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Alveolar gene expression of tight junction protein in nicotine rats treated with zinc and vitamin D

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***Abstract*---**The current study aimed to investigation the role of zinc and vitamin D in modulating alveolar response of nicotine stress as a model of mammals male Wistar rats. Thirty mature males werekept at 23 ± 2 C°, randomly assigned to five equal groups and treatment for 14 days, C = Control drenched vehicle without treatment, G1 = injected with i/p nicotine 1.5 mg/ kg b.w., G2 = administrated orally of zinc 60 mg/ kgb.w., G3 = administrated orally of vitamin D 250 µg/ kg b.w., G4 = administrated orally both of zinc and vitamin D with same doses stressed and nicotine 1.5 mg/ kgb.w.i/p. Rats were anesthetized with ketamine at the end of the treatment period with xylazine and blood samples have been collected from the optical vein for estimation of serum ferritin and transferrin, then animals were sacrificed and lung was removed and weighted.All groups of rats had lung samples extracted rapidly, dipped in DEPC solution, and frozen under liquid nitrogen for determination of tight junction protein (TJP) gene expression by RT-PCR analyses. Nicotine-stressed rats treated with zinc and vitamin D (G4) havesignificant highly increased expression of alveolar tight junction protein when compared with nicotine group (G1). While, significant increase in serum ferritin and transferrin was seen in G1 when compared to those of G4. It could conclusion that zinc and vitamin D had a protective effect antioxidant, immunomodulatory on lung oxidative stress induced by nicotine.

***Keyword*---**Nicotine, zinc, vitamin D, tight junction protein, ferritin and lung.

Introduction

Stress is thought to play a role in the development of human depression (Biala et al. 2018). Unpredictable stresses have been proven to cause behavioral alterations in animals, including changes in locomotor and exploratory behavior, as well as feeding, drinking, and aspiration behavior impairment (Biala et al. 2018; Raeeszadeh and Mortazavi 2021). The pathogenic presence of oxidative stress in the lungs has been reported in several experimental models of nicotine exposure in rats (Kalpana and Menon2004). Disruption of the mitochondrial respiratory chain, increased production of oxygen-free radicals at the microsomal level, especially increased response of polymorphonuclear leukocytes (PMN) to active cytochrome P450 and C5a (Yamazaki et al. 1999) It has been suggested that nicotine is a substance that promotes oxidative stress in the lungs. ROS then react with various biomolecules in the cell, such as lipids, proteins and nucleic acids, causing oxidative damage and ultimately cell death (Shimaa et al. 2020). When nicotine is absorbed into the systemic circulation, it causes lung and liver damage. Nicotine is a highly reactive alkaloid that can combine with adjacent molecules, causing OS (Dhouib et al. 2015). Many studies have recently revealed that nicotine is one of the most harmful substances that cause reactive oxygen species to be released in the alveolar cell (Wiegman et al. 2020). There have been a lot of studies done on the involvement of OS and reactive oxygen species (ROS) in the etiology and/or progression of a variety of disorders (Shimaa et al. 2020).

Vitamin D potential function protective effects of the COVID-19 infections and mortality (Grant, et al.,2020).These include maintaining cell connections and gap junctions, increasing cell development by reducing the stroma of cytokines by interferons and tumor necrosis factors, and regulating defense by suppressing helper T cell 1 and T cell regeneration (Rondanelli et al., 2018; Ali 2020).Vitamin D is involved in three major mechanisms that reduce the risk of respiratory infections and maintaining tight to prevent immune cells from infiltrating the lungs and other respiratory tissue, killing some viruses through antiviral mechanisms, and reducing the synthesis of pro-inflammatory cytokines .It regulates the immune system and prevents the development of pneumonia (Grant et al., 2020). Zinc was an essential trace element that helped to avoid metabolic syndrome, such as atherogenic dyslipidemia, hyperglycemia, insulinemia, and high blood pressure, by inhibiting pro-inflammatory cytokines and protecting cells from oxidative stress damage by ROS neutralization (Olechnowicz et al. 2018). As a structural component of non-mitochondrial enzyme activity, enhances action of zinc is to reduce the formation of OH from H_2O_2 by production sulfhydryl protein groups or inactivating redox-activated transport metals such as iron and copper (Yousef et al. 2002). Therefore, because zinc and vitamin D were used in the treatment of lung disease, the current study was designed toidentify the role of zinc and vitamin D in ameliorating the deleterious effects of nicotine in adult rats**.**

