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Bioscience Research

Print ISSN: 1811-9506 Online ISSN: 2218-3973

Journal by Innovative Scientific Information & Services Network

RESEARCH ARTICLE BIOSCIENCE RESEARCH, 2021 18(4): 3316-3325. **OPEN ACCESS**

Caffeine modulates the effect of nicotine on CD68-positive macrophage and apoptosis in thymic and ileal lymphoid tissues

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Contradictory data are available on the effect of nicotine and caffeine on the function and structure of the immune system. This study was designed to investigate the effect of the combined administration of nicotine and caffeine on the CD68-positive macrophages in the thymus gland and ilial payer patches of the rat. Twenty four male albino rats are assigned into 4 groups (n=6); the control, nicotine-treated (10 mg/kg/body weight), caffeine-treated (100mg/kg/bodyweight) and combined nicotine- and caffeine-treated groups. After 4 weeks, the thymus gland and ileum were dissected out and processed for immuno histochemical examination using anti-CD68 and anti-caspase-3 antibodies for detection of macrophages and apoptosis, respectively. The number of CD68+ve macrophages as well as the density of CD68 immuno expression significantly decreased in the thymus and ilial payers patches following the single and combined administration of nicotine and caffeine. The number of apoptotic cells significantly increased in the thymus and ilial payers patches of the groups treated with nicotine, caffeine and the combination of them compared to the control. The combined administration of nicotine and caffeine significantly increased CD68+ve macrophages and significantly reduced apoptosis compared to the group received nicotine alone. This ameliorative effect of caffeine might be attributed to its antioxidant and anti-inflammatory potentials.

Keywords: Nicotine, caffeine, macrophages, apoptosis, thymus, immunohistochemistry, ilium.

INTRODUCTION

Nicotine, and caffeine are described to be the most psychoactive drugs consumed worldwide HAGEN TUSHINGHAM (2019). Nicotine, 3-(1-methyl-2-pyrrolidinyl) pyridine, is a psychostimulant agent and is addicted by millions of peoples worldwide (Pesta *et al.* 2013). Nicotiana tabacum is the plant source of this alkaloid (Lina et al. 2014).

Caffeine (1,3, 7-trimethylxanthine), present in the universal drinks included tea and coffee, is consumed worldwide on a daily basis. It is reported to improve the mood and concentration and increase physical performance and enhanced energy (Tauler et al. 2016). Based on several meta-analyses studies, coffee and caffeine are now considered among the healthy antioxidants supplements that were proved to be of value in decreasing the risk of many neurodegenerative, cardiovascular diseases and many types of cancers (Espinosa et al. 2013, Grosso et al. 2017, Postuma et al. 2012, ZHANG et al. 2017).

It is known that most smokers used to drink coffee more frequently compared to nonsmokers (NGUYEN, 2017).

Smoking as well as exposure to nicotine was reported to induce immunosuppression. Chronic administration of nicotine was described to decrease T cell proliferation upon exposure to any foreign antigen (Singh et al. 2000). In addition nicotine was described to have an immunosuppressive effect in experimental rodents as it inhibits splenic-T cell proliferation in response to oncanavalin A as well as antibody-formation by splenic cells in response to antigenic stimulation (KALRA et al. 2004). Upon reviewing the literature, scarce information were available on the effect of nicotine on the resident macrophages in the lymphoid organs. Nicotinic acetylcholine receptor alpha7 subunit (α-7 nAChRs) was reported to be expressed in other non –neuronal cells such as macrophages (Chao et al. 2017).

A contradictory data was reported on the effect of caffeine on the immune system function (Ortiz-Villanueva et al. 2018). In a previous study, caffeine was reported to modulate the function of immune system cells including T, B lymphocytes, macrophages and natural killer (NK) cells (LI et al. 2020).

Epidemiological and experimental studies have shown a positive interaction between caffeine and nicotine consumption. The pattern of interaction whether synergistic or antagonist varies according to species and organ function (GIACOMIN et al. 2008). Moreover, smoking was reported to increase the metabolism of caffeine in humans (Lashein et al. 2016).

This paper was conducted to investigate the effect of combined administration of nicotine and caffeine on the CD68-positive macrophages in the thymus gland and ilial payer patches of rat. We hypothesized that caffeine has a possible role in protection against nicotine-induced impact on the CD68-positive cell population in the thymus gland and payer patches of rat.

MATERIALS AND METHODS

Chemicals

Nicotine hydrogen tartrate and Caffeine anhydrous extra pure (99%) were provided by Sigma Aldrich Inc, USA. Nicotine was dissolved in saline and stored in glass bottle wrapped with foil at 4°C in order to prevent exposure to day light. The caffeine anhydrous was dissolved in saline and stored in glass bottle and filtered before injection.

Study design

The experiment was performed according to animal care ethics recommended by the biomedical research ethical committee, Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia (Reference No 293-19) in January 8, 2019. Twenty four male albino rats (200 to 250 gm), were purchased from experimental animal unit at King Fahd Medical Research Center (KFMRC) and left to acclimatize for one week under the standard laboratory conditions (Temp=22C^0, 12/12 h light/dark cycle) before starting the experiment.

The animals were sorted into 4 groups (n=6). Rats of the control group were injected subcutaneously with 2ml normal saline. Rat of nicotine-treated group were injected subcutaneous with pure nicotine dissolved at a dose of 10 mg/kg/body weight daily for 4 weeks to produce a chronic plasma level of nicotine as was previously described (Leach et al. 2013). Rats of caffeine-treated group were injected intraperitoneal with caffeine anhydrous at a dose of 100 mg/kg/bodyweight daily for 4 weeks. The choice of this dose was dependent on the protective effect reported in a study (Kagami et al. 2008).

