/dark cycle. Standard commercial rat chow (1st Milling Company, Qassim) and drinking water were provided *ad libitum*. The animal experiment was approved by the Committee of Research Ethics in the Qassim University (20-02-01) and carried out according to NIH and the university guidelines for the care and use of laboratory animals.

Experimental protocol

The estrous cycle of the female rats was recorded for two estrous cycles (ECs) according to Abdulghani et al. (2012) before the administration of MSG. The animal with irregular EC was excluded from the experiment. Animals with regular EC were assigned randomly into two groups (each group n = 6 animals); MSG group treated with 2 g/kg of MSG (Calis et al., 2016) dissolved in distilled water (DW) and control-group 10 mL/kg DW. MSG (200 mg/1 mL of distilled water) was orally administered for 14-16 days. The effective doses of MSG were selected in this study according to the LD_{50} value of MSG in a rat model as reported by Chakraborty (2019) and pilot study of MSG effects on estrous cycle regularity (Abdulghani unpublished). At the end of the experiment, vaginal lavage was performed for treated and control animals. The animal with estrus phase was anesthetized with ketamine and xylene, and a blood sample was collected between 10-11 am by cardiopuncture using 27-gauge needles. The collected blood was put in a non-heparinized sample test tube for serum estrogen analysis. The animals were sacrificed by cervical dislocation, and each left and right oviduct were isolated for the counting of oocytes, according to Donadio et al. (2007) and Raineki et al. (2008). The weight of ovaries and oviducts were recorded and immediately preserved in 10% formalin for histological evaluation.

Oocytes counting

Oocytes of female rats were counted following the methods described by Donadio et al. (2007) and Raineki et al. (2008) with slight modifications. At the end of experiment, females with estrous phase were sacrificed, and each left and right oviduct and ovary were removed, and their weights were taken. Each oviduct was dissected and subsequently pressed slightly between two microscope slides. The ampulla was identified in both left and right dissected oviducts in each animal. The number of oocytes from both oviduct ampulla was counted under a light microscope (Optika, Bergamo, Italy) with 4× magnifications. Photo of the ampulla with oocytes was taken (Fig. 1).

Estrogen level measurement

The collected blood samples from animals were centrifuged at 5000 rpm for 10 min, and the serum was separated. The separated serum was kept refrigerated at -80°C until analysis. The serum samples were sent to the Al Safwa Medical Laboratory to measure estrogen levels for both groups of animals, and the chemiluminescent immunoassay (Beckman coulter Access II, USA) was used. The mean and SEM were calculated (Fig. 2).

Gross morphological evaluation of the ovaries

At the end of treatment, the ovaries were dissected from the sacrificed rat for histological evaluation. Ovaries were fixed in 10% formalin immediately after removal for one week. Each fixed tissue was subjected to tissue dehydration processing and paraffin wax embedding. The prepared ovarian tissues in paraffin blocks were sent to the Histopathology Section of Al-Thobhani Specialized Medical labs for further process (section cutting and staining). Two histological slides were made from each ovary for each rat. The hematoxylin and eosin-stained ovaries were observed for the two major features, follicles corpora lutea (newly formed and old) and (atretic- and large-follicles) under a light microscope (Labomed, Inc. Losangeles, USA) attached with an image analysis system (TCapture, Tucsen Photonics Co., Ltd, Fujian, China). The photos of ovarian sections were taken with 4× and 10× magnifications (Labomed, Inc. USA) (Fig. 3). The newly formed corpora lutea are defined after ovulation and observed at estrus to consist of luteinized granulosa cells of follicles. The old corpora lutea are defined as large size, proliferation of fibrous tissue, appearance of vacuolated and apoptotic cells. Atretic follicles are characterized by a very dark staining and shrunken granulosa cells with loss of granulosa cells and segmented oocytes. The large follicles (preantral and antral) are whole grown oocytes surrounded by many layers of granulosa cells. The number of newly formed-corpora lutea and old-corpora lutea, atretic-follicles and large-follicles were counted twice for each rat, and the mean was calculated (Fig. 4). The relative weight of each ovary and oviduct were also calculated (Fig. 5).

