Long term effects in ovaries of the adult mice following exposure to Monosodium glutamate during neonatal life – a histological study

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ABSTRACT

Monosodium Glutamate (MSG) has been in use since long as a flavour enhancing substance. Its widespread use has also earned it a bad name to be harmful for human health. It had been incriminated in wide range of effects comprising retinal degeneration, metabolic disorders, endocrinal disorders including reduced fertility rate in both in both sexes in mice. However there were many contradicting views too, which have prompted us to undertake the present study. For our study eight female newborns of Swiss Albino mice were injected subcutaneously with MSG (2mg / gm of body wt. in a dilution 40 mg of per ml. of distilled water) on completion of 2nd, 4th, 6th, 8th and 10th day of life. Another five mice pups were injected with same volume of distilled water and taken as control. On completion of 75 days the mice were sacrificed of, ovaries were collected through dissection, 5 micron thick sections were cut and stained by H and E and PAS stain and studied under light microscope. It was observed from the quantitative analysis of the ovarian tissue that there was increase in the number of the primary follicle without increase in number of Graffian follicle in the experimental group.

Keywords: Monosodium Glutamate (MSG), Swiss Albino mice, ovary, primordial follicle, primary follicle.

INTRODUCTION

Monosodium glutamate (MSG) commonly known as Azinomoto is a widely used food additive. For thousand of years, the Japanese cooks have used a flavour enhancer from seaweed called "Sea Tangle" which was later found to be MSG.[1] MSG triggers the taste bud sensitive to 'UMAMI' taste,[2] referred as the fifth primary taste.[3] MSG's great property of enhancing the taste of the food and overwhelming popularity prompted the Scientists to analyse its possible effects on human. Since 1960s, the research data began to show dangers of MSG, though, differences in observations surfaced from time to time.[1] The experimental animals showed some adverse effects, simultaneously, the experiments showed that the neonates of mice were the most sensitive of all animals.[4] These effects involved the central nervous system, retina, endocrine glands including gonads etc. The hypothalamus, particularly its arcuate and preoptic nuclei developed lesions following exposure of the experimental animals to MSG during neonatal period.[5,6]

Several studies were done to see the effects of MSG on the tissues concerned with the reproduction eg. testis, ovary, uterus etc. in the neonatal animals (rats, mice), following exposure to MSG (dose varying from 2.2 to 4 mg / kg of body wt.). The researchers, though reported reduction in weight of both testes and ovaries, they did not comment on histological changes of these organs[5] Some workers reported few histomorphological alterations, like, increased number of atretic follicles, reduced number of graffian follicles,[7] no corpora lutea etc.[8] Fertility rate has been reported to be reduced in both sexes.[9] However, only few literatures were available detailing the changes in histomorphology following MSG administration in mice.

Since experimental and clinical studies showed some adverse effects on use of MSG in multiple organ system we have undertaken this study of the histological changes in an adult ovary after administration of MSG in neonates.

Biochemical aspect - MSG is a salt of sodium and (L-)

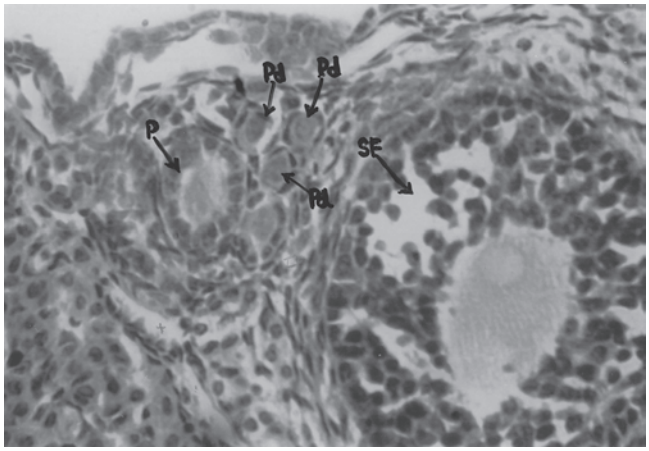

Fig. 1. Photomicrograph of an ovarian section of a Control animal showing several primordial (Pd) and one primary follicle (P) with cuboidal follicular cells and a secondary follicle (SF). H.E.: 400X.

