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ORIGINAL ARTICLE

Effects of varenicline on lung tissue in the animal model

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ABSTRACT

Objective: This study aimed to investigate acute and chronic effects of varenicline on lung tissue in an experimental study. **Methods:** A total of 34 rats were randomly allocated into study (varenicline) and control groups. The rats were divided into two groups (i) control group, (ii) varenicline group. Then, the rats in the each group were sub-divided equally in turn as acute (C1; V1) and chronic (C2; V2) ; all rats of acute and chronic groups were sacrificed under the anesthesia on the 45th day for acute group [C1 (n=5) and V1 (n=12)] and the 90th day for chronic group [C2 (n=5) and V2 (n=12)], respectively. Thus, biochemical and histopathological analysis were carried out. **Results:** Thirty four rats completed the study, 24 were in varenicline group and 10 were in control group. In chronic exposure to varenicline, oxidant levels comprising of malondialdehyde (MDA), and myeloperoxidase (MPO) increased and superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and glutathione peroxidase (GPx) levels, named as antioxidants, decreased significantly when compared to the control group. MDA and MPO levels were also significantly higher and SOD, CAT, GPx, GSH levels were also significantly lower in chronic varenicline group when compared to acute varenicline group. These findings were also supported by histopathological observations. **Conclusion:** This is the first study, which evaluated pulmonary effects of varenicline experimentally on an animal model. It was observed that chronic varenicline treatments cause inflammation and lung cell injury.

Keywords: Varenicline; Lung tissue; Oxidative stress; Pulmonary toxicity.

INTRODUCTION

Smoking tobacco is the most important and preventable cause of pulmonary diseases such as chronic obstructive pulmonary disease, lung cancer, and interstitial lung diseases. Furthermore, it has been associated with approximately 50% of deaths due to cardiovascular and pulmonary diseases. The most important pathological changes associated with smoking tobacco are inflammation and oxidative stress of the respiratory tract. Metaplastic and dysplastic changes in bronchial epithelium are accompanied by elevated expression of adhesion molecules and secretion of many cytokines and oxidative stress products.[1-3]

Quitting smoking is the most effective method of prophylaxis and treatment of pulmonary diseases. The effects of nicotine are mediated through a variety of mechanisms including actions at various nicotinic receptor subtypes (nAChR) and modulation of the neurotransmitters release such as dopamine, serotonin and glutamate.[2] Varenicline is the most widely used agent for quitting smoking and it is a highly selective partial agonist at the α4β2 nAChR and a full agonist at the α7 nicotinic acetylcholine receptor.[3,4]

In randomised clinical trials varenicline has been shown to increase the chances of successful long-term results in 2–3 times compared to pharmacologically unsupported attempts to quit smoking.[3] Varenicline has been reported to suppress the withdrawal symptoms and feeling of enjoyment derived from smoking.[5] Smokers treated with varenicline reported that cigarette smoking was less satisfying and rewarding than those given a placebo.[6] The most common side effects of varenicline include nausea, insomnia, abnormal dreams, headache, agitation, anxiety, tachycardia, dyspepsia, and constipation.[7]

Although there are lots of studies evaluating the non-smoking related effects of varenicline, but the number of studies evaluating the effects on tissues is extremely low, whether or not it has a negative effect on rats' lung tissue which has never been evaluated. To this end, it was aimed to explore the acute and chronic effects of varenicline using histopathological examinations to assess oxidative stress and apoptosis

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in the lung tissue of rats treated with varenicline. It was also detected biochemical parameters and weighed individual lungs.

METHODS

The current study protocol was approved by the Animal Ethics Committee (Number: 2012/A-61). In the present study, 34 male Wistar Albino rats, 10-12 weeks of age and weighing 250–300 g were supplied from the Inonu University Laboratory Animals Research Center. The rats were kept in a temperature (21±2°C) and in humidity (60±5%) controlled room in which a 12:12 h light:dark cycle was provided during the trial. The rats were randomly divided into varenicline (V: n=24) and control (C: n=10) groups and the rats in each group were sub-divided equally as either acute (C1; V1) or chronic (C2; V2). The rats in the control group were administered distilled water orally. Modified animal-to-human dosing tables were used to adjust the duration and dose scheme of varenicline (Champix 1 mg Tb®, Pfizer Corporation, Istanbul, Turkey) according to the human therapy protocol.[8,9] Accordingly, the dosage of oral varenicline was adjusted as 9 µg/kg daily on days 1–3, 9 µg/kg twice daily on days 4–7, and 18 µg/kg twice daily on days 8-90 (total of 83 days). The rats in the acute group were sacrificed on day 45 [C1 (n=5) and V1 (n=12)] and the rats in the chronic group [C2 (n=5) and V2 (n=12)] were sacrificed on day 90 by using ketamine and xylazine. The lung tissues were stored for biochemical and histological studies.