Statistical analysis

Data represent mean ± SEM (n = 6) of the number of ovulated oocytes, atretic-follicles and large-follicles, newly formed-corpora lutea old-corpora lutea, estrogen level, and relative weights of ovaries and oviducts of the MSG-treated groups and control. The student's *t*-test was used to compare MSG-treated groups and control, and differences were considered significant at $p<0.05$ by SPSS version 23 (SPSS, New York, USA).

RESULTS

Effect of MSG on oocytes number and estrogen level

The ovulated oocytes were counted under low magnification 4× (Fig. 1A). We observed the numbers of ovulated oocytes to be significantly lower in both left ($p<0.01$) and right ($p<0.05$) ampulla of animals treated with MSG when compared to control animals (Fig. 1B).

The estrogen level non-significantly decreased in the MSG-treated group compared to the control group (Fig. 2).

Effect of MSG on histomorphometric changes of ovarian tissues

The histology of ovarian tissues of oral MSG-treated female rats in estrus phase revealed observable significant ($p<0.01$) differences in the average number of newly formed corpora lutea (CLs) but not in average number of old corpora lutea (CLs) when compared with control rats (Figs. 3 and 4A). Similar pattern for both large-follicles and atresia-follicles, where the average number of large follicles showed observable significant ($p<0.01$) differences while the average number of atresia follicle did not, when compared with control rats (Figs. 3 and 4B).

Effect of MSG on relative weights of ovary and oviduct

The average relative weights of left and right ovaries of MSG-treated group showed no significant differences compared with the respective controls (Fig. 5A). The average relative weights of the left and right oviducts are non-significantly higher than in the respective controls (Fig. 5B).

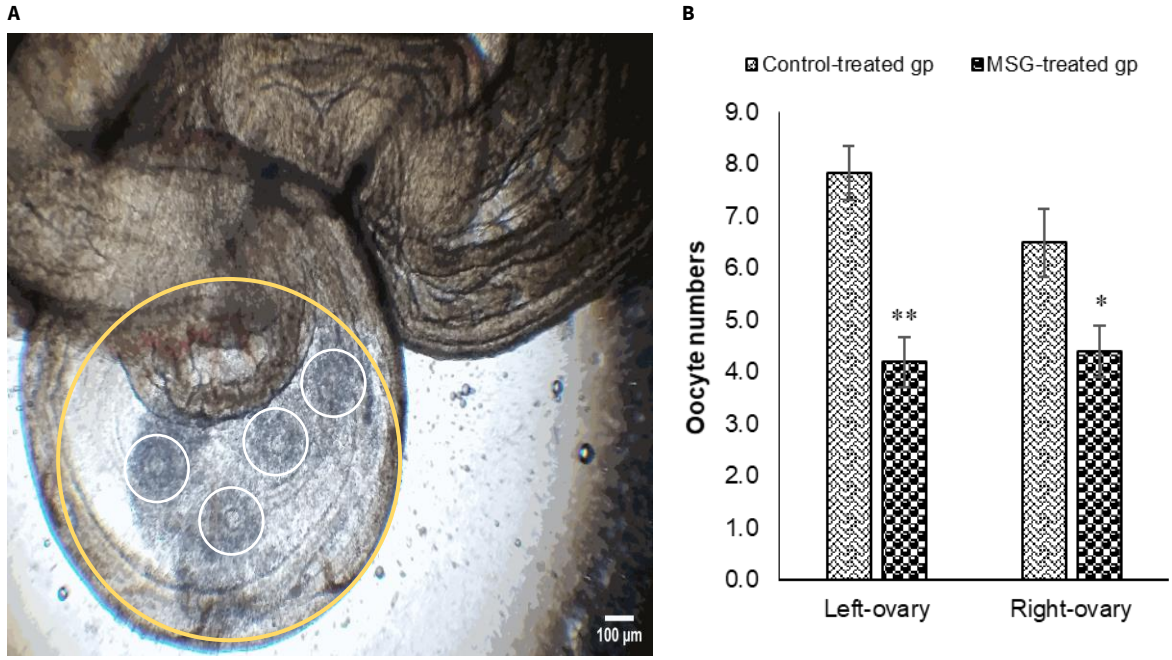

Figure 1. (A) Photomicrograph of the ampulla of oviduct of female SD rats. Oviduct ampulla (large yellow circle), oocytes (small white circles); **(B)** Effect of oral MSG (2 g/kg) on numbers ovulated oocytes in the oviduct of female SD rats.