Table-1: Study design of the animal experiment

Particulars	Group A (Experimental)	Group B (Control)
1) Weight of the pups	Weighing of each neonate of both group was done before drug administration as well as on 28th and 75th day after birth	
2) No.of neonates	Female – 8	Female – 5
3) Administration of dose.	Inj. Monosodium glutamate (2mg of MSG/0.05 ml of distilled water was injected subcutaneously (s.c.) on 2, 4, 6, 8 and 10 days of postnatal life in the dose of 2mg/gm of body weight.t	Littermate control was injected with same amount of distilled water s.c. on same days of postnatal life like tha of the experimental group.
4) Nursing and rearing	On 28th day, the pups of both the groups were weaned.	
5) Histological study	At two and half months of age (puberty at 50 – 60 days of life)[12] the animals of both groups were sacrificed after injection of Thiopentone Sodium given intraperitoneally in a dose of 50 mg / kg of body wt. Prior to dissection, perfusion was carried out with 10 ml solution of Formal saline through intracardiac catheterization in each animal. The ovaries were collected; blotted, weighed, volume was measured by water displacement method, then immersed in fixative, and subsequently processed for routine paraffin embedding. 5 micron thick sections were cut and stained and studied under light microscope.	

Glutamic acid. In pure form it appears as a crystalline powder, when dissolved in water (or saliva) it rapidly dissociates into Sodium and L – Glutamic acid. L – Glutamic acid is a ubiquitous amino acid present in most foods, either in free form or bound to peptides or proteins. It has been calculated that in a 70 kg man has a daily Glutamic acid (GA) intake of about 28 gm that is derived from the diet and from the breakdown of gut proteins. The daily GA turnover in the body is about 48 gm. Despite this large turnover, the total pool of GA in blood is quite small, about 20 gm, because of its rapid excretion and utilization by various tissues, particularly muscle and liver [1]. Today, it is widely accepted that GA is the major excitatory neurotransmitter within the brain.[10]

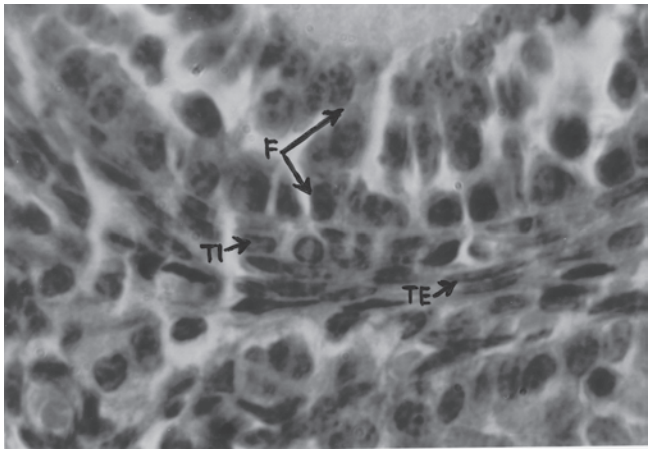

Fig. 2. Photomicrograph of a maturing follicle from ovary of a Control animal showing several layers of follicular cells (F) cells of theca interna (TI) and externa (TE). The cells have distinct nucleus and cell membrane. The cells of theca interna are polyhedral with round to oval nuclei while theca externa cells are ellipsoidal with elongated nuclei. H.E.: 1000X

mediating fast synaptic transmission and active in perhaps one third of all CNS synapses.[11]

MATERIALS AND METHODS

In this study, the newborn pups of the Swiss - Albino mice have been selected as the animal model. This animal model has been extensively used eg. for observing the sensitivity of drugs on various systems.[1]

Summary of the procedure – The neonates were exposed to natural day and night sequence. Proper cleanliness of the cages was maintained. Rat feed and cool drinking water (filtered) were provided ad libitum. The animals were divided into two groups, Group A (Experimental) and Group B (Control). The design of the study is mentioned in Table-1.

A stock solution of MSG was prepared by dissolving 4 gm of MSG crystals in 100 ml of dist. water, so that 0.05 ml of the said solution contained 2 mg of MSG. The dose schedule was so adjusted that the amount of MSG injected per pup as per their respective weight.

Gross study – As stated above, before putting into fixative, the ovaries from both groups were blotted, weighed and measured for volume.

Table-2: The weight of the pups of both the groups

Particulars	Control Grp.	Expt. Grp.
Mean wt. at birth	1.43 gm	1.49 gm
Mean body wt. on 28th day	11.54 gm	12.76 gm
Mean body wt. on 75th day	22.79 gm	24.78 gm

Table-3: Mean of the volume and body wt. of the ovaries of the Control and Expt. (MSG treated) mice at autopsy (i.e.75th day)

Group	No. of animal	Avge vol. of ovary (cc)	Avge. wt. of ovary (gm)
Control	5	0.01	4.4
Expt.	8	0.01	4.3
Significance		P > 0.05 (NS)	P > 0.05 (NS)

Ovarian Histological study procedure – After paraffin embedding, 5µ thick sections were cut and stained with H&E and PAS stain.[13] The sections were studied for qualitative assessment.