Spectrophotometric analyses of TBARS ingredient of the homogenates were performed by thiobarbituric acid reaction use.[10] Three millilitres of 1% phosphoric acid and 1 ml of 0.6% thiobarbituric acid solution were added to 0.5 ml of plasma in a tube. The mixture was heated in boiling water for 45 minutes and after cooled, and extracted into 4 ml of n-butanol. The absorbance was measured spectrophotometrically (UV-1601; Shimadzu, Kyoto, Japan) at 532 nm. The amount of lipid peroxides was calculated as per TBARS of lipid peroxidation. A standard graph prepared for evaluation of standard fluids was used and the results were expressed in nanomoles per gram (nmol/g tissue) (1,1,3,3-tetramethoxypropane).

In this study, it was used the method developed by Sun et al.,[11] which involves inhibition of nitroblue tetrazolium (NBT) reduction by the xanthine/xanthine oxidase system as a superoxide ($O2^{\bullet-}$) generator in order to evaluate total SOD (EC 1.15.1.1) activity (Cu-Zn and Mn). The enzyme amount that caused 50% inhibition in the NBT reduction ratio was described as one unit of SOD. SOD activity was expressed as units per gram protein (U/g protein).

It was used the method defined by Paglia and Valentine[12] in order to evaluate GPx activity (EC 1.6.4.2). It was initiated an enzymatic reaction by adding H_2O_2 to a tube, which contained nicotinamide adenine dinucleotide phosphate (NADPH), reduced glutathione (GSH), sodium azide, and glutathione reductase. The change in absorbance at 340 nm was monitored by a spectrophotometer. The results were expressed as U/g protein.

It was used a 4-aminoantipyrine/phenol solution as the substrate for MPO-mediated oxidation by H_2O_2 in order to evaluate the MPO (EC 1.11.1.7) production and recorded the change in absorbance at 510 nm.[13] The amount causing degradation of 1 µmol H_2O_2/min at 25°C was described as one unit of MPO. The results were expressed as U/g protein.

It was used Aebi's method in order to assay catalase activity (CAT, EC 1.11.1.6).[14] This method is based on the determination of the rate constant (k, s−1) or the H_2O_2 decomposition rate at 240 nm. The activity was expressed as k per gram protein (k/g protein).

A previously defined method was used in order to analyse the GSH ingredient in the lung tissue as non-protein sulphydryls.[15] It was mixed aliquots of tissue homogenate with distilled water and 50% trichloroacetic acid in glass tubes and centrifuged at 3000 rpm for 15 min. The supernatants were mixed with Tris buffer (0.4 M, pH 8.9) and added 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB, 0.01 M). It was assayed absorbance of reaction content at 412 nm within 5 min of the addition of DTNB against a blank with no homogenate. It was extrapolated the absorbance levels from a glutathione standard curve and defined as GSH (µmol/g tissue).

At the end of the study, all animals were sacrificed under ketamine anaesthesia. The lung tissues were removed. Tissue samples were placed in 10% formalin and prepared for routine paraffin embedding. Paraffin blocks were cut into slices 5 µm thick, mounted on slides, and stained with hematoxylin-eosin (H-E). It was examined lung sections histopathologically for severity of alterations including haemorrhage, inflammatory cell infiltration, thickened alveolar wall, and congestion. Lung injury was classified semi-quantitatively for each criterion as (0) normal, (1) mild, (2) moderate, (3) or severe, with scores ranging between 0 and 12. Lung sections were observed by using a Leica DFC280 light microscope and a Leica Q Win and Image Analysis system (Leica Micros Imaging Solutions Ltd., Cambridge, UK).

Statistical power analysis was used in order to determine the necessary sample sizes required to detect even minor effects. It was employed the NCSS packet program in order to calculate the necessary of sample sizes for a power of 0.80 was. The analyses were performed by using SPSS software, 22.0 version (SPSS Inc., Chicago, IL). Kolmogorov-Smirnov test was used in order to examine normality of distribution. And the Kruskal-Wallis H test was used in order to examine the data that were not distributed normally. After a significant Kruskal–Wallis H test, a Conover test was also utilized for biochemical and histopathological analyses. A value of $P<0.05$ was accepted as statistically significant. The results were presented as medians (min–max).

