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Original Article

Teratogenic Effect of Smokeless Tobacco on the Gonads of Albino Rats

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Abstract

Background

Teratogens are substances that, when exposed to a pregnant woman, may result in a physical or functional abnormality in the human embryo or fetus. Caffeine, cocaine, tobacco and alcohol are common teratogens. Teratogenecity depends upon the duration of exposure and amount of teratogens. Smokeless tobacco is a tobacco product used other than smoking such as chewing, sniffing or placing the product between the gum and the cheek or lips.

Materials and Methods

This is a experimental study conducted from September 2023 to August 2024 where sixty healthy adult albino rats (11 - 13 weeks old) weighing between 150 -200 gm were bought and reared in the animal house of Anatomy department of Nobel Medical College and all the rats were randomly divided into three equal groups; a control group and two experimental groups (E1 & E2) where E1 were fed with Rajnigandha and E2 were fed with Bhola gutkha as the test materials. Statistical analysis was done in SPSS version 19. All the data were expressed as mean±SD.

Results

Reduction in gross body weight from initial (163±10.59gm) to (149.50±13.83gm), volume from (3.50±0.52cc) to (2.50±0.52cc) and diameters of seminiferous tubules from (348.05±23.33µm) to (208.30±12.30µm) and ovarian follicles from (21.35±3.83µm) to (20.74±2.55µm) and reduction of sex ratio in the experimental groups.

Conclusion

The study conclude that the smokeless tobacco have a wide range of effects on the overall reproductive functions of the Albino rats and low birth weight baby along with reduction in sex ratio which implies that it directly have bad effects on the human reproductive health.

Keywords: *Gonads, Microanatomy, Fetal weight, Smokeless Tobacco, Teratogens*

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Introduction

Teratogens are substances that have the potential to cause a human embryo or fetus to have a physical or functional abnormality when they are exposed to a pregnant woman. Common teratogens include cocaine, alcohol, and caffeine. This phenomenon is known as embryo-toxicity, and it is the study of harmful reactions in progeny by drugs or the environment when the mother is exposed to them at any point of time from conception until delivary [1]. Any forms of aberrant development brought on by environmental damage are referred to as developmental toxicity, these can include congenital diseases without apparent morphological abnormality, delayed mental development, or growth retardation [2]. Different forms of smokeless tobacco products include chewing tobacco, mouth or spit tobacco, snuff or dipping tobacco, snuff, dissolvable tobacco, and heated tobacco [3].

Smokeless Tobacco (ST) is the most commonly used tobacco products in the Nepalese markets in the daily life of general people. Its use specially during the reproductive period is of critical as it effects the weight and the reproductive function. So study related to the adverse effects of ST is necessary to create awareness against the uses of ST among the Nepalese adults.

The objective of the study was to study the teratogenic effect of smokeless tobacco on the gonads (testis & ovary) of Albino rats to specifically know the effects on gross weight of the Albino rats diameters of gonads (seminiferous tubules and ovarian follicles) and overall effect on the sex ratio.

Materials and Methods

This is a experimental study conducted in the animal house Anatomy department of Nobel Medical College Teaching Hospital, Biratnagar, Nepal from September 2023 to August 2024 with a sample size of sixty healthy males and females adult albino rats (11 - 13 weeks old) weighing between 150 -200 gm. They were given standard pellet diet and were kept in a well ventilated room at favourable temperature (25±5c) and natural day light cycle. Animals were kept in groups consisting of < than 5 rats per cage made up of propylene (40cm×25cm×15cm) with husk bed. Smokeless tobacco (ST) were purchased from Pan store. Ethical clearance were obtained prior to undertaking research from the Institutional Review Committee (IRC) of Nobel Medical College, Biratnagar. Rajnigndha and Bhola gutkha was used as the test material for the current study. The samples were grounded and stored at 4°C. After that, distilled water was used to dissolve the sample powder. Samples were stirred during a 20-minute centrifugation. After being filtered, the extract administered orally to the experimental rats.

Sample size and sampling methods: 60 Albino rats with random sampling methods by using the formula, $n= [Z^2 x\ p(1-p)]/e^2$ where z is confidence level at 95% (1.96); e is margin of error taken as 5% and p is expected prevalence from literature. Healthy male and female adult rats weighing between 150- 200 gm were included in the study and unhealthy rats, rats below 150 gm and above 200 gm were excluded from the study.

