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Protective Effect of Withania Somnifera Bleomycin Induced Pulmonary Fibrosis in Experimental Rats

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Abstract

Pulmonary fibrosis is a progressive lung disease with a high fatality rate. Bleomycin (BLM) is a common chemotherapeutic drug used to treat a wide variety of carcinomas. It has been stated that BLM is one of the most regularly used medications for producing experimental lung fibrosis because of its severe pulmonary toxicity.
Methods: Lung fibrosis was induced in rats by a single administration of bleomycin (on day 0). The lung fibrosis model was validated by comparing treated and control rats for levels of inflammatory cytokines and indicators of oxidative stress. Cytokine and oxidative marker levels were analyzed in the aforementioned rat lung fibrosis model to determine the effects of Withania somnifera.This study looked at whether or not Withania somnifera may reduce lung fibrosis caused by BLM. Intratracheally administered BLM was given for four weeks, with Withania somnifera given orally at 200 and 400 mg/kg. Withania somnifera not only greatly decreased MDA in lung tissue homogenate and increased GSH in the lungs, but it also significantly decreased TGF-1 and IL 13 in BALF and serum.Since the effects of Withania somnifera were similar to those found with conventional therapy, the findings support its use as a therapeutic agent for the management of idiopathic pulmonary fibrosis in rats exposed to bleomycin-induced lung fibrosis.

Keywords: Lung, Biomarkers, Fibrosis, and Withania somnifera

Introduction

Idiopathic pulmonary fibrosis is a fatal lung ailment with an unknown cause [1]. The prevalence of IPF has been increasing [2] according to epidemiological research during the last two to three decades. Despite the fact that the aetiology and pathophysiology of IPF are still unclear [3, 4], two anti-fibrotic medicines, pirfenidone and nintedanib, have recently been discovered to be helpful in slowing disease development and have been licensed as therapies. Due to the absence of defined markers of [5], clinical treatment of IPF remains challenging.

Pulmonary fibrosis is a chronic, ultimately deadly lung disease. It's the end result of a number of lung inflammatory illnesses. Pulmonary fibrosis is characterized by the disappearance of alveoli, the accumulation of myofibroblasts, the alteration of the lung parenchyma, and the deposition of an abnormally high amount of extracellular matrix [6]. Over 5 million individuals throughout the world are affected with pulmonary fibrosis, making it one of the most common interstitial lung conditions [7]. The average survival time for those diagnosed is around 3 years. Bleomycin is one anti-neoplastic drug that has been linked to BLM, or pulmonary fibrosis. Pathogenesis-related factors include cigarette smoking and exposure to mineral dusts or asbestos [8].

Researchers have shown that BLM-induced pulmonary fibrosis in rats and mice may be utilized to study the progression of human pulmonary fibrosis and the impact of various medicines. It is hypothesized that BLM sets off an inflammatory and fibro-proliferative response by producing reactive oxygen species (ROS) that bind to DNA and cause DNA damage. It has also been suggested that BLM contributes to the worsening of tissue damage caused by oxidants by encouraging the breakdown of endogenous antioxidant defenses. [9].

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Ayurveda, India's ancient medicinal practice, was first documented in the sixth millennium BCE (Charak Samhita, 1949). Over the course of over 6000 years, the Rasayana herb ashwagandha (Withania somnifera) has been widely used. Tonic, narcotic, diuretic, anthelmintic, astringent, stimulating, and thermogenic are just few of the benefits associated with Ashwagandha root. Anti-inflammatory, analgesic, anti-tumor, antioxidant, immunomodulatory, and other therapeutic characteristics are among its many attributes [10, 11]. The purpose of this research was to determine whether or not Withania somnifera might mitigate BLM-induced pulmonary fibrosis in rats.

Materials & Methods

Drugs and Chemicals

The drug and chemicals are taken from different suppliers like Withania sominefera provided by Natural Remedies Bengaluru, Bleomycin and Pirfenidone was purchased from Cipla Ltd., and ketamine from pharmacy shop. Other chemicals were taken from SRL, New Delhi. Elisa kits were purchased from Elabscience.

