Long term effects of monosodium glutamate on spermatogenesis following neonatal exposure in albino mice – a histological study

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ABSTRACT

Monosodium glutamate (MSG), popularly known as *Azinomoto* has been in use since long as a flavour enhancing substance. Its widespread use has also earned it a bad name as hazardous for human health. It has been incriminated to cause wide range of effects comprising retinal degeneration, metabolic disorders, endocrinal disorders including reduced fertility rate in both male and female experimental mice and rats following neonatal exposure. However there are many contradicting views too regarding the above effects which have prompted us to undertake the present study. In our study seven 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. Similar number of controls were injected with same volume of distilled water. Testes were obtained through dissection on completion of 75 days of life. 5 micron thick sections were cut, stained by H.E. and Heidenhain's Iron Haematoxylin and studied under light microscope. It was observed from the quantitative analysis of the seminiferous tubules that there was increase in the number of the pachytene stage of primary spermatocyte in the experimental group as compared to that of the control animals of corresponding age.

Keywords: Monosodium glutamate (MSG), swiss albino mice, testes, primary spermatocyte (pachytene stage).

INTRODUCTION

Monosodium glutamate (Azinomoto) is a well known food additive used throughout the world. This flavour enhancer, not very long ago, was isolated in the laboratory, is called Monosodium Glutamate (MSG) At a later stage this flavour gained immense popularity worldwide. Since then it has been in use, widely in restaurants (particularly mixed in noodles, soups etc.), packaged food industries (eg. instant meals) and household kitchens. Modern commercial MSG is produced by fermentation of starch, sugar, beet, sugarcane or molasses.[1]

MSG's great property of enhancing the taste of the food and overwhelming popularity prompted the Scientists to analyse its possible effects in human. Since 1960s, the research data began to show dangers of MSG, though, differences in observations surfaced from time to time. The experimental animals showed some adverse effects. At the same time, the experiments showed that the neonates of mice were the most sensitive of all animals.[2] 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.[3]

There were several studies in respect of the effect of MSG in the tissues related to 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.). Though there were reduction in weight of testes and ovaries, the former did not show any changes on histological aspect. Fertility rate had been reported to be reduced in both sexes,[4] or in males only.[5] However, we couldn't get any literature regarding the histological studies of the testes following neonatal treatment of animals with MSG.

Hence we have embarked on this journey v to study the histological changes in the testes, in neonate mice.

Biochemical aspect: This compound MSG is a salt of sodium and (L -) Glutamic acid. In pure form it appears as a crystalline powder. L – Glutamic acid is an ubiquitous amino acid present in most foods either in the 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 ~ 28 gm that is derived from the diet and rest from the breakdown of gut proteins. The daily GA turnover in the body is ~ 48 gm. Despite this large turnover, the total pool of GA in blood is quite small, ~ 20 gm, because of its rapid extraction from and utilization by various tissues, particularly muscle and liver.[6]

Table-1: Study design of annual 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	
2) No. of neonates	Male – 7	Male – 5
3) Administration of dose.	Monosodium glutamate dissolved in distilled water (for injection) was injected subcutaneously (s.c.) on completion of 2, 4, 6, 8 and 10 days of life through disposable insulin syringe and needle.	Littermate control was injected with same amount of distilled water s.c. on same days of postnatal life like that of the experimental group.
4) Nursing and rearing	Neonates of both group (Expt. And Control) were kept with their dams till 27 th day. On 28 th day, the pups were separated from their mother and they were segregated on sex basis and they were given rat feed and water ad libitum.	
5) Histological study	At two and half months of age 7 nos. of male from Group A (Expt.) and 5 nos. of male from Group B (Control) were sacrificed after adminis tering Inj. Thiopentone Sodium intraperitoneally. Following dissection, the testes were collected and weighed and volume was measured.Tissues were processed for routine paraffin embedding and 5 micron thick sections were cut and stained with H & E and Heidenhens iron haematixylin stain. Stained sections were studied under light microscopy.	