Materials and Method

Nicotine 72290 (-) nicotine (-)- nicotine >97%(GC); KP 243-248° $C10H_{14}N_2$ Mr. 162.24. Switzerland .Vitamin D Switzerland –acino and zinc

Experimental design

Thirty (30) adult male rats were kept at (23±2C°) have been at random divided into five groups equally of experiment and treatment for 14 days were maintained at room temperature Rats. Control group: administered orally and injected) with sterile distilled water, G1: injected with nicotine 1.5mg/kgb.w. I.P.,G2administered 60 mg /kg.b.w. of zinc orally, G3: administered 250 µg /kgb.w vitamin D orally andG4: administered both zinc and vitamin D with same doses orally and injected with nicotine 1.5mg/kg. I.P at 14 days. Rats were anesthetized with ketamine at the end of the treatment period. (kitamine 100 mg/ kg I.P) with xylazine (10mg/kg I.P) (Veilleux-Lemieux et al. 2013), and blood samples have been collected from the optical vein, then animals were sacrificed and lung was removed. Blood samples have been use for serum ferritin and transferrin) assessment. All groups of rats had lung samples extracted rapidly, dipped in DEPC solution, and frozen under liquid nitrogen for determination of tight junction protein (TJP) gene expression by RT-PCR analyses. **Molecular study VI**: RNA Isolation from lung tissues according to the Surzcki method for estimation tight junction protein expression in the alveolar cell (Surzcki 2000).

ELISA kit

Ab137993- Transferrin Rat ELISA kit abcamand rat ferritin catalog Number: MBS564109Rats serum transferrin and ferritin concentration was measured using ELISA technique ferritin and transferrin kit.

Result

Figure 1 explain the tight junction protein gene expression of all experimental groups stressed rats treated with (zinc 60 mg/kgb.w. and vitamin D 250 µg/kgb.w) significant ($P < 05$) increase in fold changes of tight junction protein gene expression of alveolar cells G1 when compared with control group, whereas highly significant ($P<0.05$) gene expression of G3 and G2 in comparison with control and G1. In contrast the effective doses of zinc and vitamin withnicotine 1.5 mg/kgb.w.I.P) recorded highest significantwhen compared with nicotine group G1 and , due to antioxidants effects of zinc and vitamin D on oxidative stressed induced by nicotine 1.5mg/kg.

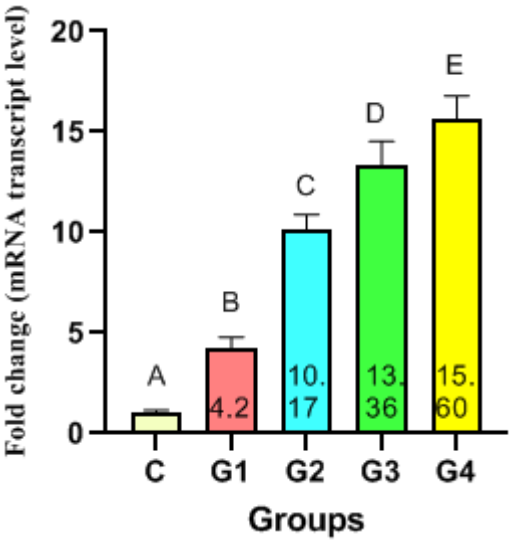

Figure 1 : Effect of nicotine, zinc and vitamin D on lung TJP gene expression in adult male rats after 14 days

C = Control drenched vehicle without treatment for 14 days.
G1 = injected with i/p nicotine 1.5 mg/ kgb.w.I.P.
G2 = administrated with orally of zinc 60 mg/ kgb.w.
G3 = administrated with orally of vitamin D 250 µg/ kgb.w.
G4 = administrated with orally of zinc 60 mg/ kg and vitamin D 250 µg/ kg stressed with nicotine 1.5 mg/ kgb.w.I.P.
Values are expresses as mean ± SD, n=5
Different capital letters mean significantly (p≤ 0.5) different between groups.

