



## Chemical Composition, Antioxidant Activity and Preventive Role of Milk By-Products Against Nicotine-Induced Alteration in Sexual Hormones and Organs Pathology in Rats



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### Abstract

This study aimed to evaluate the antioxidant activity of skim milk (SM) and permeate (P) *in vitro* and to evaluate their protective role against nicotine (NT)-induced oxidative stress, disturbances in sex hormones and histological changes in different organs of female rats. Four groups of female rats were treated orally for 4 weeks included the control group, NT-treated group (0.6 mg/kg b.w), NT plus SM-treated group (100 mg/kg b.w) and NT plus P-treated group (200 mg/kg b.w). Blood and tissue samples were collected at the end of the experiment for different analyses. The *in vitro* results showed that SM and P have a diphenyl 1-2 picryl-hydrazil (DPPH) scavenging activity in a dose-dependent and SM was more effective than P. The *in vivo* results showed that NT administration induced a significant reduction in superoxide dismutase (SOD), reduced glutathione (GSH), follicle-stimulating hormone (FSH) and luteinizing hormone (LH) accompanied by significant histological changes in ovary, colon, and urinary bladder. Co-administration of NT plus SM or P could alleviate the histological changes, improved the antioxidants enzymes activity and normalized FSH and LH. The overall results showed that SM was more effective than P. It could be concluded that these milk by-products can be used as a dietary supplement to reduce the risk of tobacco smoking or tobacco chewing.

**Keywords:** Nicotine; skimmed milk; permeate; sex hormones; antioxidant; pathological changes

### 1. Introduction

Nicotine (NT) and its metabolites are the highly addictive alkaloid and the main characterized chemicals in tobacco and tobacco smoke[1]. NT is generally self-administered via chewing tobacco or inhalation of tobacco smoke. It absorbs rapidly by the circulatory system and metabolized by the liver[2] resulting in disturbances in the balance between the circulatory antioxidants and the prooxidants[3]. The average yield of nicotine in a cigarette is 2 mg and it acts as a stimulant in mammals; however, high dose (50-100 mg) is considered harmful to human[4]. The nature addictive of nicotine includes drug-reinforced behavior, psychoactive effects, relapse after abstinence, compulsive use, tolerance and physical dependence[5]. Epidemiologic studies have shown that smoking of cigarette has many harmful effects on the visceral tissues in the females[6]. Necrosis, fibrosis, congestion, follicular and endometrial degeneration were observed

in the brain, kidney, pituitary, ovary and uterus, respectively. Nicotine induces harmful effects on several vital visceral organs and showed several observations typical to those reported in the smokers women[7].

The symptoms of NT poisoning caused by excessive stimulations of cholinergic neuron against an NT acetylcholine receptor which are present in central and autonomic nervous system, and the neuromuscular junction [8]. These common processes may be classified into the impact of tobacco smoking on the protein binding, glucuronidation and cytochrome P450-mediated metabolism[9]. Additionally, tobacco may contain trace metals such as arsenic and cadmium which are known to cause endogenous metallothioneins and theoretically could alter the pharmacokinetic[10]. Alpha 1- acid Glycoprotein (AAG) is the major serum protein in humans[11] which has a direct correlation between its concentration and the frequency of cigarette

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smoking[10]. On the other hand, NT addictive triggers the generation of free radicals, including the hydroxyl radical ( $\bullet\text{OH}$ ), the hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and the superoxide anion radical ( $\text{O}_2^{\bullet-}$ ), thus it crushes the antioxidant defense system and ultimately induces oxidative stress *in vitro* and *in vivo*[12]. Therefore, the antioxidant application may be acts as possible counteractive or preventive agents for the therapeutic of NT toxicity.