Today, it is widely accepted that GA is the major excitatory neurotransmitter within the brain, mediating fast synaptic transmission and active in perhaps one third of all CNS synapses.[7]

MATERIALS AND METHODS

In this study, the newborn pups of the Swiss – Albino mice were selected as the animal model.

Summary of the procedure – The neonates were exposed to natural day and night sequence, each on 12 hours basis. The animals were divided into two groups, Group A (Experimental) and Group B (Control). Each group was further subdivided on the basis of the date of birth of the litters of each mother. The design of the study is shown in Table-1.

A **stock solution** of injection was prepared by dissolving 4gm of MSG crystals in 100 ml of distilled water (D.W.) 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.

Table-2: The weight of the pups of both the groups on 28th and 75th days

Control group		Experimental group
Mean birth wt. -	1.43 gm	1.49 gm
Mean body wt. on 28th day	11.66 gm	13.38 gm
Mean body wt. on 75th day	26.82 gm	27.9 gm
Mean birth -wt. -	1.49 gm	
Mean body wt. on 28th day	13.38 gm	
Mean body wt. on 75th day	27.9 gm	

Procedure for injection to the experimental group -

The newborns of the Experimental group were given the subcutaneous injection of Monosodium Glutamate (MSG) at the dose of 2 mg / gm(8) of body weight on day 2, 4, 6, 8 and 10 of postnatal life through insulin syringe after weighing each pup on those respective days and calculating the dose accordingly. The newborns of the Control group were given corresponding volume of distilled water for injection on similar scheduled days of postnatal life.

General Observations: All the animals were examined for any gross abnormalities or changes eg. general appearance, condition of skin and fur, structural defects etc. The weight of the pups were taken on 28 and 75 day after birth. After the testes were dissected there volume were majored by water displacement method.

Histological study: At two and half months of age of the aninmals were sacrificed after anaesthetizing with 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 (10.0%) through intracardiac catheterization . The testes were collected from respective animals dried then weighed, and volume of each was measured. The testis of one side of each animal were ly immersed in 10% formal saline while that of the other side were put in Zenker – Formol (Helly's Fluid) solution and the tissues were processed accordingly.[8]

Quantitative and qualitative assessment -

The present investigation was aimed at quantitative assessment of the seminiferous tubules. In case of the testis the parameters being the tubular diameter, volume proportion of the tubules and count of pachytene stage of the primary spermatocyte (as an index of spermatogenesis).[9] To estimate these stereology and morphometrical methods were used.

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

Group	No. of animals	Average Volume of testes (cc)	Average Weight of testes (gm)
Control	5	0.1	93.4
Experimental	7	0.1	93.2
Significance		P > 0.05, NS	P > 0.05, NS

Five tubules from each section were considered.Every fifth section were studied.Total number of twenty tubules were studied for each animal.

Testicular histological study -

(a) Volume proportion of the tubule:

Thus the volume proportion of the components "tubule" were calculated by superimposing Haug's graticule grid, randomly and repeatedly and subsequently counting the number of points which fall on the tubule. The volume proportion will thus be,

$$p = h / (h + m)$$

where, p is the volume proportion component, h is the number of points falling on the component and m is the number of points falling elsewhere.

(b) Volume of the testes:

(c) Diameter of the seminiferous tubule – it is measured by recording the shortest distance two diametrically opposite points on the border between basement membrane and germinal epithelium.[9] We did it by projecting the tubule on the screen of the Ermascope 202 and then measured with the help of eyeipiece micrometer, standardized by projecting on the stage micrometer before calculation. The total number of 20 tubules from five sections of each animal were taken for consideration in both control and experimental animals.

(d) Measurement of the tubular area and Nuclei count – Measurement of the tubular area was done with the help of the 'hit method'.[9] To gain an impression of spermatogenetic activity, we considered only the pachytene spermatocytes.[9] These cells stands out because it is present in almost every spermatogenic cycle and its characteristic nucleas and size were unmistakable. The nuclear count of pachytene spermatocytes were regarded as a criterion of the regenerative capacity of the germinal epithelium.