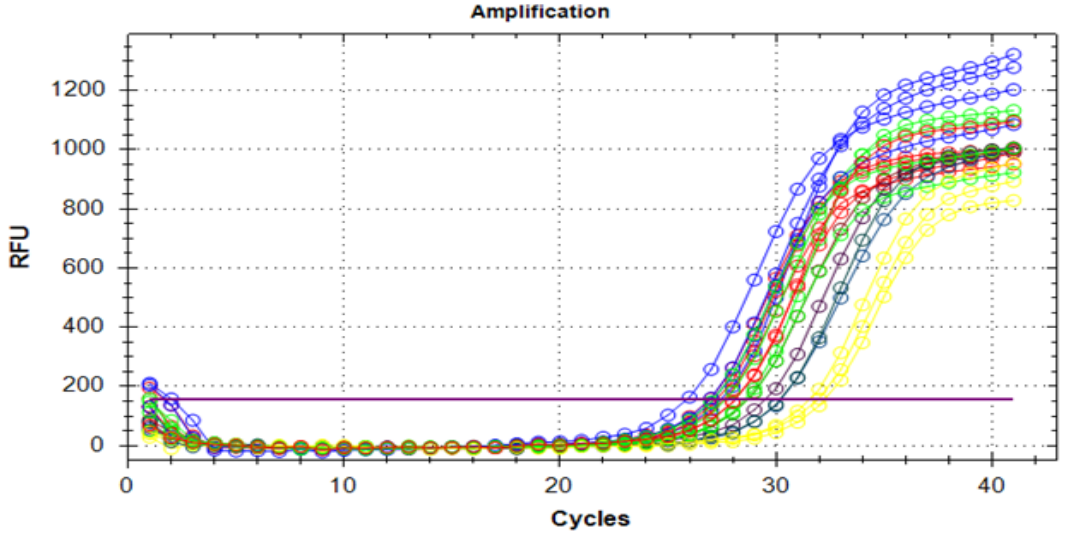

Figure (2): Real Time PCR amplification plots for TJP gene in experimental rats lung samples. Where, the red plots G3, the green plots G2, the black plots G1, the blue plots G4 and the yellow plots C

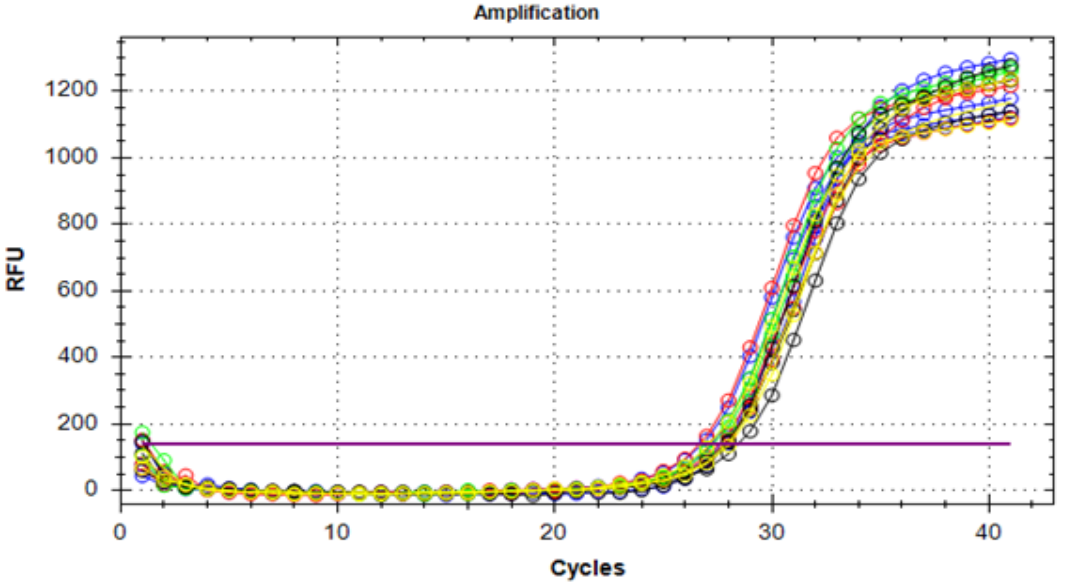

Figure (3): Real Time PCR amplification plots for GAPDH gene in experimental rats lung samples. Where, the red plots G3, the green plots G2, the black plots G1, the blue plots G4 and the yellow plots C.

