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Original Article

Histological effects of the use of chewing tobacco during pregnancy on ovaries of the offspring of rats.

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Abstract:

Introduction: Exposure to smokeless tobacco products (Nicotine) is associated with the generation of reactive oxygen species, modulation of inflammatory mediators, inhibition of collagen synthesis and impairment of DNA repair capacity which in some way may cause harmful effects on the ovaries of the offspring.

Objectives: To observe the effects of chewing tobacco on the gross morphological and histological alterations on the ovaries of offspring's rats.

Methodology: Twenty pregnant rats were divided in two groups: Group. A (Control) 10 pregnant rats received normal diet and water ad libitum. Group. B (Experimental) 10 pregnant rats received 5% tobacco mixed with normal feed and water ad libitum. After 21 days, the female offspring of both control and experimental groups were sacrificed, ovaries removed and stained with H&E stains for microscopic examination.

Results: The results clearly showed that smokeless tobacco consumption during gestation reduces the weight and size of ovary along with the congested blood vessels, compact stroma and degenerative primordial follicles. Hence the practice of chewing tobacco in mothers is directly related to the adverse effects in offspring health and ovaries.

Key words: Chewing Tobacco, Offspring, Ovaries, Rats

Introduction:

Tobacco chewing tobacco is wide spread potentially dangerous social propensity all through the world, yet it is more prevalent in South East Asia. Currently, tobacco use is expanding rapidly throughout the world and present a threat to present and future world wellbeing.[1,2] South Asian people most commonly use an addictive type of chewing tobacco that is Gutkha.[3] Over the past many years, tobacco utilization and the chewing tobacco among various places of the world has most prominently changed with the presentation of many new forms of chewing tobacco.[4] The utilization of other types of tobacco is gaining popularity worldwide along with the reduction in the general pattern of cigarette smoking.[5-7] The two principle types of smokeless tobacco are snuff and biting tobacco. Biting tobacco is the form which arises from leaves while snuff is ground or shredded tobacco leaves and may be dry or moist. Moist is generally placed between cheeks and gums while dry snuff tobacco is inhaled through the nose.[8] The propensity for oral utilization of smokeless tobacco, for example, "biting tobacco" or "spit tobacco" is turning into a worldwide risk to human health with each passing day because of its various injurious impacts. [9] Smokeless tobacco items in addition to nicotine contain other different constituents as well for example benzopyrene, cadmium, lead, arsenic, chromium and nickel.[10] Chewing Tobacco has additionally been involved in causing diseases of cardiovascular system, respiratory system and oral tumors.[11,12] The essential reproductive wellbeing concerned with smokeless tobacco is mainly nicotine having vasoconstrictive effects

produces an unfavorable impact influencing pregnancy, its progress and outcome. Tobacco contains many aggravating factors, some of which are known to affect conceptive well-being, for example, carbon monoxide (CO), nicotine, and metals. One of the troubling viewpoint is the acknowledgment of these substitute types of tobacco through social media which is expanding particularly in females.[13] The conceivable unfavorable wellbeing impacts of smokeless tobacco amid pregnancy have received far less consideration with a limited number of publications addressing this practice. In rodents, nicotine introduction amid pregnancy brought about decreased birth weights, expanded fetal mortality, abnormal bone advancement, and diminished activity levels. A huge number of pregnant ladies utilize smokeless tobacco, commonly in Africa and Asia.[14]

As different risks of smoking in pregnancy are now widely established; most women are switching to smoke-free nicotine products with a hope that it will not badly affect fetus.[15] The current clinical scenario of chewing tobacco during pregnancy is much more relevant making chewing tobacco during pregnancy a real clinical issue.[16] Pregnant lady are a vital population for tobacco aversion endeavors since its utilization causes genuine hazard issues to fetal and maternal wellbeing. [17] In a few reviews, it was observed that the recurrence of spontaneous abortion, stillbirth, preterm delivery and low birth weight infants were substantially higher among mothers who utilized smokeless tobacco items during the term of pregnancy.[18] The health hazard due to use of smokeless tobacco during pregnancy and especially on the offspring have received less attention in the literature. The females of our society have less knowledge to the possible adverse effects about the use of the chewing tobacco during pregnancy. On the use of smokeless tobacco (chewing tobacco) and its adverse effects on the fetal organs along with its histological features the literature available is very limited.

Methodology:

Animal protocol: Twenty female albino wistar rats of an average weight of 200 to 300 grams were purchased from Animal Lab, Karachi. The rats were acclimatized for 10 days prior to the start of the experiment. The rats were kept in plastic cages made of polyethylene. Optimum temperature of 25 ±2^{o}C and light dark cycle of 12 hours was maintained. The rats were provided with balanced chow diet and tap water ad libitum. All the rats received proper care in compliance with the Animal Ethical Committee in the Animal Husbandry of Sindh Agricultural University Tando Jam. The study design is quasi experimental study and was conducted during the period between April 2020 to September 2020.

