

Immunosuppressive Activity of Goat Kefir in a Rat Model with Bleomycin-induced Pulmonary Fibrosis

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ABSTRACT

Objective: This study aimed to investigate the immunomodulatory capacity of goat kefir on pulmonary fibrosis rat model. **Material and Methods:** Twenty-five male rats were randomly divided into five groups: one group only received induction with bleomycin (0.3 mg/rat) to induce pulmonary fibrosis; three groups were treated with different doses (2.5, 3.5, and 4.5 mL/200 g BW) of goat kefir, following the induction with bleomycin, for 30 days; and one group served as negative control, did not receive bleomycin induction as well as kefir. On day 30, all the animals were sacrificed. Plasma levels of TGF- β , IL-4, and IFN- γ were measured using the ELISA method, and the expression of α -SMA in myofibroblast cells was examined with the help of immunohistochemistry assay. **Results:** Induction with bleomycin significantly elevated the expressions of TGF- β , IL-4, and IFN- γ in comparison to the control group. Following the administration of kefir (3.5 and 4.5 mL/200 g BW), the concentration of TGF- β was significantly decreased ($p < 0.05$); whereas, the concentration of IFN- γ increased slightly ($p < 0.05$) only in the group that received the 4.5 mL/200 g BW dose of kefir. In contrast, IL-4 exhibited increasing levels with higher doses of kefir ($p < 0.05$). The expression of α -SMA in myofibroblasts showed a tendency to decline following the administration of kefir, although this decline was not statistically significant. **Conclusions:** Goat kefir caused a reduction in the TGF- β levels in fibrosis conditions; however, the kefir elicited an immunosuppressive effect during the progression of the pulmonary fibrosis.

Key words: Bleomycin, Cytokine, Immunomodulator, Kefir, Pulmonary fibrosis.

INTRODUCTION

Pulmonary fibrosis is a condition caused by various pulmonary disorders and often culminates in death.¹ The development of fibrosis occurs mainly in response to tissue damage caused by an injury, through several phases i.e the coagulation, the inflammatory, the fibroblast proliferation/migration and the final remodeling phase. The damaged epithelial cells, during the inflammatory phase, produce inflammatory mediators, causing migration of the immune cells from the circulation both in and around the damaged lung tissue. The immune-activated cells then produce proinflammatory and anti-inflammatory cytokines which further initiate fibroblast recruitment and proliferation, as well as the conversion of alveolar epithelial cells into mesenchymal cells that resemble the fibroblast cells.² The differentiation of fibroblasts into myofibroblasts stimulates the secretion of extracellular matrix components, causing accumulation of extracellular matrix at the site of the damaged tissue, leading to gradual thickening and rigidification of lung tissue rendering it unable to function properly.³

Transforming Growth Factor- β (TGF- β) is a cytokine produced by alveolar macrophages. TGF- β plays a pivotal role in the differentiation of fibroblasts into myofibroblasts, which in turn express α -smooth muscle actin (α -SMA) and produce excessive extracellular matrix.⁴⁻⁶ Another cytokine,

Interleukin (IL)-4, is responsible to stimulate the proliferation of fibroblasts and initiate myofibroblast differentiation.⁷ IL-4 is able to induce the production of TGF- β from lung fibroblasts, thereby increasing the production of extracellular matrix.^{8,9} Interferon (IFN)- γ , a proinflammatory and profibrotic cytokine produced by the immune cells during inflammation, decreases the fibroblast procollagen mRNA levels, thereby inhibiting fibroblast proliferation as well as collagen production.^{10,11}

