ORIGINAL ARTICLE

Subchronic kretek smoke exposure induces myocardial p53 protein expression and associated inflammatory and fibrotic alterations in rats

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ABSTRACT

Kretek smoke contains various toxic substances that are harmful to human health and has been associated with inflammation and structural alterations in multiple organs. However, its effects on cardiac tissue remain incompletely understood. This study aimed to evaluate myocardial histopathological alterations and p53 protein expression following subchronic kretek smoke exposure in rats. Male Rattus norvegicus Sprague–Dawley rats were randomly divided into two groups. Group I was exposed to normal air, while Group II was exposed to kretek smoke at a dose of 1 kretek/rat/day for 30 days. Cardiac tissues were examined using H&E and MT staining. IHC analysis was performed to assess myocardial p53 protein expression, while ultrastructural observations of the myocardium were conducted using SEM. Group II rats exhibited myocardial inflammation characterized by lymphocyte infiltration and increased collagen accumulation, whereas Group I rats showed no inflammatory changes and minimal collagen deposition. Myocardial p53 protein expression was significantly higher in Group II (224.68 ± 5.44 μm²) than in Group I (199.87 ± 13.35 μm²; $P = 0.005$). Ultrastructural myocardial alterations were observed in Group II but not in Group I. In conclusion, subchronic kretek smoke exposure increases myocardial p53 protein expression accompanied by inflammatory and fibrotic alterations.

Key words: Fibrosis, inflammation, kretek smoke exposure, myocardium, p53 protein expression

INTRODUCTION

Most smokers in Indonesia use clove-mixed tobacco cigarettes known as kreteks.[1] This condition increases the number of individuals exposed to secondhand smoke.[2] Both active smokers and passive smokers remain at risk of smoking-related diseases.[3] Smoking prevalence in Indonesia remains high and represents an important public health concern in the ASEAN region.[4] Tobacco exposure also contributes to cancer burden and premature mortality, including among individuals exposed to secondhand smoke.[5] Smoking is also associated with oral mucosal lesions such as leukoplakia in exposed individuals.[6] These conditions highlight the importance of investigating smoke-related injury in the cardiovascular system.

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Cigarette smoke exposure induces cellular signaling alterations in airway smooth muscle cells through TRPA1-mediated calcium influx.[7] Cigarette smoke combustion also produces particulate emissions that contribute to biological exposure during inhalation.[8] Smoke particles deposit in the respiratory tract under realistic inhalation conditions and contribute to tissue exposure mechanisms.[9] Previous studies demonstrated decreased density of pyramidal cells in the cerebral cortex and Purkinje cells in the cerebellum after exposure to filtered kretek cigarette smoke in rats.[10] Additional studies also reported histometric abnormalities of Purkinje cells following low-dose kretek smoke exposure.[11] These findings indicate that kretek smoke exposure produces measurable structural alterations in neural tissue.

The heart is also vulnerable to cigarette smoke exposure because smoke-derived toxic compounds can reach systemic circulation and contribute to myocardial injury processes. Experimental studies demonstrated that cigarette smoke exposure induces cardiac hypertrophy and vascular inflammation in animal models.[12] Short-term cigarette smoke exposure also induces cardiac remodeling in rats.[13] Smoke exposure is further associated with inflammatory mechanisms that contribute to tissue injury processes.[14] Our previous study demonstrated increased p53 gene expression in lung tissue after exposure to low doses of kretek cigarette smoke in rats.[15]

Evidence linking kretek smoke exposure with myocardial histopathological alteration remains limited. Information regarding myocardial p53 protein expression after kretek smoke exposure is also still scarce. Therefore, this study evaluated myocardial histopathological alterations in rats exposed to subchronic kretek smoke. The study also assessed collagen accumulation in myocardial tissue. In addition, p53 protein immunopositivity was examined in exposed rats.

MATERIALS AND METHODS

Type of research

This randomized controlled study used male *Rattus norvegicus* rats of the Sprague–Dawley strain. Animals were randomly assigned to two groups, Group I (control) and Group II (kretek smoke exposure).

Nicotine content in kretek

Nicotine content in kretek was measured using high-performance thin-layer chromatography. A CAMAG Linomat 5 instrument (CAMAG, Switzerland; Linomat 5_220727, S/N 220727, version 1.00.13) was used. Kretek samples were extracted and analyzed in triplicate. Nicotine concentration was expressed as mean ± standard deviation (SD) (µg/g) to ensure accuracy and reproducibility.