Quantitative assessment - The present investigation was aimed at quantitative assessment of the ovarian components. The following estimations were done – a) number of primordial follicle, b) number of primary follicle and c) number of graffian follicle per unit area respectively.

For histological study we selected every 10th (tenth) ovarian section for the study to avoid overlapping of same follicle. To find out the unit area, Haug's graticule was used. First the Haug's graticule was calibrated by putting the graticule in the eye piece of the microscope and a linear micrometer on the stage. The length of each side of Haug's graticule and area were calculated in the following manner –

Under 10X obj., each side of the small square of Haug's graticule = 6 small divisions of stage micrometer = 6 x 0.01mm = 0.06 mm or 60µ. So area of each small square B_o (Grid area for ovary) = 0.06 x 0.06 mm = 0.0036 sq.mm. Hence area of the ovary (in a particular section) = no. of hits x 0.0036 sq.mm.

Statistical analysis: We analysed the data with the help of Student's t – test (unpaired).[14]

Observation: *1. Gross observations* – 5 female pups born out of 2 females on different dates (Control group - B) and 8 female pups born out of another 3 females on different dates (Experimental group - A) were selected.

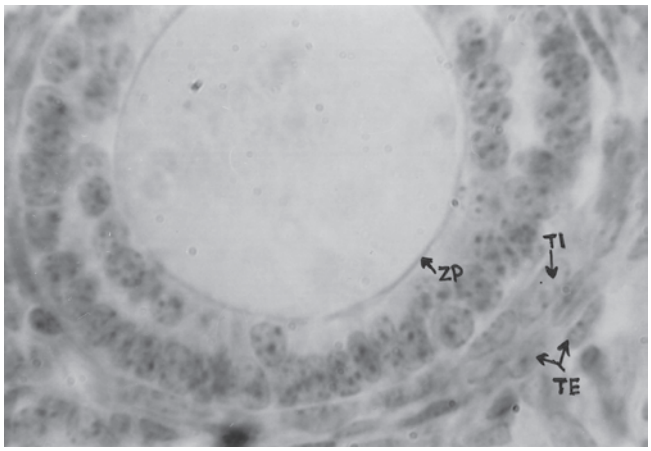

Fig. 3. Photomicrograph an ovarian section of a Control animal showing a growing primary follicle (P) with multilayer of follicular cells, a distinct PAS + ve zona pellucida (ZP), theca interna (TI) and externa (TE) are seen too. PAS Stain: 1000X.

The pups survived throughout the period of experiment. The pups of either group did not show any external abnormalities at birth. The average hair growth and eye opening were recorded on 7th day and 10th day respectively in all pups. We measured the weight of the pups at birth, on 28 th day (when they were separated from the mother – weaning) and on 75 th day. The average weights of the Group B animals were 1.43 gm, 11.54 gm and 22.79 gm on those three days respectively while weights recorded for Group A animals were 1.49 gm, 12.76 gm and 24.78 gm respectively (Table-2).

2. Weight and volume of ovaries – On 75 th postnatal day the ovaries were collected following dissection and wt. and volume of each ovary was recorded before processing it further (Table-3). The average weight of the ovaries of Gr. B animals was 4.4 mg while that of Gr. A animals were 4.3 mg. Volume of ovary of were same for both group at 0.01 ml.

3. Histological findings - A. Ovaries of Control Group – The sections of the ovary showed an outer covering of a single layer of cuboidal to columner germinal epithelium. Enclosed within this, the ovarian parenchyma showed an outer, highly cellular zone, the cortex with number of follicles at different stages of maturation (Fig. 1).

Table-4: Count of primordial and primary follicles in average area and per unit area of each animal of control group

Sl. no. of the animals	Average area (mm^2)	Average no. of follicles per sections			Follicles per unit area		
		Primordial	Primary	Graffian	Primordial	Primary	Graffian
1st female	1.877	3.8	5.9	0.6	2.02	3.14	0.3
2nd female	1.780	3.8	5.0	0.5	2.13	2.80	0.2
3rd female	1.775	3.8	4.0	0.5	2.14	2.25	0.2
4rth female	1.983	1.6	3.1	0.6	0.80	1.56	0.3
5th female	2.527	2.4	5.3	0.7	0.95	2.09	0.2

Table-5: Count of primordial and primary follicles in average area and per unit area of each animal of experimental group.

