PROTECTIVE EFFECTS OF AJWA ON NICOTINE INDUCED CHANGES IN BLOOD VESSELS AND CORPUS LUTEUM

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ABSTRACT
Introduction: Nicotine adversely affects ovarian functions and Ajwa (Phoenix dactylifera) being an antioxidant prevents nicotine from causing toxicity.
Aims & Objectives: To assess the effects of Ajwa on nicotine induced vascular and luteal changes in adult albino rats.
Material & Methods: Study was conducted in the Anatomy Department of Federal Postgraduate Medical Institute, Shaikh Zayed Medical Complex Lahore. Four equal groups were made of 40 adult female albino rats. 1ml distilled water was given to the rats of control (A) group by gastric intubation, experimental group B was given nicotine injection intraperitoneally (0.1 mg/kg body weight) for 28 days. The rats of experimental group C were given Ajwa fruit extract 1gm/kg body weight daily by gastric intubation, while rats of experimental group D were given 0.1mg/kg body weight nicotine intraperitoneally plus 1gm/kg body weight Ajwa fruit extract by gastric intubation for 28 days.
Results: Vascular congestion was found in one rat of group A & D and in all animals of group B. Corpus luteum of group B revealed congestion and was focally infiltrated by inflammatory cells.
Conclusion: Ajwa improves nicotine induced changes in blood vessels and corpus luteum of adult albino rats, so it may be useful to improve infertility or subfertility induced by smoking in women.

Key Words: Nicotine, Phoenix dactylifera, vascular congestion

INTRODUCTION
Nicotine is member of a family named ''solanaceae''.[1] Nicotine applies anti-inflammatory effects in several cell kinds and has been shown to profit various conditions in which an inflammation-related device is connected.[2] Higher quantities of nicotine are found in smokeless tobacco than ordinary cigarettes.[3]

Nicotine stimulates the autonomic nervous system by catecholamine release. Nicotine increases lipid peroxide, reactive oxygen species and oxidative stress. Action on nicotinic receptors produce cell multiplication and toxic effects on various systems throughout the body.[4] In nicotine exposed individuals, wound healing is delayed and risk of infection is increased. Nicotine causes immunosuppression.[5] One cigarette decreases human life by 11 minutes.[6]

Nicotine causes hypoestrogenic state by inhibiting 21 hydroxylase, chronic anovulation, menstrual irregularities and early onset of menopause. Cervical inflammation and cancer has also been linked with nicotine use. Harmful effects of smoking were reported on ovary.[7]

Smoking in women lowers progesterone, estrogen and raises follicle stimulating hormone.[8] Mothers who smoked during pregnancy gave birth to female offspring with impaired fertility and ovarian dysfunction.[9] Maternal smoking increases incidence of miscarriage, low birth weight in new born, still birth, mental retardation. and retarded fetal growth. [10,11,12]

Date palm is a monocotyledonous plant. It is dioecious in that they have distinct male and female plants. Only female plant bears fruit.[13] Antioxidants in fresh dates are carotenoid, anthocyanins, phenolics, free and bound phenolic acids. A study of comparison of fresh and dried phoenix dactylifera shows that on drying at higher temperature, high level of phenolics have been found in *Phoenix dactylifera*. This high temperature and hot climate causes maturation of degrading enzymes leading to degradation of tannins.[14]

Antioxidants are dynamic substances, which retain the capability to defend the body from harms during drug metabolism, pollution, introduction to ionizing radiation and ultraviolet light. Excessive creation of reactive oxygen and nitrogen species lead to many

unhealthy states like hypertension, atherosclerosis, cardiovascular disorders, aging, cell death, inflammation, cancerous mutation, Alzheimers and Parkinsons disease. Aqueous extract of dates significantly inhibits protein oxidation and lipid peroxidation and has a radical scavenging activity for hydroxyl and superoxide.[15] Antioxidants act together and deactivate free radicals and stop them from producing injury.[16] The antioxidants present in dates improve immunity and decrease the hazard of heart conditions and tumor. From ages, all portions of date palm are used as drug for the control of fever, inflammation, paralysis, nervous and memory disorders.[17]

