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Effect Of Folic Acid And Vitamin B_{12} On Nicotine Mediated Ovarian Dysfunction

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***Abstract*:**

Nicotine is an abundant and most significant component of cigarette smoke. Epidemiological evidence strongly suggests an association between cigarette smoking and misalignment of female reproductive function. In the present study we examined the impact and underlying mechanisms of action of folic acid and vitamin B_{12} on nicotine-induced damage in ovary of rats. Female Wistar rats were treated with nicotine (3 mg/kg body weight/d, intraperitonealy) with or without folic acid (36 µg/kg body weight/d, orally) and vitamin B_{12} (0.63 µg/kg body weight/d, orally) for 28 days. Serum estrogen and oxidative stress parameters were measured. Folic acid and vitamin B_{12} blunted the nicotine-induced impairment in serum estrogen level. Moreover, folic acid in combination with vitamin B_{12} also attenuated the nicotine-induced changes in markers of oxidative stress in the ovarian tissue of rats as demonstrated by reduced level of nitric oxide (NO) and malondialdehyde (MDA), increased activities of superoxide dismutase (SOD) and catalase (CAT) as well as elevated reduced glutathione (GSH) level. The present study shows that folic acid and vitamin B12 supplementation can reduce nicotine-induced impairment in serum estrogen level and ovarian function by modulating oxidative stress.

Key words: Nicotine, ovary, oxidative stress, folic acid, vitamin B_{12}

Introduction

Tobacco smoking poses a significant global public health challenge, with projections indicating that if present patterns persist, one billion individuals may succumb to tobacco-related illnesses in the 21st century (Yin *et al.*, 2019). Nicotine, the chief addictive component in tobacco, is primarily consumed through cigarette smoking. Nevertheless, smoking tobacco emits numerous toxic substances known to inflict harm on various organs, including the ovaries (Morishita *et al.*, 2022). Globally, there is a consensus on the importance of smoking cessation, and the popularity of e-cigarettes as a potential substitute for traditional tobacco in smoking cessation efforts has risen (Bordel *et al.*, 2006). This is attributed to their lower adverse effects on the human body and the diverse array of flavors and nicotine concentrations available.

Data from animal studies present indications that nicotine exposure may have detrimental effects on female reproduction (Blackburn *et al.,* 1994; Patil *et al.,* 1998). For instance, exposure to nicotine in utero leads to impaired fertility, disrupted ovarian steroid hormone and protein levels, and an increased number of atretic follicles in adult female rat offspring (Neal *et al.,* 2007; Holloway *et al.,* 2006). Notably, exposure to nicotine during fetal, neonatal, and adult stages results in altered morphology and apoptosis in the ovaries of mice and rats (Holloway *et al.,* 2006, Mohammadghasemi *et al.,* 2012). In their study, Petrik et al. (2009) identified the expression of nAChR-2 and nAChR-7 in ovarian tissues and isolated granulosa cells. They suggested that one potential mechanism by which nicotine induces folliculogenesis defects in adult female rats is through the activation of receptors, leading to apoptosis in granulosa cells and/or oocytes.

Pro-oxidant effects of nicotine tempted us to suggest that treatment with an anti-oxidant might prevent nicotine-induced oxidative stress and damage in the present study. It has been shown earlier that folic acid supplementation increases total serum antioxidant capacity (Song *et al.,* 2009) which could be beneficial in smokers, who have increased markers of oxidative stress and damage than non-smokers. Moreover, the anti-inflammatory effect of folic acid is manifested by a decrease in the levels of interleukins and C-reactive proteins. Furthermore, smokers (as well as individuals exposed to second-hand smoke) have decreased levels of folate than non-smokers (O'Callaghan *et al.,* 2002). With this background, we hypothesize that folic acid supplementation in combination with vitamin B_{12} could be a good therapeutic agent for the prevention of ovarian dysfunction caused by nicotine exposure.

