Title page

Gender-Based Effects of Chronic Nicotine Administration on Hypothalamic Pituitary Adrenal Axis and Indoleamine 2,3 dioxygenase Activity in Rats

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Abstract

Activation of indoleamine 2,3 dioxygenase (IDO) and thus kynurenine production exerts immunosuppressive effects resulting in T lymphocyte inhibition. Stress response as well as nicotine addiction may account for an augmented hypothalamic-pituitary-adrenal (HPA) axis activity. As there is a paucity of studies regarding gender-based differences in HPA axis and IDO activity in relation to chronic nicotine administration, the present study was designed. Adult Albino Wistar male (n=12) and female rats (n=12) were divided into two groups viz control group and nicotine treated group. Control groups received drinking tap water while nicotine-treated groups received nicotine by oral gavage (3.08mg/ml/kg) daily for 21 days. Body weight, serum cotinine and tryptophan concentrations were found to be significantly lowered whereas brain tryptophan and serum corticosterone were significantly raised in nicotine treated females as compared to the male rats. Nicotine treatment decreased brain kynurenine only in males but not in females. However, female control group showed low kynurenine levels than their male counterparts. IDO activity was significantly inhibited in both nicotine treated male and female rats as compared to their respective controls with no significant gender-based differences after drug treatment. Therefore, it could be concluded that chronic nicotine administration results in an inhibition of IDO but an augmented HPA axis activity, latter effect being more pronounced in females. These findings could be targeted for pharmacological interventions against inflammatory disorders and cancers.

Keywords: Nicotine, Tryptophan, Kynurenine, Indoleamine 2,3 dioxygenase, Corticosterone.

Introduction

There is a high comorbidity of anxiety, depression and tobacco smoking in both men and women [1, 2]. However, studies have shown that women are not only more prone to tobacco usage [3] they also suffer from more severe withdrawal symptoms and have far greater relapse rates as compared to men [4, 5]. Moreover, women begin tobacco usage at a much earlier age [6]. These aspects make women more susceptible to develop illnesses related to smoking and hence this requires ascertaining biologically based gender variations in response to nicotine administration. Also, studies have reported conflicting findings regarding serum cotinine, the biomarker of nicotine exposure, with some indicating higher cotinine levels in males [7] whereas some report higher levels in females [8].

Nicotine dependence entails both positive and negative reinforcement properties of the drug. Positive reinforcement refers to the rewarding effects or psycho stimulant properties of nicotine whereas negative reinforcement comprises the alleviation of the withdrawal symptoms [9]. Central serotonergic processes have been reported to be responsible for the drug cravings and physical dependence and its hypoactivity underlies relapse following withdrawal. Nicotine has been shown to cause the release of 5-hydroxytryptamine (5-HT) from the hypothalamus, cortex, striatum, spinal cord, hippocampus and dorsal raphe nucleus. Availability of L-tryptophan constitutes a rate limiting factor for 5-HT biosynthesis as only less than 5% of tryptophan is utilized through this pathway [10]. Most of the tryptophan (about 95%) is metabolized to kynurenine (KYN) [10,11]. The cleavage of the tryptophan's indole ring leads to the synthesis of N-formyl kynurenine and subsequently to kynurenine by the rate-limiting enzymes TDO (Tryptophan 2,3 dioxygenase) mainly in the liver and IDO (indoleamine 2, 3 dioxygenase) extrahepatically [10], latter comprises only 5-10% and becomes significant only when immune system is activated. IDO is not inducible by glucocorticoids and gets inhibited by high tryptophan levels [50]. IDO activity could be determined by the ratio of L-kynurenine to L-tryptophan. Although both IDO and TDO are responsible for the metabolism of tryptophan to kynurenine but the ratio of L-kynurenine to L-tryptophan determines the IDO activity in inflammatory conditions only with IFN-γ being the main inducer of IDO [12]. IDO activation exerts immunosuppressive effects by causing depletion of tryptophan and thus increasing KYN levels that in turn result in inhibition of T lymphocytes activation [13]. However, lowered serum IDO levels have been reported in smokers [45] and this could be responsible for the immunostimulatory effects of smoking seen in diseases involving inflammatory processes such as Sjogren's syndrome and ulcerative colitis [14]. Nevertheless, no study to date has depicted the effects of chronic nicotine administration on brain IDO activity.

