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# MELAMINE IMPAIRS THE FUNCTIONS OF FEMALE REPRODUCTIVE SYSTEM IN RAT

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## ABSTRACT

The objective of the present study was to examine the probable toxic effect of melamine on female reproductive system functions in rat. Melamine is white colour crystalline nitrogen rich organic base. It is soluble in water and used in organic synthesis and in manufacture of resins. It is commonly used in a variety of common consumer products, such as laminates, resins, adhesives, glues, plywood packaging, floorings, plastics molding compounds, paint pigment, furniture, food packaging, dinnerwares and food utensils. The toxic effect of melamine on different bodily system has been reported but the harmful effect of melamine on female reproductive system has not been reported till to date. In our study, we observed significant changes in duration and cellular characteristics of estrous phases, body weight and organ (uterus and ovary) weight parameters, uterine smooth muscle contraction and histological changes of ovary and uterus of melamine exposed groups of rat compared to control group of rats. From these results, it may be concluded that melamine impairs the female reproductive system functions in rat by impairing the normal physiological set point.

**KEYWORDS:** Melamine; Ovary; Uterus; Estrous cycle; Uterine smooth muscle contraction.

## INTRODUCTION

Melamine is an organic base and a trimer of cyanamide with a 1,3,5 – Triazine skeleton. It is a chemical compound that has a number of industrial uses, including the production of laminates, glues, dinnerware, adhesives, molding compounds coating and flame retardant. Melamine is food an adulterants, it is added into milk to increase the protein count at less cost.[1] It is a white colour crystalline nitrogenous organic compound containing high amounts of nitrogen, which is interpreted as high protein in various standard protein measuring tests therefore added to foods to boost the protein content.[2] It is used primarily in the synthesis of Melamine formaldehyde resins for the manufacturer of laminates, plastics, coating, commercial filters, glues or adhesives and moulded compounds such as dishware and kitchenware.[3,4] When mixed with resins melamine has fire retardant properties owing to its release of nitrogen gas when burned or charred. It is also used in paints and as a fertilizer. Melamine is metabolite of cyromazine, which can be used as a pesticide or veterinary drug. In 2007, in North America, widespread pet illness and deaths were attributed to the formation of melamine-cyanurate crystals in the kidneys of these animals.[5] In 2008, in China, more than 50,000 infants have been hospitalized and resulted in 6 deaths after eating milk powdered baby food tainted with melamine.[6] Melamine is a harmful substance if swallowed, Inhaled or absorbed through the skin.[7] It causes renal and urinary problems and even infant death when it reacts with cyanuric acid inside the body.[8] The harmful effect of melamine is considered to increase in combination with its analogues, particularly cyanuric acid. FDA reported that when Melamine and cyanuric acid are absorbed into the blood stream, they concentrate and form large numbers of round, yellow crystals, which in turn block and damage the renal cells.[9] and chronic exposure may cause reproductive damage, excretory problem, cardiovascular and gastro intestinal damage.[10-14] The probable toxic effect of melamine in female reproductive system function has not been studied till to date. So the aim of the present work was to evaluate the effect of melamine on female reproductive functions in rats.

## MATERIALS AND METHODS

### *Reagents and Chemicals*

All the reagents used were of analytical grade. Melamine (≤99%) was purchased from Sigma-Aldrich, USA, Sodium chloride (NaCl), Potassium chloride (KCl), magnesium chloride ($MgCl_2$), Calcium chloride ($CaCl_2$), Sodium bicarbonate ($NaHCO_3$), Sodium dihydrogen phosphate ($NaH_2PO_4$), Dextrose, Eosin and Hematoxylin were procured from EMerck, India and SRL, India respectively.