Sample size and animals

Sample size was guided by the resource equation, with five animals per group considered sufficient for exploratory studies.[16] Similar designs using Sprague–Dawley rats for histological and p53 analyses have been reported previously.[11,15] All rats were male, aged 2–3 months, and weighed 200–250 g. Rats were anesthetized with ketamine 100 mg/kg and xylazine 10 mg/kg, intraperitoneal, prior to euthanasia, as previously described.[10] Hearts were collected and fixed in 10% neutral buffered formalin (NBF) for histological preparation and p53 immunohistochemical analysis.

Animal treatment

Rats were housed individually with *ad libitum* food and water. Group I breathed normal air, and Group II was exposed to 1 kretek/rat/day for 30 days in a custom chamber. At the end of exposure, rats were anesthetized, and hearts were collected and fixed in NBF. A schematic of the study design is shown in Figure 1.

Histological analysis

Heart tissues were sectioned at 5 µm. H and E staining evaluated myocardial histomorphology and inflammation, while Masson's Trichrome (MT) staining assessed collagen deposition. Observations were performed at ×400 by three independent observers blinded to group allocation. Five random fields per section were captured per animal. Collagen area fraction was quantified using ImageJ with threshold-based segmentation. The mean per animal was used as the biological replicate for analysis.

Scanning electron microscopy

Tissue samples were coated with platinum using a Turbo Pumped Sputter Coater Q150T (Quorum Technologies, United Kingdom). Ultrastructural analysis was performed using a FEI Nova NanoSEM 450 (Thermo Fisher Scientific, Czech Republic) at accelerating voltages of 2–5 kV. Scanning electron microscopy (SEM) was used to evaluate ultrastructural myocardial morphology.

Immunohistochemistry for p53 protein

p53 protein expression was evaluated by immunohistochemistry. Deparaffinized sections underwent antigen retrieval in citrate buffer (pH 6.0) at 95°C for 20 min, followed by incubation with anti-p53 primary antibody (mouse monoclonal, clone DO-7, NCL-L-p53, Leica Biosystems; 1:550). horseradish peroxidase-conjugated secondary antibody and 3,3′-diaminobenzidine chromogen were applied for 10 min at 25°C. Sections were counterstained with hematoxylin. Brown nuclear staining indicated positive p53 expression. Quantification was performed in five random fields per section using ImageJ with color deconvolution; the mean per animal was used as a biological replicate. Observers were blinded to group allocation. Results are expressed as mean ± SD.