Cortisol downstream of the HPA axis has a regulatory function on innate immunity and inflammation. The neuroendocrine system could affect the immune system resulting in variations in the peripheral cytokine production. An aggravated hypothalamic-pituitary-adrenal (HPA) axis underlies both the response to stress and nicotine addiction [15]. However, studies have reported varied results related to the levels of cortisol in smokers with some showing increased levels [15] while others depict no variations [16].

There is a common perception that smoking reduces weight and this has been employed especially by women and adolescents as an important triggering factor for the initiation of smoking [17]. However, studies have reported conflicting findings regarding interrelationship between smoking and weight gain with some reporting no association [18] while others have reported low BMI in light and current smokers [19] and an increased BMI in ex-smokers [20].On the other hand, there are studies who have reported weight gain in current smokers [21] and increased waist circumference resulting from raised cortisol levels in heavy smokers [22]. However, there is a general paucity of studies showing gender-based differences related to weight gain and chronic nicotine administration.

The present study was designed to ascertain the gender-based effects of chronic nicotine administration on the HPA axis as well as the immunoregulatory effects of IDO through the kynurenine pathway of tryptophan metabolism.

Materials and Methods

Animals

Locally bred adult Albino Wistar male and female rats (n=24), weighing 150–170 g, were used to carry out the experiments. They were purchased from HEJ research institute of Chemistry, University of Karachi and maintained under standard lab conditions (i.e.12 hour natural light-dark cycle at 23 ± 2 ºC) for 3-weeks with 6 rats/cage plus free access to lab chow (purchased from Agha Khan University, animal care unit) and drinking tap water. Procedures regarding animal's treatment were carried out in accordance with the laws (1996) of national research council for the usage and care of lab animals. Ethical approval for the study was taken from the ethics committee for animals, University of Karachi. All strategies were employed to reduce the number of rats as well as pain to them.

Drug and treatment

Male and female rats were divided randomly into 2 groups viz control and nicotine-treated groups (n=12/group). Control groups (n=6/group) received nicotine-free drinking water only while nicotine-treated groups (n=6/group) received nicotine hydrogen tartrate orally at a dose of 3.08 mg/ml/kg-body-weight/day for 21 days. The dose of nicotine was based on published work of our laboratory [23]. Body weight was monitored twice a week daily during the treatment.

Sample collection

All rats were killed by decapitation, and brain was removed within 30 sec from skull. The brain then taken out using spatula was rinsed in ice cold saline (0.9%) and stored in at -70^{o}C until analysis. Trunk blood from sacrificed rats was collected in an appropriate collection tube and was let to sit for 30minutes at room temperature to clot, then centrifuged at 3000 rpm for 15 minutes to obtain the serum, which was stored at -20^{o}C until use.

Fluorometric determination of serum tryptophan, corticosterone and cotinine

Serum tryptophan concentrations were estimated using spectrofluorometer model FP-6200 by a modified version of Denkla and Dewey method [24], corticosterone levels were determined fluorimetrically as described previously [25] and cotinine levels were analyzed by using human cotinine Elisa kit, Bioassay Technology Laboratory

High performance liquid chromatography (HPLC) for IDO enzyme activity

Tryptophan and kynurenine concentrations in brain were determined by using HPLC coupled with UV/FL detector (SHIMADZU) as described previously [26]. After weighting, frozen brain tissues of each rat were homogenized in 12 % $HClO_4$ plus ice-cold HPLC-grade water for 5 sec in a homogenizer. Homogenates were then sonicated in a bench-top ultrasonic cleaner (Powersonic 603) filled with ice-cold distilled water for 10 min. Centrifugation of homogenates was done for 15 min at 10,000 rpm in a refrigerated centrifuge machine (multifuge 3 S-R Heraeus) at 4 ^{o}C. Supernatants were immediately used for the measurements. A reverse phase column (6 mm internal diameter x 15 cm, 5 µm average particle size, Shim-pack CLC-ODS) and a mobile phase (pH=2.8) of 10 mM Sodium Phosphate Buffer: Methanol; 73:27 v/v, with a constant flow rate of 1.1 ml/min were used. After the determination of both tryptophan and kynurenine contents in samples through HPLC, their ratio (KYN/tryptophan) was used as an index of IDO enzymatic activity.