Values are expressed as mean ± SEM, (n = 6), *p< 0.05, **p< 0.01 *vs.* control.

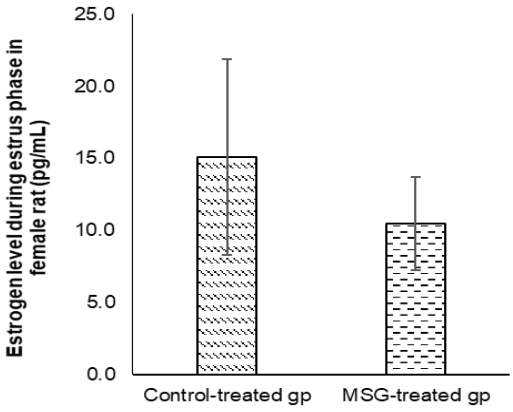

Figure 2. The effect of oral MSG (2 g/kg) on estrogen level in female rats in estrus phase.

Values are expressed as mean ± SEM (n = 6).

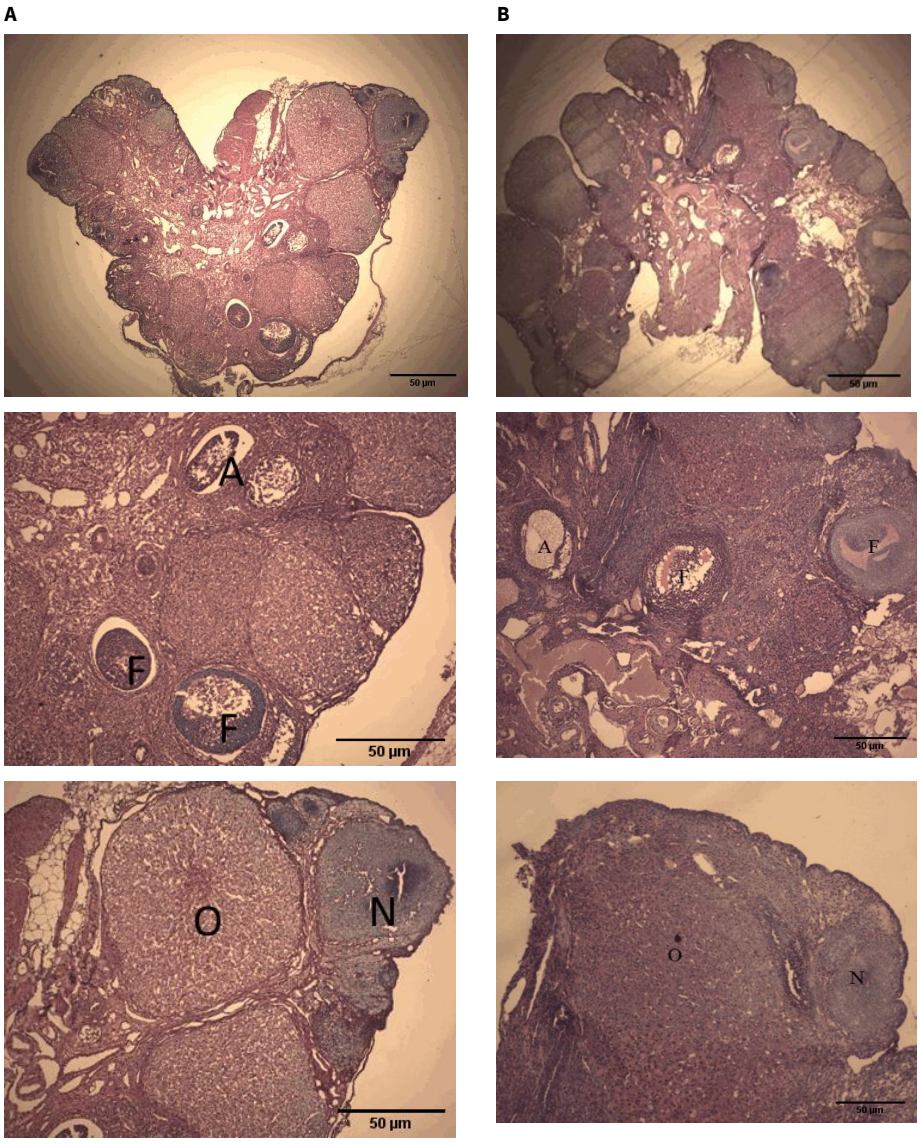

Figure 3. Cross sections of the ovaries of female SD rats; **(A)** Control; **(B)** MSG-treated.