Table-4: Measurements of the diameter of the seminiferous tubules

Group	No. of animals	No. of the tubules observed @ 20 tubules per animal	Mean diameter (in micron)	Standard deviation (SD)	Significance (one tailed student's t-test)
Control	5	100	196.24		P > 0.05 i.e. NS
Experimental	7	140	193.46	14.94	

Statistical analysis: We analysed the data with the help of **Student's t - test (un-paired)**.[10]

OBSERVATION:

A. Gross observations – 5 male pups born out of 2 (two) females **(female** pups born were excluded**)** on different dates were selected as Control group (Group B). All the pups survived throughout the period of experiment. We collected the required tissues by performing the dissection on scheduled dates. In the second set of experiment, 7 male pups born out of 3 (three) mothers **(**female pups born were excluded**)** on different dates received MSG injection as per schedule and kept as Experimental group (Group A). However there were no casualties in these group also.

The pups of either group did not show any external abnormalities at birth. The average hair growth and eye opening were recorded on 7 days and 10 days respectively. The nutritional effect of MSG on the pups (if any) were gauged by the gain in weights. In this context we measured the weight of the pups at birth, on 28 th day and on 75 th day. The weights measured for animals of each group were put in tabulated form (Table-1). There were increase in the weight of the experimental group of pups. Mean body weight of control group were 26.8 gm and those of experimental group was 27.9 gm (75 days) Table-2.

B. Weight and volume of testis – The weight and volume of the each gonad were measured and the data were tabulated. There were not much difference in the mean weight and volume of the testes of two groups of animals (Table-3).

Histology of testes of control group – The light microscopic study was carried out. The tubules were of different shapes in the cross sections. Individual tubule was surrounded by interstitial connective tissue with capillary network and interstitial cells. When the cellular constituents of the tubules were studied we observed mostly three different spermatogenic cellular associations according to the cell cycle. The more frequent one seen was consisting of spermatogonia (dark and pale types), 2 to 3 layers of primary spermatocytes at different stages of division, mostly at the pachytene stage, several layers of spermatids and spermatozoon. The second cellular association were seen consisting of spermatogonia, several layers of primary spermatocyte and spermatids, and immature spermatozoa with elongated head and residual cytoplasm at the other end.

Nuclei of few sertoli cells in each of the tubule were seen.

Table-5: Measurements of the area of the seminiferous tubules

Group	No. of animals	No. of the tubules observed @ 20 tubules per animal	Mean diameter (in micron)	Standard deviation (SD)	Significance (one tailed student's t-test)
Control	5	100	0.0285	0.0302	P > 0.05 i.e.NS
Experimental	7	140	0.0277		

The nucleus appeared very pale and irregular outline, some with a centrally placed nucleolus. The nuclei were located away from the basement membrane. The interstitial tissue between the tubules contained connective tissue with capillaries and clumps of interstitial cells (Leydig cells). The cells were ellipsoidal with oval nuclei. The cytoplasm looked vacuolated, but strongly eosinophilic. The cells were grouped around capillaries.

Testes in Experimental Group – Tubules were cut in different cross sectional view. Different types of spermatogonia cells were seen. The diameter of the tubules were of different size in the same section similar to that of Control animals. The spermatogenic cells were several layers thick but lesser than that of Control animals. They were little dissociated and disarrayed, though cellular associations like that of the control group were seen. Even, spermatozoa with well formed tail were seen in the lumen of some tubules. But most important observation within the tubules were that of increased number of pachytene stage of primary spermatocyte. The interstitial cells appeared to be more in number and larger than that of the control animals. They were round to oval, with oval nuclei and vacuolated eosinophilic cytoplasm.

Quantitative assessment of testes: The quantitative record is an important evulationary guide for defining the functional status of the testis. We have selected the following parameters eg. diameter of the tubules, area of the tubules and number of pachytene stage of the spermatocytes per tubule. The comprehensive quantitative data along with statistical analysis were presented in (Table 4-6).