RESULTS

No mortality occurred due to varenicline exposure or anaesthesia, none of them caused any animal mortalities. All of the animals survived until the end of the study.

Body weight (BW), lung weight (LW), oxidant/antioxidant parameters (MDA, SOD, CAT, GPx, GSH, and MPO), and lung injury scores are presented in Tables 1, 2, and 3. Acute exposure to varenicline did not lead to a significant decrease in LW and BW in comparison to the control group, whereas, a numeric increase was observed in MDA and MPO levels and a significant decrease was observed in SOD, CAT, GSH, and GPx content. On the other hand, chronic exposure to varenicline significantly increased MDA and MPO and decreased SOD, CAT, GSH and GPx levels in comparison to the control group. In addition, significantly higher levels of MDA and MPO and CAT, SOD, GPx were observed in the lung tissues of the chronic varenicline group and GSH levels were significantly lower than the acute varenicline group.

The histological appearance of the lung tissue was normal in the acute and chronic control groups (Figures 1A and B). The lung sections from V1 and

Table 1. The histopathological damage score of all groups.

Groups	C1 (n=5)	V1 (n=12)	C2 (n=5)	V2 (n=12)
Lung injury score	0 (0-1)	6 (5-7)[a]	0 (0-1)	7 (6-8)[b,c]

Data are expressed median (min-max). [a]p =0.001 vs C1; [b]p =0.001 vs C2; [c]p =0.001 vs V1.

Table 2. Changes of SOD, CAT, GPx activities and GSH, MDA contents in the lung tissue of rats administered by acute and chronic varenicline [Median (min-max)].

		MDA nmol/g tissue	SOD U/g protein	CAT K/g protein	GPx U/mg protein	GSH µmol/g tissue	MPO U/g protein
Acute	Control Group (n=5)	7.37 (6.39-9.12)	46.99 (36.64-50.49)	14.17 (10.82-17.75)	4.28 (2.76-5.66)	0.47 (0.43-0.55)	31.32 (24.69-37.78)
	Varenicline Group (n=12)	7.09 (4.62-15.90)	44.40 (32.15-59.05)	13.34 (18.83-7.73)	3.93 (2.05-6.96)	0.46 (0.28-0.67)	31.65 (22.14-40.26)
Chronic	Control Group (n=5)	7.12 (6.44-8.02)	45.01 (34.12-54.29)	13.20 (8.14-21.81)	3.98 (2.90-4.65)	0.46 (0.41-0.50)	30.90 (20.71-35.43)
	Varenicline Group (n=12)	18.75 (11.19-21.60)[a,b]	14.89 (6.04-18.15)[a,b]	4.87 (3.15-5.12)[a,b]	1.11 (0.7-1.92)[a,b]	0.15 (0.10-0.20)[a,b]	90.78 (70.46-109.12)[a,b]

[a]Significantly different compared with both control groups (p≤0.05); [b]Significantly different compared with acute varenicline group (p≤0.05).

Table 3. Lung weight changes.

		Lung Weight (g)	Body Weight (g)
Acute	Control Group (n=5)	20.1±1.62	300±10
	Varenicline Group (n=12)	18.8±2.81	295±12
Chronic	Control Group (n=5)	19.0±1.47	310±8
	Varenicline Group (n=12)	19.4±2.90	318±12

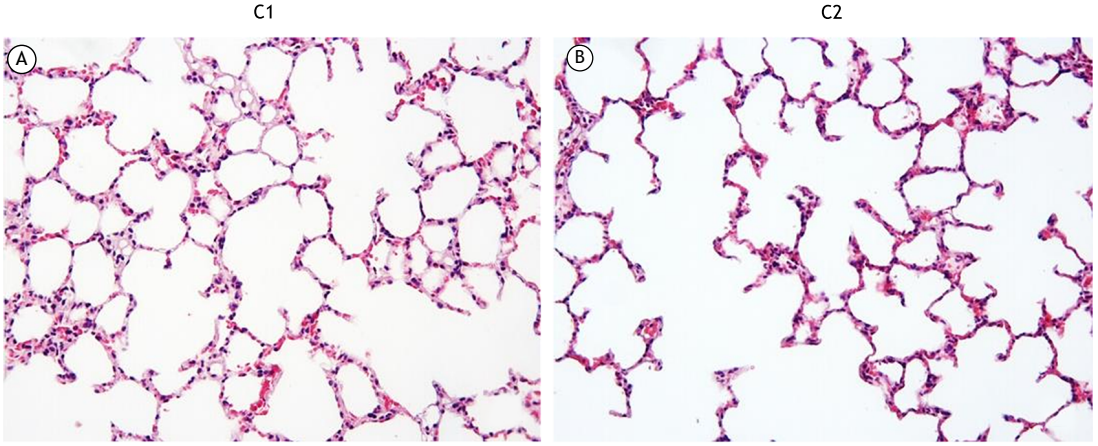

Figure 1. Normal appearance of lung histology in C1 **(A)** and C2 **(B)** groups, H-E; X20. GROUPS. C1: Acute Control (n=5); C2: Chronic Control (n=5).