Experimental protocol: Twenty female rats were divided into two groups. Each group comprises of 10 rats. The adult male rats were restricted from having sex for seven days in order to increase their sexual desire and then were allowed for mating with the female rats. 3 female rats were mated with 1 male rat considering the day of mating as gestational day 0. The vaginal mucus plug confirmed the pregnancy between 1-10 days of pairing. The males were removed once the pregnancy was confirmed. Throughout the experiment, the 10 rats in control group (A) received normal feed and clean water ad libitum while the 10 rats in experimental group (B) were treated with 5% tobacco mixed in normal feed from day 1 till parturition. The duration of gestation, number of offspring and size, along with lactation were determined.

Data collection procedure: The female offspring from both the groups were sacrificed and ovaries were removed. The method for sacrifice used was cervical dislocation. The animals were dissected. After the removal of ovaries their weight was measured and were kept in 10% formaldehyde. Ovaries of the offspring were kept in labeled jars and were further processed for paraffin embedding. The sections that were cut had thickness of Four micron (4um) & were stained with Hematoxylin & Eosin.

Data analysis procedure: Data was analyzed on SPSS version 22.0 (IBM, incorporation, USA). Continuous variables were analyzed using Student t-test. Microsoft excel sheet was used for frequency and % data and graphing. Statistical significance was taken at 95% confidence interval (P≤ 0.05).

Results:

Body weight distribution: The mean ± SD of body weight in the control and experimental groups was noted as 41.0±1.94 and 38.50±0.74 respectively (P=0.01). The findings suggest the smokeless chewing tobacco has adverse effects on the somatic formation of offspring as the body weight was found low in experimental rats as compared to the rats in control group (P=0.01). This result is shown in table I.

Ovary weight: The mean ± SD of ovary weight in the control and experimental groups was noted as 16.79±0.76 and 14.44±1.05 mg respectively. The findings suggest the smokeless chewing tobacco has adverse effects on the ovarian development of offspring as the gross ovarian weight was found significantly decreased in experimental rats as compared to the rats in control group (P=0.01). This result is shown in table II.

Histopathological findings:

Ovarian stroma: The normal and compact stroma findings are shown in the table III. Control rats showed normal stroma in 90% compared to 10% in experimental rats, while compact stroma in controls was found in 10 in contrast to 60% in experimental rats (p=0.01)

Ovarian blood vessels congestion**:** Control rats showed congestion in 10% compared to 70% vascular congestion in

Table I. Body weight (grams) in animal groups (n=20)

Groups	Mean	±SD	t-value	P-value
Group A. Control Rats	41.0	1.94	3.79	0.01
Group A. Experimental rats	38.50	0.74		

Table II. Ovary weight (mg) in animal groups (n=20)

Groups	Mean	±SD	Df	t-value	P-value
Group A. Control Rats	16.79	0.76	19	5.72	0.01
Group A. Experimental rats	14.44	1.05			

Table No 3: Comparison of various histopathological findings between control and experimental off springs albino wistar rats

Histological variables		Control group A (offspring)	Experimental group B (offspring)	P-value
Ovary stroma	Compact	1	6	0.01
	Normal	9	4	
	Total	10	10	
Blood vessels	Congested	1	7	0.006
	Normal	9	3	
	Total	10	10	
Primordial follicles	Damaged	1	6	0.01
	Normal	9	4	
	Total	10	10	
Oocytes	Damaged	1	5	0.05
	Normal	9	5	
	Total	10	10	

experimental rats (p=0.006). Table III shows the findings of blood vessels in controls and experimental rats.

Primordial follicles**:** Control rats showed normal primordial follicles in 90% compared to 40% normal primordial follicles in experimental rats (p=0.01). Frequency of normal and damaged primordial follicles is shown in table III. Damaged Primordial follicles were observed in 60% of experimental rats, indicating the deleterious effects of tobacco on ovaries of offspring.

Oocyte findings**:** Control rats showed damaged Oocytes in 10% compared to 50% in experimental rats (p=0.01). Frequency of normal and damaged Oocytes is shown in table III. Normal Oocytes were observed in 90% controls rats, compared to only 50% in experimental animals; this shows the ovarian damage caused by tobacco.

Discussion: The results of the present study clearly showed that smokeless tobacco consumption during gestation reduces the primordial follicles population and its degeneration hence resulting in diminished fertility and decreased reproductive efficacy in the off springs of Wistar albino rats. These alterations in the ovaries were associated with significant growth retardation of female off springs.