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, parenteral etc. We used mice because of several advantages eg. easy handling and availability, continuous reproductive cycle independent of seoson, reproducibility of the experiments owing to short reproductive cycle etc. Studies also established that mice is one of the most susceptible species to the MSG. The similar pattern of experiment were conducted by others.[2,11]

We performed this experiment with 2 mg/gm of body weight of MSG administered subcutaneously (s.c.) on alternate day starting from 2 nd day of neonatal life (48 hr.) for 5 days. Others had used different dose schedule, mostly a gradually increasing dose over the days. Olney[3] 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 others.[4,12,13] While the first author[4] used infant mice, the other two authors[12,13] used infant rat as animal model. Few authors[14,15] 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. In the present study pups getting MSG at a dose of 2 mg/gm of body wt survived through the period of observation while Diniz *et al*[16] 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. They further added that infant mice were not equipped with enzymes to metabolise MSG. According to them 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.[3] In the present study the pups received MSG at a dose of 2mg/gm of body wt survived through the observation. The dose schedule followed by us was much lower than that of experiment conducted by the others.[3,4,12,13]

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 food, because of neuroendocrinal disturbance. Similar finding were reported by other authors.[3,17]

In the present experiment we studied the histology of the testes after H & E staining for general histology, Heidenhain's iron haematoxylin staining for nucleus, the H & E stained sections of the testes of the experimental group showed tubules apparently similar in size as that of the control animals. We noticed three types of cellular associations in both groups of animals. In one of the

Table-6: Nuclear count per unit of the seminiferous tubules

Group	No. of animals	No. of the tubules observed @ 20 tubules per animal	Mean diameter (in micron)	Standard deviation (SD)	Significance (one tailed student's t-test)
Control	5	100	256.4	43.56	P > 0.05 i.e. Significant
Experimental	7	140	364.26		

cellular associations we noticed spermatogonial layer, resting stage of primary spermatocyte and spermatids while the other had the spermatogonial layer, pachytene stage of primary spermatocyte, spermatids and maturing spermatozoa with residual cytoplasm. In many of the tubules of the experimental group the layers of cells of the spermatogenic epithelium were less. Interestingly we observed that the pachytene stage of primary spermatocytes appeared to be more and the interstitial cells (Leydig cells) were larger in testicular sections of the experimental group compared to that of control. However, we could not find any literature describing detail histological features of testes in MSG treated mice hence these finding could not be compared. We also assessed the seminiferous tubules quantitatively eg. diameter and area of the tubules in a section, no. of the pachytene stage of primary spermatocyte in the tubules. There were little difference in the diameter of the tubules between control group and the experimental group. The mean diameter of experimental group were little less, though they were not statistically significant. (196.24 micron and 193.46 micron respectively). However there was a significant finding of increased no. of the pachytene stage of primary spermatocyte (Table-5) in the tubules of the experimental group (364.26/unit area in experimental group compared to that of 256.4/unit area of control group). The significant increase in the pachytene spermatocyte with attenuated layer possibly suggested compensatory mechanism for the more loss of speramtogenic cells in the experimental group.

The enlarged size of the Leydig cells observed in case of the testes of the experimental group might be due to compensatory hypertrophy since testosterone level showed a decreased l in MSG treated males.[15] No other worker commented on the size and number of the Leydig cells.

Summary: Our observation of MSG induced histological changes in the testes showed that both the germinal epithelium and Leydig cells were affected. There was fewer spermatogenic cells in many of the tubules with significantly increased pachytene stage of primary spermatocytes. The Leydig cells were hypertrophied. This was possibly due to disruption of the pituitary-adrenal axis leading to low serum LH and testosterone level. As reported by some authors, testosterone level showed a decreased level in MSG treated males.[15,17]

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.[1] From our observation we conclude that MSG in the dose schedule used by us in infant mice caused minor changes in seminiferous epithelium e.g increase in the pachytene stage of primary spermatocytes, apparent increases of Leydig cells and also gain in weight of the pups. These suggest some damage to the seminiferous tubule by use of MSG and its long term effect should be evaluated further.

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