Rats of nicotine- and caffeine-treated group were injected nicotine and caffeine at the same dose and route as in the previous two groups for 4 weeks.

Histopathological techniques

At the end of experiment, animals were anaesthetized by deep ether before cervical dislocation. The chest and abdomen were opened and the thymus gland and ileum were rapidly dissected out and fixed in formalin (10% in phosphate buffer) then processed into paraffin blocks. The latters were sectioned at 5 micron thickness (Suvarna et al. 2018). The paraffin sections were stained immuno histo-chemically using an avidin–biotin-complex (ABC) technique. The primary mouse anti-CD68 monoclonal antibody (Dako A/S DK-2600Glostrup, Denmark) and anti-caspase-3 mouse monoclonal antibody (Dako Company, Cairo, Egypt) were used for detection of macrophages and apoptosis, respectively

The slides were fixed, deparaffinized and hydrated, treated with 0.5% hydrogen peroxide in methanol for 10 min to block endogenous peroxidases, and washed in tap water. Sections were incubated with 10 mM citrate buffer, pH 6.0, and heated in a microwave oven to 95 _C during two cycles of approximately 5 min each for

antigen recovery. After cooling, sections were washed in phosphate-buffered saline (PBS) (PH 7.6) for 5 min. and placed in diluted, normal saline. Sections were incubated with

the primary antibodies (diluted in bovine serum albumin 1:50) for 1 h. at 25 _C. Samples were treated with the Dako EnVision+ System for 30 min, followed by incubation with 1 mg/ml DAB solution (3,3tetrahydrochloride diaminobenzidine, Sigma, USA) in PBS pH 7.4 and 1% hydrogen peroxide (Merck) for 5 min.

The primary antibody was omitted during staining of some slides to be used as a negative control. The nuclei were counterstained with hematoxylin. Brown cytoplasmic staining was considered positive reaction.

Photographing of stained slides was done by using Olympus Microscope BX-51 provided with a digital camera. Pro Plus image analysis software version 6.0 was used for assessment of number of CD68-positive and Caspase-3 positive cells in 20 fields from 3 slides of each animals as was previously described (Zhan *et al.* 2006). In addition, semi-quantitative assessment of integrated optical densities (IOD) of CD68- and Caspase-3 immunoexpression was determined in 20 fields of germinal center of payer's patches and thymic lobules.

Assessment of caspase-3 gene expression using real-time polymerase chain reaction (RT-PCR)

The RT-PCR assay was performed on Real Time PCR system (Applied Bio-systems, 7500, USA) and 2X SYBR Green PCR Master Mix (Applied Bio-systems, USA). Paraffin processed samples underwent the process of RNA extraction as was previously described (PATEL *et al.* 2016, PIKOR *et al.* 2011). Extraction of total RNA using Trizol was performed according to the supplier instruction (Invitrogen Life Technologies, Carlsbad, CA, USA). Reverse transcription was performed using oligo-dT primers (Bioneer Inc., Daejeon, Republic of Korea) in a 20-ll reaction including 5 ll RNA. The resulted cDNAs were amplified using PCR Master Mix with primers (PIKOR *et al.* 2011). Caspase-3 (forward 5′-*TGTATGCTTACTCTACCGCACCCG*-3′, reverse 5′-*GCGCAAAGTGACTGGATGAACC*-3′), β- actin, forward: 5′-CCC ATC TAT GAG GGT TAC GC and reverse: 5′-TTT AAT GTC ACG CAC GAT TTC-3′ were used. The results analysis was done using the Light Cycler 480 software.

Optimization of the primer concentration was performed to determine the primer concentration that gives the lowest threshold cycle (CT). This optimization was performed by the creation of a matrix of reactions to test a range of concentrations for each primer against different concentrations of the partner primer. A Nano Drop 2000 Spectrophotometer (Thermo Fisher Scientific, USA) was used to ensure the purity of the extracted RNA and minimize the nonspecific amplification in real-time RT-PCR reactions. The relative levels of mRNA were analyzed using the ΔΔCt method. The CT values were figured out after that relative quantification using the ΔCT method with reference gene.

Statistical analyses

The Statistical Package for the Social Sciences (SPSS) version 16 was used to perform statistical analysis of data obtained in this study. The normality of the data was checked by Kolmogorov–Smirnov test and it revealed that the data was parametric. The parametric data were compared using analysis of variance, followed by Bonforoni post hoc test to avoid a multiple-comparison effect. The sample size was determined in advance before starting the experiment using power analysis. p value < 0.05 is considered significant.

RESULTS

Effect of nicotine and caffeine on CD68+ve macrophages in Peyer's patches of the Ileum:

Control rats showed average number of CD68+ve cells in Peyer's patches, which was observed to be markedly reduced in nicotine-treated rats and moderately reduced in both caffeine- and combined caffeine and nicotine-treated rats. Statistical analysis of the number of the CD68+ve cells and IOD on CD68 immuno expression in the payers patches of the studied groups was performed using one way ANOVA test followed by Bonforoni post hoc test. The number of CD68+ve cells showed significant decrease in nicotine- (23±6, p=0.002), caffeine- (27±7, p=0.003) and combined nicotine and caffeine (34±10, p=0.02) treated groups compared to the control (58±19). The number of CD68+ve cells was significantly higher (p=0.04) in combined nicotine and caffeine treated group compared to nicotine-treated group. Not only that, the IOD of the CD68 immuno expression was significantly decreased in Peyer's patches of nicotine-, caffeine-, combined nicotine and caffeine groups (p=0.01, p=0.02, p=0.04) compared to control, respectively Fig. (1).

