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Effect of Moringa *Oleifera* Extract on Nicotine Induced Neurotoxicity in Female Rat and Their Embryos

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Abstract: Nicotine is a neuroteratogen that disturbs brain development by stimulating nicotinic Acetylcholine Receptors (nAChRs) that control neural cell differentiation. Plant is known as common source of natural medicine, either in the form of traditional preparations or as pure active principles. *Moringa oleifera* (MO) is known as a vegetable, a medicinal plant. MO has various biological actions, including regulation of thyroid hormone, control of diabetes, gastric ulcer, cancer and hypertension. Also it is, used for treating various inflammatory, cardiovascular and liver diseases. In the present work therapeutic efficacy of oral administration of MO extract (10, 50 and 100 mg/kg body weight,) was investigated in femal rats exposed to1.5 mg/kg nicotine given subcutaneously twice daily from 11^{th} to 20^{th} days of gestation. After treatment, the brain of female rats and embryos were collected to examine the effect of MO against nicotine induced neurotoxicity by using analyses of neurohormones (growth hormone-releasing hormone (GHRH) and somatotropin release-inhibiting hormone (SRIH)), antioxidant enzymes (peroxidase, cholinesterase activities and glutathione GSH levels) as well as lipid malondialdehyde content (MDA) and protein carbonyls (PC) oxidation. The results revealed that nicotine treatment modulated the levels of GHRH and SRIH in the hypothalamus, increased lipid / protein oxidation of brain tissues. Also, it decreased the antioxidant activities. MO extract inhibited the nicotine neurotoxic effects in female rat brains and their embryos specially in the group given the high nicotine concentration. Our results suggest that MO extract could be used as a useful antioxidant against neurotoxicity induced by nicotine treatment.

Key words: *Moringa oleifera* · Nicotine · Neurotoxicity

INTRODUCTION

Smoking in developing countries is increasing in which at least 15% of women are reported to smoke during pregnancy. Smoking during pregnancy is associated with reduced birth weight, disturbance in cognitive function and increased incidence of attention deficit-hyperactivity disorder (ADHD) in offspring [1]. However, it has been difficult to establish the relationship between smoking exposure and cognitive impairment.

Nicotine is a major contributor to neurodevelopmental and autonomic abnormalities [2]. It is the main bioactive components in tobacco smoke which may increase cancer risk through activation of nicotine acetylcholine receptors [3]. The exposure to nicotine contributes to cardiovascular disorders such as cardiac arrhythmias, hypertension, and vasospasm, via both direct and indirect mechanisms [4]. This effect mediated by nicotine's direct interaction with cardiac inward rectifier K^+ channels, which may alter cardiac action potential or indirectly, by stimulating catecholamine release from the adrenal medulla and sympathetic nerve endings [5]. During pregnancy, nicotine crosses the placenta to the fetal circulation having various effects in fetal organs [6]. These include decreases in blood flow in brain, synaptic function, and neuronal cell proliferation and differentiation [7]. Prenatal exposure to nicotine has been shown to increase contractile responsiveness of the aortae to norepinephrine and potassium chloride KCl [8] and increase the susceptibility to ischemia/reperfusion injury in male rat offspring [9].

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Nicotine activates nicotinic Acetylcholine Receptor nAChR [10]. The binding sites are detected during embryonic development in both human and animal fetuses [11, 12]. These receptors are functional in prenatal and early postnatal rat pups [13]. Therefore, exposure to nicotine during pregnancy could directly influence brain development.

Moringa oleifera Lam. Syn. Moringa pterygosperma Gaerth (Family: Moringaceae) is commonly known as drumstick or horseradish tree. It is indigenous to Northwest India but, at present, it is widely distributed in the tropics throughout the Pacific, West Africa, as well as Central America and the Caribbean [14, 15]. It is a typical multipurpose tree. Leaves and seeds of MO represent an important source of nutrients for rural populations in certain areas of India and West Africa [14]. Most of the parts of the plant are used for the traditional treatment of diabetes [16], hepatotoxicity, rheumatism, venomous bites and also for renal disorders [15, 17]. Although MO leaves have been used for the traditional treatment of some disorders, their neuroprotective activity has not yet been investigated.