Animals

The study used both sex Wistar rats (180-220 g). Animals were taken from the Central Animal House Facility, Hamdard University and kept in a controlled environment. They were provided with food and drink. Animals were cared for according to CPCSEA criteria for animal usage, which were approved by the Institutional Animal Ethics Committee (IAEC) protocol number 1444 (Registration number 173/GO/ReBi/S/2000/CPCSEA).

Bleomycin induced lung fibrosis in rats

In this model animals are randomly divided into 5 groups of 6 rats each. Group I served as normal control. Group-II experimental control was given single dose of bleomycin(intratracheal injection 1.5 U in 0.3ml saline) zero day [12]. Group-III and Group-IV given Withania somnifera (WS) at two different doses (200 and 400 mg/kg p.o.,) started next day after bleomycin. Group-V was given as standard (pirfenidone dose 10mg/kg) started same on first day. After 28days, blood samples were collected from all animals by retro-orbital under mild anesthesia, after those animals have been sacrificed. The blood samples analysed for lung fibrosis markers. Lungs were harvested, rinsed in ice-cold saline. The left lobes from all the lungs were isolated for preparation of lung homogenate.

Collection of bronchoalveolar lavage fluid (BALF)

The tracheas were exposed, cannulated, and the thoracic cavity was opened. 3 times, 2 ml of sterile 0.9% saline was slowly infused into the lungs. After gently squeezing the chest numerous times, 50–70% of the recovery was obtained.The BALF was centrifuged using a cooled centrifuge at 2000 rpm; 4°C for 10 min. Supernatant was then isolated and placed in a deep freezer for various tests.

Assessment of lung interleukin-13 (IL-13) and TGF-β 1

Lung content of IL-13 and TGF-β 1 was quantified using commercially available enzyme-linked immunosorbent assay (ELISA) kit, according to the ELISA manual instructions.

Estimation of MDA levels

Malondialdehyde (MDA) is widely used as oxidative stress biomarker in biomedical research. Lipid peroxidation is measured spectrophotometrically as 2-thiobarbituric acid-reactive substance (TBARS) in supernatant of liver homogenate. 0.1 ml of supernatant was mixed with 0.2 mlof sodium dodecyl sulfate (8.1 %), 1.5 ml of 20 % acetic acid and 1.5 ml of 2-thiobarbituric acid (0.8 %). The reaction mixture was finally made up to 4.0 ml with distilled water. After vortexing, samples were incubated for 1 h in 95o C and after cooling with tap water; 1.0 ml of distilled water and 5.0 ml of mixture of butanol–pyridine 15:1 (v/v) were added. The mixture was shaken for 10 min. and then centrifuged at 4000 rpm for 10 min. Then Butanol–pyridine layer was taken and measured spectrophotometrically at 532 nm. TBARS values are expressed as MDA equivalents. 1, 1, 3, 3-tetramethoxypropane (TMP) was used as the standard [13].Protein estimation by lowry method.

Assay of reduced glutathione (GSH)

Glutathione (GSH) levels were estimated by the method of Ellman [13]. This assay is based on the enzymatic recyclingprocedure in which glutathione was sequentially oxidized by the DTNB and reduced by NADPH in the presence of glutathione reductase. For assay, an equal quantity of samplewas mixed with 10% trichloroacetic acid and centrifugedto separate the proteins. To 0.1 ml of this supernatant, 2 ml of phosphate buffer (pH 8.4), 0.5 ml of 5'5-dithiobis (2-nitrobenzoic acid) and 0.4 ml of double distilled water was added. The mixture was vortexes and absorbance was read at412 nm within 15 min. The concentration of 2-nitro-5-benzoic acid formation was measured and reduced glutathione isexpressed as µmol/mg protein.

Statistical Analysis

The values were expressed as mean ± standard error of the mean. One-way analysis of variance (ANOVA) followed by appropriate post hoc test (Tukey test) were used for analysis. $p < 0.05$ was considered as statistically significant.