Sl. no. of the animals	Average area (mm^2)	Average no. of follicles per sections			Follicles per unit area		
		Primordial	Primary	Graffian	Primordial	Primary	Graffian
1st female	1.078	2.7	9.1	0.3	2.5	8.44	0.2
2nd female	2.212	2.0	7.8	0.7	0.90	3.52	0.3
3rd female	1.017	0.9	6.9	0.3	0.88	6.78	0.2
4rth female	1.553	2.5	8.0	0.5	1.60	5.15	0.3
5th female	2.301	2.6	9.0	0.7	1.12	3.19	0.3
6th female	2.349	1.8	7.3	0.7	0.76	3.10	0.3
7th female	2.106	1.5	6.2	0.7	0.71	2.94	0.3
8th female	2.106	1.0	5.0	0.4	0.84	4.24	0.3

Some capillaries were extending from the medulla to the cortical area between the follicles. The stroma of the ovary was cellular with fibroblasts like cells with ellipsoidal nuclei and forming a capsular zone (theca). The follicles were limited by a thecal zone. The primordial follicle consisted of a large spherical oocyte enveloped by a single layer of squamous epithelium (follicular cells). Nucleus of the ova was pale stained with eosinophilic cytoplasm while primary follicle having a cuboidal lining with big ova inside (Fig. 1). The oocyte was proportionately larger with accumulation of more ooplasm in the primary follicles whose follicular cells were cuboidal, forming initially a single layer (Fig. 1). Maturing primary follicles showed multiple layer of follicular cells and well differentiated theca interna and theca externa (Fig. 2).

Further development of the secondary follicles was marked by appearance of PAS + ve zona pellucida in between the oocyte and granulosa cells (Fig. 3). There were many secondary follicles (antral follicles) with formation of cavities within the interstices of the follicular cells. In mature follicle the oocyte was situated at one pole of the follicle being encompassed by cumulus oophorus cells. We observed several mature (Graffian) follicles occupying the cortex. The epithelium of that region was thinned out forming stigma. There was presence of several corpus luteum in most of the ovaries. The cells were larger, paler, arranged in clusters and separated by minimum amount of connective tissue. The zona pellucida was thicker and well formed. The two zones of the theca could be differentiated as well.

B. Ovaries of Experimental Group –We examined the 5 micron thick sections of the ovary like that of the Control animals. The germinal epithelium was cuboidal to low columner type. The appearance of the ovarian stroma and the vascular network and different developmental stages of the follicles within it were similar to that of Control group. The theca interna and externa could be seen distinctly. The corpus luteum was well formed and encapsulated (Fig. 4). The zona pellucida was thinner and at places follicular cells were less in number and separated from each other (Fig. 5).

The only interesting difference we noticed that the number of the primary follicles appeared more in almost every sections of the ovary of each individual experimental animal while that of the primordial, antral (secondary), corpus luteum (Fig. 6) and Graffian follicles were almost of similar number. The primary follicles otherwise displayed all the features as that of the follicles of the Control animals.

Quantitative assessment of ovary: Quantitative study was carried out in order to assess the functional status of the ovary. To do this job histologically we have selected some parameters eg. number of primordial follicles, primary follicles and Graffian follicles in each section observed; area of that section etc. As mentioned in the chapter of Material and Method under the section of Statistical analysis we have done the calculation accordingly and put in the respective tables. The

Table-6: Statistical analysis of primordial follicles in Control and Experimental animals

Group	No. of animals	No. of the sections observed @ 10 per animal	Mean of primordial follicle per unit area	Combined variance	SD	Significance
Control	5	50	1.608	0.359	0.63	P > 0.05 i.e. NS
Experimental	8	80	1.163			

Table-7: Statistical analysis of primary follicles in Control and Experimental animals

Group	No. of animals	No. of the sections observed @ 10 per animal	Mean of primordial follicle per unit area	Combined variance	SD	Significance
Control	5	50	2.36	2.53	1.59	P < 0.05 i.e. NS
Experimental	8	80	4.76			significant at 5 % level

assessment was done by comparing the different parameters of the Experimental group with that of the Control (Tables 4-8).