The findings revealed a decrease in the expression of tight junction protein genes in the lung tissues of nicotine treated rats as compared to zinc groups at 60 mg/kg, vitamin D 250 µg/kg and G4 group (zinc and vitamin D with nicotine) indicating exposure to nicotine, cause alteration of transcriptional program necessary and down-regulating of TJP (which is necessary for the maintenance of healthy lung), associated with up-regulation of oxidative stress-related genes, evidenced by the occurrence of oxidative stress of rats in this group. These results agree with (Shaykhiev et al. 2011). In contrast, Tatsuta et al. (2019) who studied the apical junctional complex (AJC) in the airways of humans smoking cigarettes. Also, cigarette smoking extract caused dysfunction in the epithelial barrier airway associated with downregulation of tight junctions (TJs) and adherens junctions (AJs) proteins. The epithelial barrier function is maintained (as the first-line defense against a wide range of inhaled exogenous substances) by apical junctional complexes that form between neighboring cells, including apical tight junctions (TJs) underlying adherens junctions (AJs)(Tsukita et al. 2019; Roehlen et al. 2020).

Weak expression of ZO-1, occludin, and E-cadherin was observed in bronchial epithelium and lung tissue sections from patients with chronic obstructive pulmonary disease (COPD) compared with healthy individuals(Nishida et al. 2017; Aghapour et al. 2018; Li et al. 2019). Recently, Mo et al. (2022) explained that nicotine treatment-induced pyro-ptosis, (a unique form of inflammatory cell death), of epithelial cells (16HBE cells) mediated by the activation of caspase-1 and the NOD-like receptor protein-3 (NLRP3) inflammasome, was involved with the progression of COPD. A temporary increase in the activity of tight junction protein expression of male rats by zinc and vitamin D were agreement previously (Hawkins et al. 2004 and Brzóska, et al. 2021). In alveolar cells rises of GSSG

concentration and lowering the GSH/GSSG ratio is implicated in numerous antioxidant actions because of down regulation of GPx level (Son et al. 2020; Brzóska, et al. 2021). Nicotine effects on the biological activity of the alveolar cell by incrementing free radical formation and also causing a decrement in biological cell antioxidants. attenuating of antioxidant enzymes such as GSH- reductase and GSH-Px , with depression in gene expression of tight junction protein leading to alveolar cell damage (Mcgilligan et al. 2007; Paul et al. 2020). An oxidative stress was induced by liberation of a high level of H_2O_2 results from the administration of nicotine caused activation of protein kinase c PKC leads to increases in tight junction permeability and reorganization of the cytoskeleton. In our system, suppression of PKC is deleterious to tight junction function (Schuller et al. 2003). This result of oxidative stress triggered by intraperitoneal nicotine leads to high liberation of H_2O_2. Oxidative stress causes inhibition of tight junction protein expression by effects on protein kinase C PKC (Wang et al. 2020; Wu 2020). Furthermore, the role of zinc and vitamin D regulatory pathways on intraperitonial nicotine in male rats via regulation of antioxidant enzymes, particularly glutathione peroxidase and MDA, as well as differential regulation of protein kinase, all of which lead to regulating tight junction protein activity (Fan et al. 2013; Wu 2020).

Intestinal epithelial cells can also be activated by oxidative stress, causing occludin and ZO-1 redistribution and loss of inhibitory activity (Shah et al. 2012; Wang et al. 2012; Sharma et al. 2021). Many reports suggested that PI3K is a negative pathway for the activation of solid bonds in the respiratory epithelium exposed to new tobacco smoke, and lowers albumin permeability. As a result, PI3K may interact with protein tyrosine kinase and .influences tight junction integrity in smoke-exposed epithelial cells (Shah et al. 2012; Wang et al. 2012, 2020). Essentially, the permeability caused by the activity of serine protease in various cell types due to oxidant stimulation of serine proteases circulation of the transduction routes outlined cannot be ruled out (El-Sokkary et al. 2007). Stimulation of PI3K/Akt can lead to increased production of IL-6 through activating the NF-B pathway through loss of regulation of an antioxidant enzyme. Furthermore, this pathway is involved in the enhanced expression of IL-6 as a result of free radicals (Shah et al. 2012).