Statistical analysis was done in SPSS version 19. All data were expressed as mean ±SD. Independent t test and one way ANOVA was applied to test for statistical significance at 95% confidence interval. A value of $p<0.05$ were considered stastically significant.

Experimental groups

Group A: control group (10 male and 10 female)
Group B: were given sample A (10 male and 10 female)
Group C: were given sample B (10 male and 10 female)
Group A (the control group): were not given any sample. They were just given their normal feed.
Group B: were given sample A (10 mg/kg body weight) twice a day along with their normal feed for 56 days.
Group C: were given sample B (10 mg/kg body weight) twice a day along with their normal feed for 56 days.

The following parameters were studied

- Histomorphological changes in testes and ovary following the oral administration of smokeless tobacco (ST).
- Comparision and determination of morphometric parameters such as;
  1. Diameter of seminiferous tubules.
  2. Weight, volume and dimensions of testes.
  3. Diameter of ovarian follicles.
  4. Weight, volume and dimensions of ovary.
- Gross anatomical abnormalities in the off springs.
- Sex ratio: No. of male and female off springs.

During the time of sacrifice weight of the animals were taken and anesthetized with chloroform soaked in cotton and the gonads (testes and ovary) were fixed by 'in vivo perfusion' described as follows. As soon as the various dose applications were finished, all treated rats were put to sleep, along with the corresponding control and

experimental research. With their ventral surfaces facing upward and all four limbs pinned, they were stored on the dissecting tray. The inferior vena cava and abdominal aorta were visible once the abdomen was opened. The abdominal aorta was punctured with an 18-gauge needle, which was then secured in place with thread. The needle was fastened to a sterile flask tube and linked to two bottles that held physiological saline and Bouin's fluid independently. After that, the chest was opened, and a scissor or knife (scalpel) was used to puncture the right atrium to allow blood to drain out. To drain away the blood, physiological saline was then perfused. The remedy after that, Bouin's fluid was transfused using a three-way stopcock. For each fluid, about 200ml were perfused. When the animal began to leak clear fixative drips from its snout, perfusion was halted. Following the conclusion of the perfusion, the testes were removed from the scrotum using a knife and forceps, and the weight was recorded. Next, the volume was determined using the water displacement method, and the testes were post-fixed for 24 hours using the same fixative. After 18 hour fixation in bouin's fluid, the tissue was processed and histological slides were prepared from vertical sections from the polar and the equatorial regions of each testis. Micrometer was used for quantitative measurement of parameter; diameter of seminiferous tubules, and interstitial spaces, germ cells, sertoli cells and leydig cells were observed qualitatively in histological slides. Diameter (D) of each seminiferous tubule and ovarian follicles were measured by two directions and mean of two was taken as a diameter of that seminiferous tubules and ovarian follicles. The cross-sectional area of the seminiferous tubules and ovarian follicles were determined by formula; Area (A)=πD2/4. Two slides per rat were observed for both control and experimental groups.

Results

It was observed that the morphological (qualitative) discrepancies were observed such as discontinuity of basement membrane of seminiferous epithelium, reduction in the number of spermatocytes and primary follicles in the experimental groups. Loss of hair was observed in the experimental rats. It was also observed that the experimental rats suffered from diarrhoea and conjunctivitis in first week of tobacco administration. There was loss of appetite among them. The experimental rats of sample B showed drastic decrease in body weight. Among the experimental rats of sample B, three rats died but none of the rats died among the experimental rats of sample A.

The findings of this study support the hypothesis that tobacco use or its compounds have a deleterious effect on sex hormones. Furthermore, our findings unequivocally demonstrate that adult male rats given tobacco aqueous extract had a variety of pathological alterations in their testes. The current study has shown that some of the seminiferous tubules appeared to be devoid of sperm, most likely as a result of modification or inhibition of the spermatogenesis process. These changes may vary depending on the stage of the process at which the tobacco extract acts.

Similar spermatogenesis stage deterioration and focal seminiferous tubule disorder with pronounced spermatogenic cell population loss were seen in experimental group B. Additionally, the deteriorated seminiferous tubules demonstrated the exfoliation of damaged germ cells with adipose tissue in the lumen. Some of the seminiferous tubules were devoid of secondary spermatocytes and spermatids. The size of ovarian follicles were reduced in the rats treated with ST.