***Protocol for Maintaining Rat***

Studies were performed on 3-4 months old female virgin albino rats of Charles Foster strain weighing about 90-100 gm. Animals were maintained in Animal House at Physiology Department as per national guidelines. The Animals were kept in equal light-dark cycle (12L: 12D) at a room temperature of 25ºC±2ºC and fed standard laboratory chow and water *ad libitum*. The animals were sacrificed by cervical dislocation on the 24$^{th}$ hour after the completion of last dosage.

***Experimental Design***

After one week of acclimatization to the laboratory environment, the animals were randomly distributed into three groups (each group contains seven animals) for chronic melamine exposure. The different effective dosages of Melamine were selected in this study according to the graded percentage of $LD_{50}$ value of Melamine in rat model.[9,15-17] During administration of melamine, daily vaginal fluid was collected for estrous cycle study. **Control**: received distilled water, **Treated I:** received 5% of $LD_{50}$ of Melamine, **Treated II:** received 10% of $LD_{50}$ of Melamine for 28 days exposure durations by oral gavage.

***Vaginal Smear Collection, Staining and Microscopic Observations***

For smear collection mainly used the Pipette smear Technique. The vaginal lavage was collected with a plastic pipette filled with normal saline (0.9% NaCl). By introducing a small amount of fluid into the vagina and placing one or two drops of the resulting cell suspension onto a glass slides in every morning (11.00-11.30a.m) during the exposure period.[18] A different glass slide was used for each rat housed in a cage for the specific dose. A comparative study of estrous cycle was carried out according to the method of Marcondes et al., 2002 with slight modifications.[19] Then dried vaginal smear were stained with eosin- hematoxylin following the protocol of Bancroft et al., 2002 with slight modifications.[20] The smears were analyzed under light microscope. Images were obtained by digital Camera fitted with light microscope. The Diestrus index was calculated as follows: Diestrus index= (number of day with clear diestrus smear/total duration of treatment) x 100.[21]

***Measurement of Body Weight and Organ Weight***

The body weight of the rats was measured from the first day of the treatment up to the last day of the treatment period in every ten alternate days. The weight of rats taken on the day of the application of first dose was considered as the initial body weight, and the body weight taken on the day of sacrifice was considered as the final body weight. The body weight and organ weight (Uterus and ovary) of each rat was assessed using a sensitive balance (Wensar-Electronic balance, India).

***Recording of Uterine Smooth Muscle Contraction in Single Dose Ex Vivo Experiment***

After overnight fasting, each rat was sacrificed by cervical dislocation. The abdomen of the sacrificed rat was then opened immediately and the uterus was removed by transverse incision. A segment of uterine tube was placed longitudinally in 40 ml organ bath of Dale's apparatus (Recording has been taken through Kymograph instrument) containing Tyrode's solution consisting of 8.0 g/l NaCl, 0.2 g/l KCl, 0.2 g/l $CaCl_2$, 0.1 g/l $MgCl_2$, 1.0 g/l $NaHCO_3$, 0.05 g/l $NaH_2PO_4$ and 1.0 g/l glucose (pH-7.4). The temperature of the bath was maintained within a range of 37±0.5ºC and continuously bubbled with 95% $O_2$ and 0.5% $CO_2$.[18,22]

***Histological staining technique for morphological study***

Neutral buffered formalin (NBF) fixed and paraffin impregnated uterine tissue sections were stained with hematoxylin-eosin stain according to the method of Bancroft et al, 2002[20] with slight modifications. Briefly, 5µm paraffin section of uterine tissue was kept sequentially in xylene and graded ethanol and stained with hematoxylin for 3 minutes. After removing the excess color the slide was counterstained with eosin for 1 minute and then the stained slides were dehydrated with graded ethanol, cleared with xylene and mounted with DPX and were observed under the microscope (100X magnification). Images were obtained by digital Camera fitted with light microscope.

***Statistical Analysis***

All the data obtained from this study were expressed as mean ± SEM. Statistical comparisons between the values obtained in control and in treated rats were evaluated by paired Student's t test or analysis of variance (ANOVA) whichever is applicable. $p \leq 0.05$ was considered as significant.