Vitamin D increased the expression of the G6PD antioxidant pathway gene and oxidized glutathione levels, and may protect lung cells and airways in asthma pathology with anti-inflammatory and antioxidant effects when Vitamin D is exposed to air pollutants (Ali 2020; Giménez et al. 2020). Vitamin D treatment significantly reduced inflammatory cell infiltration in the airways, serum levels of IL6, TNF, and (IL) 1, as well as apoptosis-binding protein Bcl2, caspase-3 expression (CASP3), and GPx and MDA with expression of tight junction protein activity (Giménez et al. 2020).

In comparison to the control group, our findings show that a complex blend of fresh, whole intraperitoneal nicotine stimulates regulatory of tight junction mechanisms in a different pattern in the pulmonary epithelium, while highly significant increment of zinc and vitamin D stressed by nicotine treatment in G4 group when compared with the G1 group. This result of nicotine effects is due to high increased of free radicals because of liberation of H_2O_2 and a high decrease

in antioxidant levels, especially GPx and GSH, with increased lipid peroxidation by a high increment of serum MDA that effects on mechanism pathway cell signaling specifically, this leads to increased permeability to ions and macromolecules by effectivity on local myosin contraction and actin proliferation (Olivera et al. 2007; Schweitzer et al. 2021).

Furthermore, nicotine enhances ZO-1 and/or occludin by activating tyrosine phosphorylation, either by inhibiting GPx activity or boosting MDA activity, causing redistribution of these proteins away from tight junction complexes and greater permeability to macromolecules (Li et al. 2019). The substantial reduction in tight junction integrity exhibited in respiratory epithelium after nicotine infusion has a molecular explanation. The effect of oral administration of zinc group G2 and vitamin D group (G3) or combination of both with intraperitoneal nicotine on serum transferrin was clarified in figure 4 there are significant ($p < 0.05$) decrement in the mean values of serum transferrin level in all treatment group when compared with control group, and decreased in the level of serum transferrin in combination group G4 (Zinc 60 mg/kg, vitamin D 250 µg/kg and nicotine 1.5 mg/kg)when compared with positive control nicotine group**.**

Results of serum ferritin level showed in figures 4 revealed of male rats treated with zinc (60 mg/ kg b.w) and vitamin D (250 µg/ kg b.w)for14 daysshowed significant ($P < 0.05$) decreased level of serum ferritinin all treatment groups (G2, G3 and G4) compared with that of non-treated group (C) and with nicotine group G1. On the other hand, insignificant ($P < 0.05$) among all treatment groups (G2, G3 and G4).

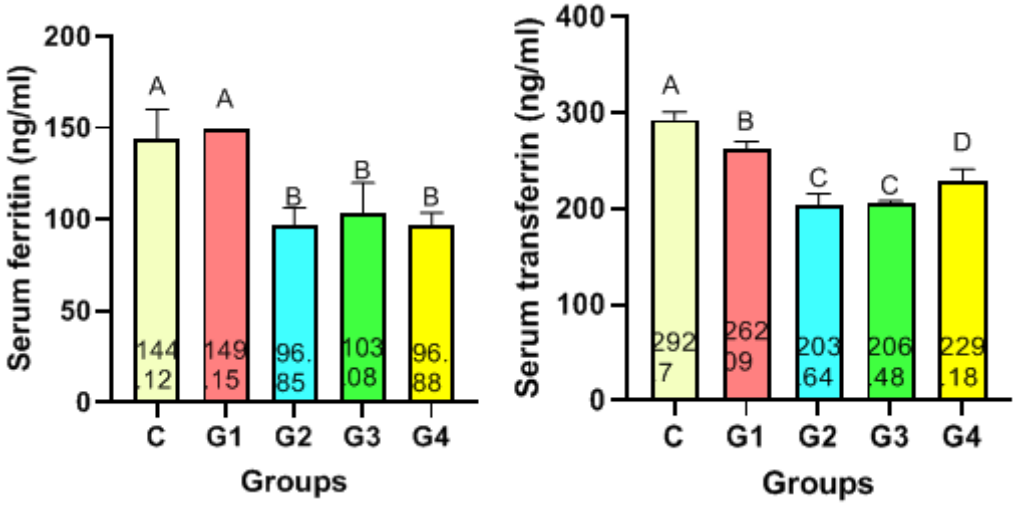

Figure (4): Effect of nicotine, zinc and vitamin D on serum ferritin and transferrin in adult male rats after 14 days