Materials and methods

Animal Model

The ethical guidelines recommended by the Institutional Animal Ethics Committee (IAEC) of Serampore College, West Bengal, India, were strictly adhered to conduct all animal experiments. The experiments involved female Wistar albino rats with a weight range of 110-125 g each. The rats were housed in plastic cages within a controlled animal house environment (maintained at a temperature of 24 ± 3°C) and subjected to a 12-hour light/dark schedule, with unrestricted access to water.

Experimental design

The animals were checked for normal oestrous cycle and randomly assigned to four groups, each comprising five rats: Group A (control), Group B (nicotine-treated), Group C (nicotine + folic acid), and Group D (nicotine + folic acid + vitamin B_{12} supplementation). All groups were fed a control diet consisting of 71% carbohydrates, 18% protein, 7% fat, and 4% salt mixture. The dosage and administration route of nicotine were consistent with previous reports (Mukherjee et al. 2006). Rats in Groups B, C, and D received a daily intraperitoneal injection of nicotine tartrate (dissolved in 0.9% physiological saline) at a dose of 3.0 mg/kg body weight for 21 days. Additionally, in Group C, animals received oral treatment with folic acid at a dose of 36 µg/kg body weight per day for 28 days. In Group D, animals were treated with both folic acid and vitamin B_{12} at doses of 0.63 µg/kg body weight per day for 21 days, administered concurrently, following the nicotine treatment (Abu-Taha *et al.,* 2019).

Preparation of serum

All groups of animals underwent anesthesia using pentobarbitone sodium (60 mg/kg body weight, administered intraperitoneally) and were subsequently euthanized through cervical dislocation, a recommended physical method. Blood samples were collected from the heart, and serum was isolated for the assessment of estrogen.

Measurement of serum estrogen level

Serum estrogen level was measured by using commercially available ELISA kit obtained from Wuhan Fine Biotech Co., Ltd., China. All samples were run at a time to avoid intra-assay variation. Inter-assay variation was 3.9%.

Preparation of ovarian tissue extract

The experimental animals underwent ovarian removal for the evaluation of different oxidative stress markers. Ovarian tissue extracts were prepared using ice-cold Tris-HCl buffer (pH 7.4). The assessment of Superoxide Dismutase (SOD) and Catalase (CAT) activity involved homogenizing the tissues in ice-cold isotonic phosphate buffer saline (PBS) at two distinct pH levels (7.0 and 8.0).

Measurement of oxidative stress parameters

In aerated solutions, nitric oxide (NO) undergoes rapid decomposition, leading to the formation of stable nitrite/nitrate products. In this study, nitrite accumulation, indicative of NO production, was determined through the Griess reaction (Raso *et al.*, 1999).
The quantitative evaluation of lipid peroxidation employed the thiobarbituric acid (TBA) test (Wills, 1987), with the generation of malondialdehyde (MDA) serving as an indicator of lipid peroxidation levels in this experiment.
Superoxide dismutase (SOD) activity was determined using the nitroblue tetrazolium (NBT) method, which relies on the inhibition of NBT reduction by SOD (Sun *et al.*, 1988).
Catalase activity was assessed following the method outlined by Aebi (Aebi, 1984), involving the monitoring of hydrogen peroxide (H_2O_2) decomposition at 240 nm.
The level of glutathione (GSH) was determined using 5,5'-dithiobis-2-nitrobenzoic acid (Ellman, 1959).

Estimation of protein

Protein quantification in the ovarian tissue extract was conducted following the procedure outlined by Lowry et al. (1951), utilizing bovine serum albumin (BSA) as the standard.

Statistical analysis

The data were expressed as mean ± standard error (SE). To evaluate potential significant differences among the scores of various groups, the Kruskal–Wallis nonparametric analysis of variance test was conducted. Intergroup comparisons were carried out using the Mann–Whitney U multiple comparison test to identify correlations between study variables. Statistical analysis was performed using StatsDirect 2.7.2, with differences considered significant if $p < 0.05$.