Chemicals

Nicotine hydrogen (+)-tartrate, L- tryptophan, L- kynurenine, corticosterone were purchased from Sigma-Aldrich, St. Louis, MO, USA and all other chemicals were of analytical grade either from BDH laboratories UK or Merck, Germany.

Statistical analysis

The data is presented as the means ± SEM (n=6). Statistical analysis was done by using Student's t-test (when using 2 groups) and Two-way analysis of variance (ANOVA) followed by Newman-Keuls q-test (when comparing 4 groups) by using GraphPad Prism. Pearson's correlation coefficient was applied to assess correlations between various parameters. $p<0.05$ was considered statistically significant.

Results

Effect of Chronic Nicotine Administration on Body Weight of Male and Female rats.

Chronic nicotine treatment significantly increased the body weight of both male and female rats when compared with their respective controls. Data studied using two-way ANOVA showed a significant effect of sex ($p<0.01$), drug ($p<0.01$) on body weight with significant effect of sex x drug interaction ($p<0.01$). Newman-Keuls q statistics showed significant decrease (12.69%, $p<0.01$) in the body weight in females (199.2±2.21) as compared to similarly-treated male rats (226.3±1.88) (Fig1).

Effect of Chronic Nicotine Administration on Brain Tryptophan Metabolism in Male and Female Rats

Table 1 depicts the effect of chronic nicotine administration on brain tryptophan, kynurenine (KYN) and IDO activity (KYN/tryptophan ratio) in male and female rats. Data analyzed through two-way ANOVA showed that there is no effect of drug on brain tryptophan and brain KYN while sex had a significant effect on brain tryptophan ($F=698.52$, $p<0.01$) but no effect on brain KYN. However, the influence of sex × drug interaction is significant on brain tryptophan ($F=4.91$, $p<0.05$) and KYN ($F=10.06$, $p<0.01$). Additionally, a significant effect of sex ($F=109.99$, $p<0.01$), drug ($F=7.17$, $p<0.05$) and sex x drug interaction ($F= 26.33$, $p<0.01$) on IDO activity was found. Newman-Keuls q statistics showed significantly decreased brain KYN levels as well as decreased brain IDO activity in female controls as compared to their male counterparts. However, no significant gender-based differences in brain KYN and IDO activity were depicted between nicotine treated male and female rats. On the contrary, brain tryptophan

levels were found to be significantly increased in nicotine treated females as compared to the similarly treated male rats.

Effect of Nicotine on Serum Tryptophan as well as Serum Corticosterone Concentration in Male and Female Rats

Table 2 shows the effect of 21 days of nicotine administration on serum tryptophan and serum CORT. Data analyzed using two-way ANOVA showed a significant effect of sex ($F= 42.17$, $p<0.01$), drug ($F=8.51$, $p<0.01$), and sex x drug interaction ($F=6.44$, $p<0.05$) on serum tryptophan. Similarly, a significant effect of sex ($F=14.65$, $p<0.01$) and drug ($F=28.03$, $p<0.01$) on serum CORT was found. However, the influence of sex × drug interaction was not significant on serum CORT. Newman-Keuls q statistics showed a significant reduction in serum tryptophan (17.47%, $p<0.01$) but significant increase (14.72%, $p<0.05$) in serum CORT in nicotine treated females as compared to similarly-treated male rats. However, it was also noted that the serum CORT was also significantly raised in female controls as compared to their male counterparts.

Serum Cotinine in Nicotine Treated Male and Female Rats

Serum cotinine was found to be significantly decreased in female rats (26.47±1.64) as compared to male rats 38.08±1.12 ($p<0.01$) (Fig 2)

Correlation Between Cotinine and Body Weight in Nicotine Treated Male and Female Rats

Figure 3 depicts significant positive correlations between cotinine versus body weight in both male ($r=0.821$, $p=0.04$) and female rats ($r= 0.826$, $p=0.04$).

Correlations Between Various Parameters in Nicotine Treated Male and Female Rats

No significant correlations were determined between brain kynurenine and brain tryptophan nor between IDO and brain kynurenine in nicotine treated male and female rats. Similarly, no significant correlations were depicted between IDO and brain tryptophan in nicotine treated male and female rats (Table 3).

