



Research article

Effect of multiple doses of N-methyl-N-nitrosourea, an end product of methylguanidine (found in processed food), on the fertility of female Swiss albino mice



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ABSTRACT

Methylguanidine, an originator of carcinogenic methyl nitrosourea, has been found in many animal meats and processed stored food often in high concentration. The present study was designed to understand the multiple dose effect of N-methyl-N-nitrosourea (MNU), an end product of methylguanidine, in Swiss albino mice fertility as well as cancer induction. Accordingly, a total of five experimental groups of animal (female Swiss albino mice) were taken, considering group-I as vehicle control and group-II-V as treatment groups (whereas group-II-V were treated with single to quadruple doses of 50 mg/kg of MNU respectively in a three weeks interval). After accomplishment of MNU injection, each female mice was mated with male mice to check the fertility efficiency. The results of the study indicated that, mice treated with highest number of MNU doses were 42.85% less efficient in getting pregnant than the control mice. There were noted changes in body weight, food and water intake upon MNU-exposure compared to control group. A significant increase in cumulative weight of vital female organs like uterus and ovary were also observed in mice injected with quadruple doses of MNU (50 mg/kg) compared to control mice. The findings of the study suggest the direct effect of MNU in pregnancy, without any cancer incidence in the vital female organs of Swiss albino mice.

1. Introduction

Urbanization, lifestyle changes and increased population are fuelling the demand for processed food (Clark et al., 2019; Satterthwaite et al., 2010). During this process, food materials are exposed or contaminated with diverse ranges of carcinogens (Clark et al., 2019; Christian, 2007). In recent years various reports has been published on the role of food carcinogens in increased cancer incidence and infertility in human population (Clark et al., 2019; Koriech 1994). Methylguanidine, an originator of carcinogenic methyl nitrosourea, has been detected in many animal meats and processed stored food often in high concentration (Fujinaka et al., 1976). The carcinogens might not be directly present in raw food materials but presence of some originator like methylguanidine promotes the production of methyl nitrosourea like carcinogen during processing or storage (Fujinaka et al., 1976). N-nitroso compounds are

known food carcinogens and are easily produced by reaction between nitrogen oxides and secondary and/or tertiary amines or amides (Janice et al., 2009). On the other hand exposure of these carcinogens causes hormonal disturbances in body along with severe reproductive problems and other health disorders. Human population are generally exposed to these compounds through diet like preserved meats, smoked fish, sausages, dried milk, pickles, etc. (Liu et al., 2009). People working, as hairdresser, in rubber industry, with metal, leather and fertilizer are also prone to get contaminated with these carcinogens (Raj et al., 2003; Lewis et al., 2013). There are also chances of getting exposed through cosmetics and with few pharmaceutical products (Carpenter and Bushkin-Bedient 2013). N-methyl-N-nitrosourea (MNU) is one such carcinogen found in various processed food materials significantly (Donnelly et al., 2004). MNU is a direct alkylating agent that damages DNA by binding to it which causes genetic mutation and ultimately increases the risk of cancer

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incidence and progression (Houten and Sancar 1987; Gorbacheva et al., 1988). MNU induced retinal degeneration and/or degenerative disease models are very popular to study the therapeutic trials (Tsubura et al., 2011). For example, a single dose intra-peritoneal injection (50 mg/kg body weight) of MNU in Sprague Dawley (SD) rats induces estrogen (ER+) dependent breast mammary tumors (Rivera et al., 1994). MNU treated retinal degeneration and/or degenerative disease models are very popular to study the therapeutic trials (Chen et al., 2014; Maurer et al., 2014). The altered organotropic effects of MNU in different animal model depends not only on dose but also on route, age and sex (Thompson and Singh 2000; Chan et al., 2007; Chang et al., 2012). The hormonal imbalance treated by MNU might affect the fertility by damaging the reproductive organs (Iannaccone 1984). Considering the above factors we are intended to study the effect of multiple doses of MNU on reproductive system in female Swiss Albino mice. Accordingly, primary objectives of our study was to check the effect of MNU on fertility of Swiss albino mice and thereafter the effect of MNU on developing cancer incidence in female vital organs.