Effect of nicotine and caffeine on apoptosis in Peyer's patches of the Ileum:

Ileal Peyer's patches of control rats showed few Caspase-3+ve apoptotic cells, which were obviously increased in nicotine- and caffeine-treated rats whereas they were moderately increased in the combined nicotine and caffeine-treated group. Statistical analysis showed that the number of Caspase-3+ve cells significantly increased in nicotine-treated (48±14, p<0.001), caffeine-treated (32±12, p=0.001) and combined nicotine and caffeine-treated group (24±8, p=0.003) compared to the control (10±3). The number of Caspase-3+ve cells was significantly lower (p=0.01) in combined nicotine and caffeine-treated rats compared to nicotine-treated rats. The results of IOD of Caspase-3+ve cells were consistent to that of the apoptotic cell number Fig (2)

Effect of nicotine and caffeine on CD68+ve macrophages in the thymus:

Thymus gland of control rats showed some CD68+ve cells resident specifically in the medulla and interlobular septa. The number of thymic CD68+ve cells was significantly reduced in nicotine-treated (7±2, p=0.001), caffeine-treated (6±2, p<0.001) and combined nicotine and caffeine-treated groups (15±4, p=0.03) compared to the control (20±6). Combined administration of nicotine and caffeine resulted in a significant (p=0.001) increase CD68+ve cells in the thymus compared to the nicotine-treated group. Statistical analysis of the IOD confirmed that CD68 immuno expression was reduced significantly (p<0.001, p<0.001, p=0.04) in nicotine-, caffeine- and combined nicotine and caffeine-treated groups compared to the control, respectively Fig. (3).

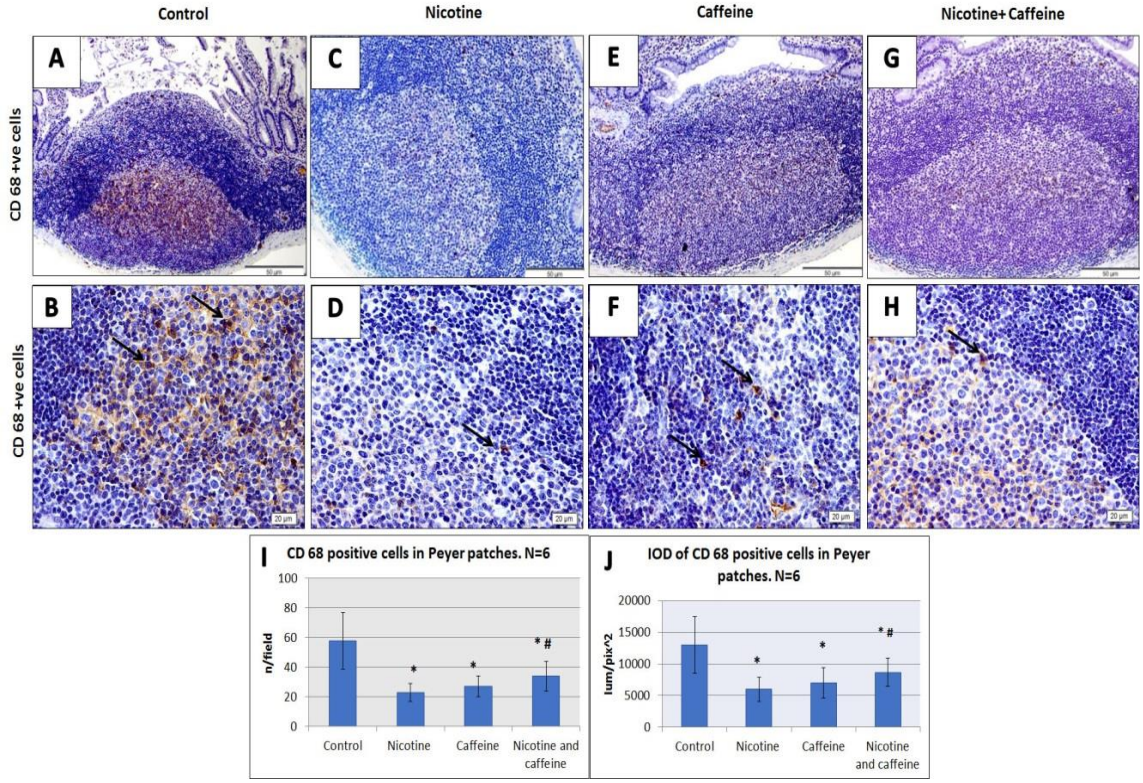

Figure1 :Sections of Peyer patches of rat showing large number of CD68+ve cells (arrow) **in the control group (A, B) that appears to be markedly reduced in nicotine-treated group (C, D) and moderately reduced in caffeine-treated group (E, F) as well as in the group treated with the combination of nicotine and caffeine (G, H) (Immunohistochemical staining with anti CD68, A, C, E, G x100, B, D, F, H x 400). The number (I) and the IOD (J) of the CD positive cells in payers patches of the studied groups are compared using one way ANOVA test followed by Bonforoni posthoc test. Results are expressed in the form of mean ±SD. IOD: integrated optical density. * significance versus control group, # significance versus Nicotine.**