## RESULTS

***Effect of Melamine on Estrous Cycle Physiology in rat***

***a. On durations of Estrous cycle in rat***

To study the effect of melamine on the function of the ovary, the estrous cycle physiology has been studied in melamine exposed rats compared to control rats. We observed a significant increase in the durations of proestrus and metestrus phases and decrease in the duration of diestrus phase in comparison with control groups of rats. The estrus phase was increased (not significantly) in melamine exposed groups of rat compared to the estrus phase of control rats (Table 1 and Figure 1). Further, we found a significant decrease in Diestrus index dose dependently for both exposure groups compared to control group of rat (Table 1 and Figure 1).

**Table 1: Showing the changes in phases of Estrous Cycle of melamine exposed rat and control rat.** *The values are as mean ±SEM (n=7) for statistical analysis (*p<0.05 Vs Control).*

| Phases of Estrous cycle | Control | Treated I | Treated II |
|---|---|---|---|
| Proestrus | 3.6±1.05 | 6.8±0.80 (*) | 7.8±0.86(*) |
| Estrus | 5.8±1.24 | 7.4±0.67 | 7.4±0.74 |
| Metestrus | 6.2±0.49 | 8.4±0.92 | 7.4±0.60(**) |
| Diestrus | 10±0.63 | 5.4±1.12(***) | 4.4±0.67(***) |
| Diestrus Index | 31.46±2.26 | 19.288±4.01(***) | 15.68±2.45(***) |

***b. On cellular characteristics in Estrous smear***

In proestrus, estrus, metesturs and diestrus phases of the estrous cycle, the thickness of the smear were change due to changes in the number of nucleated epithelial cells, cornified epithelial cells, non-nucleated epithelial cells as revealed from the stained representative slides in a dose dependent manner in both melamine exposed groups in comparison with control groups of rats.

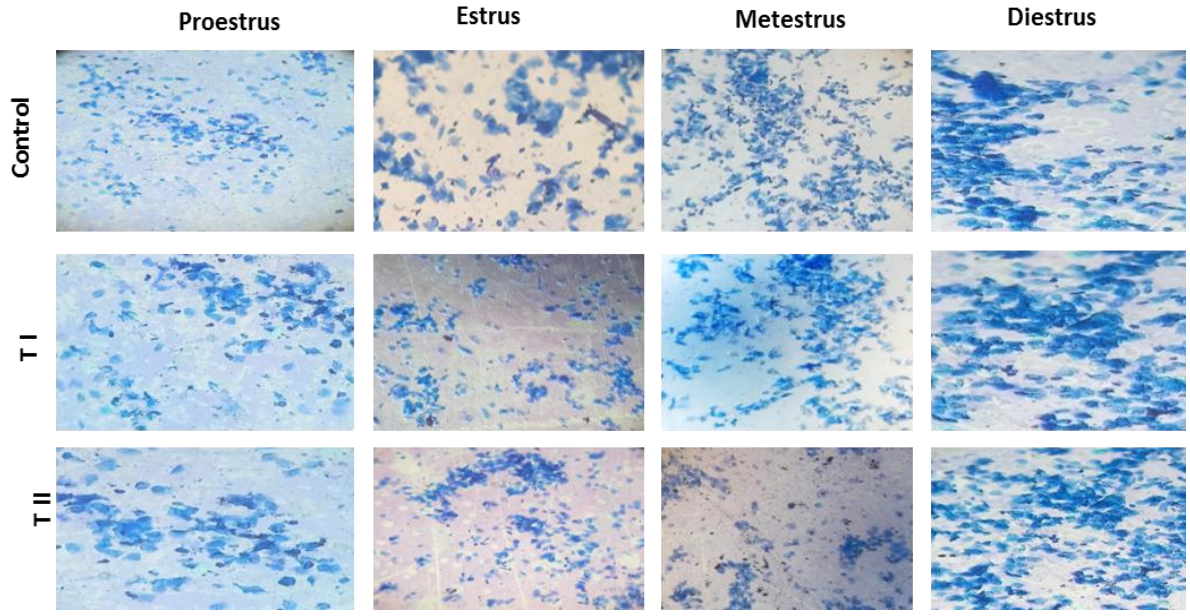


**Figure 1: Cytological change of phases (proestrus, estrus, metestrus and diestrus) of estrous cycle of control and melamine exposed groups of rat (Magnification 10x) for 28 days exposure duration.**