A: atretic follicles; F: large follicles; O: old corpus luteum; N: newly formed corpus luteum.

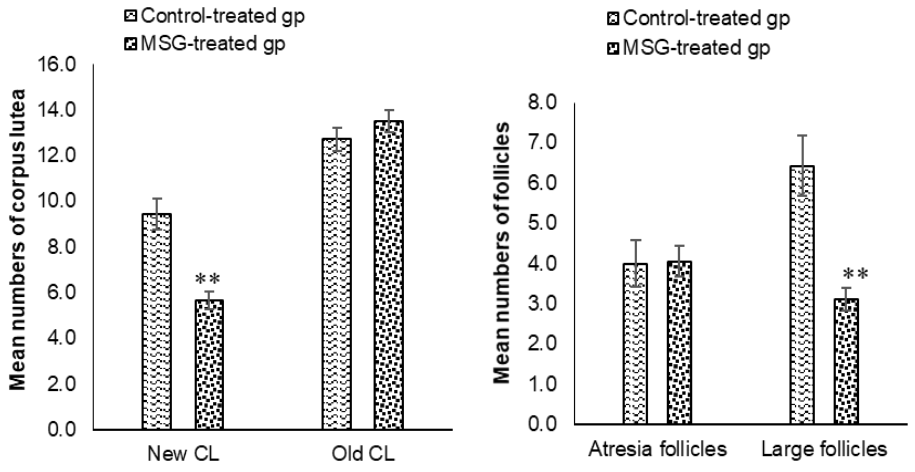

Figure 4. The effect of oral MSG (2 g/kg) on histomorphometric changes of ovaries of female rats.

(A) The effect of oral MSG (2 g/kg) on the numbers of corpora lutea; **(B)** The effect of oral MSG (2 g/kg) on the numbers of follicles.

Values are expressed as mean ± SEM (n = 6). **P< 0.001 *vs.* control. CL: corpora lutea.

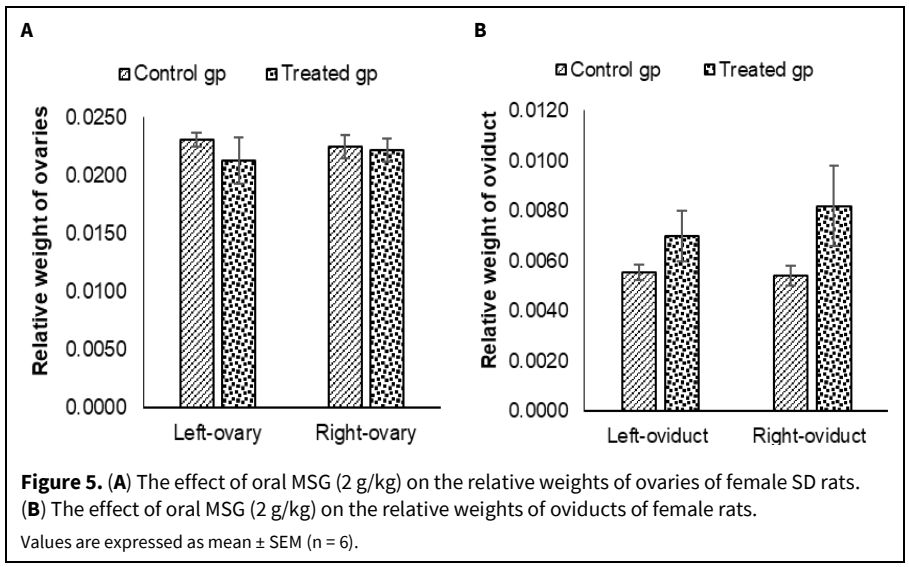

Figure 5. (**A**) The effect of oral MSG (2 g/kg) on the relative weights of ovaries of female SD rats. (**B**) The effect of oral MSG (2 g/kg) on the relative weights of oviducts of female rats.