C = Control drenched vehicle without treatment for 14 days.
G1 = injected with i/p nicotine 1.5 mg/ kg b.w.I.P.
G2 = administrated with orally of zinc 60 mg/ kgb.w.
G3 = administrated with orally of vitamin D 250 µg/ kgb.w.

G4 = administrated with orally of zinc 60 mg/ kg and vitamin D 250 µg/ kg stressed with nicotine 1.5 mg/ kgb.w.I.P
Values are expresses as mean ± SD, n=5
Different capital letters mean significantly (p≤ 0.5) different between groups.

The results showed that rats receiving nicotine (G1) revealed a significant decrease in transferrin concentration when compared with control, while a significant increase when compared with all treatment groups (G2, G3 and G4), on the contrary, insignificant increased of serum ferritin in the stressed group (G1) when compared with control, while highly significant when compared with all treatment groups (G2, G3 and G4). Similar, results were obtained by other investigators (Ghio et al. 2008; Zhang et al. 2019). It is believed that pulmonary oxidant/antioxidant balance is considered important for lung function. Pulmonary oxidative stress may adversely affect respiratory health. Based on previous studies which confirmed that nicotine causes oxidative stress in animal models by producing powerful reactive oxygen species (ROS) and nitrogen–oxygen species (Ahmad et al. 2019).

The conversion of nicotine into OH• is responsible for the majority of its harmful effects on tissues, including lipid peroxidation (Mohammadghasemi et al. 2021). DNA, proteins, and enzymes, particularly lipid peroxidation enzymes, are damaged by severe oxidative stress. As a result of the damage, cells die, resulting in a variety of pathological diseases (Hamza and El-shenawy 2017). In the same manner during the respiratory burst, the effect of nicotine on male rats' lung tissue causes them to produce hypochlorous acid (HClO), which reacts with unsaturated fatty acids, proteins, and any oxidizable group, causing protein adducts and genetic mutations, as well as influencing signaling pathways. This is caused by a decrease in serum transferrin, and ferritin levels (Chattopadhyay 2016 andHamza and El-shenawy 2017).

The current study's findings further indicated that nicotine's adverse effect on pulmonary function may be due to decreased serum transferrin, and ferritin concentrations. Other research has confirmed this (Zhang et al. 2019). Ferritin reduces oxidative stress very quickly by catalytic release of O2 or reactive oxygen species (these are made from Fe3 + Fe2 + substrate and release Fe2 + ions or iron-producing enzymes such as catalase. Oxidative stress is associated with high H2O2 imbalances, but the effect of reducing oxidative stress is clear (AL-Okaily and Nowfel, 2015; Bradley et al. 2016; Al-Okialy, 2018). Generally, it is not always been proven whether the upregulated ferritin reduces oxidative stress by catalyzing the removal of O2 or reactive oxygen species as Fe3+ is produced from a Fe2+ substrate, emptying Fe2+ ions, or causing an increase of iron for the production of enzymes like catalase, which directly decreases oxidative stress by massive imbalance of H2O2, but the function in decreasing oxidative stress is evident (Bradley et al. 2016). The main function of ferritin is to reduce the oxidative stress caused by the rapid removal of O_2. Assuming, of course, that the key residues for these two activities are not the same, the key residues associated with this activity may be more resistant to mutation than the correspondent residues of a ferritin whose primary function is to isolate spare iron in a type that can be quickly rallied when the cell requires it (Bresgen and Eckl 2015; Ghio et al. 2008; Zhang et al. 2019). Since macrophages are a major source of serum ferritin

and serum ferritin levels indicate iron stores in the body, serum ferritin is clinically used as a reliable indicator of systemic iron status (Lee, et al. 2016, Fabiano et al. 2018 andHiroshi Kawabata 2018).