2. Materials and methods

2.1. Chemicals

N-methyl-N-nitrosourea (MNU) was procured from Toronto research chemicals, Canada. Sodium chloride was purchased from Merck, Germany. Hematoxylin, eosin, ethanol, and other chemicals otherwise not mentioned were purchased from Sigma Aldrich.

2.2. In vivo MNU treatment

Use and care of mice for the experiment described herein was approved by the Institute of Advanced Study in Science and Technology Animal Ethical Committee (IASST/IAEC/2018-19/11). Four weeks old female Swiss Albino mice (n = 31) were purchased from Chakraborty enterprise, Kolkata, India. Animals were randomly divided into five groups (n = 6 in each group; whereas in group-V: n = 7) and vehicle or vehicle containing MNU (50 mg/kg) was administered through intra-peritoneal (i.p.) (for better absorbance possibility) route at a fixed time interval on the basis of our previous study (Das et al., 2019; Samanta et al., 2016). The dose of MNU was selected on the basis of our previous experimental data; as the mouse dose is almost double of rat dose, due to difference in metabolic system we used MNU at 50 mg/kg body weight in multiple time (single to quadruple time) to fulfill our research objectives (Das et al., 2019). MNU was dissolved in 0.9 % sodium chloride solution for injecting in mice (Samanta et al., 2016). The groups were divided as; Group-I: mice received 0.9 % sodium chloride in every 21 days till the end of the experiment, Group-II: mice received single dose of 50 mg/kg MNU, Group-III: mice received two doses of 50 mg/kg MNU at three week interval for each dose, Group-IV: mice received three doses of 50 mg/kg MNU at three week interval for each dose, Group-V: mice received four doses of 50 mg/kg MNU at three week interval for each dose. Weekly body weight changes and daily food and water intake was noted over 12 weeks-time period. Throughout the experimental period mouse were sustained under controlled atmosphere (24 ± 2 °C and 55 ± 5% relative humidity) and supplied standard rodent pellet diet (Provimi animal nutrition Pvt. Ltd.) with water ad Libitum. All the statistical data analyzed were two sided.

2.3. Fertility experiment

After completion of 12 weeks, animals from each group were divided into equal number and kept in two separate cages (no. as A & B; one extra in Gr-VB). A healthy male mouse was introduced in to each cage to start the mating process and confirmed by vaginal smears. On alternative days

male mice from each cage were replaced with other healthy male mice to nullify the male mice with infertility problem. After 10 days, male mice from all the groups were removed. All the female mice were kept under observation for next one month to determine the fertility rate and to count the number of litter born (Table S4, Graphical abstract/Figure S1). To figure out any signs of teratogenicity in the MNU treated mother, the pups were separated at 22nd day and three mother mice from each groups were sacrificed under euthanasia (Cervical dislocation) and vital organs like uterus along with ovaries, liver and kidney were collected in 10 % formaldehyde solution and stored until pathological changes observed (Monsees and Opuwari 2017).

2.4. Histopathology of vital organs

To study the pathological changes in the tissues, all the organs were removed from the formaldehyde, dehydrated in different concentrations of ethanol, cleared in xylene and embedded in paraffin. The paraffin blocks were sectioned at 5 µm size using microtome and mounted on glass slides. Further the slides were stained using hematoxylin and eosin and observed under light microscope to study the pathological changes. Photographs (10 X) were taken using Zen software of Zeiss microscopy.

2.5. Statistical analysis

All statistical analysis were two-sided with corresponding 95% confidence interval (CI). The two way ANOVA followed by Bonferroni test was used to examine the treatment effects of MNU on body weight changes, food and water intake capacity and organ weight changes. A significance level was set at P < 0.05. Statistical analyses were performed in GraphPad Prism 6.05.