Previous reports described the antioxidant activity of different sources of animal and plant proteins such as milk proteins[13]. Free radicals formation induce damage to the organism and causes a variety of potentially fatal diseases[14]. Bioactive peptides derived from proteins such as milk by products, namely skimmed milk and permeate have been shown to have antioxidant activates against free radicals originated from environmental pollutions[15]. Due to its low content of fatty acids, skimmed milk consider safe for those with high cholesterol who need to reduce their cholesterol levels, as well as those who wish to maintain a healthy cholesterol level. Moreover, the antioxidant activity has attributed to some amino acids such as histidine, some hydrophobic and free amino acid[16]. Therefore, milk components and milk by products can play an important role against the health hazards induced by pollutants such as smoking inhibition oxidation and scavenging the free radicals[17]. Additionally, it contains approximately all essential of human nutrition[15]. Permeate represents a by-product of dairy technology resulted from the ultrafiltration of milk and has bioactive peptides of specific protein fragments that have a positive impact on the body function and ultimately influence human health[18]. The current study was designed to evaluate the antioxidant activity of skim milk (SM) and permeate (P) *in vitro* and to examine the prophylactic and antioxidant effects of skimmed milk and permeate against NT toxicity in female albino rats.

## 2. MATERIALS AND METHODS

### Materials and chemicals

Permeate was obtained from Dairy Department, Faculty of Agriculture, Cairo University, Egypt. Skimmed milk and diphenyl 1-2 pieryl- hydrazil (DPPH) were purchased from Sigma Company (St. Louis, MO, USA). Pure Nicotine (PurNic™) was purchased from River Supply Co. (CA, USA). Super oxide dismutase (SOD) and reduced glutathione (GSH) were supplied by Bio Diagnostic Co. (Cairo, Egypt). ELISA kits for follicle stimulating hormone (FSH) and luteinizing hormone (LH) were purchased from IBL International GmbH (Hamburg, Germany). The amino acids content in skimmed milk and permeate are presented in Table (1) as supply by the manufactures.

All chemicals used were of the highest analytical grade available.

### DPPH scavenging activity of skimmed milk and permeate

The antioxidant activity of the skimmed milk and permeate compared to vitamin C was determined *in vitro* by the DPPH radical scavenging activity as described by Kalucka et al[19]. The absorbance was recorded at 517 nm against a blank and the scavenging activity was calculated as follows:

$$\text{Inhibition percentage} = (A \text{ blank} - A \text{ sample}/A \text{ blank}) \times 100$$

Where:

A blank: absorbance of control reaction which contain all reagents without the test compound)

A sample: absorbance of the tested compound.

### In vivo study

#### Experimental Animals

Thirty two adult female albino rats weighting about  $150 \pm 15$  g were obtained from the Animal House Lab., National Organization for Drug Control and Research (NODCAR), Giza, Egypt. Animals were fed a standard rodent diet purchased from Meladco Feed Co., Auber City, Cairo, Egypt. The animals were housed in filter-top polycarbonate cages in a room free from any source of chemical contamination, artificially illuminated (12 h dark/light cycle) thermally controlled ( $25 \pm 1^\circ\text{C}$ ) and humidity ( $50 \pm 5\%$ ) free from any chemical contamination at the Animal House Lab., NODCR, Giza, Egypt. All animals received humane care in compliance with the guidelines of the Animal Care and Use Committee of the National Organization of Drug Control and Research (NODCR) and the National Institutes of Health (NIH publication 86-23 revised 1985).

#### Experimental design

The animals were distributed into four equal groups (8 rats/group) and treated orally for four weeks as follows: the control group, the group treated with nicotine (NT, 0.6 mg/kg b.w) in corn oil; the group treated with NT plus skimmed milk (SM, 100 mg/ kg b.w) and the group treated with NT plus permeate (P, 200 mg/kg b.w). At the end of the experimental period, blood samples were collected through the retro-orbital venous plexus of each animal, under light anesthesia by diethyl ether after the animals being fasting for 12 h. The serum was separated using cooling centrifuge (3000 rpm for 15 min) and was used to estimate the GSH, SOD, FSH and LH according to the manufacturer's instructions. All animals were sacrificed after the collection of blood samples and samples of ovary, colon and urinary bladder were collected, fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at a thickness of 5  $\mu\text{m}$

and stained with H&E for histological examination. The sections were analyzed using an Olympus BX51 microscope equipped with camera according to Lillie and Fulmer[20].