Discussion

The present study aimed to assess the gender-based effects of chronic nicotine administration on the HPA axis as well as the immunomodulatory effects of IDO. The study revealed that chronic nicotine administration resulted in significantly increased corticosterone levels that were more pronounced in females as compared to the male rats. Additionally, significant inhibition of IDO activity was also revealed with no significant gender-based differences after nicotine administration.

The finding of significantly raised serum cotinine levels in nicotine treated males as compared to similarly treated females is consistent with other previous studies [7, 27]. Raised salivary cotinine levels have also been reported in males as compared to females [28]. It has been reported that males have reduced CYP2A6 activity as well as reduced rate of clearance of cotinine as compared to its formation. Also, CYP2A6 activity can be induced by estrogens [29] and enhanced cotinine clearance has been shown in states of elevated estrogen levels [30].

Nicotine affects immunity and causes T cell anergy which is mediated via activation of HPA axis [31]. Raised corticosterone levels reported in the present study are consistent with the previous studies that have also reported raised serum CORT levels in rodents upon nicotine administration [32]. Additionally, raised basal cortisol levels have been reported in both acute and chronic smokers [15] as well as in consumers of smokeless tobacco [33]. Elevated levels of serum CORT might be due to the overactivation of HPA-axis leading to raised dopamine levels in the brain's nucleus accumbens [34] that in turn has been shown to be a mediator of the nicotine's rewarding effects [35]. Raised serum CORT levels in females resulting from administration of nicotine have also been reported by other studies [36] and might be due to the phenomenon of sexual dimorphism [37] resulting from the activation of the HPA-axis by the ovarian estrogen [38]. However, basal serum CORT levels have been shown to be raised in females as compared to males [39] as revealed in the female control group of the present study. Serum CORT has also been reported to increase the production of anti-inflammatory cytokine, IL – 10 [40]. Thus, nicotine treatment could be employed for various inflammatory disorders.

In the present study both nicotine treated male and female rats experienced weight gain as compared to controls which is consistent with the findings of Basterra-Gortari et al. [21] as well as of the Nurses' Health Study [41] who has also reported weight gain in current smokers. However, many studies support the perception of low body mass index (BMI) associated with smoking [19]**.** The increased weight gain in the present study could be attributed to the increased release of corticosterone in response to nicotine administration. However, female rats gained lesser weight as compared to males which might be due to the lowered bioavailability of estrogen owing to the anti-estrogenic effects of nicotine [42]. Significant positive correlations between cotinine versus weight in both female and male rats could be linked to the finding of increased risk of obesity being positively associated with the number of cigarettes smoked per day [43].

The finding of increased serum tryptophan levels is similar to the previous work done in our lab on a male population of Naswar (smokeless tobacco) users [33]. However, lower levels of serum tryptophan have also been

reported previously in cigarette smokers as compared to non-smokers [44]. Significant inhibition of brain IDO activity is being reported for the first time in the present study. Significantly raised brain tryptophan levels could be responsible for this inhibition as it has been reported that brain IDO undergoes substrate inhibition by large doses of tryptophan [50].

Increased serum tryptophan levels and inhibition of IDO activity would result in shifting the synthesis of tryptophan metabolites from kynurenine to 5-HT synthesis in brain that in turn has been shown to be involved in rendering feelings of calmness, satisfaction and relaxation [46] and therefore could be responsible for the enhanced anxiolytic-like effects of chronic nicotine administration in females. The level of 5-HT in brain was not measured in the present study but our findings are supported by a previous study that depicted that decreased IDO activity is accompanied by increased 5-HT levels [47].

Conclusion:

Thus, it could be inferred that chronic administration of nicotine results in decreased levels of IDO and an augmented HPA axis activity, latter being more pronounced in females. The anti-inflammatory effect of nicotine via raised CORT levels could be employed in various inflammatory diseases. Additionally, the inhibition of IDO activity could be applied in pharmacological interventions [48] designed for diseases such as cancer where the immunosuppressive effects exerted by increased IDO expression have been reported to be responsible for the progression of the disease process [49].

Author contributions: Samina Bano contributed to the concept and design of the study. Sumaiya Binte Hamid and Humaira Sharif conducted the experiment under supervision of Samina Bano. Faiza Sajid, Sumaiya Binte Hamid and Humaira Sharif analyzed the data. Sumaiya Binte Hamid wrote the manuscript. Samina Bano and Faiza Sajid revised and approved the final version of the manuscript.