V2 exhibited some histopathological changes including inflammatory cell infiltration, haemorrhage, thickened alveolar wall, and congestion (Figures 2A, B, C and D). In addition to these changes, it was also observed lipid-laden macrophages (Figures 3A and B) and intrabronchial macrophages in some areas (Figures 3C and D). When C1 and V1 as well as C2 and V2 were compared, significant differences were detected (p=0.001, for all). Significant differences were also noticed between V1 and V2 (p=0.001).

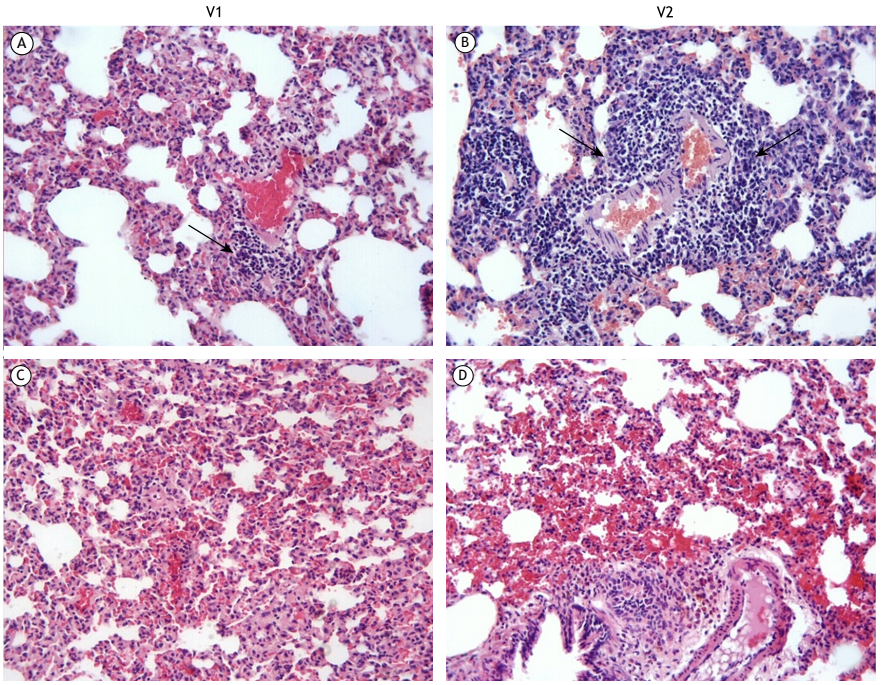

Figure 2. Visible lymphocytes accumulation around the blood vessels **(A** and **B)** (arrows) and congestion of the parenchyma **(C** and **D)** in V1 and V2 goups. It was noticed that inflammation and congestion are more prominent in V2 group than V1 group. H-E; X20. V1: Acute Varenicline (n=12); V2: Chronic Varenicline (n=12).