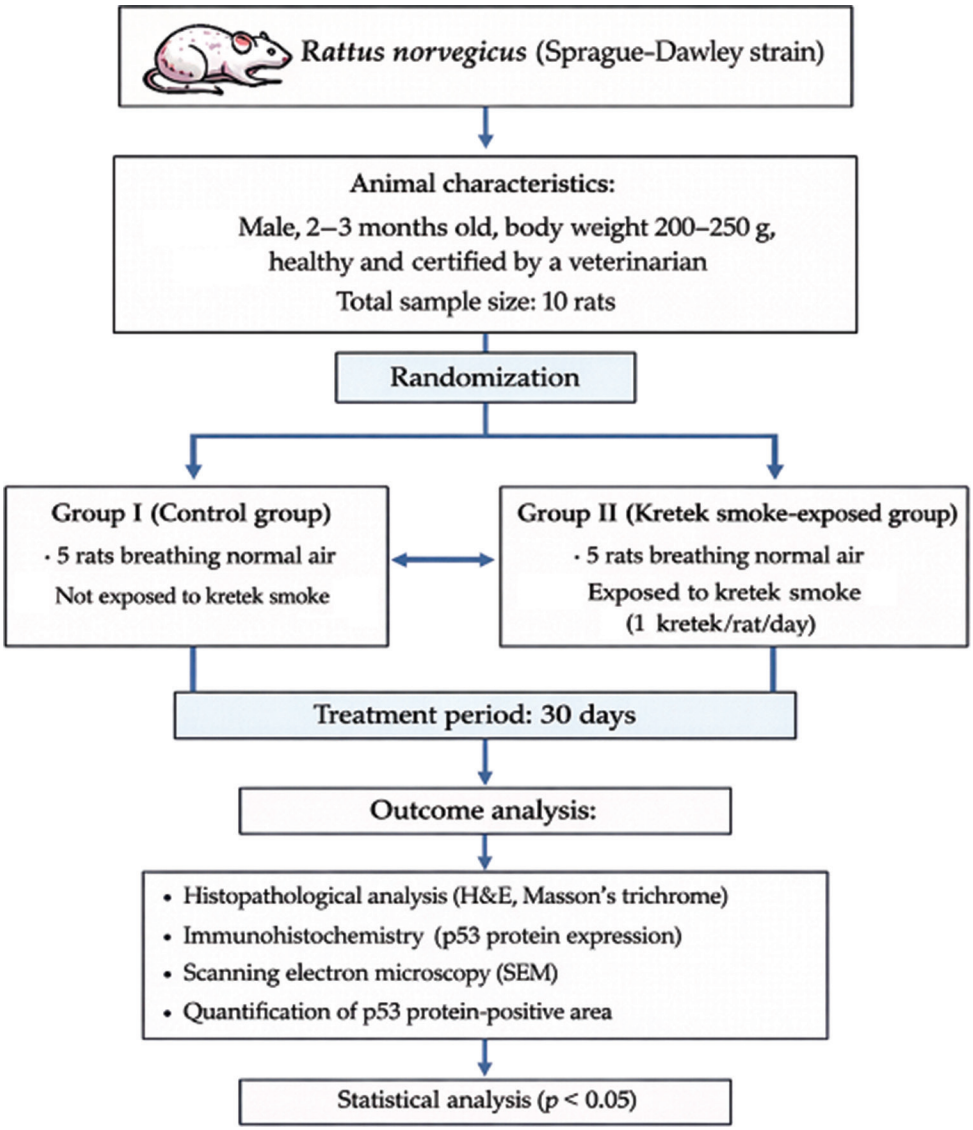

Figure 1: Experimental design of kretek smoke exposure in rats. Group I (control group) rats breathed normal air. Group II (kretek smoke-exposed group) rats received smoke exposure (1 kretek/rat/day) for 30 days. H and E: Hematoxylin-eosin

Statistical analysis

Five random fields per heart section were analyzed for collagen deposition. The mean collagen area fraction per animal was used as the unit of analysis ($n = 5$ per group). Data normality was assessed using the Shapiro–Wilk test and homogeneity of variances using Levene's test. Because assumptions were met, independent *t*-tests were used to compare collagen area fraction and p53 expression between groups. $P < 0.05$ was considered statistically significant.

Ethical approval

This study was approved by the Research Ethics Committee, Faculty of Medicine, Universitas Trisakti (No. 011/KER/FK/03/2025). All procedures followed internationally accepted guidelines for laboratory animal care.

RESULTS

Heart morphology

Gross examination showed normal cardiac shape and uniform dark red coloration in both groups, with no visible surface abnormalities. The average heart length was 3.1 cm, and the average width was 2.3 cm, with no apparent differences between Group I and Group II [Figure 2].

Nicotine content in kretek

Nicotine concentration in kretek extract was 3.88 ± 0.05 µg/g.

Histological features of the rat myocardial tissue

Histological examination of Group I is shown in Figure 3a-c. The myocardial tissue exhibited normal structure, with preserved endocardium, myocardium, epicardium, and pericardium. No vascular inflammation or lymphocyte infiltration was

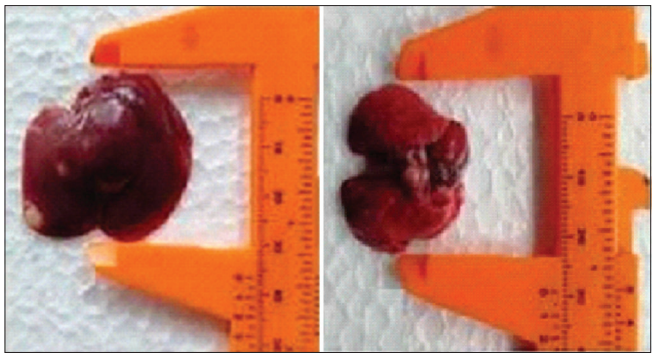

Figure 2: Gross morphology of the heart from *Rattus norvegicus* (Sprague-Dawley strain) rats

observed (blue arrows). In contrast, Group II rats showed structural alterations [Figure 3d-f], including vascular inflammation and lymphocyte infiltration (red arrows). The severity and extent of lesions varied among animals.

Scanning electron microscopy of rat myocardium

SEM images [Figure 4] revealed morphological differences in myocardial fibers between Group I (control) and Group II (smoke-exposed) rats. Because SEM primarily shows surface morphology, qualitative sarcomere enlargement in Group II should be interpreted with caution, and quantitative assessment requires TEM or fluorescence-based imaging.