Values are expressed as mean ± SEM (n = 6).

DISCUSSION

This study evaluated the probable subacute toxic effects of oral MSG (2 g/kg/day) treatment for 14-16 days (three-four ECs) on the ovulated oocytes, estrogen level and histomorphometric changes ovaries of female SD rats. We observed that MSG impaired the ovarian function in regular estrous cycle of SD rats by significantly decreasing the number of ovulated oocytes, large follicles, and newly formed corpora lutea and non-significantly reducing the serum estrogen level.

Oral administration of MSG (2 g/kg/day) for three-four estrous cycles may affect follicles' maturation, which is strongly dependent on coordinated hypothalamic-pituitary-ovary axis function. Folliculogenesis occurs during adulthood in the ovaries and can be classified into two phases. The first phase refers to activate the primordial follicles to grow to preantral follicles and is assumed not dependent on the hypothalamic-pituitary-ovary axis. The second phase refers to the maturation of preantral follicles to Graafian follicles and is dependent on the hypothalamic-pituitary-ovary axis. Estradiol plays a crucial role in regulating the hypothalamic-pituitary-ovary axis by exerting negative and positive feedback on pituitary FSH and LH release (Moenter et al., 2020). The follicle stimulation hormone (FSH) secreted from the anterior pituitary and estradiol secreted from the ovary facilitates the maturation of the ovarian follicles and prepares the endometrium of the uterus physiologically. The LH secreted from the anterior pituitary and high estradiol blood level trigger the release of

oocytes from the mature Graafian follicle to the fallopian tube in humans and the oviduct in animals. LH facilitates the development of corpus luteum gradually from post-ovulated Graafian follicles. Key events include inhibited estradiol production by ovarian granulosa cells and altered ERα activation in neuronal cells leading to alterations in ovarian cyclicity. Correct ovarian cyclicity is essential for successful ovulation (Barbieri, 2014). Our findings agreed with previous studies (Obochi et al., 2009; Koffuor et al., 2013; Mondal et al., 2017; Abdel-Aziem et al., 2018), showing that oral administration of MSG at different doses and durations can cause pathological alteration in ovaries, leading to reduced fertility. These studies showed that MSG (0.01 g/kg for 60 days) (Obochi et al., 2009), (0.6 and 0.8 g/kg for 30 days) (Koffuor et al., 2013), (0.8, 1.6 and 2.4 g/kg for 30 and 40 days) (Mondal et al., 2017), and (1.2 g/kg for 28 days) (Abdel-Aziem et al., 2018) showed altered on estrogen levels. Disrupted ovarian cyclicity and modified follicle maturation can directly impact female fertility. Although the previous studies have elucidated certain aspects of MSG's ovarian disruption in rodents, no study correlated the number of the ovulated oocyte with other effects of MSG on the ovary (histological and hormonal effects). However, in the current study was performed.