Male flowers of Phoenix dactylifera or date palm pollen were regularly used as an aphrodisiac in old-style medicine. Antioxidant property of date palm increases reproductive function and fertility. It can produce a suitable condition for oogenesis and maintains effective fecundity in female mice and is a useful nutraceutical for potentiation of fertility.[18] Gonad stimulating potency of Date palm pollen (DPP) have already confirmed by Egyptian scientists. DPP suspension contains cholesterol, carotenoids, rutin and estrone which increase FSH and LH and exhibits gonadotrophic activity.[19] Considering all these properties of Ajwa, this study was planned to assess its special effects on nicotine induced vascular and luteal changes in adult albino rats.

MATERIALS AND METHODS

The study was done in the Anatomy Department of Shaikh Zayed Medical complex, Lahore in cooperation with Anatomy Department of Punjab PGMI, Lahore. 40 adult healthy female albino rats aged 3-4 month (average body weight 200-250 gm) were kept at 23±2ºC and a 12 hour light and dark cycle was maintained. They were fed on normal food and given water ad libitum. They were allowed to acclimatize for a period of two weeks before the experiment started. Ajwa Dates were purchased from Madinah Al-Monawarah and their extract was prepared by following method.[20]

3000 gram of date fruits were by hand separated from pits and 1 liter distilled water was added to this crudely crushed date fruit (3:1). This was left for 48 hours in freezer (4°C) with occasional stirring. This aqueous extract of Ajwa contains Phenolic content (1.752 mg Gallic Acid Equivalent /g of extract) and Flavonoid Content (0.1239 mg Quercetin equivalent/g of extract) quantified by Chemistry Department, PCSIR, Lahore. Nicotine synthesis grade *(*99% pure*)* loose liquid Fluka, was purchased from Sigma-Aldrich chemical company (USA).

The rats were casually divided into four groups by lottery method, each containing ten animals. Rats of control group A were treated with 1ml/kg b.w of distilled water by gastric intubation for 28 days. To experimental group B, nicotine was given 0.1mg/kg b.w/day intraperitoneally for 28 days. Experimental group C, received Ajwa fruit extract 1 g/kg b.w/day by gastric intubation for 28 days. In experimental group D, each rat was given nicotine 0.1mg/kg b.w/day intraperitoneally and Ajwa fruit extract 1g/kg b.w by gastric intubation for 28 days.

48 hours after giving the last dose, only those rats which were in estrous phase, were dissected. Other rats were left till their estrous phase. Rats were humanly sacrificed by using intraperitoneal administration of morphine at a dose of 0.3-0.5mg/kg and the ovaries were dissected out, kept in 10% neutral buffered formaldehyde solution for 48 hours. Small pieces were cut and paraffin blocks were made. Serial sections of 5 µm thickness were taken. Hematoxylin and Eosin stained slides were observed under the light microscope for changes in blood vessels and corpus leutum.

Statistical Analysis: Data was entered and evaluated by using Statistical Package for Social Sciences (SPSS), version 20. Data for qualitative variables like appearance of blood vessel and corpus luteum was described by using percentage of each group. Comparison among groups was made by using Fisher's exact test. P-value ≤ 0.05 was considered significant.