The damage caused by fibrosis is unrepairable. However, appropriate treatment may be able to improve the quality of life of the patient. Goat milk kefir is a dairy product produced by fermentation of goat milk with the help of kefir "grains" containing of a mixture of lactic acid bacteria, yeasts, and fungi.¹² Kefir has been proven to exhibit immunomodulatory activity in several diseases, through the stimulation of cytokine production by the immune cells. A study demonstrated that kefir decreased the TGF- β expression in HT-29 cell culture.¹³ Another study indicated that the administration of kefir increased IFN- γ expression in the mice infected with *G. intestinalis*.¹⁴ The expressions of the mRNAs of IL-4 genes were downregulated following the administration of kefir in murine breast cancer cells.¹⁵ The objective of the present study was to observe the potency of goat milk kefir in regulating the concentrations of plasma TGF- β 1, IL-4, and IFN- γ , as well as α -SMA expression, in the rat model with bleomycin-induced pulmonary fibrosis.

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MATERIAL AND METHODS

Material

Etahwah goat milk kefir was purchased from Inti Jaya Makmur (PIA STPP Malang, Indonesia). Kefir was obtained immediately after production, divided into aliquots, and rapidly frozen and stored at -80 °C until use.

Animals

The present study was approved by The Ethical Research Commission of the Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia (Approval number: 39/EC/KEPK/01/2017). Twelve to sixteen-week-old Wistar male rats ($n = 25$), weighing 150-200 g each, were obtained from the animal house of the Faculty of Science and Technology, State Islamic University Maulana Malik Ibrahim. The rats were randomly divided into five groups: group one received only bleomycin, group two to four were induced with bleomycin (2 mg/kg BW), followed by administration of kefir at different doses (2.5, 3.5, and 4.5 mL/200 g BW, respectively for each group), and group five did not receive bleomycin as well as kefir, served as negative control. All the rats were housed in plastic cages on hardwood shavings, kept at an ambient temperature of 24 ± 2 °C under a 12-h normal phase light-dark cycle, and were fed on BR1 Comfeed pellets (PT. Charoen Pokphand, Indonesia). Drinking water and food were freely available to all the rats. Acclimatization to the aforementioned conditions was performed at least one week prior to the induction with bleomycin.

Establishment of bleomycin-induced pulmonary fibrosis in rats and the kefir treatment

Rats were anesthetized with 0.05 mL of ketamine (Ketamine 10% inj KEPRO B.V., Holland), followed by 0.05 mL xylazine (Xyla, Interchemie, Holland), through intramuscular injection. Bleomycin solution was prepared by mixing Bleomycin hydrochloride (Bleocin, Nippon Kayaku, Kalbe Farma, Indonesia) and saline (0.9%), divided into aliquots and then stored at 4 °C. The rats were induced with Bleomycin (a dose of 0.3mg in 100 μ L, 50 μ L per nostril) using a micropipette. The animals were allowed to recover immediately afterward. This procedure was performed nine times consecutively, once each day. A similar administration protocol has also been reported previously in order to induce substantial and sustained pulmonary fibrosis in mice.¹⁶ Kefir was administered, using oral gavages, for 30 days following the first day of bleomycin induction.

On Day 30, all the rats were sacrificed using cervical dislocation. The chest cavity was opened up by performing the dissection. A blood sample was collected from the heart, preferably from the ventricle; the process was performed gradually in order to avoid heart collapse. The collected blood sample was immediately placed into a vacutainer tube containing EDTA. Blood plasma was obtained by centrifugation (15 min at 3000 rpm), apportioned into 0.5-mL aliquots, and stored at -20 °C for use in the ELISA experiments. Following the removal of the heart, the lung was reserved for the histopathological examination and the immunohistochemistry assays.

ELISA

Plasma samples were assayed for the measurement of TGF- β 1, IL-4, and IFN- γ , using solid-phase ELISA kits (Rat TGF- β 1 ELISA Kit Cat. No. ER1378 and Rat IL-4 ELISA Kit Cat. No. ER0041, China) and Elabscience kit (Rat IFN- γ ELISA Kit Cat. No. E-EL-R0009, China), following manufacturers' guidelines. The ELISA kits were purchased from FineTest. The outcomes were measured using an iMark™ Microplate Absorbance Reader (Bio-Rad).