Results

Impact of folic acid with or without vitamin B_{12} on nicotine-mediated changes in estrogen level and ovarian oxidative stress parameters were demonstrated in Figure 1. Results revealed that nicotine exert a suppressive effect on serum estrogen level as evidenced by significant reduction in estrogen level ($p<0.001$) of nicotine treated rats. However, folic acid alone did not sufficient to mitigate nicotine-induced effect on serum estrogen level. Folic acid in combination of vitamin B_{12} effectively counteracted the nicotine's ill effect on serum estrogen level.

Furthermore, both NO and MDA level were increased in nicotine-treated rats leading to generation of oxidative stress in these animals. Likewise folic acid with vitamin B_{12} markedly dampened the nicotine's toxic effect on ovary in terms of creating oxidative stress. Further, activities of both the anti-oxidant enzymes, superoxide dismutase (SOD) and catalase (CAT) and the level of GSH were reduced in the ovary of nicotine treated rats revealing compromised ovarian antioxidant system. However, combined supplementation of folic acid and vitamin B12 registered their beneficial effect against toxic effects on ovary by restoring the activities of SOD and catalase as well as the level of GSH.

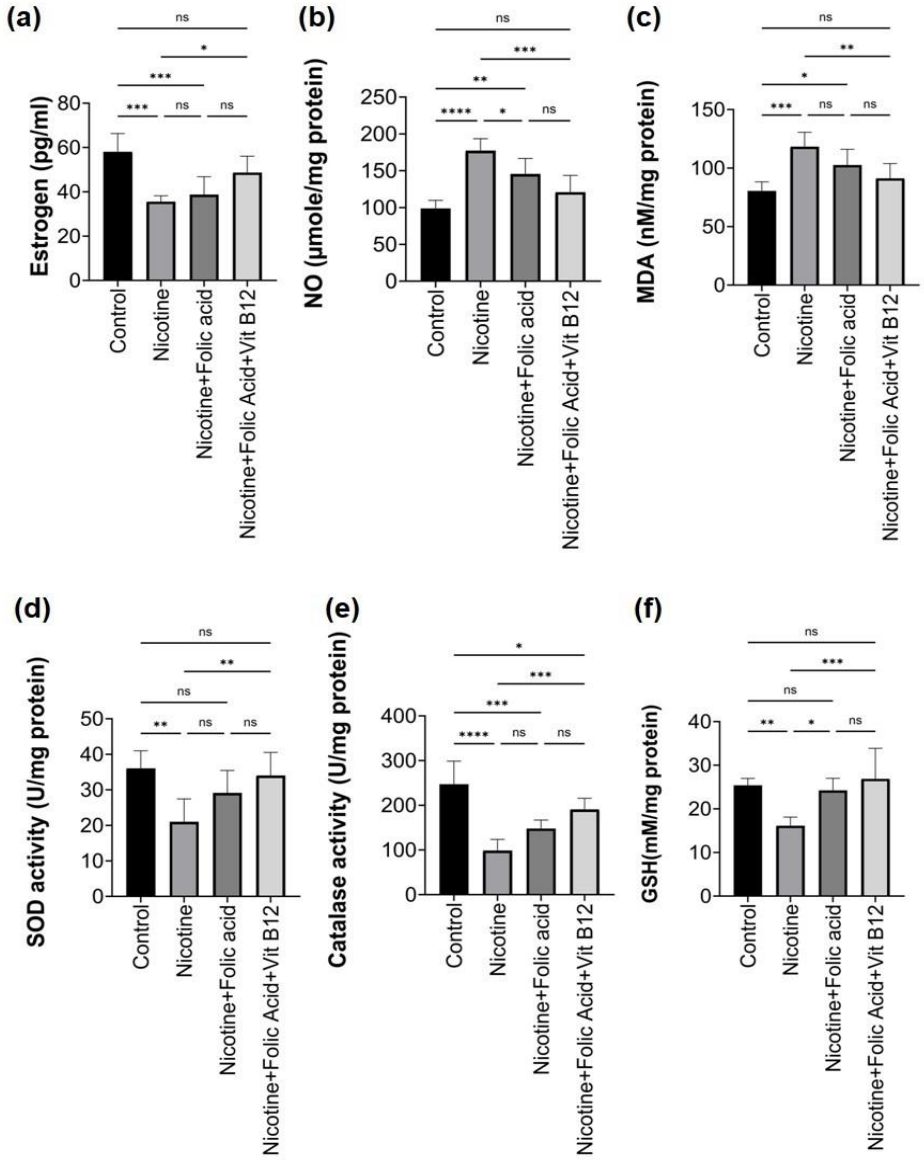

Figure 1: Effect of Folic acid (36 µg/kg body weight/day for 21 days) and Folic acid+ Vitamin B_{12} (0.63 µg/kg body weight/day for 21 days) on Nicotine (3 mg/kg body weight/day for 21 days) induced changes in (a) Estrogen level (b) NO level (c) MDA level (d) SOD activity (e) Catalase activity (f) GSH level . Significance level based on Kruskal Wallis test [p<0.05, #]. Significance level based on Mann-Whitney U multiple comparison test: *p<0.05, **<0.01, ***P0.001, ns – not significant

Discussion

Estradiol, the primary active hormone from ovaries, is pivotal in female reproduction. Variations in its levels can lead to specific ailments (Fan *et al.*, 2019). Prior research demonstrated nicotine's inhibition of estrogen production in lab settings (Miceli *et al.*, 2005). Confirming these findings, Fan et al. (2019) showed nicotine's impact on reducing estradiol and certain gene expressions. Our study aligns, revealing nicotine's influence on estrogen reduction, potentially affecting ovarian functions. This disruption might impair reproductive cycles and suggests nicotine's direct interference in steroid production, leading to various complications. Encouragingly, folic acid and vitamin B_{12} restored estrogen levels in nicotine-treated rats, hinting at their potential protective role in ovarian functions.