The morphometric histological characteristic of the ovarian section of MSG-treated and control groups showed a significant decrease in the numbers of newly formed corpora lutea and the large follicles but not in old corpora lutea and atresia follicles. Previous studies reported that MSG caused the degeneration of ovarian and fallopian tissues of marine animals (Eweka et al., 2010; Mondal et al., 2017). Mixed MSG (0.08 mg/kg) with food for 14 days (Eweka et al., 2010) and orally different doses (0.8, 1.6 and 2.4 g/kg) showed alteration in histological features of ovary and oviducts of rodents. However, the duration of treatment with MSG in our study is shorter (3-4 ECs) than the period (8-9 ECs) reported previously (Eweka et al., 2010; Mondal et al., 2017). The duration of exposure of toxic chemicals to ovaries in humans and rodents can influence the different phases of folliculogenesis (Gougeon, 1986; Zheng et al., 2014). If the exposure duration of toxic substances to ovaries is more than 180 days in humans (≈ 6-9 MSs) and more than 36 days (≈ 9-12 ECs) in rodents, the chemicals can influence both phases, growth and maturation of folliculogenesis. Both phases' completed folliculogenesis takes several menstrual cycles (≈ 6-9 MSs) in humans (Gougeon, 1986) and several estrous cycles (≈ 9-12 ECs) in rodents (Zheng et al., 2014). So, if the exposure duration of toxic chemicals to the ovary is around 60 days in humans (≈ 1-3 MSs) and 14- 20 days (≈ 3-4 ECs) in rodents, the toxic chemicals can influence the follicles in the second phase of folliculogenesis, which is dependent on hypothalamic-pituitary-ovary axis. Several studies with different duration of treatment with MSG longer than in our study reported increased estrogen levels (Obochi et al., 2009; Koffuor et al., 2013; Mondal et al., 2018; Agbadua et al., 2020), which do not concord with our study. In contrast, Abdel-Aziem et al. (2018) reported a decrease in estrogen level, which concord with the results of our study show decrease without statistical difference. Studies on orally administered MSG (0.1 g/kg) for 60 days in female Wistar rats reported a two-fold increase in serum total estradiol level (Obochi et al., 2009) and a 59.2% increase in plasma estradiol level when MSG (0.6 and 0.8 g/kg) for 40 days (Koffuor et al., 2013). Another study showed a significant increase in serum LH, FSH, and estradiol levels in treated groups of rats (Mondal et al., 2017). A recent study reported an increase in estrogen level (Agbadua et al., 2020) following a single oral of MSG (0.2 g/kg) to Wistar rats for 28 days. The possible mechanism involved in causing the estrogen upsurge was suggested to include activating the aromatase catalyzed conversion of testosterone to estradiol and aromatization of ring A of estradiol, which increased the enzyme activity, resulting in increased estradiol synthesis (Obochi et al., 2009). Moreover, Abdel-Aziem et al. (2018) and Mondal et al. (2018) reported that MSG's-induced decrease in antioxidant enzymes in ovarian tissues of rats may be responsible, although other possible MSG pathways have not been ruled out. Subacute effect of oral MSG showed significant effects on the physiological function of ovaries in young adult female SD rats, which concord with Ali et al. (2014), who reported that subcutaneous injection of MSG (4 mg/kg) daily for 14 days to female adult albino rats showed histological changes in ovaries. Calis et at. (2016) who reported that intraperitoneal administration of MSG (2 g/kg) for seven days does not cause significant histological changes on the kidneys, liver or brain cortex of adult Sprague-Dawley female rats. It is noteworthy that the reported variations of the effects of MSG in different studies has been linked to variations in kinetics of MSG (Bizzi et al., 1977).

Generally, the results obtained in the current study show MSG (2 g/kg) administration can impair directly or indirectly ovarian function. The direct effect of MSG on ovaries, it can cause unbalance of oxidative status in ovarian tissue and other organ tissues (Sharma et al., 2014; Umukoro et al., 2015). The indirect effect of MSG on ovarian function can be related to a metabolic abnormality and energy generation in the body (Kazmi et al., 2017; Kayode et al., 2020). All these effects may impair the function of reproduction in females, which can reduce fertility.

CONCLUSION

Subacute oral administration of MSG with 2 g/kg/day may negatively influence the ovarian function of young female rats via reduction of ovulated oocytes and the attenuation of estrogen level. Further study on *ex vivo* and *in vitro* effects of MSG on aromatase enzyme, MPF and MARK gene expression during rat oocyte maturation may suggest.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGMENTS

The researcher would like to thank the Deanship of Scientific Research, Qassim University, for funding the publication of this project. The author would also like to thank Dr. Adel Al-Thobhani for arranging histology processing of the tissue samples, Dr. Sattam Alenezi for arranging the biochemical analysis and Prof. Bala Yauri for critically reviewing the final draft of this manuscript. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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AUTHOR CONTRIBUTION:

Contribution	Abdulghani MAM
Concepts or ideas	x
Design	x
Definition of intellectual content	x
Literature search	x
Experimental studies	x
Data acquisition	x
Data analysis	x
Statistical analysis	x
Manuscript preparation	x
Manuscript editing	x
Manuscript review	x

Citation Format: Abdulghani MAM (2022) The ovulation assessment of regular cyclic rats following subacute oral administration of monosodium glutamate: An *in vivo* study. J Pharm Pharmacogn Res 10(3): 397–405. https://doi.org/10.56499/jppres21.1312_10.3.397