3. Results

3.1. Changes in body weight and food intake in MNU treated female Swiss Albino mice

To determine the effect of MNU at different exposures in female Swiss Albino mice, the change in body weight was recorded weekly and is depicted in Figure 1A. Experimental observation suggest that animals of the control group without MNU (Group-I) gained weight concurrently to that of the MNU treated mice of Group –II to Group V (Raw data are available as Supplementary Table S1). The relative weight increased, until the final week in all the groups. This establishes that MNU has no consistent effect on the body weight. Result showed in Figure 1B illustrates the difference in cumulative intake of food between the control and the MNU treated mice (Raw data is available as Supplementary Table S2).

It was measured that compared to the control group, the food intake record of the MNU exposure group (Group –II to Group V) was lower. Whereas, among the different MNU treated group, the group with quadruple shot were measured with less food intake. The relative food intake in the experimental group increased up to week 8, after which it declined until week 10; and thereafter it increased and peaked at the final week. Similar results were measured for water intake and are represented in Figure 1C (Raw data are available as Supplementary Table S3). The relative water consumption was higher in control group than in the MNU treated groups. However, in Group –I (control), maximum water intake was noted on week 10 and in the MNU treated groups the intake was higher on week 11. In the following weeks till the end of the experimental observation, it declined gradually. The findings indicated that the implemented long term dose of MNU non-significantly affect the food and water intake compared to that of the control. But, no difference in the food and water intake was noted between the single, double, triple and quadruple shot of MNU group.