Therefore, the present study was carried out to investigate the possible useful effect of MO leaves extract on nicotine-induced neurotoxicity in pregnant rats and their embryos.

MATERIALS AND METHODS

Chemicals: Nicotine and solvents were of analytical grade and purchased from the Sigma–Aldrich Co., LLC (St. Louis, MO, USA).

Plant Material and Sample Extraction: *Moringa oleifera* leaves were collected from a cultivation site located in Eastern Almadinah Almunawarah farms. The collected leaves were air-dried and ground into a fine powder in a mill. The powder of MO leaves (1000 g) was extracted with ethanol/water (80/20) by shaking for 2 days at room temperature. The extract was filtered over Whatman No. 1 paper and the solvent (ethanol/water) was removed using a rotary evaporator at 40-50°C. Afterwards, the concentrated extract was then oven-dried (50°C) before being used for the experimental study.

Experimental Animals: Eighty adult albino pregnant female rats (140-160 g) and their embryos, purchased from the animal provider and were maintained on standard laboratory diet (protein, 16.04%; fat, 3.63%; fiber, 4.1%; and metabolic energy, 0.012 MJ) and water *ad libitum* at the Animal House Laboratory, King Fahd Medical Research Center, King Abdulaziz University. After an acclimation period of 1 week, animals were divided into several groups (10 rats/ group) and housed individually in filter-top polycarbonate cages, housed in a temperature-controlled (23±1°C) and artificially illuminated (12 h dark/light cycle) room free from any source of chemical contamination. All animals received human care in compliance with the guidelines of the Animal Care and Use Committee of King Fahd Medical Research Center, King Abdulaziz University.

Experimental Design: The pregnant female rats were randomly allocated in 8 groups (n=10 per group) as follows: Group 1: Pregnant animals served as untreated control group. Group 2: Pregnant rats were subcutaneously administered 1.5 mg/kg of nicotine (Sigma, St. Louis, MO) twice daily at 8:00 am and 2:00 pm from the day 11th to 20th of gestation. Groups 3, 4 and 5: Pregnant rats were orally administered 10, 50 and 100 mg/kg body weight of MO, respectively during the same period of gestation. Groups 6, 7 and 8 were pregnant rats treated as in groups (3, 4 and 5) plus 1.5 mg/kg of nicotine. The dose of nicotine and MO were selected according to Lv *et al*. [18] and Luqman *et al*. [19], respectively.

All animals were sacrificed by cervical dislocation after 24 h of the last injection. Hypothalamus was collected from pregnant females for hormonal analysis. Brain tissues were collected from fetal rats for biochemical analyses.

Assays for the Neurohormones GHRH and SRIH Rat Hypothalamus

GHRH Assay: Hypothalamic growth hormone-releasing hormone GHRH was determined in hypothalamus tissues by ELISA kit (USCN Life Science Inc., Wuhan, China) according to the manufacturer's instructions on day 20 of gestation.

SRIH Assay: Hypothalamus tissues were used to determine the level of somatotropin release-inhibiting hormone SRIH. The lyophilized perfusates were reconstituted with 450 µl of an assay buffer (0.1% bovine serum albumin, 100 mM PBS, 0.1% sodium azide, and 0.1% Triton X-100, pH 7.4) and subjected to radioimmunoassay RIAs (Middle Eastern Regional Radioisotope Centre for the Arab Countries). A 100 µl aliquot was applied to the assay. The neuropeptides of SRIH was measured using specific RIA kits purchased from Peninsula Laboratories, LLC (San Carlos, CA USA). The sensitivity

of this assay was 1.0 pg for SRIH. These peptides were also measured in reconstituted lyophilizates from blank perfusates (5 samples per rat) containing 450 µl of the pure aCSF, and their mean values were subtracted from the levels in all the actual perfusates from every animal.

Determination of Antioxidant Enzymes Activities

Estimation of Glutathione Concentration: The glutathione (GSH) concentration in fetal brain tissues was estimated using standard method of Beutler *et al*. [20] and Luqman *et al*. [21] with slight modification.