Results

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Effects of Withania somnifera on cytokine (TGF-β1)and IL-13 in bleomycin induced lung fibrosis in rats

In experimental control, bleomycin given single dose resulted in significant increase in levels of TGF-β1 both in serum and BALF ($p<0.01$) as compared to that in normal control group. Similarly, in Group-III and IV treatment with two different doses of withania somnifera (200 and 400mg/kg) attenuated the effect as it reduced significantly the levels of serum TGF-β1 ($p<0.05$ at 400mg/kg dose), in BALF it reduced significantly both the doses of WS ($p<0.01$ at 200mg/kg and $p<0.05$ at 400mg/kg) as compared to that

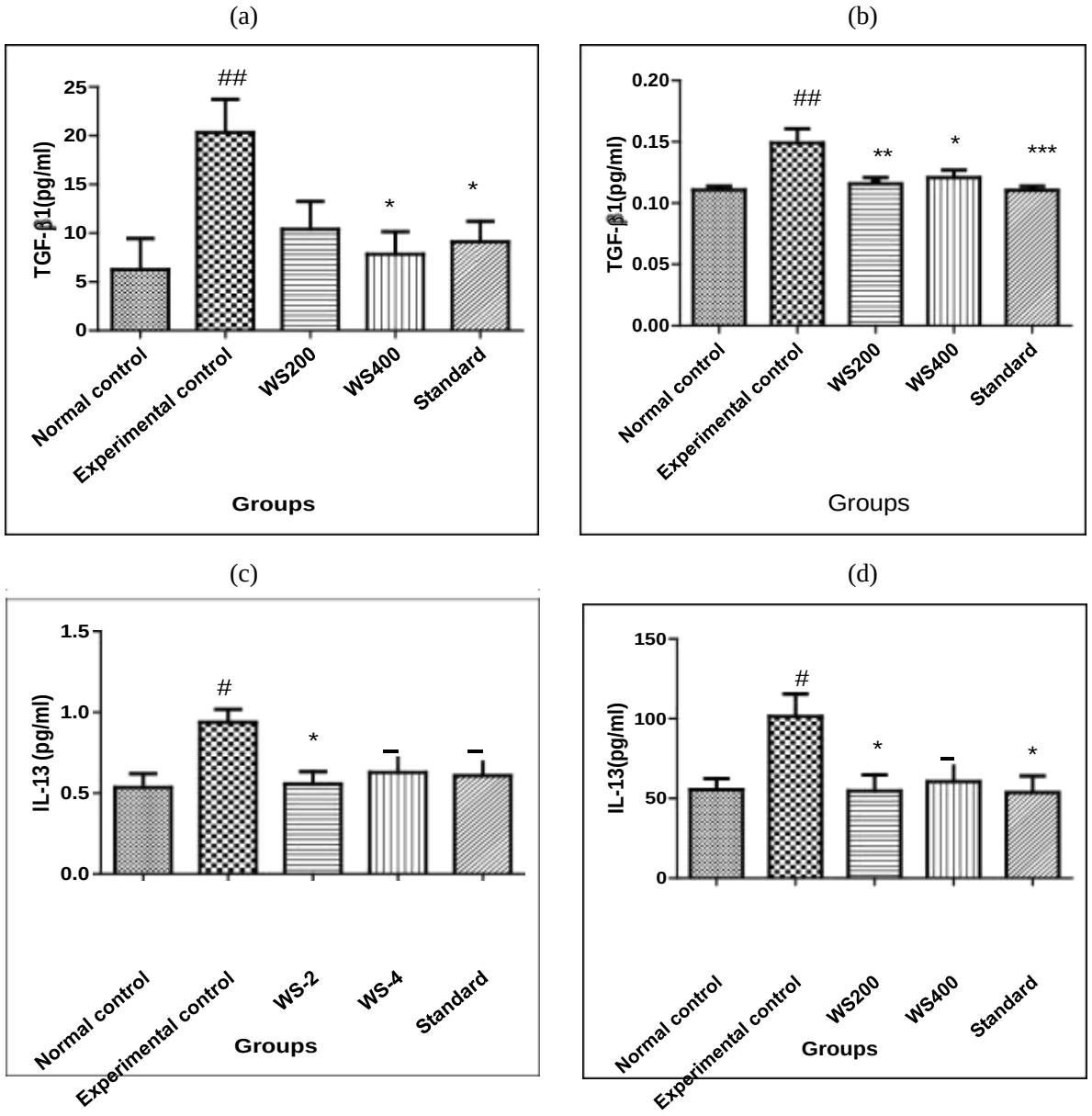

Figure 1: (a-d) Effects of Withania somnifera on (a) TGF-β1 serum (b) TGF-β1 BALF (c) IL-13 BALF and (d) IL-13 serum. WS-Withania somnifera, the values are expressed as mean ± SEM; (# $p<0.05$ & ##<0.01 compared with normal control); *$p<0.05$, **$p<0.01$ & ***$p<0.001$ compared with experimental control)

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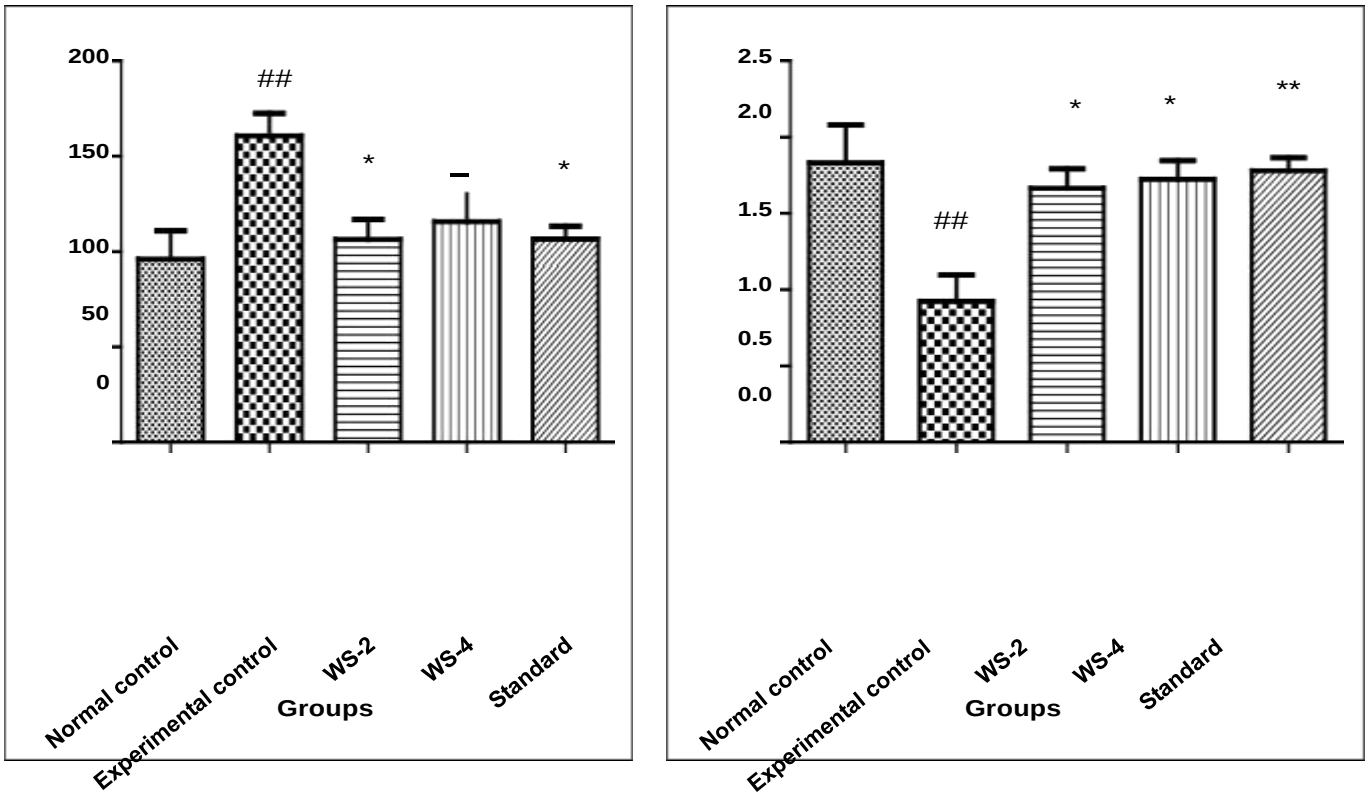