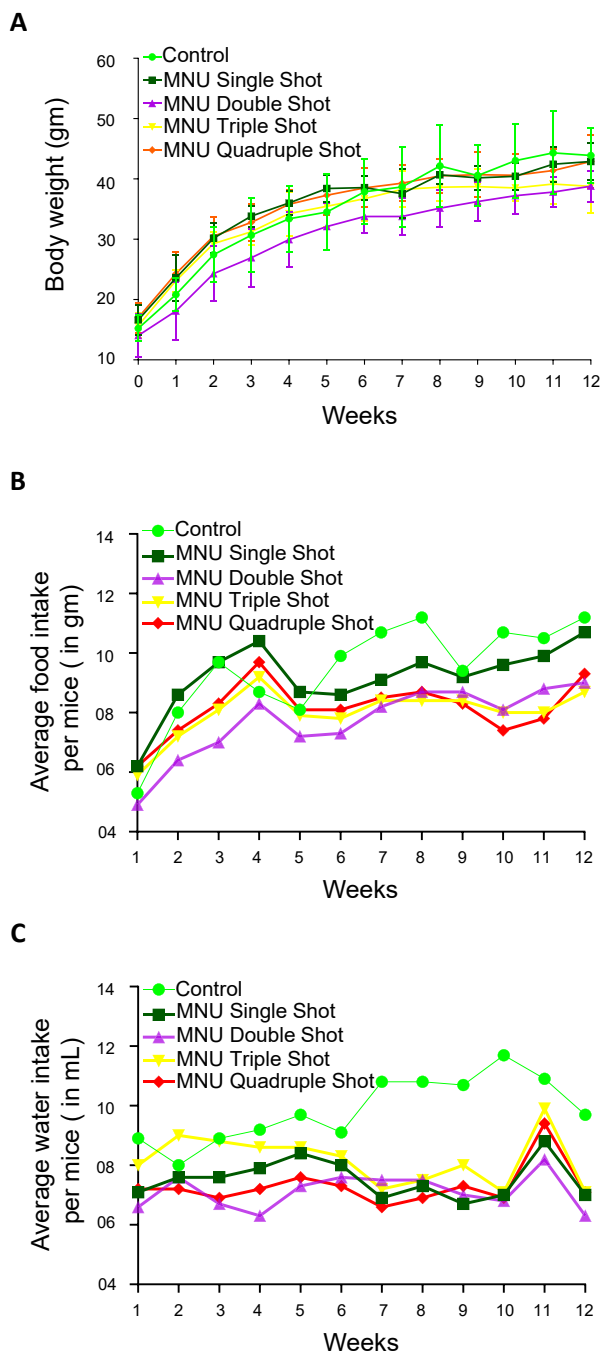


Figure 1. Effect of MNU on physiological changes. (A) Body weight changes in Swiss albino mice treated with single to multiple doses of MNU over a period of 12 weeks. (B & C) Average food and water intake by mice per group injected with various doses of MNU throughout 12 week time period. All the results were expressed in mean \pm S.D. ($n = 6$ for both control and MNU treated group; $n = 6$ for each group and $n = 7$ for group V at conclusion of the study). All the results were expressed on calculation the data available in each week. Statistical significance was determined by two way ANOVA followed by Bonferroni test.

3.2. Effect of MNU in sexual maturity and tumor incidence on mammary pad

To determine the effects of MNU in reproduction and fecundity of the female Swiss albino mice, an adult male mouse (~ 4 months old) was introduced for the breeding process in each sub divided groups. To increase the chances of litter production, the male mice were randomly shifted within the sub divided groups on alternative days. After 4 week of

observation, the percentages of pregnant mice per group were recorded (Table 1). Result showed that the percentage of pregnancy in the Group-I was significantly higher as compared to that of the MNU treated groups (Raw data is available as Supplementary Table S4). In Group -I, the rate of pregnancy was 100 %, whereas in Group -II, III, IV and V it were 66.66 %, 50 %, 66.66 %, 42.85 % respectively (Table 1). These findings suggest that the 12 week exposure of MNU in the female mice affect the reproductive system of the female Swiss albino mice significantly, and reduces the chance of litter birth.

To note the effect of MNU in birth outcome, the fecundity rate was also examined during the breeding period in both control and MNU treated groups. The total numbers of babies born per group were recorded (Table 1) (Raw data is available as Supplementary Table S4). Result reveals that the no. of baby born in Group -I was greater than that of the MNU treated groups, however were not statistically significant. In Group-I, 36 nos. of litter were born, whereas, in MNU treated groups no. of pups born viz. Group II, 31; Group III, 33; Group IV, 31; and Group V, 29 respectively (Table 1). But interestingly, the no. of average baby born per mice is quite high in Group V, whereas in control group it seems quite low (Table 1). These representative data indicated that MNU does not damage the reproductive system but may affect the uteri. However, the dual response of pregnancy and no. of litter born suggests that may be the female mice in the control group are producing mature follicle early in contrast to that of the MNU treated groups.

During the MNU exposure, every mice from each group was routinely checked for the palpable mammary tumor; and no mammary tumor was identified in a single mice even after sacrifice.

3.3. Relative weight of vital organs

To figure out any signs of abnormalities in the reproductive system of MNU treated mother mice, the pups were separated at 22nd day and meanwhile the vital organs (uterus along with ovaries and kidney) collected from three mother mice of each group were weighted (Raw data are available as Supplementary Table S5). Result suggests that there was no constructive difference in kidney weight between the control and MNU induced mice of different dose.

In Group-I the mean weight of kidney was 0.51 ± 0.06 g, in Group-II 0.46 ± 0.04 g, Group -III 0.45 ± 0.08 g, Group -IV 0.49 ± 0.05 g, and Group -V 0.45 ± 0.07 g respectively (Figure 2A). However, there was a notable augment in uteri weight in the MNU treated mice. The mean weight of uterus along with the ovaries of Group -V was 0.45 ± 0.25 g, while the control (Group-I) had a mean weight of 0.26 ± 0.05 g. Nearly 2 fold increase in uterus and ovary weight of Group-V compared to control suggests the direct effect of MNU in pregnancy process (Figure 2B).