Immunohistochemistry assay

The lung samples were immersed in 10% neutral buffered formalin for 24 h. Following the fixation, lungs were trimmed, and three transverse

sections that were each approximately 0.3 cm in thickness were excised through the lung sample (superior, median, and caudal parts) in order to include the main bronchi as well as the pulmonary alveoli. The sections were then dehydrated using an increasing- concentration graded series of ethyl alcohol, followed by embedding in one block of paraffin wax. Three serial histological slices (4 μ m) were obtained from each section, which were then processed for the immunohistochemical analysis. Alfa-smooth muscle actin polyclonal antibody (FineTest, Cat. No. ENT5053, China) was used in the immunohistochemical analysis. Alpha smooth muscle actin-stained slides were examined under a light microscope (Olympus BX51) connected to a color photomicrography digital camera system (DP70) and a PC HP Z600 Workstation.

Using 40x objective lens and the digital camera, the fields with all visible air-ways were selected and displayed on a monitor. The image analysis software (OlyVIA) program was used to measure the area of all stained tissue, and then divide it by the constant field of interest area, thereby calculating a bronchiole wall-area fraction value for 12 fields, which was the average for each bronchiole. Data were presented as averages.

Statistical analyses

All the results were expressed as mean \pm sem. Statistical analyses were performed using SPSS 21.0 software. Parametric one-way ANOVA and Least Significant Difference (LSD) test were performed for TGF- β 1 and IFN- γ endpoint readouts, while non-parametric Kruskal-Wallis test and Mann-Whitney U test were performed for the IL-4 and α -SMA endpoint readouts. The differences were considered significant at $P < 0.05$.

RESULTS

Effect of kefir on the plasma levels of TGF- β 1, IL-4, and IFN- γ

Measurement of blood plasma TGF- β 1 revealed that the levels of TGF- β 1 were elevated in the bleomycin-induced group (16.55 ± 0.76 ng/mL), and were significantly higher in comparison to the healthy group (10.28 ± 1.36 ng/mL). Following the administration of kefir at a dose of 2.5 mL/200 g BW, the levels of TGF- β 1 reduced slightly (though not statistically significant). However, a significantly decreased concentration of TGF- β 1 was shown in the groups that received kefir at doses of 3.5 mL/200 g BW and 4.5 mL/200 g BW (Figure 1A).

The concentration of IL-4 in blood plasma was dramatically higher in the bleomycin-induced group (164.16 ± 1.93 pg/mL), in comparison to the healthy rats (22.29 ± 1.06 pg/mL), which was further increased with the administration of higher doses of kefir (538.68 ± 6.45 pg/mL for kefir dose 3.5 mL/200 g BW and 571.08 ± 1.14 pg/mL for kefir dose 4.5 mL/200 g BW) (Figure 1B).

In contrast, the induction with bleomycin reduced the concentration of IFN- γ from 0.102 ± 0.001 pg/mL (in the healthy rat group) to 0.099 ± 0.002 pg/mL (in the bleomycin-induced group). The administration of kefir could recover the IFN- γ levels in the blood plasma only slightly (0.103 ± 0.001 pg/mL) in the group treated with kefir at dose of 4.5 mL/200 g BW (Figure 1C).

Effect of kefir on the expression of α -smooth muscle actin (SMA) in myofibroblasts

Induction with bleomycin evidently widened the area over which the myofibroblasts expressed alpha-smooth muscle actin (α -SMA) (7.08 ± 1.09 μ m), compared to the area observed for the healthy group (3.18 ± 0.29 μ m). Administration of kefir at a dose of 2.5 mL/200 g BW narrowed the area of α -SMA expression in myofibroblasts (5.79 ± 0.39 μ m). However, higher doses of kefir (4.5 mL/200 g BW) could not further narrow the area of α -SMA expression (4.88 ± 0.35 μ m) to reach a level similar to that in the healthy rats (Figure 2).