The rats of experimental groups had significant loss of weight after administration of the smokeless tobacco. The off springs produced by the group were very few in number (only two babies) of very low weight and both of them did not survived. Whereas the rats of control group had well developed normal physiology as well as the babies produced were (seven in numbers) with normal weight and all of them survived in the condition.

The weight of the animals were recorded and it was observed that there was consistent decrease in mean body weight of rats of experimental groups. When compared with initial body weight in control in comparison to its initial body weight. The weight in case of all the control groups was observed to be increased than the initial weight. The P value of final body weight among sample A experimental and control; sample B experimental and control showed a highly significant ($p<0.001$) difference. Table 1 &2.

Similarly, when the sample A experimental was compared with the sample B experimental no significant difference was observed ($p>0.05$). Table.1 The mean±SD of volume of testes in sample A experimental group is 2.20±0.42cc, whereas in control groups it is 3.50±0.52cc, which is statistically highly significant ($p<0.001$).

The weight of right testis of the sample A experimental and the control respectively shows mean value of 0.53±0.14gm and 1.17±0.13gm which is also highly significant (p<0.001). Similarly, the weight of left testis of the sample A experimental and the control with mean value 0.48±0.16gm and 1.19±0.16gm respectively is also highly significant (p<0.001). The mean±SD of diameter of the seminiferous tubules in sample A experimental groups is 241.95±6.83μm, whereas in control group it is 348.05±23.33μm which is statistically highly significant (p<0.001). The cross-sectional area of the seminiferous tubules of sample A experimental shows a mean value of 45986.76±2623.45μm^2 and the mean value 95478.66±12867.21μm^2 of the control also shows statistically highly significant (p<0.001) difference at the end of 56th day i.e. 8th week of intervention which is shown in the below table 3. The mean±SD of diameter of seminiferous tubules in sample B experimental groups is 208.30±12.30μm, whereas in control groups it is 347.30±40.33μm which is statistically highly significant (p<0.001). The cross-sectional area of the seminiferous tubules of the sample B experimental is 34167.20±3993.63μm^2 and the control groups is 95833.65±21456μm^2 which is also highly significant (p<0.001) at the end of 56th day that can be seen in the table 3.

The mean±SD of volume of ovary in sample A experimental group is 2.30±0.52cc, whereas in control groups it is 3.00±0.47cc, which is statistically highly significant (p<0.001). The weight of right ovary of the sample A experimental and the control respectively shows mean value of 0.30±0.21gm and 0.37±0.23gm which is also highly significant (p<0.001). Similarly, the weight of left ovary of the sample A experimental and the control with mean value 0.31±0.22gm and 0.39±0.24gm respectively is also highly significant (p<0.001). The mean±SD of diameter of the ovarian follicles in sample A experimental groups is 20.79±1.47μm, whereas in control group it is 21.35±3.83μm which is statistically highly significant (p<0.001). The cross-sectional area of the ovarian follicles of sample A experimental shows a mean value of 22167.20±3393.63 μm^2 and the mean value 86833.65±21456 μm^2 of the control also shows statistically highly significant (p<0.001) difference at the end of 56th day i.e. 8th week of intervention in the table 4. The mean±SD of volume of ovary of sample B treated experimental groups is 2.12±0.22cc, whereas in control groups it is 2.40±0.27cc which is statistically significant (p<0.05). The mean value of the right ovary in sample B experimental and control respectively is 0.32±0.22 gm and 0.35±0.23 gm is highly significant (p<0.001). The left ovary of the sample B experimental group mean value 0.31±0.13gm and control group mean value 0.36±0.21 gm also showed highly significant (p<0.001) difference. The mean±SD of diameter of ovarian follicles in sample B experimental groups is 20.74±2.55μm, whereas in control groups it is 21.35±3.83μm which is statistically highly significant (p<0.001). The cross-sectional area of the ovarian follicles of the sample B experimental is 24167.20±3993.63 μm^2 and the control groups is 85833.65±21456 μm^2 which is also highly significant (p<0.001) at the end of 56th day is shown in the above table 4.

Table 1: Comparison of change in body weight between experimental and control groups.