The findings of Dorostghoal M et al [19] are in agreement to findings of current study, they found degeneration of primordial follicles along with the reduced number of secondary and antral follicles in non-pregnant rats treated with one form of smokeless tobacco as compared to control. In addition to these findings there was also decreased diameter of secondary and antral follicle and decrease in the number of corpora leutea. However, duration of his study was 30 days. Prakash C Gupta[20], in his study observed the decrease in gestational age at birth due to the consumption of chewing tobacco during pregnancy along with decrease in birth weight of off springs. Daneil et al treated experimental rats with smokeless tobacco for 3 weeks and observed decrease in the body weight of rats after 3 weeks. His findings were consistent to the current study as reduction in the body weight of the off springs was observed and the duration for usage of smokeless tobacco was also identical in both the studies. [21]

Samrat Das et al observed effect of smokeless tobacco on reproductive parameters of male off spring rats. He found decreased sperm count with impaired motility and abnormal morphology.[22] Ketan Patel et al[23] reported that exposure to the chewing tobacco leads to low sperm count and increased risk of oligozoospermia. The findings of above mentioned both studies are identical to the findings of current study as ovaries also contains germ cells as is the sperm.

In contrast to findings of current study, Chelin et al [24] did not find any other pathological change except decrease in body weight of experimental animal fed smokeless tobacco at 3 different doses (0.75, 1.5 and 3 mg) for 84 days. Probable explanation might be the relatively small dose of smokeless as compared to the current study. Amy and Verena[25]observed the effects of smokeless tobacco along

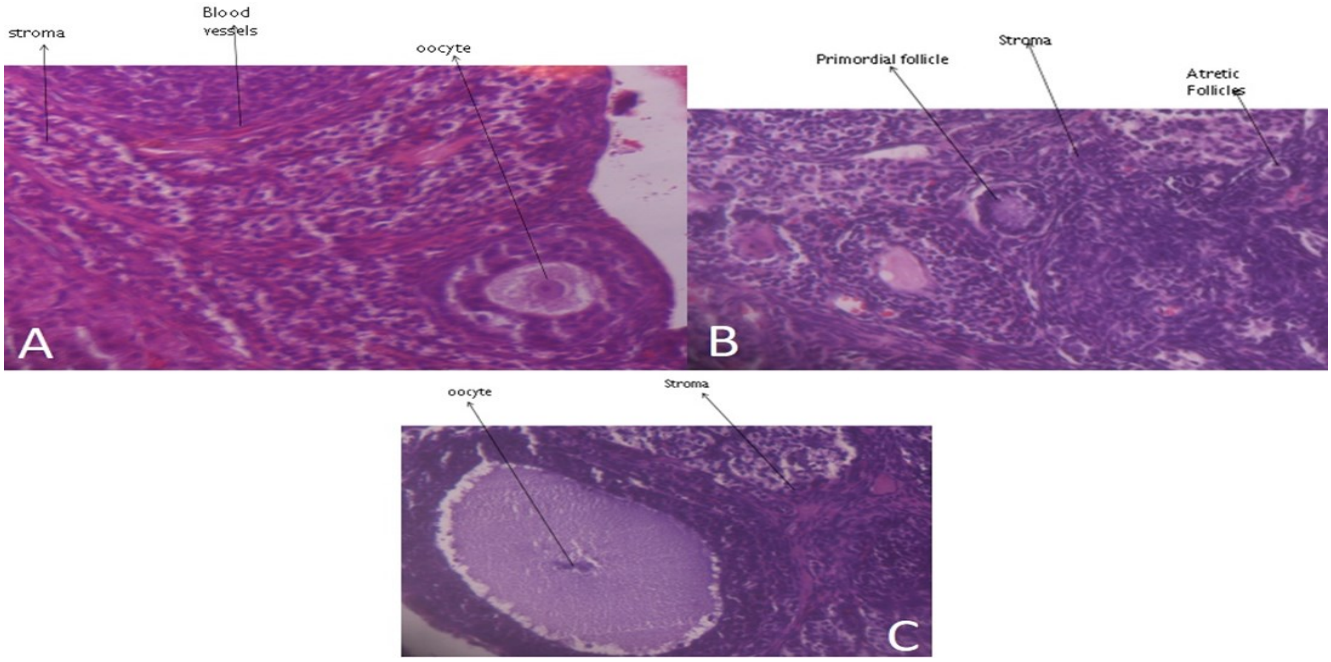

Figure 1. Photomicrograph showing histological sections of ovary of offspring albino rat in control group (H&E x 200) showing primary oocyte and normal stroma with intact blood vessels (A), primordial follicle, primordial stroma and atretic follicle (B) and oocyte and normal stroma (C) (H&E x 400)