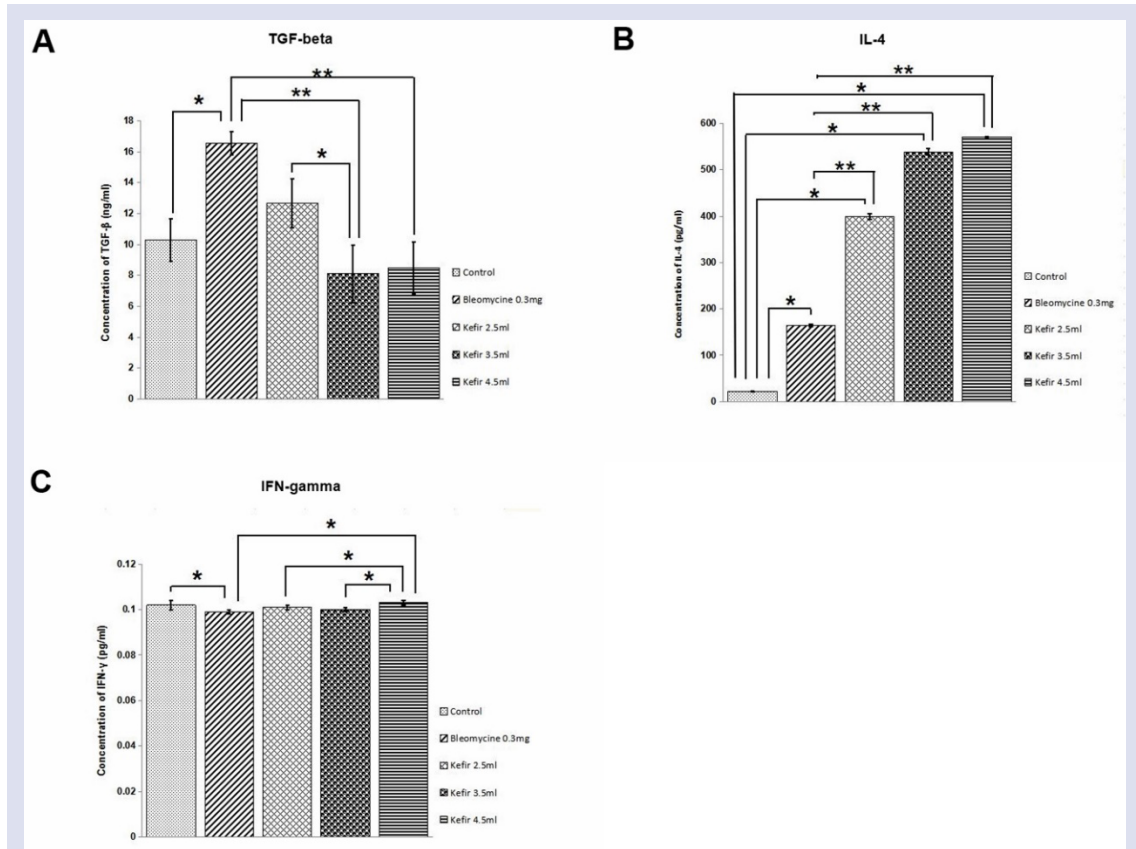


Figure 1: Effect of the administration of different doses of kefir: 2.5 mL, 3.5 mL and 4.5 mL/200 g BW on the plasma level of TGF-β1 (A), IL-4 (B), IFN-γ (C) in bleomycin-induced rat. Values are expressed as mean ± SEM, n=5. * and ** indicated p<0.05 and p<0.01 respectively.