***Effect of Melamine on mean body weight and organ weight of female rats***

The mean body weight of melamine exposed groups of rat increased significantly in a dose dependent manner compared to control groups of rats. Further, the relative organ weight of uterus and ovary (reproductive organ) has been increased significantly in melamine exposed groups of rats compared to control groups of rats (Figure 2 and 3).

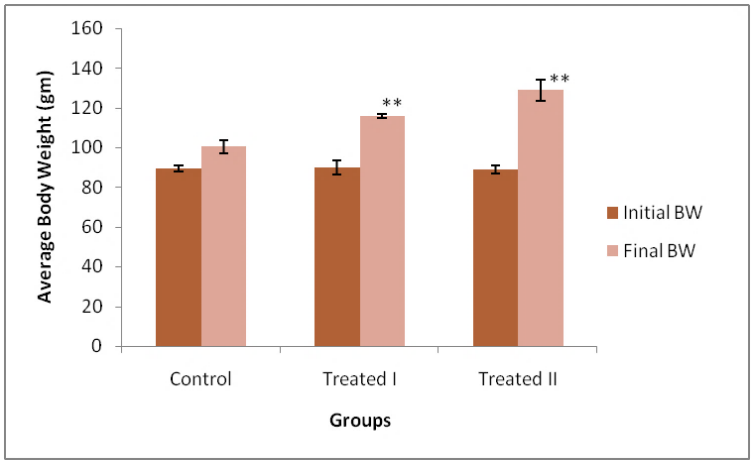


**Figure 2: Bar diagram represent the mean body weight of melamine exposed groups and control groups of rats.** *The values are as mean ±SEM (n=7) for statistical analysis (*p<0.05 Vs Control).*