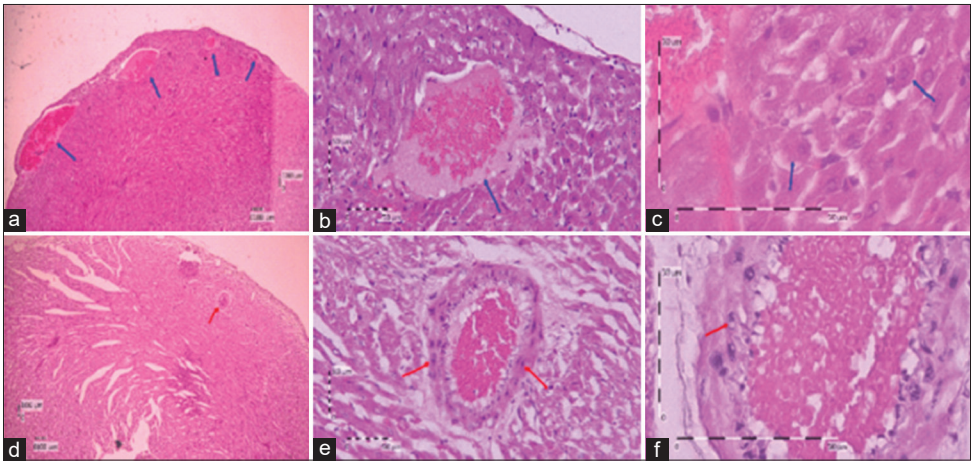

Figure 3: Comparison of rat heart histology between Group I and Group II. (a-c) Heart histology of Group I, (d-f) heart histology of Group II. All sections were stained with hematoxylin-eosin, (a and d) ×40 magnification, (b and e) ×400 magnification, (c and f) ×1000 magnification. The blue arrows indicate the absence of inflammation in cardiac blood vessels, whereas the red arrows indicate inflammation of cardiac blood vessels characterized by lymphocyte infiltration. Group I (control group) rats breathed normal air. Group II (kretek smoke-exposed group) rats received smoke exposure (1 kretek/rat/day) for 30 days

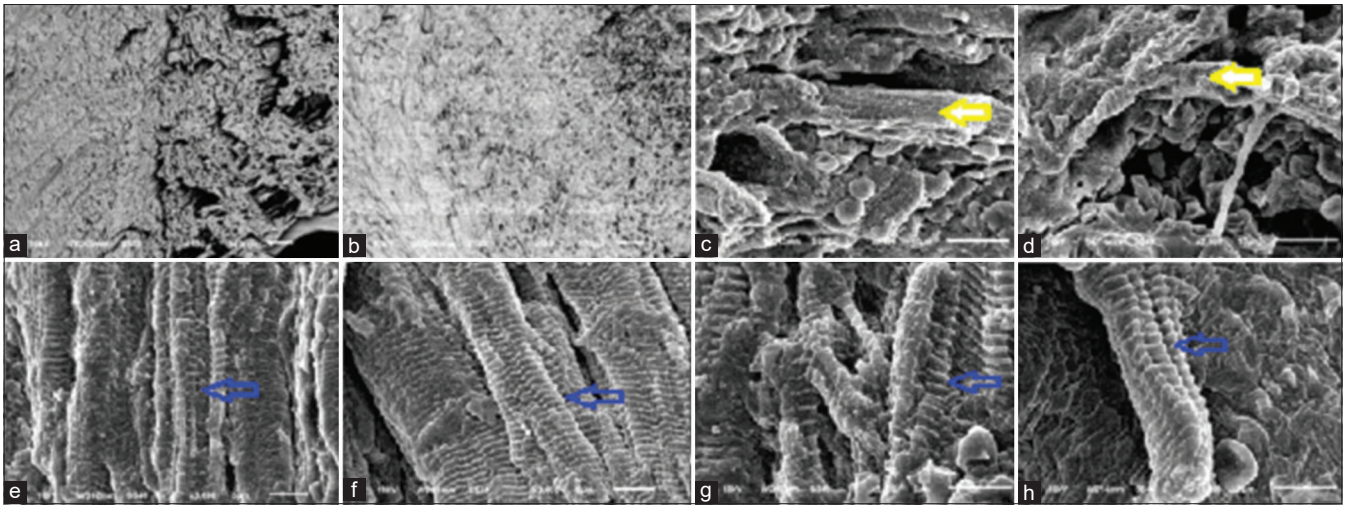

Figure 4: Scanning electron microscopy images of rat cardiac muscle, (a, c, e and g) Group I. (b, d, f, and h) Group II, (a and b) transverse sections at ×100 magnification, (c and d) ×2500 magnification, (e and f) longitudinal sections at ×3000, and (g and h) at ×5000 magnification. Yellow arrows indicate ventricular cardiomyocytes, and blue arrows indicate sarcomeres. Group I (control group) rats breathed normal air. Group II (kretek smoke-exposed group) rats received smoke exposure (1 kretek/rat/day) for 30 days