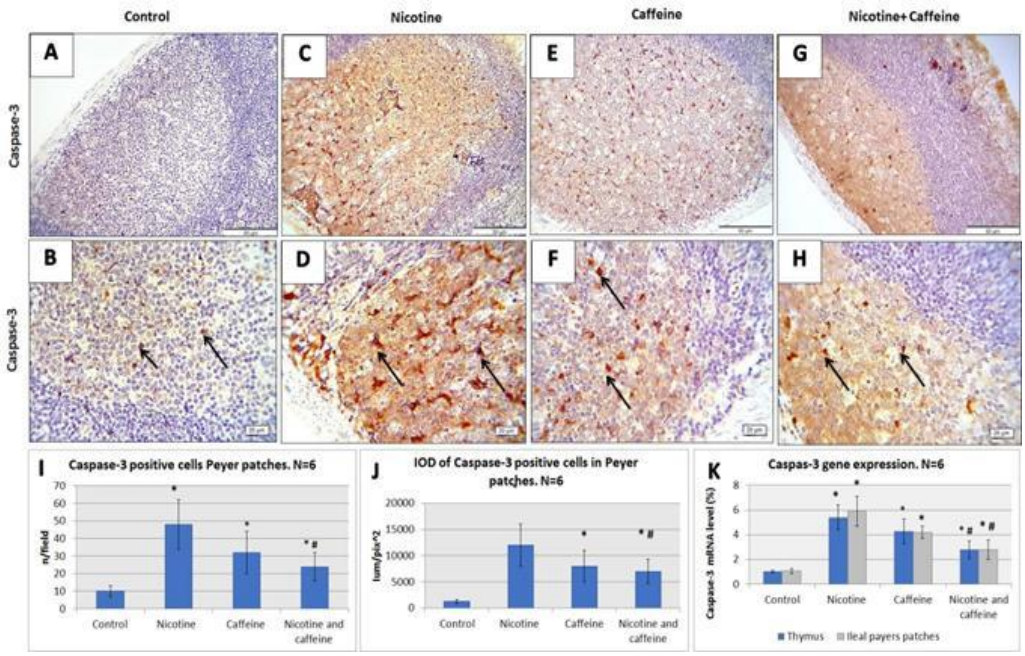

Figure 2: Sections of Payers patches of rat ileum showing few caspase-3+ve cells (arrow) in the control group (A, B) that appear to be obviously increased in nicotine-treated group (C, D) and caffeine-treated group (E, F) whereas they are moderately increased in the group treated with the combination of nicotine and caffeine (G, H) (Immunohistochemical staining with anti caspase-3, A, C, E, G x100, B, D, F, H x 400). The number (I) and the IOD (J) of the Caspase-3 positive cells in payers patches of the studied groups. Caspase-3 (K) gene expression assessed using RT- PCR. Studied groups are compared using one way ANOVA test followed by Bonforoni posthoc test. Results are expressed in the form of mean ±SD. IOD: integrated optical density. * significance versus control group, # significance versus Nicotine.

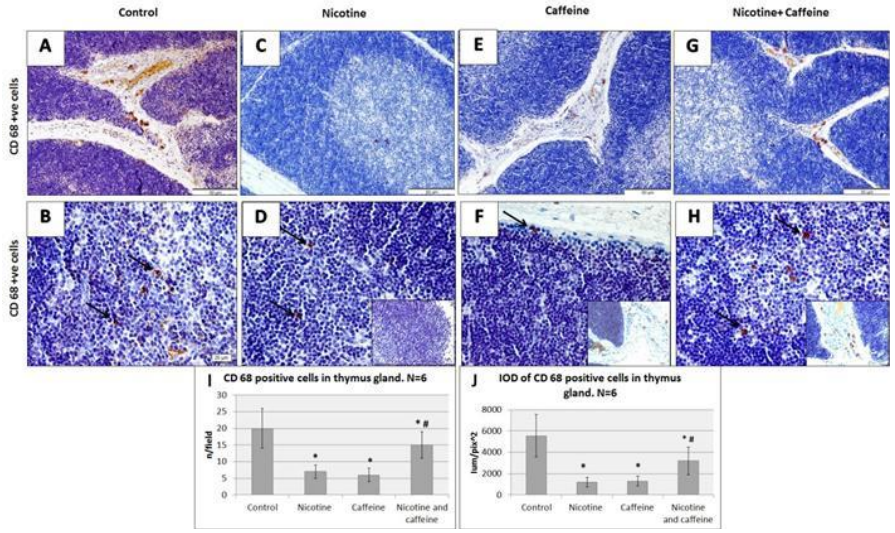

Figure 3: Sections of the thymus gland of rat showing CD68+ve cells (arrow) in the control group (A, B) that appear to be markedly reduced in nicotine-treated group (C, D) and caffeine-treated group (E, F) whereas they were moderately reduced in the group treated with the combination of nicotine and caffeine (G, H) (Immunohistochemical staining with anti CD68, A, C, E, G x100, B, D, F, H x 400). The number (I) and the IOD (J) of the CD positive cells in the thymus gland of the studied groups are compared using one way ANOVA test followed by Bonforoni posthoc test.

Results are expressed in the form of mean ±SD. IOD: integrated optical density. * significance versus control group, # significance versus Nicotine.