### Statistical analysis

The SPSS.11 program was applied for all statistical analyses. The significance of the differences among treatment groups was determined by One Way-ANOVA followed by Duncan's multiple test. The values were expressed as Mean  $\pm$  SE and the results were considered statistically significant if  $p < 0.05$ .

Table (1) Amino acids content of skimmed milk and permeate as reported by the suppliers

Amino acid	Skimmed milk (mg/ g)	Permeate (mg/ g)
Glycine	19.948	7.370
Glutamic	19.285	7.903
Aspartic	12.400	5.689
Threonine	6.469	3.410
Valine	6.735	2.791
Proline	5.846	2.587
Serine	5.135	3.579
Alanine	4.463	1.978
Tyrosine	2.457	0.857
Arginine	2.345	1.546
Methionine	1.855	1.346
Histidine	1.435	0.636

## RESULTS

The results of total antioxidant properties of skimmed milk and permeate compared to Vit C as determined *in vitro* by DPPH scavenging assay revealed that the scavenging activity was not affected by the concentration of Vit C, however; it was increased by the increase in the concentration of skimmed milk and permeate. Moreover, skimmed milk was more effective in the scavenging activity compared to permeate (Table 2).

In the *in vivo* study, the impact of different treatments on GSH and SOD (Table 3) revealed that NT caused a significant decrease in GSH and SOD by 20.33% and 38.29%, respectively compared to the control group. The animals manipulated with skimmed milk or permeate showed a significant improvement in GSH and SOD compared to the group received NT alone. However, the values of both parameters were still significantly less than the control group.

Table (2): Total antioxidant activity (TAA) of skimmed milk (SM) and permeate (P) compared to Vit C as assayed by DPPH scavenging activity

Concentration (mg/ml)	Scavenging activity %		
	Vit. C	SM	P
0.5	97	5.32	4.16
1	100	11.32	8.01
5	100	30.41	21.87
10	100	37.60	26.95
20	100	41.90	32.77

Table: (3) Effect of skimmed milk (SM) and permeate (P) on GSH and SOD in rats treated with nicotine

Parameters Groups	GSH (mg/dl)	SOD (u/ml)
Control	17.17 $\pm$ 0.64 <sup>a</sup>	58.11 $\pm$ 0.50 <sup>a</sup>
NT	13.68 $\pm$ 0.06 <sup>b</sup>	35.86 $\pm$ 1.12 <sup>b</sup>
NT plus SM	15.48 $\pm$ 0.16 <sup>c</sup>	46.80 $\pm$ 0.78 <sup>a<sup>c</sup></sup>
NT plus P	14.35 $\pm$ 0.12 <sup>c</sup>	47.1 $\pm$ 2.10 <sup>c</sup>

Within each column, means superscript with different letters (a, b, c.....) are significantly different ( $P \leq 0.05$ )

The level of GSH in the groups treated with skimmed milk and permeate was lower than the control group by 9.84 and 16.42%, respectively. However the activity of SOD was lower by 19.45 and 18.94% for the group treated with NT plus skimmed milk and the group treated with NT plus permeate, respectively compared to the control group

The results of sexual hormones (Table 4) revealed that NT administration induced significant decrease in FSH and LH. This decrease reached 13.55% and 54.34% for FSH and LH, respectively. The combined treatment with nicotine and skimmed milk induced a significant improvement in FSH since the reduction was only 7.12% and it normalized LH. Animals received NT plus permeate showed a significant improvement in FSH and the reduction reached 13.28% of the control value. However, this treatment improved LH and the recorded value was 3.4% higher than the control value.

The microscopic examination of the urinary bladder sections of the control group showed transitional epithelial cover with average five cell layer thickness, large elliptical bi-nucleated umbrella cells, loose connective tissue in the lamina propria, the occurrence of adipose tissue in the deep lamina and muscularis propria with longitudinal, circular and outer longitudinal layers of thick muscles (Fig. 1a).