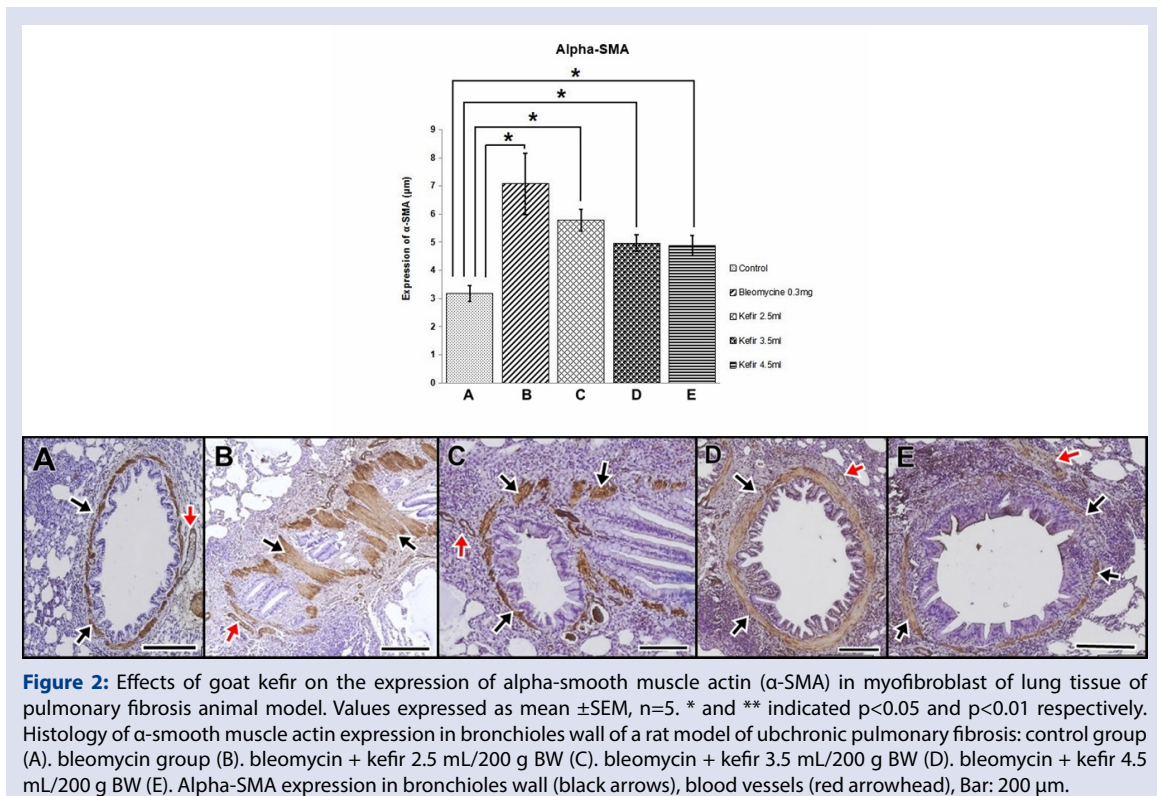


Figure 2: Effects of goat kefir on the expression of alpha-smooth muscle actin (α-SMA) in myofibroblast of lung tissue of pulmonary fibrosis animal model. Values expressed as mean ± SEM, n=5. * and ** indicated p<0.05 and p<0.01 respectively. Histology of α-smooth muscle actin expression in bronchioles wall of a rat model of ubchronic pulmonary fibrosis: control group (A). bleomycin group (B). bleomycin + kefir 2.5 mL/200 g BW (C). bleomycin + kefir 3.5 mL/200 g BW (D). bleomycin + kefir 4.5 mL/200 g BW (E). Alpha-SMA expression in bronchioles wall (black arrows), blood vessels (red arrowhead), Bar: 200 μm.