Myocardial inflammation

Kretek smoke exposure induced vascular inflammation in Group II myocardium. Inflammation areas ranged from 3455.09 to 7030.43 μm², and lesion areas from 1032.87 to 3305.50 μm². Mean inflammation (5241.57 ± 1669.60 μm²) exceeded mean lesion (2079.22 ± 946.82 μm²). The coefficient of variation was 31.85% for inflammation and 45.54% for lesion, indicating substantial inter-animal variability. Boxplot and histology [Figure 5a and b] confirmed lymphocyte infiltration; inflammation areas were larger and more variable than lesions [Supplementary Table 1].

Myocardial collagen accumulation

MT staining showed minimal collagen deposition in Group I, whereas Group II exhibited multifocal collagen accumulation, with a mean collagen area fraction of 21.98% ± 0.58% [Figure 6 and Supplementary Table 2]. Collagen deposition in Group I remained minimal and was therefore not subjected to quantitative analysis due to near-baseline staining levels.

p53 protein expression

The region of interest area positive for p53 protein expression was significantly higher in Group II (224.68 ± 5.44 μm²) than in Group I (199.87 ± 13.35 μm²) (t (8) = −3.849, P = 0.005; Figure 7c [Supplementary Table 3]). The mean difference between groups was 24.81 μm² (95% confidence interval [CI]: 9.94–39.67). Bootstrap analysis (1000 resamples) confirmed the robustness of this difference (P = 0.034; 95% CI: −38.03 to −13.37).

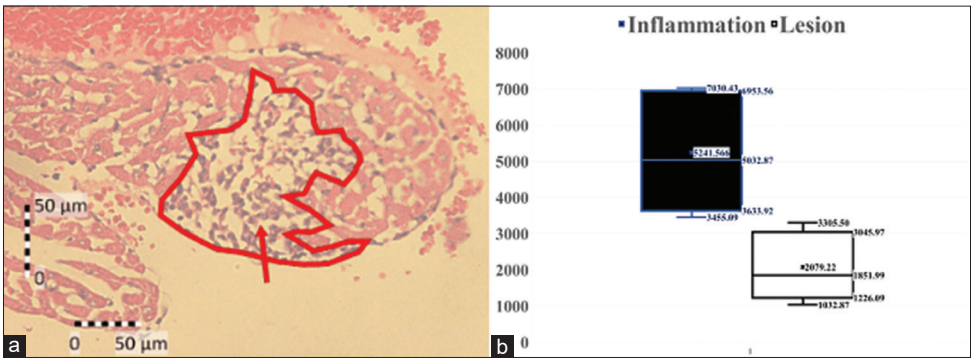

Figure 5: Histological evidence of myocardial inflammation in Group II rats, (a) photomicrograph of myocardial tissue stained with hematoxylin-eosin at ×400 magnification. Red arrows indicate lymphocyte infiltration, showing multifocal inflammatory foci, (C2) (b) Boxplot showing the distribution of inflammatory and lesion areas in Group II myocardium. Each point represents the mean area from five randomly selected fields per animal. Group II (kretek smoke-exposed group) rats received smoke exposure (1 kretek/rat/day) for 30 days