3.4. Histological analysis of liver, kidney, spleen, uterus and ovary

To understand the difference in the number of pregnant mice between control and MNU treated group the histological analysis was done of different female vital organs. For example, the liver histology (Baratta et al., 2009) shows hexagonal or pentagonal hepatic lobules with central veins and hepatic triads embedded in connective tissues in all the slides. Hepatocytes are arranged in trabeculae running radiantly from the central vein separated by sinusoids containing kupffer cells. They are regular and change does not appear in control and other groups.

In the case of kidney histology (Sadek et al., 2016) analysis, all slides show cortical labyrinth, medullary rays and also renal corpuscles, collecting tubules, renal tubules appear intact (Figure 3). Kidney architecture appears unaltered in all tissues of treated and untreated groups. Spleen histology analysis suggested no visible difference or irregularities between control and treated groups. Red pulp white pulp regions are clearly visible where red pulp constitutes the greater bulk of the splenic tissue and white pulp area takes up the bluish stain and is well marked.

On analysis of ovarian histology (Camargo et al., 2014; Gerez et al., 2005), the presence of preantral, antral and developing follicles types for

Table 1. Table depicts the effect of MNU exposure and corresponding details of % of pregnant mice, no. of litter born per group and no. of average litter born per pregnant mice.

Group description	% Pregnant mice per group	No. of litter born per group	No. of average litter born per pregnant mice
Group-I	100.00	36	6.00
Group-II	66.66	31	7.75
Group-III	50.00	33	11.00
Group-IV	66.66	31	7.75
Group-V	42.85	29	9.66

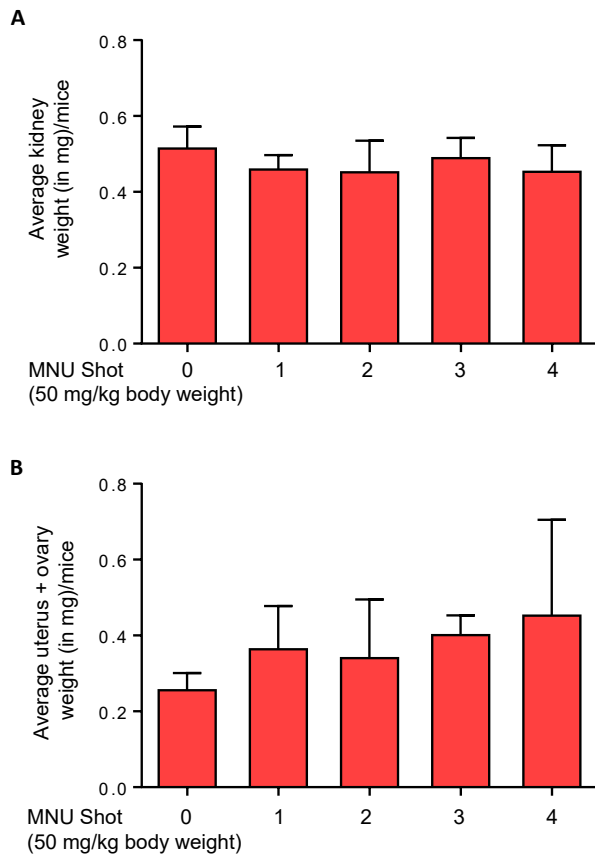


Figure 2. Effect of MNU on fertility and organ weight (A) Average weight of kidney of mice after single to multiple doses of MNU (50 mg/kg). (B) Average weight of uterus + ovary of mice after single to multiple doses of MNU (50 mg/kg). * $P < 0.05$ in comparison with only control group. Two tailed unpaired Students t-test was performed to check the statistical significance between control group and the treated group.

the experimental animal of each group was examined. They were compared among different doses of MNU exposure and confirmed altered types of follicles were present in different groups. The histological analyses showed corpus luteum, essential for maintaining the pregnancy, were present in control and a single or double shot of MNU exposure but not in triple or quadruple exposure of MNU (Figure 3). Developing follicle along with antral follicles were present in control group along with group 1 and 2, whereas degenerating follicle is seen with a reduced number of developing follicles in group 3 and 4. The decrease in the number of developing follicles reduces the availability of mature ova for fertilization thus, minimizing the chances of pregnancy. This finding suggests the alteration in an ovarian structure on multiple MNU exposure, which ultimately affects the pregnancy and baby birth (Figure 3).