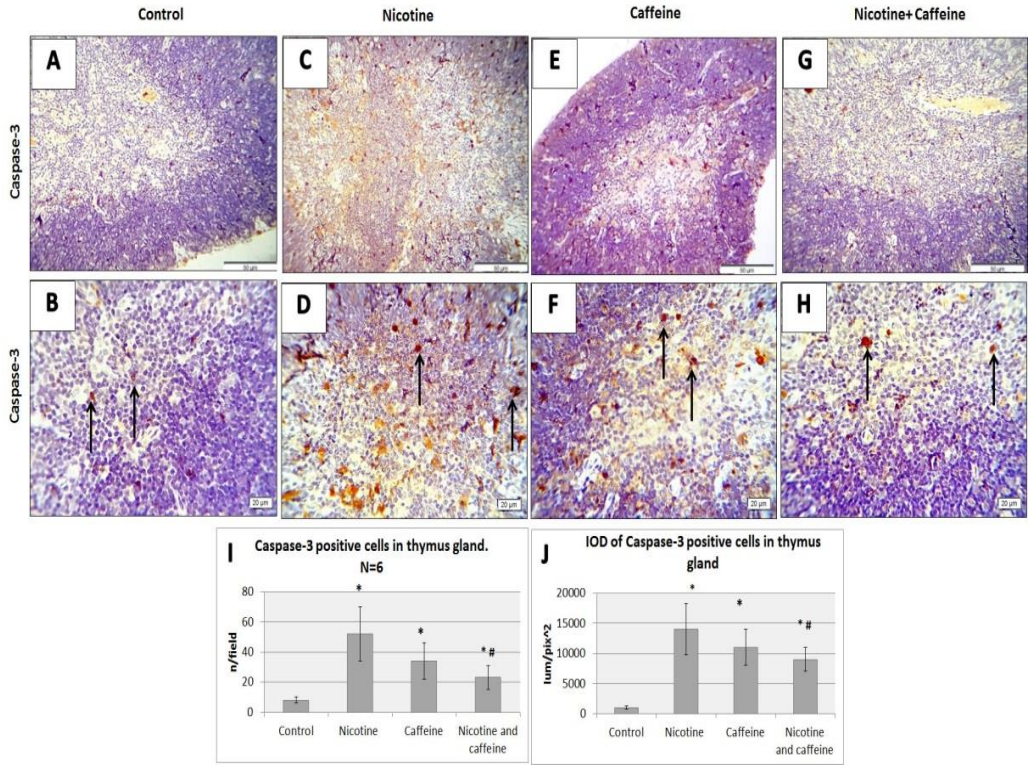

Figure 4:Sections of the thymus gland of rat showing few caspase-3+ve cells (arrow) in the control group (A, B) that appear to be notably increased in nicotine-treated group (C, D) and caffeine-treated group (E, F) whereas they are moderately increased in the group treated with the combination of nicotine and caffeine (G, H) (Immunohistochemical staining with anti caspase-3, A, C, E, G x100, B, D, F, H x 400). The number (I) and the IOD (J) of the Caspase-3 positive cells in thymus gland of the studied groups are compared using one way ANOVA test followed by Bonforoni posthoc test. Results are expressed in the form of mean±SD. IOD: integrated optical density. * significance versus control group, # significance versus Nicotine.

Effect of nicotine and caffeine on apoptosis in thymus:

Thymus gland of the control rats showed few Caspase-3+ve cells in both cortex and medulla and these cells were notably increased in nicotine-, caffeine-treated groups and moderately increased in combined nicotine and caffeine-treated groups compared to the control. The number of the Caspase-3+ve cells in the thymus were significantly increased in nicotine-treated (52±18, p<0.001), caffeine-treated (34±12, p<0.001) and combined nicotine and caffeine-treated groups (32±8, p=0.001) compared to the control (8±2). On the other hand, the number of Caspase-3+ve cells was significantly lower (p=0.01) in combined nicotine and caffeine-treated groups compared to nicotine-treated group. The statistical results of the IOD Caspase-3 immunoexpression showed significantly increase (p<0.001) in nicotine-, caffeine- and combined nicotine and caffeine-treated groups compared to the control Fig (4).

Effect of nicotine and caffeine on gene expression of caspase-3 in ileal payers' patches and thymus:

Caspas-3 gene expression level was assessed using RT PCR in thymus gland and ileal payers patches. It was observed that the level of Caspas-3 gene expression showed a significant up-regulation in nicotine, caffeine-treated and combined nicotine and caffeine administration in thymus (p<0.001, p<0.001, p=0.004) and ileal payers patches (p<0.001, p<0.001, p=0.007), respectively. On the other hand, Caspase-3+ve gene expression was significantly down-regulated (p=0.04) in thymus and ileal payers patches of the combined nicotine and caffeine-treated groups compared to nicotine-treated group (Figure 2 k).

DISCUSSION

Cigarette smoking, with its high nicotine content, was reported to have adverse impact on immune system and defense mechanism of both experimental animals and human upon continuous exposure(lindson et al. 2019, Reyes-Caballero et al. 2020, stämpfli & Anderson, 2009). Although the effect of nicotine on the monocytes function was previous reported, its effect on the amount and distribution of monocytesm in different body tissues in general and in the immune tissues in specific, was not studied yet. Nicotine was reported to block the expression of adhesion molecules (Icam-1, B7.2 and CD 40) in the monocytes following induction by IL-18 (Takahashi et al. 2006).

In this study, a significant reduction in the immunoexpression of CD68+ve cells in both thymus gland and ilieal Peyer's patches were observed in rats receiving nicotine (Bai et al. 2017). This finding was supported by previous studies on the effect of nicotine on the immune system in general. The in vivo immunosuppressive effect of chronic nicotine administration was confirmed and was thought to be mediated through specific nicotine receptors or IL-1β in the brain (Singh et al. 2000), (Razani-Boroujerdi et al. 2011). A similar effect of nicotine was reported on another set of immune cells. It was described there were nicotine-induced changes in lymphocyte populations (e.g., altered subset distribution or decreased T-cell numbers) in nicotine patch-treated animals (Kalra et al. 2004).

These results were contradicted with what was recently reported by Alotaibi and Abounasef during their study on the effect of nicotine on the muscles. They found that skeletal muscles of rats injected with nicotine showed an apparent increase in CD68-positive cells (ALOTAIBI et al. 2019). They added that combined administration of nicotine and caffeine further increased CD68-positive cells (Alotaibi et al. 2019). This could be attributed to the skewing of macrophage activation induced by nicotine exposure during smoking as it was described that smoke may induce alteration in specific functions of either M1 with subsequent direct implications on the immune system and its response to disease (Stämpfli & Anderson, 2009).