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Conflict of Interests: The authors declare that they have no conflict of interests

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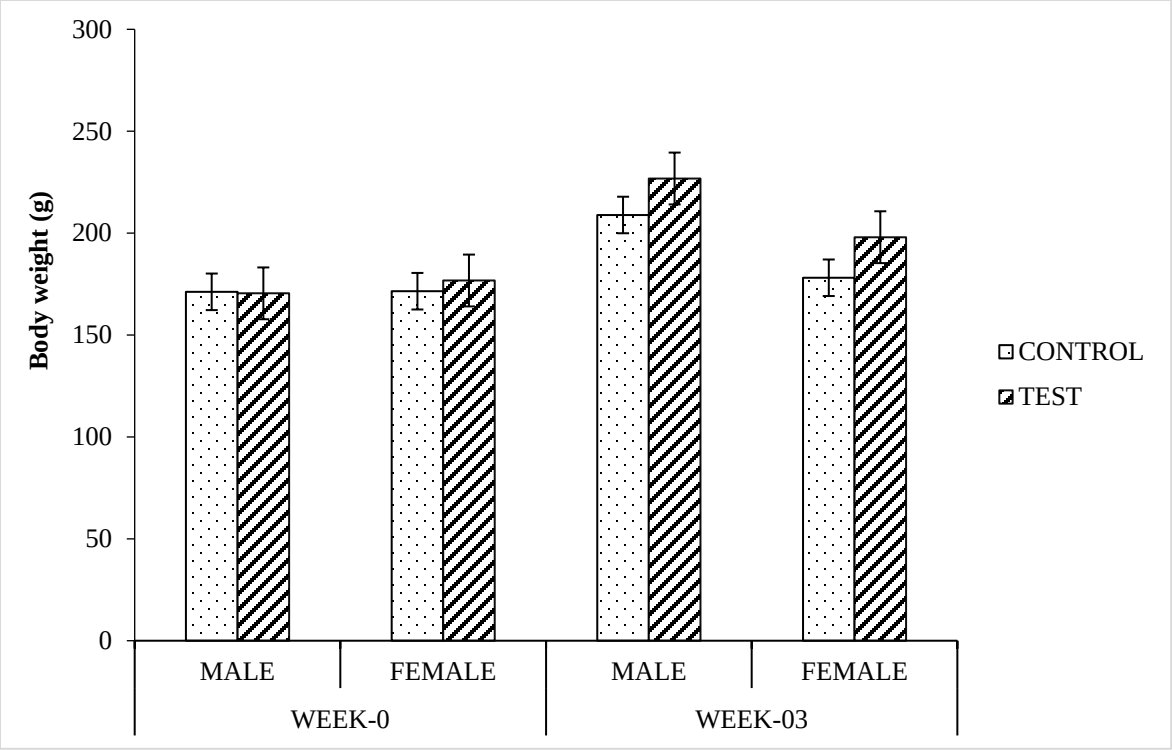


Fig. 1: Effect of Nicotine on Body Weight of Male And Female Rats

Experimental details are given in the materials and methods section. All values presented as means ± SEM for each group of 6 rats. Statistical analysis was performed using two-way ANOVA followed by Newman Keul's q-test. The significance of differences is indicated by *$p<0.01$ when drug treated group was compared with respective control group and †$p<0.05$, ††$p<0.01$ when female groups were compared with their male counterparts.