Determination of Peroxidase Activity: Peroxidase activity measurements were carried in fetal brain out according to Miranda *et al*. [22].

Determination of Cholinesterase Activity: Cholinesterase enzyme activity in fetal brain was determined according to Jakobs *et al*. [23].

Lipids and Protein Oxidation Analyses

Lipid Oxidation Analysis by Determination of MDA: Malondialdehyde formed during lipid peroxidation was measured according to the method of Esterbauer and Cheeseman [24] and Luqman *et al*. [21].

Protein Oxidation Analysis by Determination of PC: Protein oxidation was analyzed through the cellular protein carbonyl (PC) content in fetal brain tissues in which it was determined according to the method described by Baltacioglu *et al*. [25].

RESULTS

Analysis of the Neurohormones in Rat Hypothalamus

Analysis of GHRH Level

The results in Figure 1 revealed that treatment of pregnant rats with nicotine (1.5 mg/kg) decreased the level of GHRH by 43.8% compared with control pregnant rats ($P < 0.05$). However, treatment of pregnant rats with medium (50 mg/kg) and high (100 mg/kg) doses of MO increased the level of GHRH by 31.3% ($P < 0.01$) and 81.3% ($P < 0.001$), respectively, compared with control pregnant rats. So administration of MO took the values of GHRH back to normal.

Analysis of SRIH Level: The level of the neurohormone SRIH after treatment of pregnant rats with nicotine and MO is summarized in Fig. 2. The results revealed that treatment of pregnant rats with nicotine (1.5 mg/kg) increased the level of SRIH by 266.7% ($P < 0.001$) compared with control pregnant rats. However, the level of SRIH was relatively similar to that in control pregnant rat when pregnant rats treated with low (10 mg/kg) and medium (50 mg/kg) doses of MO. Furthermore, treatment of pregnant rats with high dose (100 mg/kg) of MO decreased the level of SRIH by 25% ($P < 0.05$) compared with control pregnant rats. In addition, MO decreased the level of SRIH in hypothalamus tissues induced by the nicotine treatment, since the treatment with low, medium and high doses of MO decreased the level of SRIH in hypothalamus by 31%, 47% and 69% respectively ($P < 0.01$), when compared with (1.5 mg/kg) nicotine treated-pregnant rats (Fig. 2).

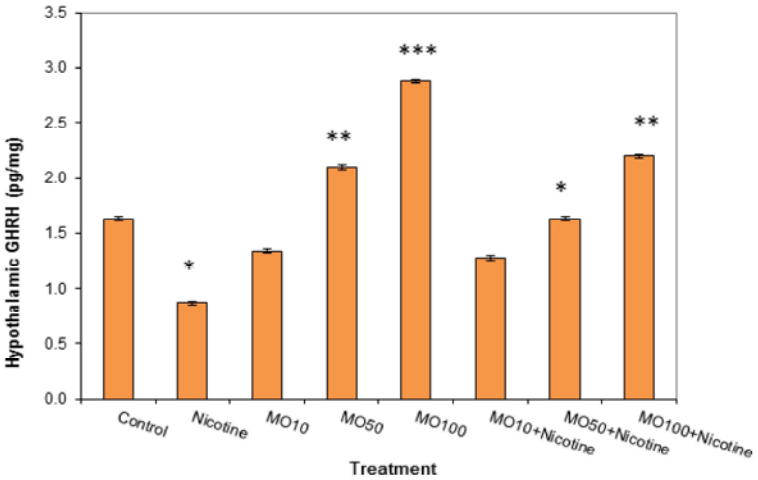

Fig. 1: Hypothalamic GHRH level in female rats treated with nicotine and MO. Data are presented as mean ± SEM. Values marked with an asterisk (*) are significantly different ($P < 0.05$), (**) are significantly different ($P < 0.01$) and (***) are significantly different ($P < 0.001$).