Figure 2: (a & b) Effects of Withania somnifera on (a) MDA (b) GSH; the values are expressed as mean ± SEM; (## p<0.01 compared with normal control); *p<0.05 & **p<0.01 compared with experimental control) in Experimental control. Pre-treatment with standard also significant attenuated the effects of bleomycin and reduced the levels of serum TGF-β1 (p< 0.05) and in BALF (p< 0.001). In IL-13, bleomycin given single dose resulted in significantincrease in levels of IL-13 both in serum and BALF (p<0.05)as compared to that in normal control group.

Similarly, in Group-III and IV treatment with two different doses of withania somnifera (200 and 400mg/kg) attenuated the effect as it reduced significantly the levels of serum IL- 13 and BALF (p < 0.05 at 200mg/kg dose), as compared to that in Experimental control. Pre-treatment with standard also significant attenuated the effects of bleomycin and reduced the levels of serum IL-13 (p< 0.05) and in BALF no statistical difference. The results are shon in [Figure 1]

Effects of Withania somnifera on oxidative stress parameters in bleomycin induced lung fibrosis

In experimental control, bleomycin given single dose resulted in significant increase in levels of MDA in tissue homgenate (p<0.01) and decrease in the level of GSH (p<0.01) as compared to that in normal control group. Similarly, in Group-III and IV treatment with two different doses of withania somnifera (200 and 400mg/kg) attenuated the effect as it reduced significantly the levels of MDA (p<0.05 at 200mg/kg dose) and increased the level of GSH significantly both the doses of WS (p< 0.05 at 400mg/kg) as compared to that in Experimental control. Pre-treatment with standard also significant attenuated the effects of bleomycin and reduced the levels of MDA (p< 0.05) and increased GSH (p< 0.01).The results are shown in [Figure 2].

Discussion

An increase in pulmonary myofibroblasts, the death of alveolar cells, the buildup of extracellular matrix, and the stiffening of the lungs are the hallmarks of pulmonary fibrosis. To stimulate the differentiation of fibroblasts into myofibroblasts, a critical stage in the development of lung fibrosis, TGF- is a well-established cytokine. Our most recent study reveals an unanticipated function for miR-133a, where it acts as an anti-fibrotic agent in response to TGF-1. Targeting a family of proteins in the TGF-1 signaling pathway in human pulmonary fibroblasts, it acts as a negative feedback regulator of profibrogenic pathways. To further understand the mechanisms by which TGF- promotes the differentiation of fibroblast cells into myofibroblast cells, a crucial component of pulmonary fibrosis, we used RNA-sequencing to identify miRNAs that were differentially expressed in HFL cells after treatment with or without TGF-1. First, we hypothesize [14, 15] that miRNAs that are up-regulated by TGF-1 may serve as mediators of TGF-1 profibrogenic signaling.

Based on our findings, miR-133a is one of a group of miRNAs previously recognized as being relevant in pulmonary disease, and its levels are increased in TGF-1-induced differentiated myofibroblasts. Another cytokine

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generated by cells during wound healing is TNF-, however it was unable to reproduce TGF-1's elevation of miR133a over time and concentration. Smad3 and p38- MAPK signaling pathway inhibitors prevented the TGF-1-induced up-regulation of miR-133a, indicating that miR-133a acts as a feedback mediator to down-regulate profibrotic genes and desensitize TGF-1 signaling pathways. TGF-1 stimulated -SMA synthesis in these fibroblasts, but miR-133a mimics attenuated this increase, whereas an inhibitor of miR-133a had the reverse effect. Extracellular matrix degradation occurs in a microenvironment that is surprisingly unaffected by miR-133a.