Table: (4) Effect of skimmed milk (SM) and permeate (P) on FSH and LH in rats treated with nicotine

Groups	Parameter	FSH (IU/mL)	LH (mIU/mL)
Control		21.92 ± 2.95 <sup>a</sup>	6.19 ± 1.12 <sup>a</sup>
NT		14.95 ± 1.22 <sup>b</sup>	2.83 ± 0.18 <sup>b</sup>
NT plus SM		20.36 ± 3.43 <sup>a</sup>	6.19 ± 0.81 <sup>a</sup>
NT plus P		19.01 ± 1.81 <sup>c</sup>	6.09 ± 0.66 <sup>a</sup>

Within each column, means superscript with different letters (a, b, c,.....) are significantly different ( $P \leq 0.05$ )

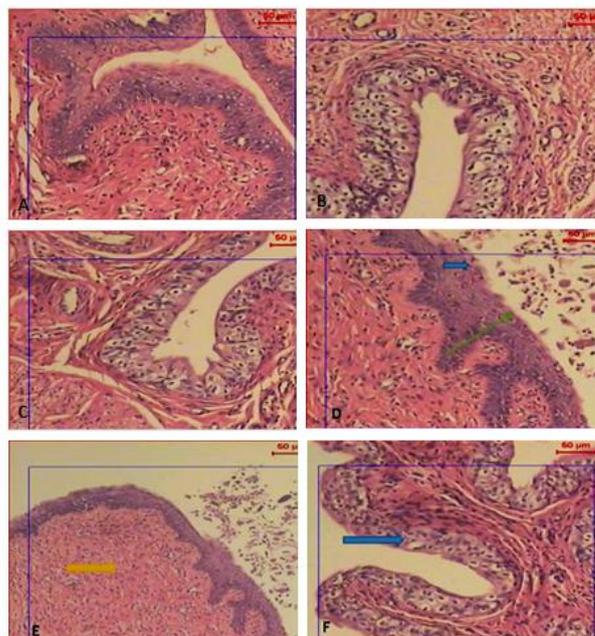
The urinary bladder of animals treated with nicotine showed sluffing of superficial umbrella cells (Fig. 1b), changes as well as decreased overall thickness of the covering epithelium and Edema of the submucosa (Fig. 1c). The examination of urinary bladder of rats treated with nicotine plus skimmed milk showed intact mucosa (Fig. 1d) and mild edema (Fig. 1e); however, the urinary bladder of rats treated with nicotine plus permeate showed intact mucosa and no edema was observed (Fig. 1f). The urinary bladder sections showed that NT administration decreased the mucosal thickness of the urinary bladder. Both skimmed milk and permeate improved the mucosal thickness, although skimmed milk was more effective than permeate (Fig. 2).

The microscopic examination of the colonic sections of the control group showed normal appearance of crypts numerous goblet cells and presence of normally present lymphoid tissue in the submucosa (Fig. 3A). The colonic sections of rats treated with nicotine showed shorting of the crypts, destruction of others and massive inflammatory cell proliferation in lamina propria (Fig. 3B) and forming collections extending to the mucosa (Fig. 3C). The colonic sections of rats treated with nicotine plus skimmed milk showed decreases in the inflammatory reaction, but some crypts were still lost (Fig. 3D). The sections of rats treated with nicotine plus permeate showed that a crypts returns to normal length (Fig. 3E) with a decrease in the inflammation or even absent in most areas (Fig. 3F). NT treatment increased the thickness of colonic crypt abscess; however, the groups received NT plus skimmed milk or permeate showed a significant increase in the thickness of the colonic crypt abscess compared to the control group (Fig. 4).