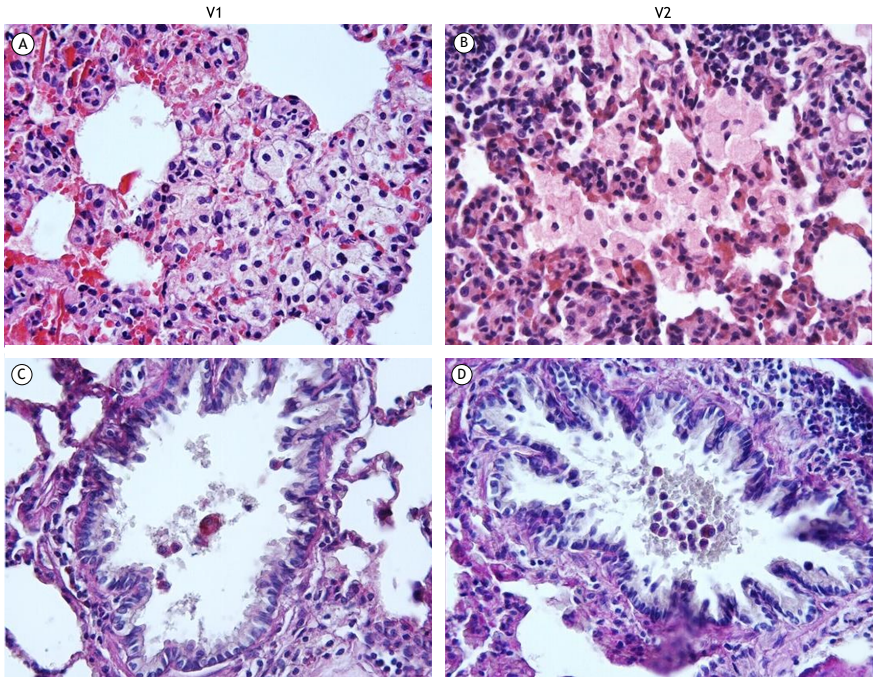

Figure 3. The appearance of lipid-laden macrophages accumulation **(A** and **B),** H-E; X40. Macrophages in bronchial lumen are observed **(C** and **D)** PAS; X40. V1: Acute Varenicline (n=12); V2: Chronic Varenicline (n=12).

DISCUSSION

The purpose of our study was to evaluate the effects of varenicline on lung tissue. In the authors' knowledge, this is the first study that demonstrates the toxic effects of varenicline on lung tissue based on biochemical and histopathological parameters.

Cigarette smoking is an important risk factor for the development of COPD and several other diseases. Additionally, tobacco smoke exposure is associated with exacerbations of pulmonary diseases.[16] Also, it is possible to notice that smoking cessation improves respiratory function and decreases pulmonary symptoms and airway inflammation; for this reason smokers are strongly advised to quit. Comprehensive programs are regarded as the most effective method of reducing tobacco-related mortality and morbidity. Without this type of support, only 6% of attempts to quit smoking are successful.[17,18]

A partial agonist for the nicotinic acetylcholine receptor α4β2 as Varenicline is the drug most widely used to quit smoking.[19] Its side effects include nausea, headache, insomnia, vivid dreams, and gastrointestinal and cardiovascular effects; however, its pulmonary side effects remain unknown.

In this study, it was demonstrated that chronic varenicline treatment increased the level of TBARS, which is an important sign of oxidative stress caused by increasing lipid peroxidation in lung tissue. However, chronic treatment with varenicline significantly reduced the levels of the antioxidants SOD, CAT, GPx, and GSH in lung tissue. MDA, which is a cursor of lipid peroxidation, was created by peroxidation by reactive oxygen species of fatty acids and leads to irreversible cell damage.[20,21] On the other hand, antioxidant defence systems protect the cell against oxidative damage under normal physiological conditions.[22] Oxidative stress results from an imbalance between TBARS and the antioxidant defence system. Membrane lipids, proteins, nucleic acids, and deoxyribonucleic acid molecules are the most sensitive cellular conformations to reactive oxygen species (ROS), which cause to cell injury, membrane damage, protease activation, DNA destruction and protein-lipid peroxidation.[23]

MPO is a highly sensitive index of tissue neutrophil sequestration included in the pathogenesis of various inflammatory diseases and is an inflammatory marker.[24,25] In this work, it was determined that chronic varenicline treatment caused a significant increase in the levels of MPO in the lung tissue leading to pulmonary inflammation.

It was also perceived that chronic varenicline exposure increased inflammatory cell infiltration and congestion histopathologically. The main role of alveolar macrophages is the phagocytosis and to eliminate the inhaled particulates, preventing damage to the delicate and highly functional alveolar epithelium. The word foamy is used generically to describe the vacuolated appearance of alveolar macrophage cytoplasm under light microscopy, which can be classified ultrastructurally based on the presence of lysosomal lamellar bodies, neutral lipid droplets, or drug particles in response to a variety of conditions.[26]

Inflammation is an essential component in the pathogenesis of lung damage that is orchestrated in part by endogenous and migrating leukocytes, which, along with pulmonary epithelial and endothelial cells, it create a feedback loop through which stimuli from cell damage activate alveolar and interstitial macrophages.[27] Activated leukocytes can deliver reactive oxygen species (superoxide, hydrogen peroxide, hydroxyl radical, hypochlorous acid, nitric oxide, and peroxynitrite) and proteases that maintain the injury/repair processes that are thought to contribute to fibrotic processes.[28] Data of this study indicate that acute administration of varenicline did not exert oxidative or inflammatory effects on the lung, in contrast to chronic administration.