Treatment Groups	Initial body weight (Mean±SD) (gm)	Final body weight (Mean±SD) (gm)	Difference in body weight(gm)
Experimental(E1) for sample A.	163±10.59	149.50±13.83	13.5
Experimental(E2) for sample B	161±10.48	143.50±30.91	17.5
Control(C)	167±13.98	181±13.70	14

Table 2: P value of Final Body weight E1 vs C;

Parameters	SampleA experimental (Mean±SD)	Control (Mean±SD)	P value
Final Body Weight(gm)	149.50±13.83	177±10.32	0.000
Final Body Weight(gm)	143.50±30.91	181±13.70	0.003
Final Body Weight(gm)	149.50±13.83	143.50±30.91	1.000

P value of 0.05 is the significance level.

Table 3: Showing the comparison of various testicular parameters between the sample A experimental and control group.

Types/parameters	Sample A Expt. (Mean±SD)	Sample B Expt. (Mean±SD)	Control (Mean±SD)	P value
Volume (cc)	2.20 ±0.42	2.50±0.52	3.50±0.52	0.000
Right Testis weight (gm)	0.53 ±0.14	0.43±0.11	1.17±0.13	0.000
Left Testis Weight(gm)	0.48 ±0.16	0.39±0.12	1.19±0.16	0.000
Diameters of Seminiferous tubule (μm)	241.95 ±6.83	208.30±12.30	348.05 ±23.33	0.000
Cross-sectional Area of Seminiferous tubules (μm^2)	45986.76 ±2623.45	34167.20 ±3993.63	95478.66 ±12867.21	0.000

P value of 0.05 is the significance level.

Table 4: Comparison of various Ovarian parameters between sample A, sample B treated experimental group and the control group.

Types/parameters	Sample A Expt. (Mean±SD)	Sample B Expt. (Mean±SD)	Control (Mean±SD)	P value
Volume (cc)	2.30±0.52	2.12±0.22	3.00±0.47	0.078
Right Ovary weight (gm)	0.30±0.21	0.32±0.22	0.37±0.23	0.000
Left Ovary Weight (gm)	0.31±0.22	0.31±0.13	0.39±0.24	0.000
Diameters of Ovarian follicles(μm)	20.79±1.47	20.74±2.55	21.35±3.83	0.000
Cross-sectional Area of ovary (μm²)	22167.20 ±3393.63	24167.20 ±3993.63	86833.65 ±21456	0.000

P value of 0.05 is the significance level.

Histological Photographs

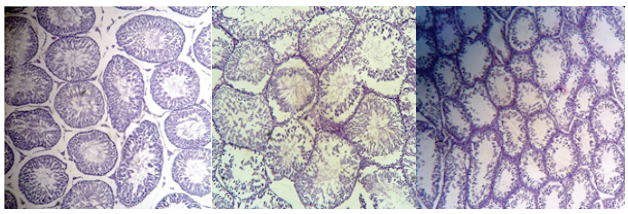

Figure 1: Histological section of Testis of Control group, treated with sample A and sample B

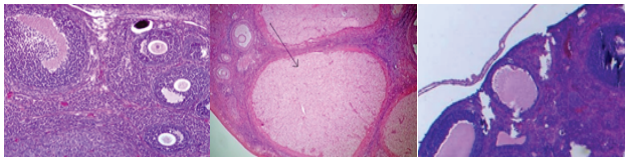

Figure 2: Histological section of ovary of control group, treated with sample A and sample B

Discussion

The current study sought to determine whether the exposure of Albino rats to smokeless tobacco during pregnancy and lactation impacted the development of the gonadal organs. The findings unequivocally demonstrated that high levels of maternal smokeless tobacco use during pregnancy and lactation dramatically decreased the body, testicular, and ovarian weights as well as the fertility, and reproductive efficiency in Albino rats. One of the important contributors to embryotoxicity is use of tobacco products in any form. It is estimated that 25% of pregnant women and 30% of all reproductive age group of women consume smokeless tobacco products [4]. The gonads are complex glands that produce gametes and sex hormones in the body. Female gametes are called eggs and male gametes are called sperm. The testicles, the male gonads, produce sperm in the form of spermatozoa. Ovaries are female gonads that produce eggs. Both gametes are haploid cells [5]. Decreases in sperm count and motility brought on by substances are positively connected with reductions in testosterone levels brought on by exposure to tobacco smoke, smokeless tobacco, and nicotine. Follicle stimulating hormone (FSH) and luteinizing hormone (LH) levels increased concurrently with this [6]. Negative reproductive outcomes, such as stillbirth, early birth, and low birth weight babies, are associated with it. Smokeless tobacco use during pregnancy may raise the chance of stillbirth and early delivery. Pregnancy-related exposure to nicotine from smokeless tobacco products can impact the developing brain of the unborn child [7]. Study done by Abdul Ghani *et al.,* showed that exposure to cigarette smoke causes impaired spermatogenesis in rats, which was partly due to the induction of DNA damage and oxidative stress [8].