RESULTS

Animals of control group, had normal blood vessels in ovaries of 9 animals (90.0%) and were congested in 1 animal (10.0%) (Fig 1A). For group B, congestion was found in medulla and extending to cortex (Fig 1B). Vessel wall was thick, edema, fibrosis and hyalinization of vessels was present in all 10 rats of group B (100.0 %). There was no blood vessel congestion seen in group C (Fig 1C) whereas in group D only 1 (10.0%) rat had blood vessel congestion (Fig 1D). Among the groups Fisher's exact test showed statistically significant, p-value<0.001*. (Table 1)

Group B showed significant changes as compared with groups A, C & D with p-value< 0.001*. Multiple comparisons showed that the comparison of group A from group B was significant with p-value< 0.001*. Comparison of group B from group C & D was significant with p-value<0.001* while group A when compared to group C showed insignificant p-values 0.305. and difference of group C from D was also insignificant with p-value 0.305. (Table 2)

Table 1: Distribution of vascular congestion among groups

Congestion of Blood Vessels	Group A n (%)	Group B n (%)	Group C n (%)	Group D n (%)	p-value
Absent	9 (90.0%)	0 (0.0%)	10 (100.0%)	9 (90.0%)	< 0.001*
Present	1 (10.0%)	10 (100.0%)	0 (0.0%)	1 (10.0%)	

Based on Fisher's exact test
*p value ≤ 0.05 is considered statistically significant

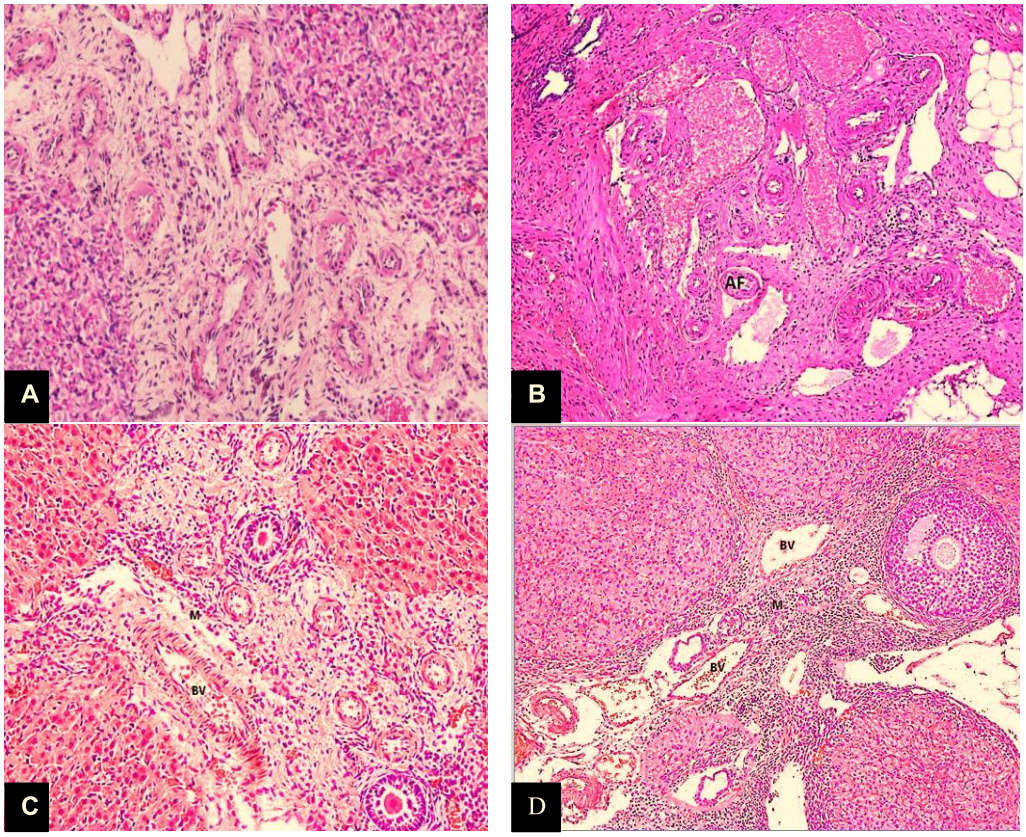


Figure 1: Photomicrograph of ovary of albino rat of control group A and experimental groups B, C and D showing blood vessels in medulla. Blood vessel congestion is seen in group B as shown by arrows. (H & E, 20X)

Table 2: Multiple comparisons for congestion of blood vessels among groups.