The uterine histology (Brody and Cunha 1989; Dixon et al., 2012) analysis appears quite normal in control and/or up to double exposure of 50 mg/kg of MNU, but in other treated groups uterine endometrial

glands are not developed and also, the luminal epithelium appears to be non-dividing and low in height, thereby further reducing the chances of successful implantation and pregnancy (Figure 3).

4. Discussion

Recent studies suggest, processed food creates several health issues including metabolic disorders, infertility and obesity (Clark et al., 2019; Liu et al., 2009). Among them infertility is the major challenge in today's society especially for women maintaining an urban lifestyle (Clark et al., 2019). The purpose of the present study was to evaluate the effect of MNU, an end product of methylguanidine generally found in processed food, on female mice organs leading to infertility and/or in cancer induction (Osowole et al., 2013). MNU is a widely used chemical for the induction of breast cancer in rodents; but, this might be species specific (Das et al., 2019; Samanta et al., 2016). Accordingly, in the present study, we have evaluated the effect of multiple exposure of lower dose (50 mg/kg) of MNU in Swiss albino mice for fertility or cancer induction in female vital organ as well as palpable tumor in mammary pad. The single dose of MNU is used to treat the mice for DNA damage response, but there was nothing in details about the effect of MNU on mice fertility (Gorbacheva et al., 1988). The present study was designed to understand the long term and/or effect of multiple exposure of MNU to cause serious health issues like, infertility, cancer induction, etc. in Swiss albino mice. MNU, a spontaneously dynamic methylating agent causes DNA damaged and subsequently genetic modification, which may affect short term as well as long term disorders (Gorbacheva et al., 1988). In current study even four doses of MNU at 50 mg/kg in three weeks interval, did not induce any noted cancer occurrence in female vital organ including mammary pad in Swiss albino mice. Neither palpable tumors or solid tumor were observed throughout the study in all the experimental groups. This observation is may be hormone related, as SD rat developed ~100% mammary tumor after single dose of MNU injection in comparison with human ER positive breast cancer (Samanta et al., 2016). But, there was a noted change in fertility of those female mice, after getting exposure to MNU. The MNU exposure mice losses their efficacy to get pregnant even ~57% lower compared to untreated MNU group. Although the data was not statistically significant because of either lower sample size or lesser experiment time, but the clear trend is there in lowering the efficacy of getting pregnant. Also, the effect of multiple doses of MNU is not very clear to create impact in pregnancy over single or double doses, may be due to sample size. On the other hand, the histopathology analysis revealed the clear alteration of the ovary size and developing follicles in multiple doses of MNU treated mice, which effectively reduces the chance of pregnancy or lesser number of litter birth. This effect further correlated with the noted change in general food and water intake in MNU treated mice compare to control group. This study also gave a conclusion that at 50 mg/kg body weight dose of MNU (even after 4 doses), was unable to induce a single tumor incidence in female vital organ or mammary pad, which provide evidences about the limited use of Swiss albino mice in MNU treated mammary cancer model, whereas SD rats widely used for screening of anti breast cancer drugs. Further, a detailed study is required to understand the exact insight mechanism of the effect of MNU in fertility but not cancer induction. In conclusion, the experimental findings suggest about the major impact of