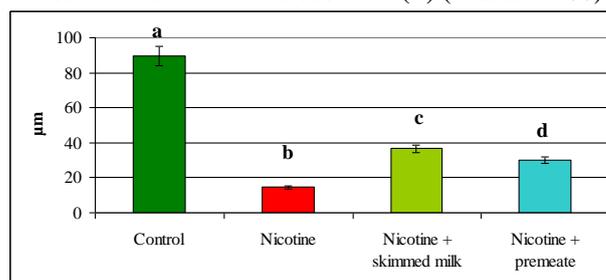
The examination of the ovary of the control group showed normal ovarian tissue with multiple follicles at variable stages of maturation with no stromal reaction (Fig. 5A). The ovary sections of rats treated with NT showed desmoplastic reaction in the stroma and gravian follicles are mostly arrested maturation at early stages and multiple hemorrhagic cysts are also detected (Figs. 5B,C).

The ovary of rats treated with NT plus skimmed milk showed attempts of follicular maturation with

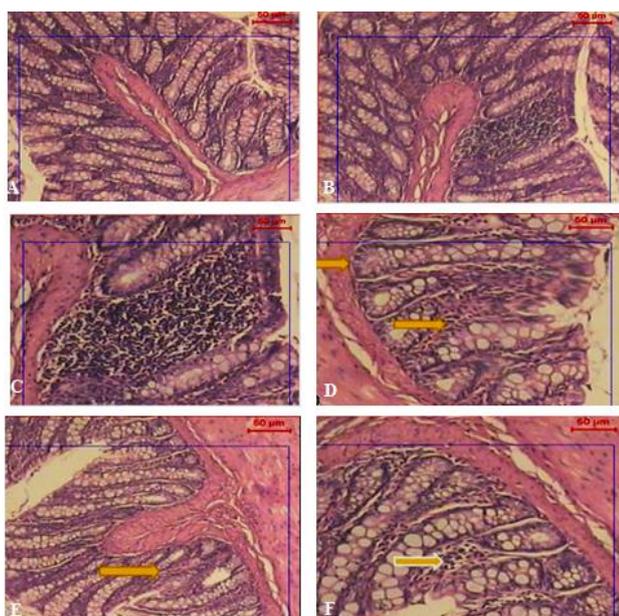
decreased stromal reaction as well as hemorrhagic cysts compared to those treated with NT alone (Figs. 5D,E). However the ovaries of the rats treated with NT plus permeate showed follicles at variable steps of maturation with minimal stromal reaction (Figs. 5F).



**Fig. 1.** Photomicrograph of the urinary bladder of control rats showing transitional epithelial cover with average five cell layer thickness, large elliptical bi-nucleated umbrella cells, loose connective tissue in the lamina propria (A). Rats treated with nicotine showing sluffing of superficial umbrella cells (B) and decreased overall thickness of the covering epithelium edema of the submucosa (C). Rats treated with nicotine plus skimmed milk showing intact mucosa (D) and mild edema (E). Rats treated with nicotine plus permeate showing intact mucosa and no edema was observed (F). (H & E X 100)



**Fig. 2.** Effect of skimmed milk (SM) and permeate (P) on bladder mucosal thickness in rats treated with nicotine, Each column, means superscript with different letters (a, b, c,.....) are significantly different ( $P \leq 0.05$ )

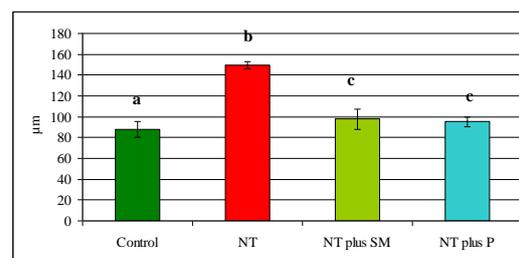


**Fig. 3.** Photomicrographs of colonic sections of: control rats showing normal appearance of crypts and numerous goblet cells and presence of normally present lymphoid tissue in the submucosa (A). Rats treated with NT showing shorting of the crypts, destruction of others with massive inflammatory cell proliferation in lamina propria (B) and forming collections extending to the mucosa (C). Rats treated with NT plus SM showing decreased in the inflammatory reaction but still some crypts were lost (D). Rats treated with NT plus P showing Crypts returns to normal length (E) with a decrease in inflammation or even absent at most of the areas (F). (H & E X 100)