Group		Chi-square	Df	p-value
A	B	16.36	1	< 0.001*
	C	1.05	1	0.305
	D	-	-	-
B	C	16.36	1	< 0.001*
	D	20.00	1	< 0.001*
C	D	1.05	1	0.305

*p value ≤ 0.05 is considered statistically significant
- Non calculable being constant

In control group A, corpora lutea were in the outer region of cortex (Fig 2A). Polygonal cells with round nucleus and eosinophilic cytoplasm were granulosa lutein cells. Theca lutein cells were oval in shape with central flattened nucleus. Numerous capillaries were present. In group B, corpora lutea were present in both outer and inner regions of cortex. Corpus luteum of group B revealed congestion of blood vessels and was focally infilterated by inflammatory cells. (Fig 2B). In experimental groups C & D, corpus luteum was normal in 10 rats (100.0%). (Fig 2C & 2D)

Fisher's exact test showed that there was an association between corpus luteum and groups. Corpora Lutea seen in groups A, C and D were normal in appearance while all rats of group B had abnormal corpus luteum. Among the groups, Fisher's exact test showed statistically significant p-value<0.001* (Table 3).

Table 3: Distribution of Corpus Luteum among groups.

Corpus Luteum	Group A n (%)	Group B n (%)	Group C n (%)	Group D n (%)	p-value
Normal	10 (100.0%)	0 (0.0%)	10 (100.0%)	10 (100.0%)	< 0.001*
Abnormal	0 (0.0%)	10 (100.0%)	0 (0.0%)	0 (0.0%)	

Based on Fisher's exact test
*p value ≤ 0.05 is considered statistically significant

Comparison of group B from groups A, C & D showed significant changes. Multiple comparison for corpus luteum among the groups showed that the difference of group A from group B was significant with p-value <0.001*. Comparison of group B from groups C & D showed significant p-value <0.001*, while comparison of group C from D and group A from groups C & D were insignificant. (Table 4)

Table 4: Multiple Comparisons for Corpus Luteum among the groups.

Group		Chi-square	df	p-value
A	B	20.00	1	< 0.001*
	C	-	-	-
	D	-	-	-
B	C	20.00	1	< 0.001*
	D	20.00	1	< 0.001*
C	D	-	-	-