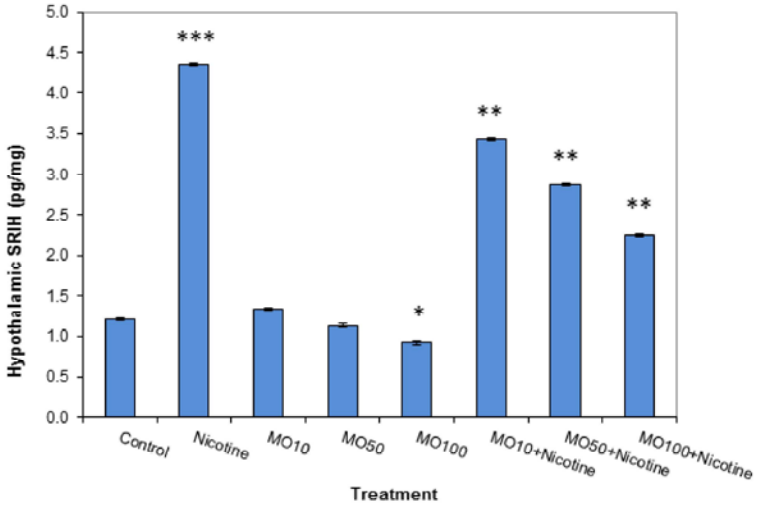

Fig. 2: Hypothalamic SRIH level in female rats treated with nicotine and MO. Data are presented as mean ± SEM. Values marked with an asterisk (*) are significantly different ($P < 0.05$), (**) are significantly different ($P < 0.01$) and (***) are significantly different ($P < 0.001$).

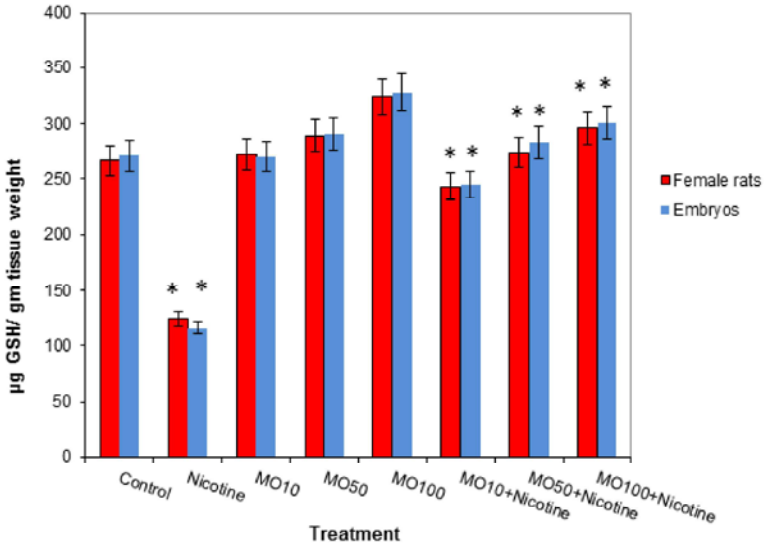

Fig. 3: The level of glutathione in brain of female rats and their embryos following exposure to nicotine and MO. Data are presented as mean ± SEM. Values marked with an asterisk (*) are significantly different ($P < 0.05$).

Effect of MO on GSH Levels: The results of the present study revealed a significant decrease in GSH levels in brain tissues of pregnant rats (53.2%) and their embryos (57.2%) when treated with nicotine (1.5 mg/kg) as compared to those in the control pregnant rats ($P < 0.05$, Fig. 3). However, reduced GSH levels in brain tissues of rats and their embryos treated with MO were relatively similar to those in control group (Fig. 3). In addition, pregnant rats treated with all doses of MO plus nicotine (1.5 mg/kg) showed an increase in the levels of GSH in brain tissues of nicotine treated rats and their embryos ($P < 0.05$, Fig. 3).