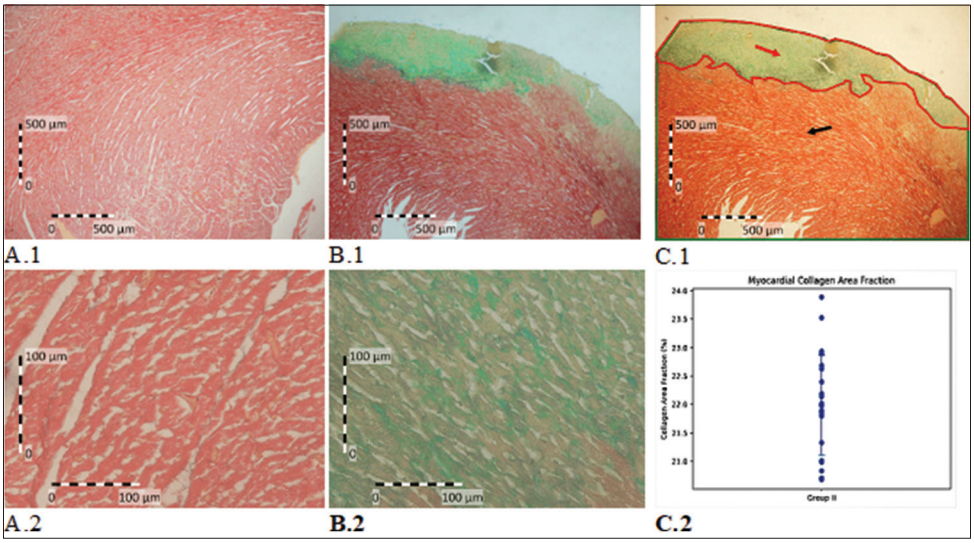

Figure 6: Myocardial collagen assessment, (A1 and A2) histology of Group I with Masson's Trichrome (×40, ×400), (B1 and B2) histology of Group II under same conditions, (C1) region of interest for histomorphometry in Group II (×40), collagen in green (red arrows), muscle fibers in red (black arrows), (C2) Scatter plot of myocardial collagen fraction in Group II (n = 5), each dot = mean of five fields per animal, central marker = mean, error bars = standard deviation. Group I (control group) rats breathed normal air. Group II (kretek smoke-exposed group) rats received smoke exposure (1 kretek/rat/day) for 30 days. A1 = Group I (×40), A2 = Group I (×400), B1 = Group II (×40), B2 = Group II (×400), C1 = Region of Interest for Histomorphometry in Group II, C2 = Scatter Plot of Myocardial Collagen Area Fraction in Group II

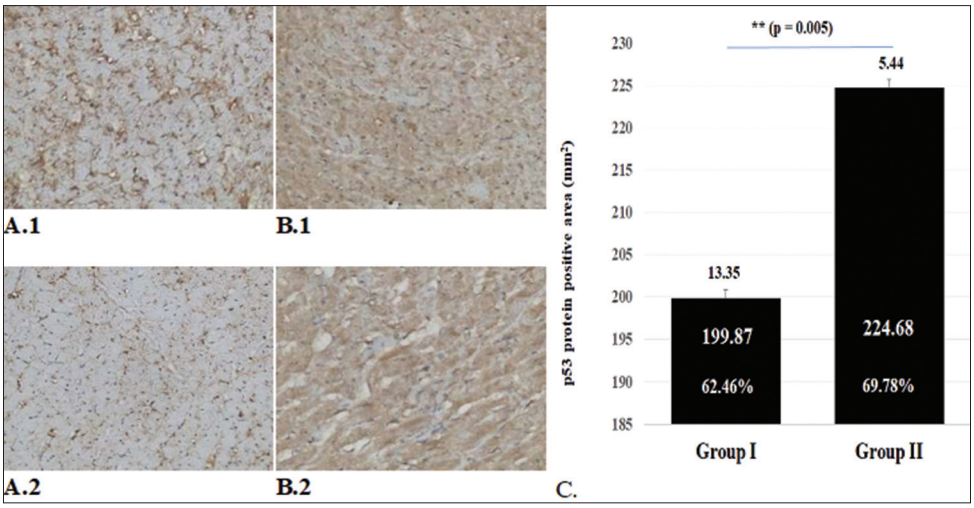

Figure 7: p53 protein expression in rat cardiac muscle detected by immunohistochemistry (IHC) (A1 and A2) IHC images of cardiac muscle from Group I, (B1 and B2) IHC images from Group II. All sections were examined at ×200 magnification, and (C) the analysis of p53 expression. Group I (control group) rats breathed normal air. Group II (kretek smoke-exposed group) rats received smoke exposure (1 kretek/rat/day) for 30 days. A1 = Group I (IHC image), A2 = Group I (IHC image), B1 = Group II (IHC image), B2 = Group II (IHC image), C = Quantitative Analysis of p53 Protein Expression