*p value ≤ 0.05 is considered statistically significant

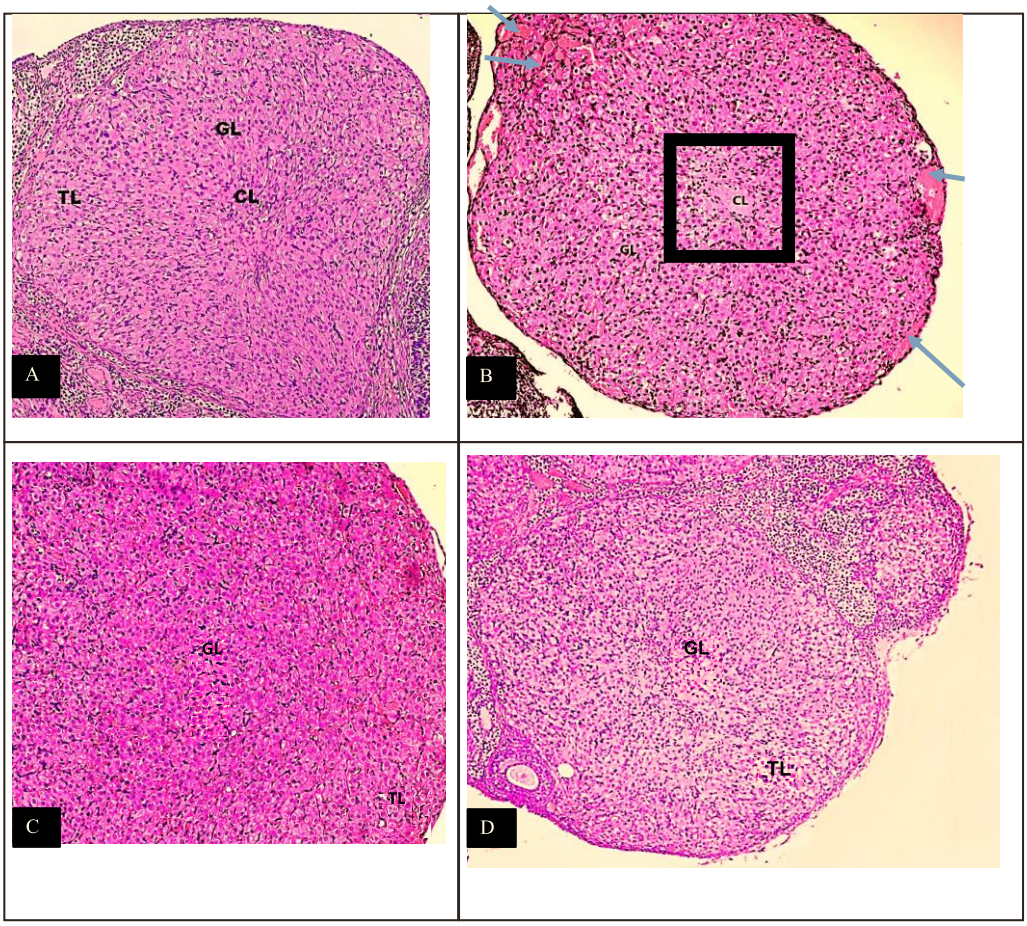
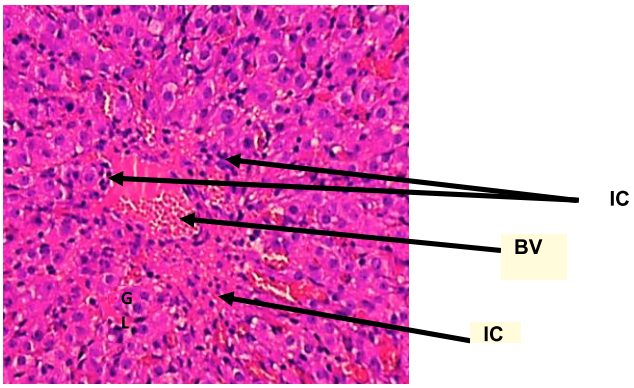


Figure 2: Photomicrograph of ovary of albino rat of control group A and experimental groups B, C and D showing Corpus Luteum (CL), Granulosa Lutein (GL), Theca Lutein (TL). Blue arrows shows blood vessel congestion in B group. (H & E, 10X)



Boxed area above shows magnified view of corpus luteum of group B, Black arrows show Congested Blood Vessel (BV) and focal infilteration of Inflammatory cells (IC). (H & E, 20X)

DISCUSSION

Poisonous consequence of smoking are due to its nicotine content. Women who smoke suffer a lowering of fecundity. Nicotine (0.5mg/kg) introduced a new phase "subestrous" and increased the length of estrous cycle. In women smokers, ovulation was late with decreased number of ova released, suggesting a likely straight action of nicotine on the ovary.[21] Nicotine acetylcholine receptors (nAChR) are present on granulosa cells.[22] Nicotine activates caspase in different tissues through nAChR-2,[23] causes apoptosis in granulosa cells and atrophy of secondary and Graafian follicles. Nicotine affects growing and developing follicles by this mechanism but also negatively affects primary follicle decreasing ovulation.[24] For growth and development of female reproductive system, steroids are needed.[25] Nicotine induced inhibition of steroidogenesis is one cause of degeneration of ovary.[26] Iranloye and Bolarinwa[27] reported that ovulation is delayed because nicotine will either inhibit estrogen creation or capacity of estrogen to control follicular growth. Reduced estrogen synthesis prolonged metaestrus and diestrus and reduced number of estrus cycles.[28]