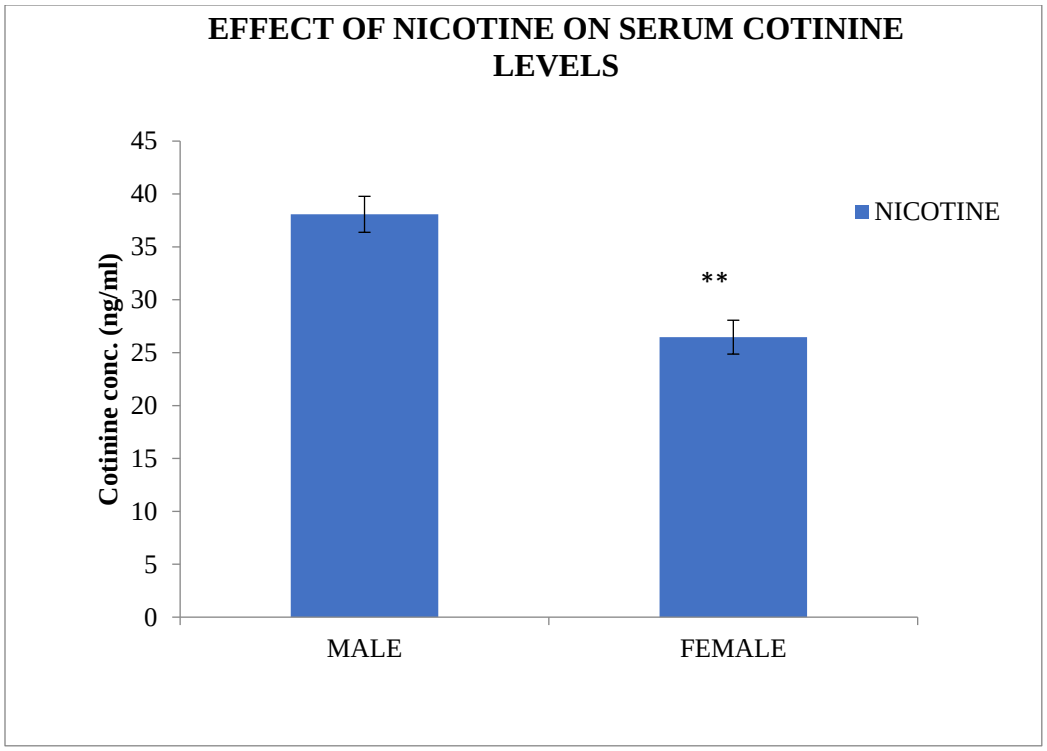
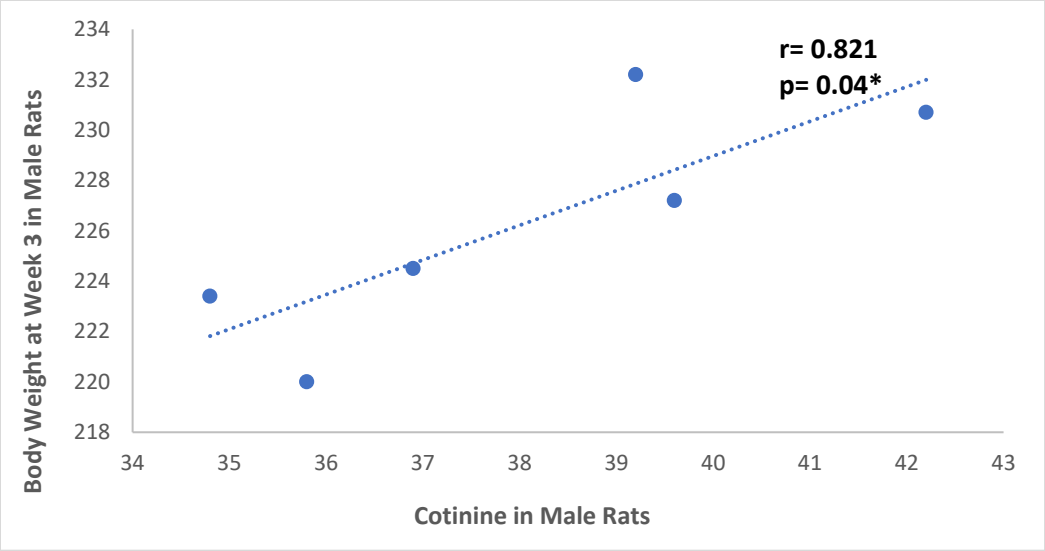


Fig. 2: Serum Cotinine Levels in Nicotine Treated Male and Female Rats

Experimental details are given in the materials and methods section. Values are given as means ± SEM. Statistical analysis was performed using student's t-test. The significance of differences is indicated by *p<0.01.