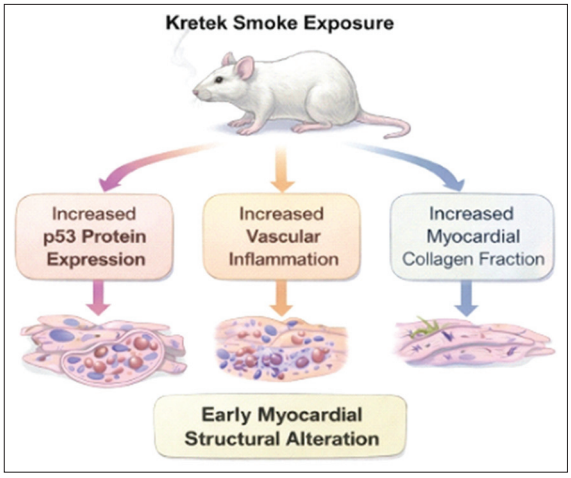

Figure 8: Schematic illustration of myocardial alterations following subchronic kretek smoke exposure in rats. Subchronic exposure to kretek cigarette smoke (1 cigarette/rat/day for 30 days) was associated with increased myocardial p53 protein expression, vascular inflammation characterized by inflammatory cell infiltration, and increased myocardial collagen area fraction in exposed rats (Group II). These findings indicate early myocardial structural alterations following kretek smoke exposure

DISCUSSION

In this study, rats exposed to kretek smoke for 30 days experienced myocardial inflammation, structural alterations, and collagen accumulation. These findings were accompanied by increased nuclear p53 expression and qualitative sarcomere enlargement. Therefore, subchronic kretek smoke exposure induces structural and molecular alterations in myocardial tissue, representing early alterations in the pathogenesis of cardiovascular disease.

This study demonstrated myocardial abnormalities in rats. This is in line with previous research showing that subchronic smoke exposure induces myocardial inflammation,[13] direct cardiomyocyte toxicity and mitochondrial dysfunction,[17] and multifocal collagen deposition.[18] These findings are consistent with smoke-induced cardiac remodeling reported in experimental models.[13] Furthermore, it contributes to the observed collagen accumulation. Structural alterations accompanied by increased nuclear p53 protein expression are also in line with studies demonstrating stress responses in cardiomyocytes.[19] Sarcomere enlargement in this study reflects ultrastructural adaptation to chronic injury, as in previous studies.[20] Collectively, our findings indicate that subchronic kretek smoke exposure induces structural and molecular alterations in myocardial tissue. These results are consistent with mechanisms of myocardial fibrosis and early remodeling in cardiovascular injury.[21]

In this study, lymphocyte infiltration and vascular inflammation were observed in Group II. These findings are consistent with previous studies demonstrating that cigarette smoke induces oxidative stress and systemic inflammation, contributing to endothelial dysfunction and cardiovascular risk.[22] Similar inflammatory responses have been reported in experimental models of cigarette smoke exposure.[13] Cigarette smoke generates reactive oxygen species (ROS) and free radicals that damage cellular membranes, proteins, and deoxyribonucleic acid (DNA), leading to cardiomyocyte injury.[23] Cigarette smoke promotes ferroptosis and inflammation in experimental models.[24] Oxidative stress is enhanced by increased circulating lipopolysaccharides after tobacco smoke exposure.[25] Clinical evidence also shows increased oxidative stress biomarkers and inflammatory

mediators in smokers, reflecting vascular dysfunction and systemic injury.[23]

Masson's trichrome staining revealed minimal collagen in rats from Group I. In contrast, rats from Group II showed increased collagen deposition, a hallmark of cardiac fibrosis. These findings are consistent with previous studies demonstrating extracellular matrix remodeling that impairs myocardial compliance and contributes to functional deterioration.[26] Increased collagen accumulation in this study is consistent with findings from other smoke exposure models.[27] Previous studies demonstrate that cigarette smoke alters lipid profiles, increases cardiovascular risk, and aggravates myocardial injury.[28] Sarcomere enlargement in this study represents an adaptive response to increased mechanical load under fibrotic conditions.[20] Furthermore, biomechanical deterioration in hypertrophied myocardium under chronic stress confirms adverse cardiac remodeling.[29] Finally, these structural adaptations observed in this study predict poor cardiac outcomes in fibrotic heart disease, consistent with previous research findings.[30]

The results of this study demonstrate increased p53 protein expression, consistent with previous studies on cellular oxidative stress induced by cigarette smoke exposure.[19] Previous studies demonstrate that oxidative stress and DNA damage regulate apoptotic and senescence pathways in cardiomyocytes.[31] ROS–mediated DNA damage directly induces p53 signaling pathways in cardiomyocytes.[32] Previous studies also demonstrate that p53 activation contributes to inflammatory and fibrotic responses in myocardial tissue.[31] However, this study evaluated p53 protein expression only. Functional modulation of p53 signaling was not performed. Therefore, further studies are required to clarify the causal role of p53 activation in cigarette smoke-induced myocardial injury.