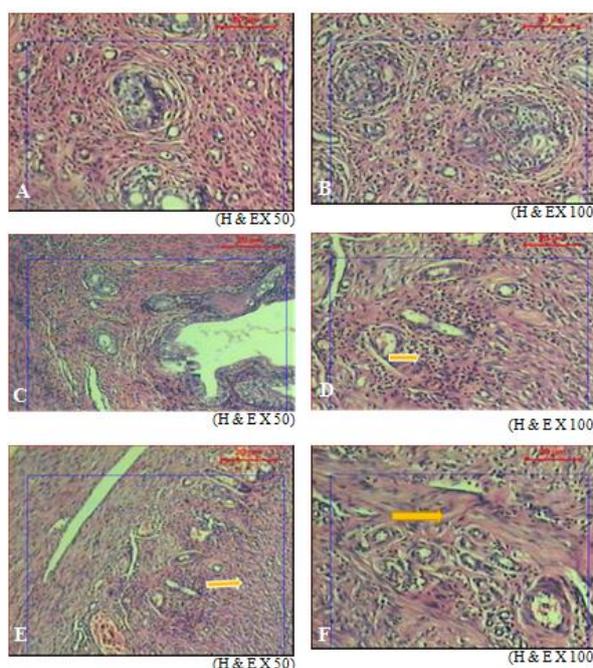
#### DISCUSSION

The *in vitro* results reported herein showed that both skimmed milk and permeate possess antioxidant activity and DPPH radical scavenging properties. These results are similar to those reported previously[21]. The antioxidant and DPPH radical scavenging activity for skimmed milk or permeate are mainly due to the higher content of certain amino acids which are able to act as free radical scavenging and/or hydrogen donor[22]. Additionally, the results also can be suggested by the higher content of the amino acids in skimmed milk compared to permeate such as tryptophan, tyrosine and phosphoserine which showed free radicals quenching[15,16].

In the *in vivo* study, the selected doses of NT, skimmed milk and permeate were literature based[8,24], respectively. It could be seen from the obtained data that milk by products protected against the toxicity of NT-induced disturbances in the antioxidant biomarkers and sexual hormones. These agents alleviated the pathological alterations in the manipulated groups after 4 weeks of treatment.



**Fig. 4.** Effect of skimmed milk (SM) and permeate (P) on colonic crypt abscess in rats treated with nicotine, Each column, means superscript with different letters (a, b, c.....) are significantly different ( $P \leq 0.05$ )



**Fig. 5.** Photomicrographs of the ovary sections of: control rats showing normal ovarian tissue with multiple follicles at variable stages of maturation (A). Rats treated with NT showing multiple atretic follicles with arrested maturation and atretic follicles (B,C). Rats treated with NT plus SM showing that the follicles started to show maturation features (D,E). Rats treated with NT plus P showing that the follicles showed more maturation better than the group treated with nicotine only (F).

Animals treated with NT showed a significant decrease in GSH, SOD, FSH and LH accompanied with significant histological changes in the urinary bladder, colonic and ovary. It was reported that besides the pharmacodynamic properties of NT, it induces immunotoxicity, genotoxicity and reproductive alterations in males and females. It also induces detrimental effects in the incidence of digestive and respiratory cancer as well as the development of cardiovascular disorders[11]. The current results also showed that

NT impairs the endocrine function and reduced the output of pituitary LH and FSH, which impair the function and morphology of reproductive organs[25].

The current study showed that NT decreased GSH and SOD which may be reflected the increase in ROS production through the breaking of the respiratory chain in mitochondria[26]. These results are in agreement with those reported previously and confirmed that NT has an inhibitory action on antioxidant enzymes in serum[27]. NT enhanced the production of ROS and impairs the steroidogenesis at the first phase of the transfer of cholesterol to the mitochondria via the suppressing of the expression of steroidogenic acute regulatory protein[28]. Although the exact mechanism of nicotine induced oxidative stress is not fully understood, it was suggested that one of the key molecular mechanisms is NADPH oxidase-dependent oxidative stress[29]. Furthermore, an increase in the biochemical parameters, tissue injury, decrease in GSH levels and increase MDA levels were reported in human primary endometrial cells and rat bladder and kidney, suggesting that NT induced oxidative stress[30].