Effect of MO on Peroxidase and Cholinesterase Activities: The results revealed that levels of peroxidase and cholinesterase activities decreased with high significant differences in brain tissues of rats and their embryos treated with nicotine (1.5 mg/kg) in comparison to those in the control pregnant rats ($P < 0.05$, Table 1). In contrast, peroxidase and cholinesterase activities levels in brain tissues of rats and their embryos treated with high dose of MO (100 mg/kg) increased significantly in comparison with those in control group (Table 1). This effect was also appeared in peroxidase activity level when pregnant rats were treated with low dose (10 mg/kg,

Table 1: Amount of peroxidase (U/mg tissues/min) and cholinesterase (U/mg tissues) activities in brain tissues of female rats and their embryos treated with nicotine (1.5 mg/kg) and MO

	Peroxidase Activity (Mean ± SD)		Cholinesterase Activity (Mean ± SD)	
Treatment	Female Rats	Embryos	Female Rats	Embryos
Control	1.82±0.02	1.88±0.02	2.31±0.03	2.42±0.02
Nicotine	0.72±0.02*	0.82±0.02*	1.14±0.08*	1.21±0.08*
MO10	1.82±0.02*	1.92±0.02*	2.64±0.03	2.73±0.03
MO50	2.49±0.03**	2.76±0.03**	2.68±0.03	2.71±0.03
MO100	4.16±0.04***	4.42±0.06***	3.32±0.05***	3.32±0.05**
MO10+Nicotine	1.11±0.02	1.24±0.02	2.17±0.04	2.25±0.03
MO50+Nicotine	1.95±0.03	2.15±0.02**	2.27±0.03	2.36±0.03
MO100+Nicotine	2.79±0.03**	3.46±0.02**	2.92±0.04	3.14±0.05*

Data are presented as mean ± SEM. Values marked with an asterisk (*) are significantly different ($P < 0.05$), (**) are significantly different ($P < 0.01$) while (***) are significantly different ($P < 0.001$).

Table 2: Levels of malondialdehyde content (MDA, nmol/mL) and protein carbonyls (PC, nmol/mg protein) in brain tissues of female rats and their embryos treated with nicotine (1.5 mg/kg) and MO

	MDA (Mean ± SD)		PC (Mean ± SD)	
Treatment	Female Rats	Embryos	Female Rats	Embryos
Control	2.14±0.2	1.83±0.2	1.21±0.03	1.10±0.03
Nicotine	5.77±0.4***	5.34±0.3***	3.73±0.08**	3.56±0.08**
MO10	2.24±0.2*	2.16±0.2*	1.16±0.03*	1.21±0.03*
MO50	2.17±0.3*	2.12±0.3*	1.11±0.03*	1.13±0.02*
MO100	1.86±0.2*	1.81±0.2*	0.92±0.03*	0.91±0.03*
MO10+Nicotine	5.81±0.5	5.13±0.4	3.13±0.06	3.21±0.06
MO50+Nicotine	4.19±0.5	4.10±0.4	2.87±0.07	2.72±0.05
MO100+Nicotine	3.62±0.4**	3.24±0.3**	2.12±0.04**	2.23±0.05**

Data are presented as mean ± SEM. Values marked with an asterisk (*) are significantly different ($P < 0.05$), (**) are significantly different ($P < 0.01$) while (***) are significantly different ($P < 0.001$).

$P < 0.05$) and medium dose (50 mg/kg, $P < 0.01$) of MO, Table 1. However, treatment with medium (50 mg/kg) of MO suppressed the negative effect of nicotine on levels of peroxidase in rat embryos but have no effect on pregnant rat. Moreover, using MO with high dose (100 mg/kg) plus nicotine (1.5 mg/kg) increased the level of peroxidase activity in brain tissues of rats and their embryos ($P < 0.01$), while produced a clear significant increase on the level of cholinesterase activity in brain tissues of embryos only ($P < 0.05$, Table 1).

Effect of MO on Lipids and Protein Oxidation Level: The results indicated that MDA and PC contents were significantly higher in brain tissues of rats and their embryos treated with nicotine (1.5 mg/kg) compared with those of control group ($P < 0.001$ and $P < 0.01$, respectively, Table 2). However, the levels of MDA and PC decreased significantly in brain tissues of rats and their embryos treated with all doses of MO compared with those in nicotine treated group ($P < 0.05$, Table 2). The levels of MDA and PC in brain tissues of rats and their embryos treated with all doses of MO reached relatively those in the control group. Treatment of pregnant rats treated with high dose of MO (100 mg/kg) plus nicotine decreased the levels of MDA and PC in brain tissues of rats and their embryos compared with nicotine ($P < 0.01$, Table 2).