Also, these results are consistent with another varenicline studies by Selcuk et al. regarding its detrimental cardiovascular effects,[9] which demonstrated adverse effects of chronic varenicline exposure on cardiovascular tissue via electrocardiographic, biochemical, and histopathological analyses. Recently, randomized controlled clinical trials and meta-analyzes of varenicline, that can be safely used in smoking cessation therapy without increased risk of cardiovascular disease have been published.[29,30] There are also clinical studies demonstrating the positive effects of varenicline on smoking cessation in patients with mild, moderate and severe COPD.[31,32] In these studies, no respiratory side effects of varenicline besides the expected side effects were found in COPD patients comparing placebo during a-year period. Tashkin et al.[33] showed that there was no difference between continuous abstainers and continuous smokers in terms of lung function and respiratory symptoms in COPD patients who quit smoking with varenicline following 1 year period, but also they showed improvement in lung function during first 12 weeks. Although it is not methodologically appropriate to compare these results with findings of this sutdy. Once, that the pulmonary function of patients using long-term varenicline for smoking cessation should be closely monitored, because it also was not detected any acute toxicity during the 45-day varenicline treatment period. Moreover, in these clinical studies, the positive effect of smoking cessation on the improvement of pulmonary function should not be ignored. There is only one study that evaluated the prolonged exposure to varenicline in rats.[34] Zaccarelli-Magalhães et al.[34] studied possible toxicity through haematological, biochemical and anatomopathological parameters of 30 days exposure to varenicline. Opposite to the present study, the authors showed no significant kidneys, heart, liver, adrenal glands, spleen and lung tissues alterations, which indicates that varenicline was not able to alter these organs either macroscopic or microscopic aspects. However, the inflammatory and oxidative stress parameters were not evaluated in this study.

It is also relevant to note that the doses and times applied to rats and the doses and duration applied to humans are different. Andreollo et al.[35] showed that rats rapidly develop during childhood and become sexually mature at about six weeks old, but reach social maturity five to six months later. In adulthood, every month of the animal is approximately equivalent to 2.5 human years. The rats of this study were 10-12 weeks of age. According to this, the use of varenicline for 3 months in rats corresponds to 7.5 years of use in humans.

Although, most recently cardiovascular safety of varenicline was reported by Benowitz et al.[29], Selçuk et al.[9] reported that in an animal study, chronic varenicline exposure caused heart weight loss and decreased mean blood pressure, induced lipid peroxidation, and reduced antioxidant activity. Both acute and chronic varenicline exposure caused impairment of mean oxygen saturation. QT interval was prolonged in the chronic varenicline group, while PR interval prolongation was statistically significant in both control and acute varenicline groups. And the authors also confirmed their results both biochemically and histopathologically.

This study had some limitations. The aim was especially on the toxic effects of chronic varenicline administration on lung tissue of rats while ignoring its exposure-dependent effects. If the antioxidant enzymes had been studied in one-week intervals in a study design with a larger sample size, high levels were detected, which would have clearly established that short-term use has no toxic effects. Additionally, the use of advanced analysis methods, molecular mechanisms, and histopathological staining could have helped to assess the effects of varenicline on lung tissue. Studies with different drug doses should be performed to determine the highest effective treatment dose with the least toxic effects. In patients using varenicline, toxic effects can be determined by assessing inflammatory markers in expired air.

In conclusion, it was demonstrated the pulmonary effects of varenicline for the first time in lung tissue of rats. Chronic (3 months in rats, equivalent to 7,5 years in human) varenicline treatment caused inflammation and injury in lung cells by changing the biochemical and histological parameters. However, there is no reference of varenicline use in humans such a long time period.