The result of the current study illustrated that, when compared to rats in control bunches, rats uncovered to smokeless tobacco with tall nicotine levels had impressively lower body weights and littler ovarian follicle and diminished seminiferous tubule distances across. Comparative inquire about by Oyeyipo *et al.* illustrated that testicular brokenness, as contradicted to a pituitary issue, is related with lower testosterone levels. In any case, nicotine's impacts on regenerative hormones were dose-dependent, and the impacts that were appeared were reduced by stopping the sedate [9].

Within the seminiferous epithelia of rats given smokeless tobacco treatment, apoptosis, disorganization, a outstanding misfortune of spermatogenic cells, and development capture were all apparent. At diverse stages of spermatocyte development, smokeless tobacco too driven to spermatocyte degeneration. These characteristics and other breaking down changes in spermatocytes and seminiferous tubules are steady with thinks about on the testicles of mice uncovered to halogenated diamines and rats with vitamin A [10]. The present study also revealed that seminiferous tubules were devoid of germ cells leading to decrease in the diameter of the seminiferous tubules. The ovarian follicles also had diminished in its size.

It has been famous that smokeless tobacco extricates can cause oxidative tissue harm and apoptosis. In assessing the ST poisonous quality in ovarian tissues, the outcomes about are reliable with those of study of Kilincet. Modifications to body lipid designs are connected to push and long-term cigarette utilize. Within the ovarian tissues of the long-term test bunches, fat buildup inside the encompassing follicular cells has moreover been seen to be a result of lipid distur-

bance which is similar to the present study showing the ovarian tissues diminishing [11]. Comparative inquire about conducted by Nawal *et al.* uncovered that Afzal had negative wellbeing impacts on Wistar rats, counting diminished basic and utilitarian results for the key regenerative organs (testicles and ovaries). The standard infusion of Afzal comes about in a impressive diminish within the levels of testosterone and estradiol and may harm a user's capacity to replicate. This study also coincides with the above study done on Wistar rat [12]. A broad, orderly audit by De Queiroz *et al.* examining the affiliation between dynamic tobacco utilize amid pregnancy and newborn child respiratory wellbeing proposed that there was the next hazard of apnoea in newborn children born to moms utilizing ST all through their pregnancy [13]. A consider conducted among the inhabitants of the Western Ghats, Southern India centered on the utilize of ST such as betel quid, areca nut and tobacco leaf to decide any genotoxic impacts. Examination utilizing genotoxicity biomarkers appeared that there was critical DNA harm in tobacco clients than non-users, and female ST clients in specific detailed higher rates of illnesses such as cardiovascular, diabetes mellitus, hypertension and unconstrained premature births [14]which is in accordance with the present study.

Existing information proposes the affiliation between incessant smokeless tobacco utilize and disability of ovarian morphology and work, oocyte quality, hormonal annoyances, fetal advancement and long-term wellbeing impacts on the hatchling. This study was also showed the similar type of affect on the overall body weights of the experimental rats treated with smokless tobacco.

Conclusion

From the present study it was concluded that the consumption of smokeless tobacco in any form leads to rapid decrease in the gross weight of the albino rats, reproductive dysfunction in both male and female. Sex ratio was also reduced along with low body weight offsprings were produced. It showed that diameter of testis and ovary was diminished after intake of smokeless tobacco Pan masala and Gutkha. So use of smokeless tobacco in any form is injurious to health and affecting the overall reproductive function. Still study on genetic level is needed to know the exact cause of the teratogenic effect of smokeless tobacco.

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Conflict of interest: None.

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