It is well known that macrophages perform different biological functions, mainly clearance of pathogens, apoptotic and senescent cells, in this context Recalcati and Cairo, (2021) referred the major targets of homeostatic phagocytosis by macrophages are old/damaged red blood cells, besides, it seem particularly adapted to store amounts of iron that may be toxic to other cells.

In contrast, Kasprowicz et al., (2020) reported that both an excess and a deficiency of vitamin D can be detrimental to the selected health indices; therefore, future studies are needed to explore the association between the various doses of vitamin D3 supplementation and iron metabolism in athletes. Our findings suggest that physiologic doses of chronic oral zinc supplementation inhibit the absorption of iron in the male rats. The adverse effects of zinc on serum iron do not exacerbate anemia, the duration and dose of supplementation should be considered (de Brito et al. 2014; Al-ghareebaw and Al-Okaily 2020).

Zinc and iron can bind due to the chemical similarities between the two trace elements. Therefore, the inhibitory effect of zinc on iron absorption (and vice versa) may be related to zinc antagonistic to iron absorption in the intestinal tract (Rolf et al., 2021). The absorption and transport mechanisms of zinc and iron are chemically similar. The quantitative effect of this relationship is tested by the amount of food concentrate used and the amount of zinc and iron when using aqueous zinc and iron solutions, foods, and in some cases, supplements. They have antagonist effects (Olivares et al., 2012; de Brito et al., 2014). High dosages of zinc in aqueous solutions have been shown to impair iron absorption, whereas zinc added to meals has had no effect (Olivares et al. 2012). This is in contrast to our findings, which showed that supplementation of zinc orally can lower serum iron levels in healthy school children for 90-day zinc supplement (Jayalakshmi and Platel 2016).

As shown in the results, significant improvements in transferrin and ferritin concentrations were observed in rats treated with zinc and vitamin D with nicotine (group G4) as compared to group G1. These results showed that zinc and vitamin D reduced respiratory systemic injury caused by nicotine, as seen by the large rise in serum transferrin profiles and decrease in ferritin and ameliorating the histopathological alterations. Zinc and vitamin D are multifunctional antioxidants and have lineal sweeping effects on ROS and chelate oxidative transmission metals while also replenishing biological antioxidants. Vitamin D also has a number of biochemical roles relating to signal transduction pathways such as insulin, NF-κB, and adenosine monophosphate protein kinase (AMPK) (Masoud et al. 2018 and Pedrosa, et al. 2022). The actions of vitamin D on the lung are essentially beneficial and include Immunomodulatory, anti-inflammatory, anti-infectious and antioxidant action, as well as maintenance of airway structure and epithelial barrier integrity (Gayan-Ramirez and Janssens 2021).

vitamin D down regulates the expression of NF-κB as well as NF-κB phosphorylation in LPS stimulated airway epithelial cells and inhibits TNF-α production, making it a potential anti-inflammatory drug (Lan et al. 2014). Recently, Chen et al., (2022) found that Vit. D supplementation reduced ICAM-1, (is an important regulator of respiratory epithelial cell inflammation), expression, monocyte adhesion, mitochondrial fission, and mitophagy via the AKT and NF-κB pathways in TNF-α-treated mice lung tissues. Thus, Vitamin D considers an effective therapeutic agent for lung inflammation. The elevation of serum transferrin profile concentrations may be referred to the effect of zinc and vitamin D to reduce the negative effect of nicotine toxicity. The findings support the idea that zinc and vitamin D are powerful antioxidants and free-radical scavengers (Chattopadhyay 2016; Skrajnowska and Bobrowska 2019). So, depending on the antioxidant activity of zinc and vitamin D in arsenic-exposed rats, the pulmonary and alveolar oxidative stress was reduced and the male respiratory health was recovered. (Lan et al. 2014; Yousef et al. 2002). Therefore, it could be suggested that the protective effects of zinc and vitamin D on male respiration in nicotine exposed rats may be attributed to the antioxidant properties of zinc and vitamin D on pulmonary function and the immunomodulatory effects of their.

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