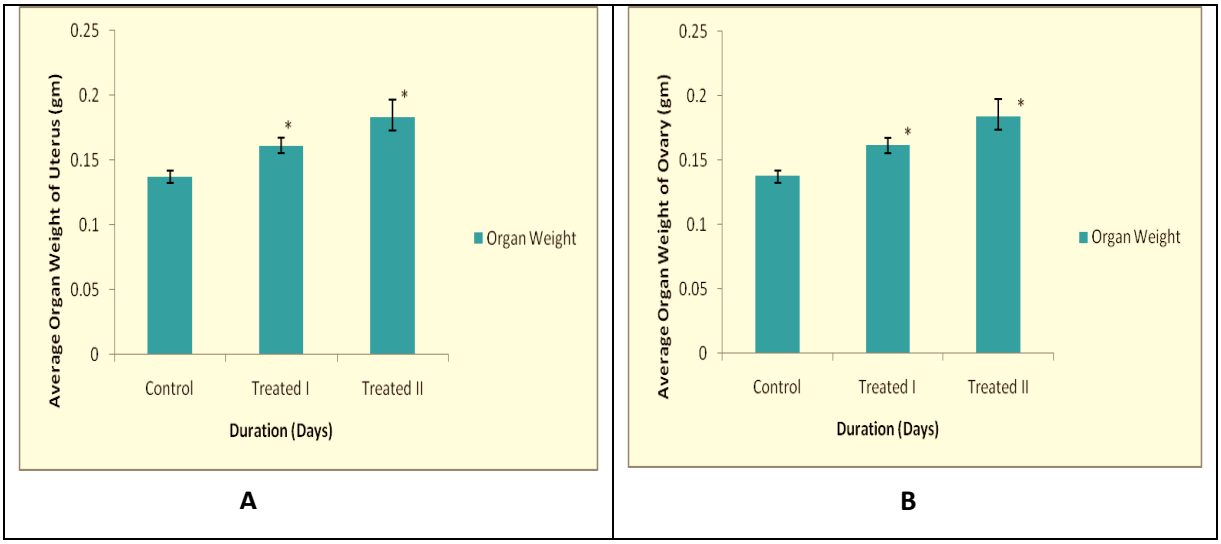


**Figure 3: Bar diagram represent the changes of organ weight (A-represent the organ weight of Uterus and B-represent the organ weight of Ovary) of melamine exposed groups and control groups of rats. *The values are as mean ±SEM (n=7) for statistical analysis (*p<0.05 Vs Control).***

***Effects of Melamine on the contraction of uterus ex vivo in rats***

We observed a significant potentiation of the amplitude of the contractions and frequency of contraction of uterine smooth muscle in melamine exposed groups of rats recorded *ex vivo* in a dose response manner compared to the amplitude of contraction and force of contraction of uterine smooth muscle in control rats (Figure 4 and Figure 5).

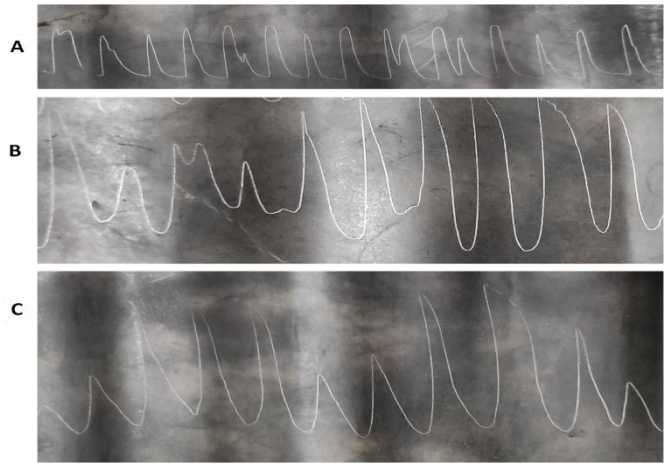


**Figure 4: Representative records of the isolated uterine smooth muscle contraction of control and melamine exposed rats *ex vivo* for 28 days exposure durations.** (A) Uterine motility of control; (B) Uterine motility of Treated I; and (C) Treated II.

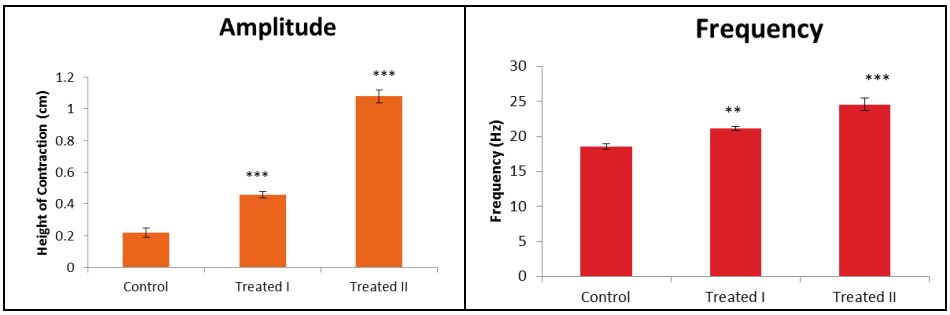


**Figure 5: Showing the changes in the amplitude and frequency of isolated uterine smooth muscle contraction *ex vivo* of rats for 28 days exposure durations compared to amplitude and frequency of contraction of control rats.** *The values are as mean ±SEM (n=7) for statistical analysis (*p<0.05 Vs Control).*

***Effect of Melamine on histological change of uterus and Ovary***

To study the melamine induced impairment, we observed the histological characteristics of ovary and uterus of melamine exposed and control groups of rats. We observed that the cell layers of uterine tissues such as endometrium, ectometrium and perimetrium were degenerated in both the exposure group compared to control group. In case of ovary, follicular development was disturbed in melamine exposed groups of rat. The histo-architectural changes in the wall structure of the uterus and ovary of the melamine exposed groups were altered compared to control groups of rats (Figure 6).