DISCUSSION

No sooner had MSG earned world wide popularity it also started earning bad names. Studies and experiments carried out in different animal model using different dose schedule, administered through different routes, e.g. oral or parenteral. We used mice because of several advantages eg. easy handling and availability, continuous reproductive cycle independent of season, reproducibility of the experiments owing to short reproductive cycle etc.[12] Studies also established that mice is one of the most susceptible species to the MSG.[1,4]

We performed this experiment with 2 mg/gm of body weight of MSG administered subcutaneously (s.c.) on alternate day starting from 2nd day of neonatal life (48 hr.) for 5 days. Others had used different dose schedule, mostly a gradually increasing dose over the days. One worker[5] used the dose of 2.2 mg to 4.2 mg/gm of body wt. in a gradually increasing dose administered through s.c. route from day 2 to day 11 of the neonatal life of the mice. Same schedule was also followed by several others.[9,15] While one author[9] used infant mice, the other two authors[7,15] used infant rat as animal model. Few workers[7,8] used an alternate day regime in a dose of 4 mg/gm given to the neonates of the rats, we however, adopted the uniform dose at the minimum level. Another worker opined that daily dietary intake of man from reasonably all possible sources did not exceed more than 0.7gm i.e. 0.01 mg/gm of body wt. in an average adult.[16] He further added that infant mice were not equipped with enzymes to metabolise MSG. According to him this dose of 2 mg/gm of body wt. introduced in infant mice was comparable to about 6 gm in human infant. It was observed that two week old mice usually convulsed to death from s.c. doses between 5 – 6 mg/gm of body wt. but this dose in 1 day old infant mice was readily lethal without convulsion.[5]

We observed that the grown up pups of the MSG treated group gained more weight than that of the control animals which might be due to higher voluntary intake of the food induced by the flavour enhancing food additive.[17]

In the present experiment we studied the histology of the ovaries after staining with H.E. for general histology and PAS method for zona pellucida. The sections of the ovary of the Experimental group showed that the stromal or follicular architecture had well delineated margin with germinal epithelium, tunica albuginea. The cortical part had both follicles and corpus lutea with a central part of fibrous tissue supporting the blood vessels. These were similar to that of the Control group.

There were several number of well developed corpus lutea suggesting release of multiple ova during ovulation.

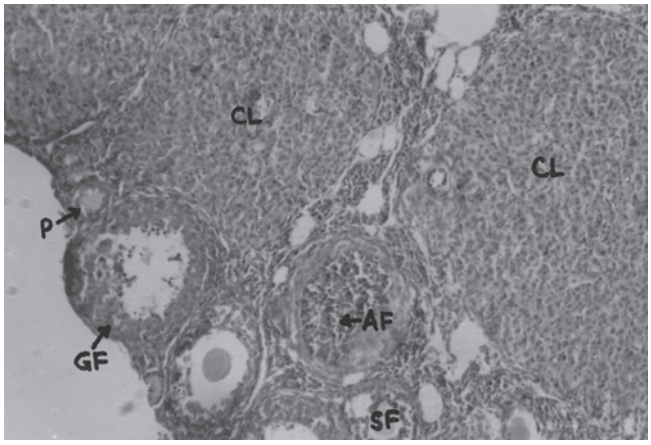

Fig. 4. A low power photomicrograph of an ovarian section of an MSG treated animal showing cortical and medullary stroma with capillaries and several follicles at different stages of development. P - primary follicle; SF- secondary follicle; GF-Graffian follicle; CL - corpus luteum; AF - atretic follicle. H.E. : 100X.

Table-8: Statistical analysis of Graffian follicles in Control and Experimental animals

Group	No. of animals	No. of the sections observed @ 10 per animal	Mean of primordial follicle per unit area	Combined variance	SD	Significance
Control	5	50	0.24	0.0036	0.06	P > 0.05 i.e. NS
Experimental	8	80	0.27			significant at 5 % level

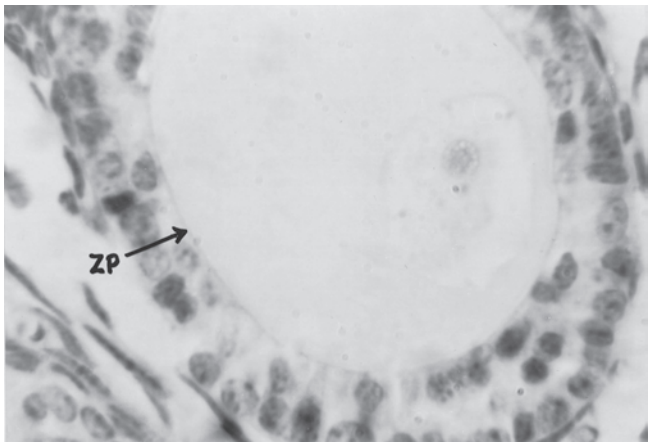

Fig. 5. Photomicrograph of a growing primary follicle (P) of a MSG treated animal with only two layers of follicular cells (F), a thin indistinct PAS + ve zona pellucida (ZP). An eccentrically placed ova could be seen. PAS technique: 1000X.