REFERENCES

1. Domagala-Kulawik J. Effects of cigarette smoke on the lung and systemic immunity. J Physiol Pharmacol. 2008;59(Suppl 6):19-34. PMid:19218630.
2. Watkins SS, Koob GF, Markou A. Neural mechanisms underlying nicotine addiction: acute positive reinforcement and withdrawal. Nicotine Tob Res. 2000;2(1):19-37. http://dx.doi.org/10.1080/14622200050011277. PMid:11072438.
3. Cahill K, Stead LF, Lancaster T. Nicotine receptor partial agonists for smoking cessation. Cochrane Database Syst Rev. 2011;(2):CD006103. http://dx.doi.org/10.1002/14651858.CD006103.pub5. PMid:21328282.
4. Hays JT, Ebbert JO. Varenicline for tobacco dependence. N Engl J Med. 2008;359(19):2018-24. http://dx.doi.org/10.1056/NEJMct0800146. PMid:18987369.
5. Rollema H, Coe JW, Chambers LK, Hurst RS, Stahl SM, Williams KE. Rationale, pharmacology and clinical efficacy of partial agonists of alpha4beta2 nACh receptors for smoking cessation. Trends Pharmacol Sci. 2007;28(7):316-25. http://dx.doi.org/10.1016/j.tips.2007.05.003. PMid:17573127.
6. McClure EA, Baker NL, Gipson CD, Carpenter MJ, Roper AP, Froeliger BE, et al. An open-label pilot trial of N-acetylcysteine and varenicline in adult cigarette smokers. Am J Drug Alcohol Abuse. 2015;41(1):52-6. http://dx.doi.org/10.3109/00952990.2014.933839. PMid:25062287.
7. Cahill K, Stevens S, Perera R, Lancaster T. Pharmacological interventions for smoking cessation: an overview and network meta-analysis. Cochrane Database Syst Rev. 2013;5(5):CD009329. http://dx.doi.org/10.1002/14651858.CD009329.pub2. PMid:23728690.
8. Sharma V, McNeill JH. To scale or not to scale: the principles of dose extrapolation. Br J Pharmacol. 2009;157(6):907-21. http://dx.doi.org/10.1111/j.1476-5381.2009.00267.x. PMid:19508398.
9. Selçuk EB, Sungu M, Parlakpinar H, Ermiş N, Taslıdere E, Vardı N, et al. Evaluation of the cardiovascular effects of varenicline in rats. Drug Des Devel Ther. 2015;9:5705-17. http://dx.doi.org/10.2147/DDDT.S92268. PMid:26543352.
10. Uchiyama M, Mihara M. Determination of malonaldehyde precursor in tissues by tiobarbituric acid test. Anal Biochem. 1978;34(1):271-8. http://dx.doi.org/10.1016/0003-2697(78)90342-1.
11. Sun Y, Oberley L, Li Y. A simple method for clinical assay of superoxide dismutase. Clin Chem. 1988;34(3):497-500. http://dx.doi.org/10.1093/clinchem/34.3.497. PMid:3349599.
12. Paglia DE, Valentine WN. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J Lab Clin Med. 1967;70(1):158-70. PMid:6066618.
13. Wei H, Frenkel K. Relationship of oxidative events and DNA oxidation in Sencar mice to in vivo promoting activity of phorbol ester-type tumor promoters. Carcinogenesis. 1993;14(6):1195-201. http://dx.doi.org/10.1093/carcin/14.6.1195. PMid:8508507.
14. Aebi H. Catalase. In: Bergmeyer HU, editor. Methods of enzymatic analysis. New York: Academic Press; 1974. p. 673-7. http://dx.doi.org/10.1016/B978-0-12-091302-2.50032-3.
15. Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophys. 1959;82(1):70-7. http://dx.doi.org/10.1016/0003-9861(59)90090-6. PMid:13650640.
16. Marco R, Accordini S, Marcon A, Cerveri I, Antó JM, Gislason T, et al. Risk factors for chronic obstructive pulmonary disease in a European cohort of young adults. Am J Respir Crit Care Med. 2011;183(7):891-7. http://dx.doi.org/10.1164/rccm.201007-1125OC. PMid:20935112.
17. Fernández E, Schiaffino A, Borrell C, Benach J, Ariza C, Ramon JM, et al. Social class, education, and smoking cessation: long-term follow-up of patients treated at a smoking cessation unit. Nicotine Tob Res. 2006;8(1):29-36. http://dx.doi.org/10.1080/14622200500264432. PMid:16497597.
18. Gratziou C. Respiratory, cardiovascular and other physiological consequences of smoking cessation. Curr Med Res Opin. 2009;25(2):535-45. http://dx.doi.org/10.1185/03007990802707642. PMid:19193001.
19. Mihalak KB, Carroll FI, Luetje CW. Varenicline is a partial agonist at alpha4beta2 and a full agonist at alpha7 neuronal nicotinic receptors. Mol Pharmacol. 2006;70(3):801-5. http://dx.doi.org/10.1124/mol.106.025130. PMid:16766716.
20. Ozbek E, Turkoz Y, Sahna E, Ozugurlu F, Mizrak B, Ozbek M. Melatonin administration prevents the nephrotoxicity induced by gentamicin. BJU Int. 2000;85(6):742-6. http://dx.doi.org/10.1046/j.1464-410x.2000.00531.x. PMid:10759677.
21. Montjean D, Menezo Y, Benkhalifa M, Cohen M, Belloc S, Cohen-Bacrie P, et al. Malonaldehyde formation and DNA fragmentation: two independent sperm decays linked to reactive oxygen species. Zygote. 2010;18(3):265-8. http://dx.doi.org/10.1017/S0967199409990311. PMid:20331908.
22. Schiller HJ, Reilly PM, Bulkley GB. Tissue perfusion in critical illnesses. Antioxidant therapy. Crit Care Med. 1993;21(2, Suppl):92-102. http://dx.doi.org/10.1097/00003246-199302001-00016. PMid:8428505.
23. Parlakpınar H, Orum MH, Sagir M. Pathophysiology of myocardial ischemia reperfusion injury: a review. Med Sci (Turkey). 2013;2(4):935-54. http://dx.doi.org/10.5455/medscience.2013.02.8082.