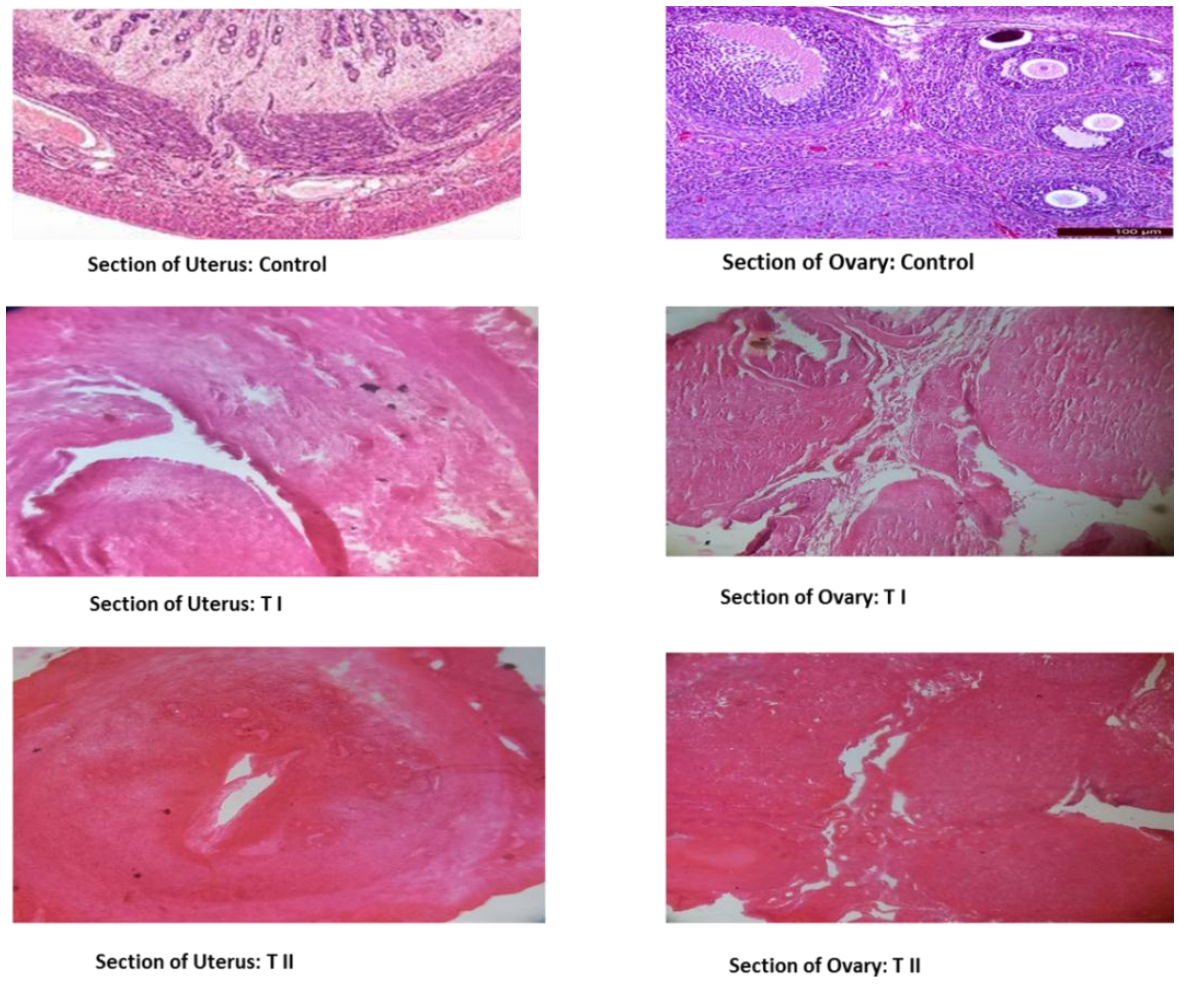


**Figure 6: Microphotographs of paraffin fixed transverse sections of the uterus and ovary stained with hematoxyline and eosin showing the morphological alterations of uterus and ovary in melamine exposed and control groups of rats of 28 days exposure durations (10X magnification).**

## DISCUSSION

The objective of the study was to examine the probable toxic effects of melamine on the female reproductive system functions. Melamine can enter into our food habit through melamine contaminated crops and leaching of melamine from plastic used in food equipment and many other routes.[23-28] In this paper we report that melamine impairs the functions of ovary and uterus in rat by altering the female reproductive functions. An estrous cycle of the rat is sequentially consisting of four phases, namely, proestrus, estrus, metestrus and diestrus.[29] The estrous cycle in rat is a significant biological tool by means of which we can study the reproductive functions of ovary in patho-physiological conditions under the influences of gonadotropins and ovarian estrogen. In our study, we observed a significant increase in the durations of proestrus and metestrus phases and decrease in the duration of diestrus phase in comparison with control groups of rats. The estrus phase was increased (not significantly) in melamine exposed groups of rat compared to the estrus phase of control rats (Table 1 and Figure 1). Further, we found a significant decrease in Diestrus index dose dependently for both exposure durations compared to control group of rat (Table 1 and Figure 1). These results suggest that the changes in the phases of estrous cycle might be due to augmentation or inhibition of the secretion of luteinizing hormone and follicle stimulating hormone from the anterior pituitary and estrogen from the ovarian follicles.

Further, the mean body weight of melamine exposed groups of rat increased significantly in a dose dependent