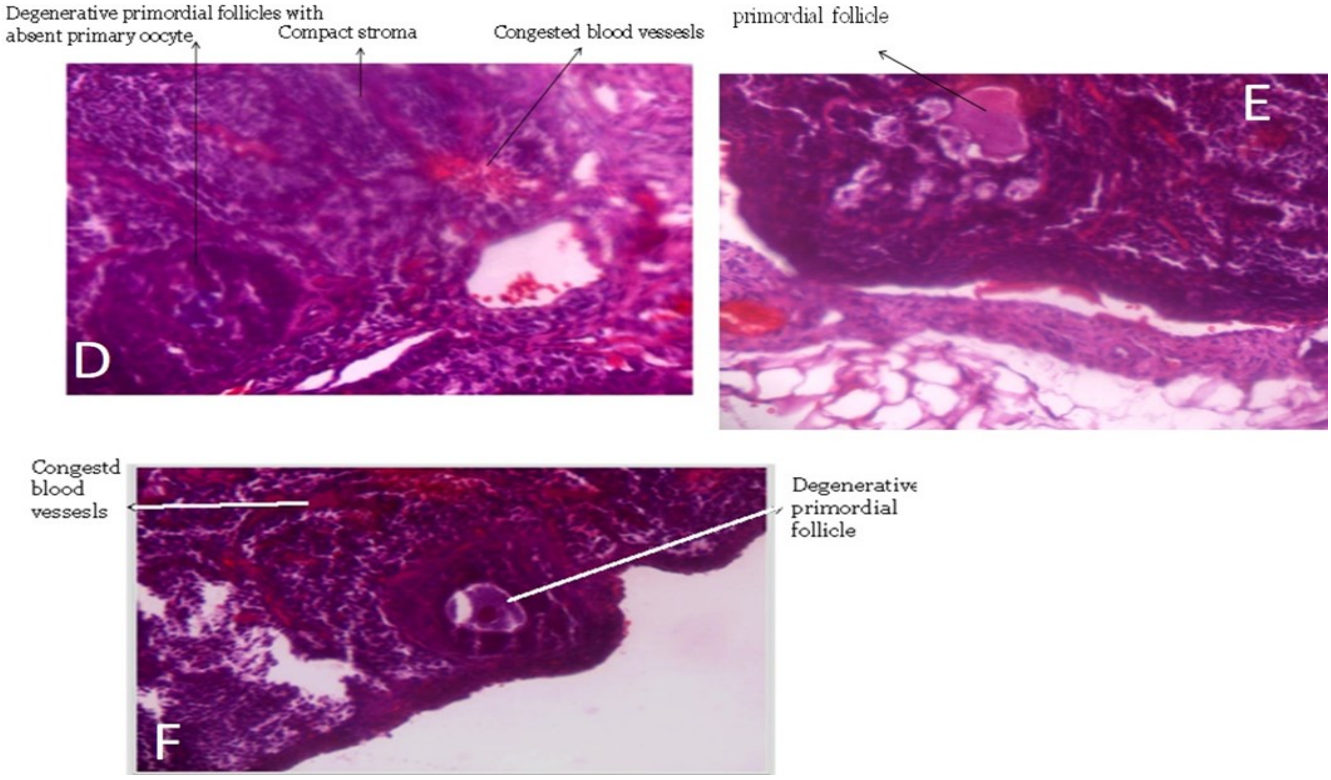

Figure 2. Photomicrograph showing histological sections of ovary of offspring albino rat in experimental group (H&E x 40) showing degenerated primordial follicle (primary oocyte absent), congested blood vessels and compact stroma (D,E and F)

with the usage of betel nuts in pregnant women. Their findings were varying to the present study as no harmful effects for mother or neonates were observed. Again the reason could be due to smaller dose and difference in the constituents of smokeless tobacco. Since their study did not mention the duration of usage of smokeless tobacco, it cannot exclude the negative long-term effects seen in chronic users. Jonah Sydney et al[26] observed that sub-acute exposure to tobacco smoke, smokeless tobacco, and nicotine does not affect sperm morphology but causes dose-related reductions in sperm motility, sperm count, and testosterone, occurring more in pre-pubertal rats than adult rats. These effects are most pronounced with tobacco use as compared to smokeless tobacco and nicotine. Study carried out for short duration and therefore long-term negative effects cannot be rule out with certainty. Again, findings are not in agreement to the findings of current study; probable explanation might be the differences of compounds, doses and duration (0.5 and 1 mg for 20 days). Since their study mentioned the small duration of usage of smokeless tobacco, it cannot exclude the negative long term effects seen in chronic users.

Conclusion: Chewing tobacco is as harmful as is the smoking itself; causes significant histopathological changes in the ovaries of tobacco treated female offspring. All anti-tobacco campaign must include smokeless tobacco as well.

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