In this study, sarcomere enlargement was observed, suggesting structural remodeling under chronic stress conditions. These findings are consistent with previous studies demonstrating adaptive and ultrastructural alterations of sarcomere organization during myocardial remodeling under increased mechanical load in fibrotic myocardium.[29] Such structural alterations are associated with impaired myocardial biomechanics and contractility and are consistent with collagen accumulation observed in this study.[26]

In this study, subchronic kretek smoke exposure induced heart inflammation and structural alterations. SEM analysis of the myocardium revealed alterations in the ultrastructural organization of cardiomyocytes and sarcomeres. Histological analysis showed collagen accumulation and increased nuclear p53 protein expression. These findings support previous evidence that short-term smoke exposure can damage the heart.[13] Tobacco smoke is linked to cardiovascular disease, including ischemic heart disease, and worsens clinical outcomes.[33] Antioxidants such as ascorbic acid reduce smoke-induced oxidative stress and heart and lung damage in rats.[34] Early prevention is essential. Public health measures, including smoking cessation and smoke-free environments, are important to protect cardiovascular health.

The overall myocardial alterations observed after 30 days of kretek smoke exposure, including increased p53 protein expression, vascular inflammation, and increased myocardial collagen area fraction, suggest early myocardial structural alteration in exposed rats. These integrated findings are summarized schematically in Figure 8.

Limitations

This study did not include functional cardiac assessments, such as echocardiography or hemodynamic measurements, limiting the interpretation of physiological impact. Ultrastructural observations were qualitative, without precise quantitative metrics. While p53 expression increased, the study design cannot establish causality between p53 activation and myocardial alterations.

CONCLUSION

Subchronic kretek smoke exposure (1 kretek/rat/day for 30 days) induced myocardial inflammation, structural alterations, and increased collagen deposition. Nuclear p53 protein expression was significantly higher in the Group II (224.68 ± 5.44 μm^2) compared to the Group I (199.87 ± 13.35 μm^2; $P = 0.005$), indicating increased cellular stress responses. SEM also revealed ultrastructural alterations of cardiac muscle fibers, supporting the presence of inflammatory and fibrotic myocardial alterations.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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Supplementary Table 1: Area of inflammation and lesion in the myocardium of rats in Group II (per animal)

Rat	Inflammation (μm^2)	Lesion (μm^2)
R1	3455.09	2786.44
R2	3812.75	1851.99
R3	6876.69	3305.50
R4	5032.87	1419.31
R5	7030.43	1032.87
Mean±SD	5241.57±1669.60	2079.22±946.82
CV	31.85	45.54

Values for each rat represent the mean area of five randomly selected microscopic fields at 400× magnification. Animal-level means (n=5) were used as the unit of statistical analysis. Group II (kretek smoke–exposed) rats received smoke exposure (1 kretek/rat/day) for 30 days. Raw data are available from the corresponding author upon reasonable request. SD: Standard deviation, CV: Coefficient of variation (SD/mean ×100%)

Supplementary Table 2: Myocardial collagen area fraction in Group II rats (aggregated per animal)

Rat	Collagen area fraction (%)
R1	21.40
R2	21.94
R3	22.28
R4	21.49
R5	22.80
Mean±SD	21.98±0.58

Values for each rat represent the mean collagen area fraction calculated from five randomly selected microscopic fields at 400× magnification. Animal-level means (n=5) were used as the unit of statistical analysis. Group II (kretek smoke-exposed) rats received smoke exposure (1 kretek/rat/day) for 30 days. SD: Standard deviation

Supplementary Table 3: Comparison of region of interest area positive for p53 protein between Group I and Group II (aggregated per animal)

Rat	ROI area positive for p53 protein (μm^2)	
	Group I	Group II
1	215.93	233.29
2	205.06	219.19
3	204.29	226.26
4	193.33	222.72
5	180.75	221.93
Mean±SD	199.87±13.35	224.68±5.44

ROI area positive for p53 protein represents the area of immunopositive p53 staining within the defined ROI. Values for each rat represent the mean calculated from five randomly selected microscopic fields per rat at 200×magnification. Animal-level means (n=5 per group) were used as the unit of statistical analysis. Group I (control group) rats breathed normal air. Group II (kretek smoke-exposed group) rats received smoke exposure (1 kretek/rat/day) for 30 days. SD: Standard deviation, ROI: Region of interest