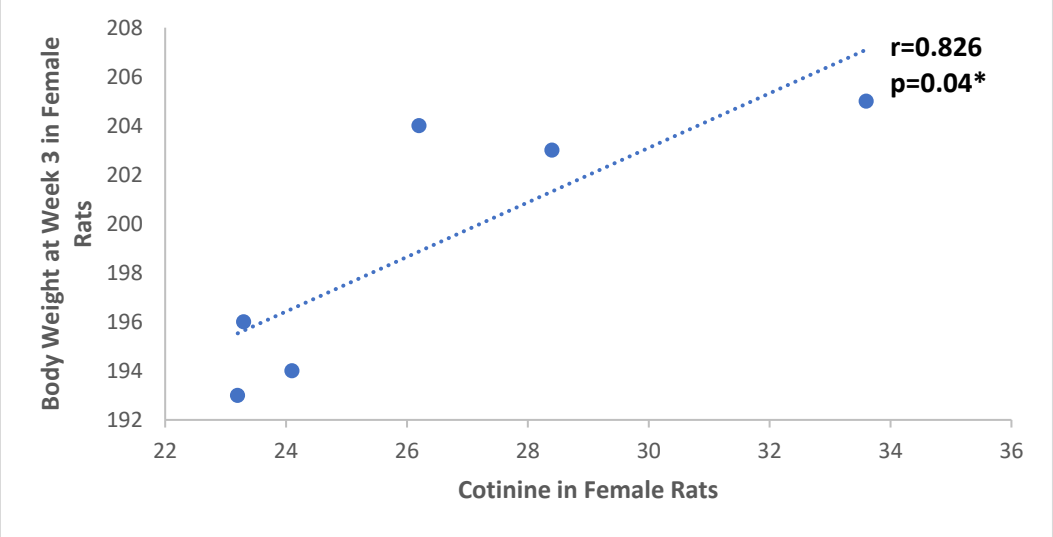


Fig 3: Scatter Plot of Cotinine Versus Body Weight in Male and Female Rats.

Table 1 Effect of Nicotine on Brain Tryptophan Metabolism In Male And Female Rats

PARAMETERS	MALE		FEMALE		Two-way ANOVA DF (1,20)		
	Control	Drug	Control	Drug	Sex	Drug	Sex × Drug
Brain TRP (nmoles/g)	17.24±0.36	23.60±0.22**	16.94±0.17	24.47±0.27**†	F=698.52 P<0.01	F=1.181 NS	F=4.911 P<0.05
Brain KYN (nmoles/g)	2.82±0.22	2.23±0.12*	2.11±0.05 ††	2.45±0.07**	F=3.82 NS	F=1.16 NS	F=10.06 P<0.01
IDO activity (KYN/TRP ratio)	0.16±0.005	0.09±0.003**	0.12±0.004 ††	0.10±0.004**	F=109.99 P<0.01	F=7.17 P<0.05	F=26.33 P<0.01

Experimental details are given in materials and methods section. All values presented as means ± SEM (n= 6). Statistical analysis was performed using two-way ANOVA followed by Newman Keul's q-test. The significance of differences is indicated by *p<0.05 & **p<0.01 when drug treated groups were compared with their respective control groups and †p<0.05, ††p<0.01 when similarly treated female groups were compared with their male counterparts.

PARAMETERS	MALE		FEMALE		Two-way ANOVA DF (1,20)		
	Control	Drug	Control	Drug	Sex	Drug	Sex × Drug
Serum Tryptophan (µg/ml)	10.46±0.34	14.71±0.82 **	10.27±0.24	12.14±0.15 *††	F=42.17 P<0.01	F=8.51 P<0.01	F=6.44 P<0.05
Serum Corticosterone (µg/ml)	14.54±0.15	20.03±0.81 **	18.67±0.73 ††	22.98±1.48 *†	F=14.65 P<0.01	F=28.03 P<0.01	F=0.40 NS

Table 2 Effect of Nicotine on Serum Tryptophan and Serum Corticosterone in Male and Female Rats

Experimental details are given in materials and methods section. All values presented as means ± SEM for each group of 6 rats. Statistical analysis was performed using two-way ANOVA followed by Newman Keul's q-test. The significance of differences is indicated by *$p<0.05$, **$p<0.01$ when drug treated groups were compared with their respective control groups and †$p<0.05$, ††$p<0.01$ when similarly treated female groups were compared with their male counterparts.

Table 3 Correlation matrix between Brain Tryptophan, brain kynurenine and indoleamine 2,3 dioxygenase**.**

	Brain kynurenine		IDO	
	Males	Females	Males	Females
Brain Tryptophan	r=0.51 p= 0.29	r= 0.22 p= 0.66	r= -0.07 p= 0.88	r= -0.11 p= 0.82
IDO	r = -0.07 p = 0.88	r= -0.25 p= 0.63	-	-

IDO= indoleamine 2,3 dioxygenase.