DISCUSSION

In the present study, pregnant rat model has been used to evaluate and compare the effects of chronic nicotine exposure on the fetal brain using neurohormones and biochemical markers. In addition, this is the first study to demonstrate the protective role of MO against nicotine induced neurotoxicity in female rats and their embryos.

The current study found that treatment of pregnant rats with nicotine decreased the level of GHRH and increased the level of SRIH in the hypothalamus tissues compared with control pregnant rats, where Morgan *et al*. [26] demonstrated that nicotine caused a 29% reduction of growth hormone, The same result was found in the present study since the growth hormone releasing hormone decreased by 43.8%. Our data also supported by Segerlantz *et al*. [27] since acipimox, which is nicotine

derivative drug, inhibited exogenous GH in GH-deficient patients. In contrast, nicotine induced significant increase of SRIH and several peptides derived from the precursors somatostatin which potentially modulate neurotransmitter release and energy metabolism [28, 29]. We are also found that the increased SRIH in the hypothalamus in female rats treated with nicotine was correlated to the decrease of the GHRH content suggesting direct contact between both peptides.

In the present work, the effect of nicotine on the antioxidant enzymes was investigated using peroxidase, cholinesterase activities and GSH levels in brain tissues. The results revealed that levels of theses antioxidant enzymes were significantly decreased in female and fetal brain tissues compared with control group [30]. Moreover, nicotine increased the lipid (MDA) and protein (PC) oxidation in female and fetal brain tissues compared with control group**.** Consistently**,** Xiao *et al*. [31] found that nicotine treatment significantly decreased the antioxidant enzyme activities (superoxide dismutase activity) and increased MDA levels in the vascular wall of rat offspring. In addition, several studies revealed that nicotine exposure are associated with increased levels of oxidative stress markers, ROS in fetal, neonatal and adult tissues [32, 33]. Xiao *et al*. [31] supported our results since the expression of one of NADPH oxidase family (Nox_2) was increased in the nicotine-treated offspring as compared with the saline control animals. This was linked to an increase in O_2^- production and oxidative damage of the vasculature as indicated by an increase in MDA levels. This finding supports our observation that the increased MDA activity in nicotine-treated offspring could be due to a reduction in antioxidant defense.

In this research, treatment of pregnant rats with MO inhibited the negative effect of nicotine to decrease the GHRH neurohormone, antioxidant enzymes (peroxidase, cholinesterase activities and GSH levels) and increase SRIH neurohormone, lipid (MDA) and protein (PC) oxidation. The highly protective effects of MO against nicotine induced neurotoxicity has been supported by Gupta *et al.* [34] who demonstrated that MO has therapeutic effect against several drugs induced toxicity. The present data also confirmed by Luqman *et al*. [19] who reported that MO increased the antioxidant activity and decrease the lipid oxidation in Swiss albino mice. Therefore, We suggested that the protective effects of MO extract on GSH and MDA concentration may be attributed to the presence of phytoconstituents that scavenge free radicals, activate the antioxidant enzymes and inhibit oxidases [35, 36].

In this study, MO extract decreased the levels of lipid and protein oxidation in nicotine treated rat, Based on our observations, MO significantly maintains the basal levels of GSH and MDA content suggesting that the extract may have a mixture of biomolecules with hydroxyl groups that prevent the abstraction of hydrogen atom from the double bond of lipid bilayer thereby preventing the damage of lipid membrane [19].

In conclusion, the present study demonstrates that nicotine induced stress associated with neurohormonal activation, increases the oxidation status of lipid and protein as well as decreases the antioxidant activity. However, the results showed concentration-dependent hydroxyl radical scavenging ability of MO leaf extract inhibited the neurotoxicty of nicotine treatment. This suggest the MO leaves extract could be used to improve the pharmacological knowledge of MO as medicinal plant, Further research is needed in order to determine the clinical application of MO extract to treat nicotine induced neurotoxicity.

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