Gene expression for MMP-2 or MMP-9, or enzyme activity of either [16]. Investigating the mechanism(s) through which IL-13 promotes tissue fibrosis, since chronic pulmonary overexpression of IL-13 induces parenchymal and airway tissue fibrosis. Using this method, we were able to look at how IL-13 induces tissue fibrosis [17, 18].

This research reveals how well Withania somnifera may shield the lungs against the pulmonary fibrosis brought on by BLM. For this research, scientists tweaked a previously published rodent model in which a single intratracheal BLM injection was used. BLM instillation has a significant effect on lung physiological processes and biochemical dynamics. Lung fibrosis is thought to result after BLM injection because oxidative stress and inflammation feed off of each other and put a halt to the injury/repair process at the same time. The reactive oxygen species (ROS) [19] that bleomycin (BLM) is hypothesized to generate attack bio- macromolecules like DNA, protein, and lipid, resulting in lipid peroxidation that causes biochemical and physiological dysfunctions [20].

Daniil Papageorgiou suggests that oxidative stress plays a significant role in the pathological progression of pulmonary fibrosis. Some research suggests that reactive oxygen species (ROS) and reactive nitrogen species (RNS) have a role in fibrosis' pathogenesis [28,29]. Oxidative stress indicators are present in the lungs of patients with pulmonary fibrosis, and aberrant antioxidant activity has been shown to exacerbate pulmonary fibrosis in animal models [21]. Current research shows that intratracheal BLM instillation severely disrupts the body's oxidant/antioxidant equilibrium, as seen by a significant rise in lung MDA content and a corresponding drop in GSH content [22]. Significant protection against BLM-induced deterioration in oxidants/antioxidants hemostasis indicated the antioxidant action of Withania somnifera. Biochemical tests corroborated the finding that BLM instilled intratracheally caused prolonged lung inflammation, adding to the list of potential explanations for BLM-induced pulmonary damage. As a result of using Withania somnifera, all of these signs and symptoms lessened.

The serum and BALF levels of TGF-1 and IL-13 rose in the bleomycin-induced experimental group. Both serum and BALF levels were dramatically decreased after Withania somnifera therapy, as they were with the control group receiving conventional care. Lung homogenate malondialdehyde (MDA) levels were estimated to measure lipid peroxidation as a result of oxidative stress. The experimental group's levels of MDA were significantly elevated due to bleomycin. This increase was statistically significant when compared to the non-bleomycin control group. Lipid peroxidation may have been hastened by highly reactive metabolic byproducts.

Withania somnifera inhibited lipid peroxidation by lowering levels of malondialdehyde (MDA) in lung homogenate in the treatment group compared to the control group. Withania somnifera has a potent protective effect against bleomycin-induced lipid peroxidation, as shown by the reduced levels of MDA in the lung homogenate of the treatment group.

Recent studies have shown that withania somnifera may reduce inflammatory cytokine (IL)-13 and transforming growth factor (TGF)-1 levels in the body. Decreased MDA and significantly higher GSH levels, as determined by oxidative stress measurements in lung homogenates, further demonstrated that Withania somnifera protected against enhanced reactive oxygen levels in response to bleomycin. These findings confirmed that withania somnifera is a therapeutic agent capable of halting the progression of pulmonary lung fibrosis.

Conclusion

Withania somnifera confers significant protective effect against BLM-induced pulmonary fibrosis. Combined antioxidant and anti-fibrotic effects are believed to be implicated in the observed efficacy.

Acknowledgement

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Authors Contributions

Mohd Rafi Reshi and Maaz Naqvi were involved in the conduct of experiments, acquisition, and analysis of data and drafting of the manuscript. Muzammil Muzaffar also conducting experiment, dosing and drafting of manuscript. Saman Anees was also involved in experiment, Drafting and analysis of the study. Arunabha Ray was involved in planning of the study, interpretation of data and critical reviewing of manuscript. All authors approved the final version of the manuscript.

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Conflict of interest

No conflict of interest

Ethical statement

Ethical approval was required as this study involve laboratory animals.

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