24. Daugherty A, Dunn JL, Rateri DL, Heinecke JW. Myeloperoxidase, a catalyst for lipoprotein oxidation, is expressed in human atherosclerotic lesions. J Clin Invest. 1994;94(1):437-44. http://dx.doi.org/10.1172/JCI117342. PMid:8040285.
25. Allegra M, Furtmuller PG, Regelsberger G, Turco-Liveri ML, Tesoriere L, Perretti M, et al. Mechanism of reaction of melatonin with human myeloperoxidase. Biochem Biophys Res Commun. 2001;282(2):380-6. http://dx.doi.org/10.1006/bbrc.2001.4582. PMid:11401469.
26. Lewis DJ, Williams TC, Beck SL. Foamy macrophage responses in the rat lung following exposure to inhaled pharmaceuticals: a simple, pragmatic approach for inhaled drug development. J Appl Toxicol. 2014;34(4):319-31. http://dx.doi.org/10.1002/jat.2950. PMid:24474237.
27. Hunninghake GW, Gallin JI, Fauci AS. Immunologic reactivity of the lung: the in vivo and in vitro generation of a neutrophil chemotactic factor by alveolar macrophages. Am Rev Respir Dis. 1978;117(1):15-23. PMid:619716.
28. Henson PM, Johnston RB Jr. Tissue injury in inflammation. Oxidants, proteinases, and cationic proteins. J Clin Invest. 1987;79(3):669-74. http://dx.doi.org/10.1172/JCI112869. PMid:3546374.
29. Benowitz NL, Pipe A, West R, Hays JT, Tonstad S, McRae T, et al. Cardiovascular safety of varenicline, bupropion, and nicotine patch in smokers: a randomized clinical trial. JAMA Intern Med. 2018;178(5):622-31. http://dx.doi.org/10.1001/jamainternmed.2018.0397. PMid:29630702.
30. Sterling LH, Windle SB, Filion KB, Touma L, Eisenberg MJ. Varenicline and adverse cardiovascular events: a systematic review and meta-analysis of randomized controlled trials. J Am Heart Assoc. 2016;5(2):e002849. http://dx.doi.org/10.1161/JAHA.115.002849. PMid:26903004.
31. Tashkin DP, Rennard S, Hays JT, Ma W, Lawrence D, Lee TC. Effects of varenicline on smoking cessation in patients with mild to moderate COPD: a randomized controlled trial. Chest. 2011;139(3):591-9. http://dx.doi.org/10.1378/chest.10-0865. PMid:20864613.
32. Sansores RH, Ramírez-Venegas A, Arellano-Rocha R, Noé-Díaz V, García-Gómez L, Pérez Bautista O, et al. Use of varenicline for more than 12 months for smoking cessation in heavy chronic obstructive pulmonary disease smokers unmotivated to quit: a pilot study. Ther Adv Respir Dis. 2016;10(5):383-90. http://dx.doi.org/10.1177/1753465816654823. PMid:27352612.
33. Tashkin DP, Rennard S, Taylor Hays J, Lawrence D, Marton JP, Lee TC. Lung function and respiratory symptoms in a 1-year randomized smoking cessation trial of varenicline in COPD patients. Respir Med. 2011;105(11):1682-90. http://dx.doi.org/10.1016/j.rmed.2011.04.016. PMid:21621992.
34. Zaccarelli-Magalhães J, Moreira N, Sandini TM, de Abreu GR, Sánchez-Sarmiento AM, Ricci EL, et al. Evaluation of prolonged exposure to varenicline in adult rats: haematological, biochemical and anatomopathological studies. Basic Clin Pharmacol Toxicol. 2018;122(3):305-9. http://dx.doi.org/10.1111/bcpt.12913. PMid:28944993.
35. Andreollo NA, Santos EF, Araújo MR, Lopes LR. Rat's age versus human's age: what is the relationship? Arq Bras Cir Dig. 2012;25(1):49-51. http://dx.doi.org/10.1590/S0102-67202012000100011. PMid:22569979.