In this study, it was observed that administration of nicotine result in a significant increase in the number of Caspase-3+ve cells in both thymus and ileal Payers patches indicating the occurrence of apoptosis in these two tissues. These finding was observed immunohistochemically and were confirmed on the molecular level using RT PCR through assessing gene expression of Caspase-3. These findings were supported by those reported by some researchers. Reduced number of cells were observed in fetal thymus following prenatal nicotine exposure along with increased apoptosis of fetal thymocyte (Chen et al. 2016) and excessive autophagy in fetal thymocytes (QU et al. 2019). The mechanism of such effect was proposed to occur through up-regulation of Fas apoptotic pathway and mediated by increased α7 nicotinic acetylcholine receptor (nAChR) expression (Liu et al. 2017). Increased apoptosis that was associated with reduced number of CD68+ve macrophages, observed in this study after administration of nicotine and caffeine, might indicate that macrophages could be the cell type that underwent apoptosis. Further investigation to confirm this explanation is required.

Contradictory data was reported on the effect of caffeine on the immune system function, however most pointed to suppression effect on immune cell function. Caffeine administration, in this study, resulted in a significant decrease of CD68+ve cells as well as a significant increase in Caspase-3 positive apoptotic cells. These finding were supported by some previous studies conducted on other immune cells. It was reported that caffeine could indirectly reduce the function of NK cell via stimulation of epinephrine release (Graham et al. 1994). Decreased cytotoxicity of lymphocytes by caffeine was attributed by Ritter et al. to its effect on suppressing cytokine expression(Ritter et al. 2005). On the other hand, Dulson and Bishop reported that caffeine has no effect on antigen-stimulated lymphocyte activation (Dulson & Bishop, 2016).

When it came to the effect on macrophages, Jafari and Rabbani had reported that caffeine has a dose-dependent effect on alveolar macrophages whereas low concentrations of caffeine prevent apoptosis compared to moderate doses that induce apoptosis of such cells (Jafari & Rabbani, 2000). He et al. reported that caffeine has dose-dependent effect on macrophage as it induces apoptosis via cleaved caspase-3 (He et al. 2003). This finding explained what was observed in the present work. In a more recent study, it was found that intake of high dose of caffeine, in contrast to low doses, resulted in an immunosuppressive status evident by reduced levels of major anti-inflammatory cytokines, TNF-α, IL-2 and IL-6 as a result of decreased activity of macrophages and

NK cells (AL Reef & Ghanem, 2018).

Although the effect of combined caffeine and nicotine on the macrophages residents in the lymphoid tissues was not previously studied; this effect was reported on other immune cell types in previous studies. In a recent study a significant enhancement of the vitality of neutrophils and their respiratory burst was reported in neutrophils co-cultured with Lipopolysaccharide (LPS)-primed Mesenchymal stem cell (MSC) and treated with a combination of nicotine and caffeine (Abbasi et al. 2018).

The combined administration of nicotine and caffeine, in this study, resulted in a significant increase in CD68+ve macrophages compared to the group treated with nicotine alone. This finding was partially supported by those of Fletcher and Bishop on the NK cells (fletcher & bishop, 2011). They found that caffeine administration either at low or high dose result in an activation of NK cells as well as stimulated NK cell after high-intensity exercise. They attributed this effect to caffeine-induced mobilization of a specific population of activated NK cells due to epinephrine release (Fletcher & Bishop, 2011). Alotaibi and Abounasef also reported an increase in the CD68-positive cells detected immuno histochemically in skeletal muscles of rats after combined nicotine and caffeine injection (Alotaibi et al. 2019). This improvement might be attributed to the antioxidant and anti-inflammatory effect of caffeine. In many studies, the release of anti-inflammatory interleukin 10 (IL-10) is potentiated by caffeine (Tauler *et al.* 2016). Stefanello and Spanevello recently confirmed that caffeine intake has been associated with antioxidant properties and leads to an increase in glutathione levels in rats (Stefanello et al. 2019).

CONCLUSION

Rats received nicotine, in this study, showed a significant reduction in the CD68+ve macrophages as well as a significant increase in apoptosis in both thymus gland and ileal Payer's patches. Caffeine administration produced a similar effect on CD68+ve macrophages but at a lesser extent compared to the nicotine-treated group. The combined administration of nicotine and caffeine resulted in a significant increase in CD68+ve macrophages and a significant reduction in apoptosis compared to those of rats received nicotine alone. This ameliorative effect of caffeine might be attributed to its antioxidant and anti-inflammatory potentials.

Among the limitation of this study, which will be tackled in a future study, was the inability to explore the mechanism behind the impact of the combination of the nicotine and caffeine of the number and distribution of CD68 macrophages of the lymphoid tissue.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

ACKNOWLEGEMENT

This project was supported by the Deanship of Scientific Research (DSR), King Abdulaziz University, Jeddah, Saudi Arabia, under grant No. (D1437-198-130). The authors, therefore, gratefully acknowledge DSR technical and financial support.

AUTHOR CONTRIBUTIONS

KAF and SSA designed and performleed the experiments and also wrote the manuscript. They collected the samples and data then analyzed it. They reviewed the manuscript and approved the final version.