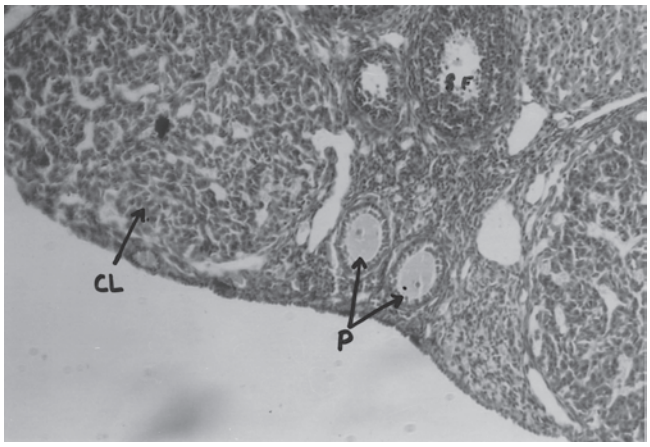

Fig. 6. Photomicrograph of an ovarian section of an MSG treated animal showing primary follicle (P) and corpus luteum (CL). H.E.: 100X.

The number of the primordial follicles too showed similarity in both these groups. We have not observed any histological differences in these structures in either group. One interesting finding was that of significant increase in number of primary follicles; however there was no histological difference from that of the primary follicles of the control group. Where as the secondary follicles and the Graffian follicles exhibited similar features in both the control and experimental groups. Though we found few atretic follicles it was apparently not different in ovaries of the two groups (case and control). However, some workers described that the ovaries of the MSG treated animals contained twice as many as atretic follicles than that of the ovaries of the control animals.[5] While another noticed increased number primordial follicles and simultaneous reaction in Graffian follicles.[7] This difference of observation (between ours and Misckowiaks') might be due to use of different criteria to distinguish primordial follicles and primary follicles, since they did not describe the different follicles. One of the scientist did not mention any histological change despite observing reduced female fertility[9] and another researcher observed that the ovaries of the MSG treated animals contained small follicles with no corpora lutea.[8]

The increase number of the primary follicles, without increase in the total number of Graffian follicles could not be explained definitely. It was observed that the FSH level remain unchanged in the MSG treated females with significant lower level of LH.[18]

Summary: Our observation of MSG induced histological changes in the ovaries showed that the changes in the ovary were minimal with increase in number of primary follicles, the reason we could not explain. It was observed that the FSH level remain unchanged while the serum prolactin and GH were increased in the MSG treated females and also there was increased number of primordial follicles and decrease in the serum oestradiol.[7] Our finding of increased primary follicle without increase in total number of Graffian follicle can only be explained by a possible transient phase of stimulation of differentiation of primordial follicles to primary follicles which waned off due to possible disruption of the pituitary – adrenal axis and this could be reversed following injection of oestradiol and progesterone.[8]

Different agencies tried to evaluate the safety aspect of MSG in respect of human health along with other food additives. Joint FAO/WHO expert committee on food additives on 1988 has recognized MSG as generally safe for human consumption.[4] It was shown that the minimum active oral dose in the weaning mice (the most sensitive species) is 0.7 gm / kg (0.7 mg /gm) of MSG (as a 10% solution) whereas in adult mice, a dose of 1.2 gm / kg (1.2 mg /gm) is required. In general, although there is little disagreement that MSG can be neurotoxic at high doses in animals. Here is substantial disagreement about the significance of this observation for human nutrition and health.[19] Even than many workers have expressed their reservations over the conclusion of WHO expert committee. From our observation we conclude that MSG in the dose schedule used by us in infant mice caused changes in the increased number of the primary follicles only and not the graffian follicle in the ovaries of the MSG treated adult following their exposure to it during neonatal life in comparison to the control females might be due to some stimulation to the oogenesis in the initial period which has subsequently got arrested later for some unknown reason. This particular dose may considered within the safe limit for human consumption but at the same time needs further evaluation.

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