Nicotine induces oxidative stress, increasing reactive oxygen species (ROS). This stress, linked to lipid peroxidation (LPO), contributes to cellular damage, affecting membrane enzymes and proteins (Helen *et al.*, 2000). This initiates a cycle of stress, tissue damage, and cell death, further elevating free radicals and reducing antioxidants (Baynes, 1991). Nicotine-treated rats displayed increased LPO in ovaries, coupled with decreased antioxidant enzyme activity. Folic acid and vitamin B_{12} supplementation reversed this trend, lowering LPO

levels by enhancing antioxidant enzyme activity, notably catalase and SOD. The study also observed higher levels of the natural antioxidant glutathione (GSH) in ovaries of rats supplemented with folic acid and vitamin B_{12}, indicating their potential in reducing LPO in nicotine-exposed tissues.

Folic acid, a member of the B-group vitamins, has demonstrated the ability to safeguard bioconstituents from free radical damage, primarily through competitive mechanisms that help prevent oxidative stress. Smokers typically exhibit lower levels of folic acid and vitamin B_{12}. The scavenging and repair of thiyl radicals by folic acid position it as a potential antioxidant (Bhattacharjee *et al.,* 2016). Therefore, our hypothesis centers on the protective potential of folic acid and vitamin B_{12} against nicotine-induced ovarian dysfunction, attributed to their antioxidant properties.

The antioxidant effect of folic acid and vitamin B_{12} is evident in this study, as it reveals an inverse correlation between antioxidant enzymes and lipid peroxidation products in the ovaries of nicotine-treated rats. These findings imply that folic acid and vitamin B_{12} function as beneficial nutritional antioxidants, mitigating tissue damage and oxidative stress caused by nicotine in the ovaries. These insights can be particularly relevant for smokers, shedding light on the potential protective role of folic acid and vitamin B_{12} supplementation.

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