REFERENCES

Abbasi, A., Kukia, N. R., Froushani, S. M. A. & HASHEMI, S. M. 2018. Nicotine and caffeine alter the effects of the LPS-primed mesenchymal stem cells on the co-cultured neutrophils. *Life sciences,* 199**,** 41-47**.**

AL Reef, t. & ghanem, E. 2018. Caffeine: Well**-**known as psychotropic substance, but little as immunomodulator. *Immunobiology,* 223**,** 818-825**.**

Alotaibi, F., Abounasef, S. K. & Fikry, H. 2019. Effects of combined nicotine and caffeine on the rat skeletal muscles: A histological and immunohistochemical study. *Journal of Microscopy and Ultrastructure,* 7**,** 147**.**

Ayuob, N. N. 2013. Can vitamin E and selenium alleviate the immunologic impact of

aluminium on pregnant rats' spleens? *Cellular immunology,* 284**,** 104-110**.**

Bai, X., Stitzel, J. A., BAI, A., ZAMBRANO, C **.**A., PHILLIPS, M., MARRACK, P. & CHAN, E. D. 2017. Nicotine impairs macrophage control of Mycobacterium tuberculosis. *American journal of respiratory cell and molecular biology,* 57**,** 324-333**.**

CHAO, R., TONG, Y.-L., LI, J.-C., LU, Z.-Q. & YAO, Y.-M. 2017. The protective effect of alpha 7 nicotinic acetylcholine receptor activation on critical illness and its mechanism. *International journal of biological sciences,* 13**,** 46**.**

Chen, T., YAN, Y.-E., LIU, S., LIU, H.-X., YAN, H.-Y., HOU, L.-F., QU, W. & PING, J. 20 **.16** Increased fetal thymocytes apoptosis contributes to prenatal nicotine exposure-induced Th1/Th2 imbalance in male offspring mice. *Scientific reports,* 6**,** 39013**.**

DULSON, D. K. & BISHOP, N. C. 2016. Effect of a high and low dose of caffeine on human lymphocyte activation in response to antigen stimulation. *Applied Physiology, Nutrition, and Metabolism,* 41**,** 224-227**.**

Espinosa, j., rocha, a., nunes, f., costa, m. S., schein, v., kazlauckas, v., kalinine, e., souza, d. O., cunha, r. A. & porciúncula, L. O. 2013.Caffeine consumption prevents memory impairment, neuronal damage, and adenosine A 2A receptors upregulation in the hippocampus of a rat model of sporadic dementia. *Journal of Alzheimer's Disease,* 34**,** 509-518**.**

Fletcher, D. K. & Bishop, N. C. 2011. Effect of a high and low dose of caffeine on antigen-stimulated activation of human natural killer cells after prolonged cycling. *International journal of sport nutrition and exercise metabolism,* 21**,** 155-165**.**

Giacomin, E., PALMERINI, E., BALLO, P., ZACA, V **,**.BOVA, G. & MONDILLO, S. 2008. Acute effects of caffeine and cigarette smoking on ventricular long-axis function in healthy subjects. *Cardiovascular Ultrasound,* 6**,** 9**.**

Graham, T. E., Rush, J. W. & Soeren, M. H. V. 1994. Caffeine and exercise: metabolism and performance. *Canadian Journal of Applied Physiology,* 19**,** 111-138**.**

Grosso, G., Godos, J., Galvano, F. & GIOVANNUCCI, E. L. 2017. Coffee, caffeine, and health outcomes: an umbrella review. *Annual review of nutrition,* 37**,** 131-156**.**

HAGEN, E. H. & TUSHINGHAM **,**S. 2019. The prehistory of psychoactive drug use. *Cognitive archaeology: Psychology in prehistory. New York: Routledge***.**

HE, Z., MA, W.-Y., HASHIMOTO, T., BODE, A. M., YANG, C. S. & DONG, Z. 2003. Induction of apoptosis by caffeine is mediated by the p53 **,**Bax, and caspase 3 pathways. *Cancer research,* 63**,** 4396-4401**.**

JAFARI, M. & RABBANI, A. 2000. Dose and time dependent effects of caffeine on superoxide release, cell survival and DNA fragmentation of alveolar macrophages from rat lung. *Toxicology,* 149**,** 101**-**108.

Kagami, K., Morita, H., Onda, K., Hirano, T. & OKA, K. 2008. Protective effect of caffeine on streptozotocin-induced beta-cell damage in rats. *Journal of pharmacy and Pharmacology,* 60**,** 1161-1165**.**

Kalra, R., SINGH, S. P., PENA-PHILIPPIDES, J. C., LANGLEY, R. J., RazanI-Boroujerdi, S. & SOPORI, M. L. 2004. Immunosuppressive and anti-inflammatory effects of nicotine administered by patch in an animal model. *Clin. Diagn. Lab. Immunol.,* 11**,** 563-568**.**

KVETNANSKY, R., RUSNAK, M., DRONJAK, S., KRIZANOVA, O **&** .SABBAN, E. 2003. Effect of novel stressors on tyrosine hydroxylase gene expression in the adrenal medulla of repeatedly immobilized rats. *Neurochemical research,* 28**,** 625-630**.**

Lashein, F. E.-D. M., Seleem, A. A. & Ahmed, A. A. 2016. Effect of caffeine and retinoic acid on skeleton of mice embryos. *The Journal of Basic & Applied Zoology,* 75**,** 36-45**.**

Leach, P. T., Cordero, K. A. & Gould, T. J. 2013. The effects of acute nicotine, chronic nicotine, and withdrawal from chronic nicotine on performance of a cued appetitive response. *Behavioral neuroscience,* 127**,** 303**.**

LI, T., GAO, N., CUI, W., ZHAO, L. & PAN, L. 2020. Natural killer cells and their function in Takayasu's arteritis. *Clinical and Experimental Rheumatology***.**

Lina, S., HASHIDA, N. & ELIZA, H. 2014. Role of Habbatus sauda towards the histological features of nicotine treated male rats seminal vesicle and prostate gland**.**

Lindson, N., Chepkin, S. C., YE, W., Fanshawe, T. R., BULLEN, C. & HARTMANN-BOYCE, J. 2019. Different doses, durations and modes of delivery of nicotine replacement therapy for smoking cessation. *Cochrane Database of Systematic Reviews***.**