The results showed that NT administration decreased the sexual hormones FSH and LH. Similarly, it was reported that nicotine causes dysregulation of reproductive hormonal systems and ovarian functions in healthy women[31] and rats[32]. These results confirmed the hypothesis that smoking alter endocrine functions of the ovary via the disturbances of the pituitary hormones release resulting in a disturbance in the development, function and structure of different organs[25].

The current histological examination was carried out on the ovary, colon and urinary bladder since these organs were reported to be affected from NT exposure in females. The changes in the colonic tissue in NT-treated rats reported in the current study confirmed the previous observations which suggested a connection between smoking and colon cancer in smoker women<sup>[33]</sup> and induce toxicity and pathological changes in the ovary of rats<sup>[34]</sup>. Moreover, the histological changes in the colon and bladder sections were similar to those reported by several investigators who showed that NT stimulates cell proliferation and suppressing physiological apoptosis<sup>[24,35]</sup>. The histological changes in the ovarian tissue reported herein in NT-treated rats were similar to those reported by Poonkodi and Eango<sup>[36]</sup> who showed distortions in the architectural integrity with vacuulations permeating the entire stroma of the ovarian cortex. In addition to the necrosis, congestion, fibrosis, follicular and endometrial degradation observed in the ovary and uterus of rats exposed to NT<sup>[37]</sup>.

It could be seen from the obtained data the prophylactic effect that milk by products reduced the toxicity of NT on the biomarkers of antioxidative parameters and sexual hormones and alleviated the pathological alterations in the manipulated groups. Administration of skimmed milk or permeate enhanced the antioxidant activity in NT-treated rats. The antioxidant properties of milk by-products were suggested in several reports which showed that the fundamental and antioxidative role of amino acids tryptophan, tyrosine in milk by-products have hydroxyl radical scavenging capacities[16] as well as their other nutritive properties[38]. Moreover, whey and its UF permeate could be used as a natural antioxidant[39,40].

Beside the antioxidant effects of peptides and different amino acids in skimmed milk and permeate, it was reported that skim milk is rich in SOD and GPx, the main antioxidant defense mechanism in the cells [41]. SOD catalyzes the superoxide free radicals and protect the cells from their harmful effects; however, GPx promotes the activities of macrophages and neutrophils and reduces the harmful effects of peroxides and protects the cell damage[42]. Previous reports also indicated that permeate is rich in antioxidants such as SOD and CAT, amino acids and peptides, carotenoids and tocopherols, ascorbic acid and phosphates which are able to reduce the peroxidation of lipid and act as free radical scavengers[21]. Taken together, the components of skimmed milk and permeate make them valuable antioxidants and may be useful to protect smokers specially women from the hazards of nicotine.

## CONCLUSION

This study indicates that nicotine disturbs the antioxidant capacity through the generation of free radicals in rats. It disturbs the female sex hormones and induced significant histological changes in the urinary bladder, colon and the ovary. Skimmed milk and permeate showed antioxidant activity as indicated by their DPPH radical scavenging properties *in vitro*. These milk by-products could alleviate the hazards effects of nicotine in female rats due to their higher content of antioxidants including amino acids peptides beside others. Moreover, skimmed milk was more effective than permeate due to its higher content of amino acids compared to permeate. These milk by-products may be suitable candidates as food supplements for smokers especially for women to reduce the risk of tobacco smoking.

### AUTHOR CONTRIBUTIONS

Authors; Mona A. Hassan, Rofanda M. Bakeer and Hagar E. Mohammed carried out the experimental work, managed the literature searches and shared in writing the first draft of the manuscript. Author Mosaad A. Abdel-Wahhab performed the statistical analysis, wrote the protocol, managed the project, managed the analyses of the study and wrote the final draft of the manuscript.

### Conflict of interest statement

The authors declare no conflicts of the interest.

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