Hypothalamus regulates gonadotrophin release from pituitary. In females, FSH stimulates the growth of Graafian follicles and LH is required for its maturation and ovulation. Nicotine inhibits the release of gonadotrophins, FSH, and LH from the pituitary, acting through the hypothalamus, blocking the neural stimulus to GnRH,[24] a decrease in gondatrophins is reflected in atrophy of both gonads in structure and function.[29] Syna PS,[3] Mohammad Ghassemi,[30] Tuttle et al[31] and Neal et al[10] noticed decrease in number of corpora lutea and Graafian follicle and increase in atretic follicle due to antagonistic action of nicotine on gonadotrophins. Patil SR[32] described that decrease in size and number of corpus luteum of rats of nicotine treated group shows anovulation or very low ovulation. Nicotine blocks ovulation by inhibiting LH surge from pituitary.[33] Blackburn CW[34] concluded that nicotine prevents estradiol manufacture, ovulation and fertilization.

In this study, vascular congestion was present in all 10 rats of group B, while only one rat of control group A and one rat of group D, showed blood vessel congestion. In group A, reason for blood vessel congestion in one rat was stasis and gravitational effect due to lying down for longer period of time because blood tends to gravitate in the dependent portion. In group B, congestion of blood vessels within ovarian medulla may be due to inhibition of prostaglandin synthesis, as these compounds are recognized to be involved in the regulation of blood flow, this commensurate with the results reported by Ismail N.H[35] who demonstrated congestion of the testicular blood vessels following monosodium glutamate treatment of male rats.

Corpus luteum is formed from the remains of follicle (from granulosa and theca interna cells) after it undergoes ovulation. Granulosa cells undergo significant hypertrophy & form granulosa lutein cells, comprise about 80% of the corpus luteum. Cells of theca lutein are half the size of the cells of granulosa lutein, are somewhat darker staining,[36] and are typically collected in the creases of the wall of the corpus luteum, which, like all endocrine glands, becomes well vascularized, as reported by F. Russel Westwood 2008 in his study of rat histological guide to staging.[37] Luteinization converts a preovulatory follicle into a highly vascular corpus luteum skilled of producing large amounts of progesterone. Neovascularization involves interaction of angiogenic and antiangiogenic substances.[38] After endothelial injury platelets adhere to subendothelial collagen fiber and to themselves and form plug. Platelets in plug release vasoactive chemical substances e.g, prostaglandin and thromboxaneA2, which further steady the plug by hurrying blood coagulation and fibrin creation.[39]

Cortex of ovary was shrunk in animals of group B. Corpus luteum revealed congestion of blood vessels and was focally infiltrated by inflammatory cells. (Fig 2B) Abnormal corpus luteum was seen in all 10 rats of group B and in none of groups A, C and D. Anti-inflammatory activities of Ajwa fruit extract could be explained, at least in part, by their antioxidant possessions.[40]

Low fecundity rates & hostile reproductive results are associated with use of nicotine. Ajwa date aqueous extract has higher concentration of polyphenols compared to sukkari and khalas.[41,42] Plant polyphenols hold properties such as anti-inflammatory activity, control of the host immune system, anti-proliferative activity, activation of procarcinogens by adjustment of cytochrome P450, upregulation of genes producing antioxidant enzymes, in addition to the ability to alter cellular signaling.[43] Ajwa dates are rich in anthocyanins[14] and flavonoids which have been reported previously to have nutraceutical properties and considered as potent antioxidants. Anthocyanins and flavonoids are potent antiangiogenic agents.[44,45]

In this study, antioxidant compounds found in aqueous extract of Ajwa prevents nicotine from causing toxicity.

CONCLUSION

Ajwa dates aqueous extract has a potent protective and ameliorative effect against nicotine induced ovarian damage and may be used to treat sexual impairment and female infertility.

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