Liu, H.-X., LIU, S., QU, W., Yan, H.-Y., Wen, X., CHEN, T., HOU, L.-F. & PING, J. 2017. α7

nAChR mediated Fas demethylation contributes to prenatal nicotine exposure-induced programmed thymocyte apoptosis in mice. *Oncotarget,* 8**,** 93741**.**

Nguyen, J. 2017. Prenatal and Neonatal Exposure to Nicotine and Caffeine Results in an Attenuation in the Respiratory Response to CO2 in Mice: A possible link to SIDS**.**

Ortiz-Villanueva, E., Jaumot, J., Martínez, R., NAVARRO-MARTíN, L., PIñA, B. & TAULER, R. 2018. Assessment of endocrine disruptors effects on zebrafish (Danio rerio) embryos by untargeted LC-HRMS metabolomic analysis. *Science of the Total Environment,* 635**,** 156-166.

Patel, P. G., Selvarajah, S., Boursalie, S., HOW, N. E., ejdelman, J., Guerard, K.-P., BARTLETT, J. M., LAPOINTE, J., PARK, P. C. & OKELLO, J. B. 2016. Preparation of formalin-fixed paraffin-embedded tissue cores for both RNA and DNA extraction **.** *JoVE (Journal of Visualized Experiments)***,** e54299**.**

Pesta, D. H., ANGADI, S. S., Burtscher, M. & ROBERTS, C. K. 2013. The effects of caffeine, nicotine, ethanol, and tetrahydrocannabinol on exercise performance. *Nutrition & metabolism,* 10**,** 71**.**

Pikor, L. A **,.**Enfield, K. S., Cameron, H. & LAM, W. L. 2011. DNA extraction from paraffin embedded material for genetic and epigenetic analyses. *JoVE (Journal of Visualized Experiments)***,** e2763**.**

Postuma, R. B., LANG, A. E., Munhoz, R. P., Charland, K., Pelletier, A., MOSCOVICH, M., FILLA, L., Zanatta, D., Romenets, S. R. & ALTMAN, R. 2012. Caffeine for treatment of Parkinson disease: a randomized controlled trial. *Neurology,* 79**,** 651-658**.**

QU, W., ZHAO, W.-H., WEN, X., YAN, H.-Y., LIU, H.-X., HOU, L.-F. & PING, J. 2019 **.**Prenatal nicotine exposure induces thymic hypoplasia in mice offspring from neonatal to adulthood. *Toxicology letters,* 304**,** 30-38**.**

Razani-Boroujerdi, S., Langley, R. J., Singh, S. P., PENA-PHILIPPIDES, J. C., Gundavarapu, S., MISHRA, N. C. & SOPORI, M. L **.**2011. The role of IL-1β in nicotine-induced immunosuppression and neuroimmune communication. *Journal of Neuroimmune Pharmacology,* 6**,** 585-596**.**

Reyes-Caballero, H., PARK, B., Loube, J., Sanchez, I., VInayachandran, V., CHOI, Y., WOO, J., EDWARDS, J., BRINKMAN, M. C. & SUSSAN, T. 2020. Immune modulation by chronic exposure to waterpipe smoke and immediate-early gene regulation in murine lungs. *Tobacco control,* 29**,** s80-s89**.**

Ritter, M., Hohenberger, K., Alter, P., HERZUM, M., TEBBE, J. & Maisch, M. 2005. Caffeine inhibits cytokine expression in lymphocytes. *Cytokine,* 30**,** 177-181**.**

Singh, S. P., Kalra, R., Puttfarcken, P., KOZAK, A., TESFAIGZI, J. & SOPORI, M. L. 2000. Acute and chronic nicotine exposures modulate the immune system through different pathways. *Toxicology and applied pharmacology,* 164**,** 65-72**.**

Stämpfli, M. R. & Anderson, G. P. 2009. How cigarette smoke skews immune responses to promote infection, lung disease and cancer. *Nature Reviews Immunology,* 9**,** 377-384**.**

Stefanello, N., Spanevello, R. M., PassamontI, S., PorciúnculA, L., Bonan, C. D., OLABIYI, A. A., DA ROCHA, J. B. T., ASSMANN, C. E., MORSCH, V. M. & SCHETINGER, M. R. C. 2019. Coffee, caffeine, chlorogenic acid, and the purinergic system. *Food and Chemical Toxicology,* 123**,** 298-313**.**

Suvarna **,**K. S., Layton, C. & Bancroft, J. D. 2018. *Bancroft's theory and practice of histological techniques E-Book*, Elsevier Health Sciences**.**

Takahashi, H. K., IWAGAKI, H., HAMANO, R., YOSHINO, T., TANAKA, N. & NISHIBORI, M. 2006. Effect of nicotine on IL-18-initiated immune response in human monocytes. *Journal of leukocyte biology,* 80**,** 1388-1394**.**

Tauler, P., Martinez, S., Martinez, P., Lozano, L., MORENO, C. & AGUILó, A. 2016. Effects of caffeine supplementation on plasma and blood mononuclear cell interleukin-1 **0**levels after exercise. *International journal of sport nutrition and exercise metabolism,* 26**,** 8-16**.**

Zhan, J., DENG, R., TANG, J., Zhang, B., TANG, Y., WANG, J. K., LI, F., Anderson, V. M., MCNUTT, M. A. & GU, J. 2006. The spleen as a target in severe acute respiratory syndrome. *The FASEB journal,* 20**,** 2321-2328**.**

Zhang, C., JIANG, H., LIU, F. & HE, Y. 2017. Application of near-infrared hyperspectral imaging with variable selection methods to determine and visualize caffeine content of coffee beans. *Food and bioprocess technology,* 10**,** 213-221**.**