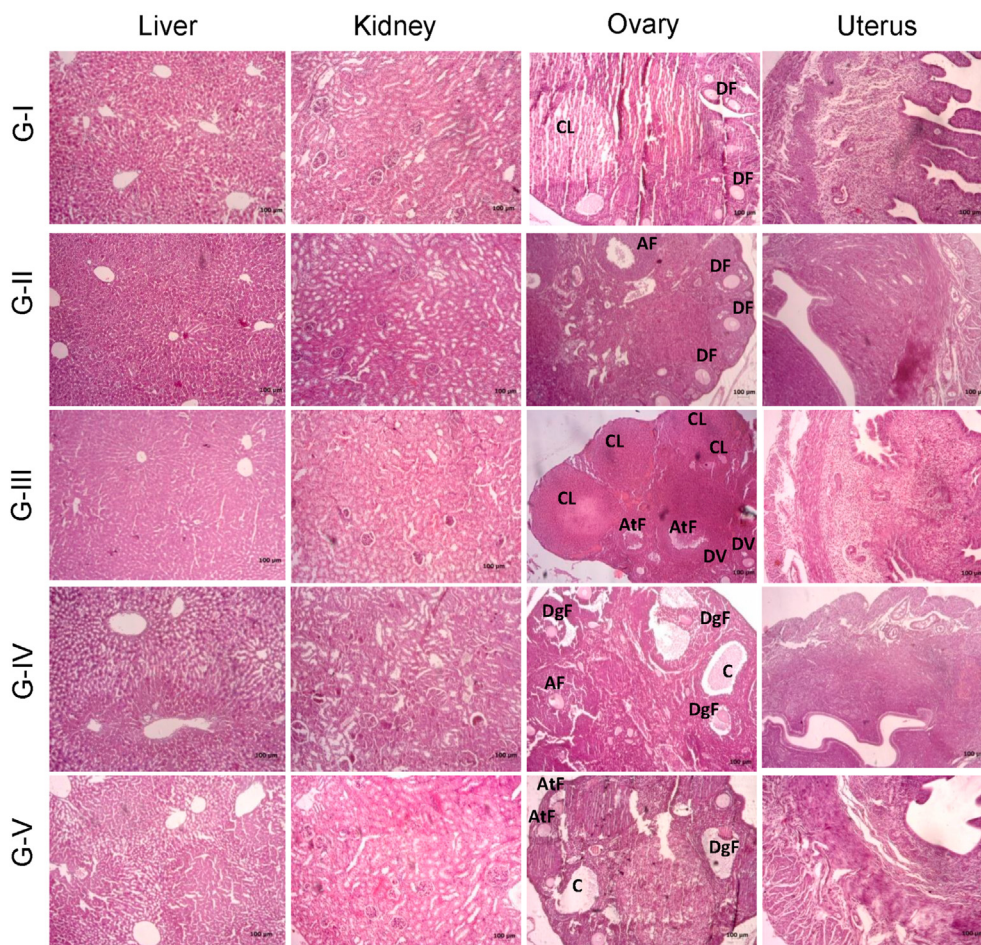


Figure 3. Effect of multiple doses of MNU on pathological changes in vital organs. The slides were stained with haematoxylin and eosin and observed under light microscope at 10 X resolution. Scale bar 100 µm. Abbreviations: C: Ovarian cyst; CL: Corpus luteum; DF: Developing follicle; AF: Antral follicle; AtF: Atretic follicle; DgF: Degenerating follicle. At least three different sections from each mice from each group have been consider. The data shown are the roughly average of total observation.

MNU on fertility and pregnancy in Swiss albino mice and alarmed the use of processed food intake on human health.

Declarations

Author contribution statement

Raghuram Kandimalla: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Momita Das, Swarnali Bhattacharjee: Performed the experiments; Wrote the paper.

Paramita Choudhury, Rajlakshmi Devi, Narayan C Talukdar: Analyzed and interpreted the data; Wrote the paper.

Suman Kumar Samanta: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

Supplementary content related to this article has been published online at <https://doi.org/10.1016/j.heliyon.2021.e06738>.

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