manner compared to control groups of rats. The relative organ weight of uterus and ovary has been increased significantly in melamine exposed groups of rats compared to control groups of rats (Figure 2 and 3). This results suggest that melamine increases the body weight probably by impairing the set-point homeostatic mechanism for the body weight and organ weights of uterus and ovary. We also observed a significant potentiation of the amplitude of the contractions and frequency of contraction of uterine smooth muscle in melamine exposed groups of rats recorded *ex vivo* in a dose response manner compared to the amplitude of contraction and force of contraction of uterine smooth muscle in control rats (Figure 4 and Figure 5). These results suggest that melamine potentiates the amplitude and force of contraction of uterine smooth muscle probably by augmenting to secretion of oxytocin which induces the contraction of uterine smooth muscle.

To study the melamine induced impairment, we observed the histological characteristics of ovary and uterus of melamine exposed and control groups of rats. We observed that the uterine muscle cell layers were degenerated in both the exposure group compared to control group. In case of ovary, follicular development was disturbed in melamine exposed groups of rat. The histo-architectural changes in the wall structure of the uterus and ovary of the melamine exposed groups were altered compared to control groups of rats (Figure 6). This result of the histological studies suggests that melamine might depress the function of the uterus and ovary by inducing the structural degenerations in the wall structure.

So, considering the above results it can be concluded that melamine impairs the function of ovary and uterus in female rat probably by altering the phases of estours cycle physiology and uterine smooth muscle contraction probably by augmenting to secretion of oxytocin which induces the contraction of uterine smooth muscle and structural degeneration in wall structure of ovary and uterus.

## CONCLUSION

In conclusion, it may be suggested that melamine impairs the functions of female reproductive system in rat probably by altering the functions of female reproductive organs.

### Conflict of Interest

There is no conflict of interest.

## ACKNOWLEDGEMENT

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