DISCUSSION

Intranasal administration of bleomycin, an alternative experimental model to study lung fibrosis in a rat model, is a method that does not cause injuries to the trachea. Bleomycin is known to stimulate inflammatory responses in the lung tissues, with increased infiltration of leukocytes following the stimulation of inflammatory cells, proliferation of fibroblasts, and the activation of myofibroblasts which secrete ECM components into the alveolar interstitial space, gradually resulting in pulmonary fibrosis.¹⁷

In the present study, the effect of kefir on the regulatory signals (TGF- β 1, IFN- γ , and IL-4) in blood plasma, as well as on the expression of α -SMA in myofibroblasts, was investigated in the bleomycin-induced pulmonary fibrosis rat model. It was observed that the expression of TGF- β 1 was 1.6-fold higher in the bleomycin-induced group compared to the healthy group, which declined with the administration of different doses of kefir and reached a value similar to that in the healthy un-induced group. There is compelling evidence from both experimental and clinical studies for an increased TGF- β 1 expression in response to bleomycin induction. Du et al. reported that the elevated levels of TGF- β and IL-4 observed in the lung tissue with fibrosis were lower for the control group compared to those in the group of Balb/c mice induced with bleomycin.¹⁸ It was observed in the present study that the levels of TGF- β 1 significantly declined following the exposure to goat kefir. TGF- β serves as a mediator in regulating cell proliferation and differentiation, as well as in the synthesis of several components of extracellular matrix. Elevated production of TGF- β 1 has been linked with the repair system in the fibrotic processes in several organs including lungs. Downregulation of lung TGF- β 1 mRNA expression in the bleomycin-induced pulmonary fibrosis in rodent has been demonstrated to be correlated with decreased extracellular matrix production in the lung, thereby ameliorating the progression of fibrosis. The decline in the TGF- β levels has been associated with the regulation of NF- κ B, which involves the production of IL-1.¹⁹ *K. marxianus* (one of the yeast species present in kefir) was able to downregulate the NF- κ B pathway in the intestinal epithelial cells *in vitro*, thereby decreasing the expression of TGF- β 1.²⁰

Surprisingly, the concentration of IL-4 in the blood plasma post the bleomycin induction showed further increase post kefir administration. The high expression of IL-4 in the bleomycin-induced fibrosis model has been demonstrated to be caused mainly by macrophages and T lymphocytes.⁹ Since IL-4 belongs to the group of proinflammatory cytokines, the elevated concentration of IL-4 leads to the progression of fibrosis. In contrast, the research conducted by Lee et al. demonstrated a decrease in the IL-4 concentration in the bronchoalveolar lavage in ovalbumin-sensitized Balb/c mice.²¹ IL-4 apparently also represses the synthesis of IFN- γ . In the present study, the plasma levels of IFN- γ observed in the healthy rats were very low. The slight increase in IFN- γ levels post administration of kefir could have been due to the repression effect caused by the high expression of IL-4. This result was in contrast to the result obtained in the study conducted by Vinderola et al., who demonstrated that the administration of kefir in healthy mice increased the IL-4, IL-2, and IFN- γ expressions in the lamina propria of the intestinal organ.²² This discrepancy could be a result of the difference in the background experimental settings between the *in vitro* and *in vivo* experiments.

Venderola performed her experiment using the peripheral blood mononuclear cells (PBMCs) obtained from normal humans treated with kefir. She suggested that increasing IFN- γ production in the peritoneal macrophage cells (*in vitro*) could be stimulated by the presence of sphingomyelin phospholipids present as a constituent in kefir.²³ Sphingomyelin is a sphingolipid present in the cell membranes of animals and bacteria, which play an important role in cell structure and function. Sphingomyelin is also responsible in several signaling

pathways. Sphingomyelin metabolism generates products, one of which is the IFN- γ cytokine, that perform important functions in the cell.²⁴ IL-4 and IFN- γ act in an antagonistic manner by suppressing each other's synthesis, while they are also able to induce the expression of each other. In a study involving mice infected with *Candida albicans*, IL-4 induced IFN- γ production from T cells.²⁵ Induction of IL-4 by IFN- γ is mediated by STAT proteins (STAT4 and STAT6).²⁶ IL-4 in synergistic association with IL-2 initiates the production of IFN- γ , which is dependent on STAT6.²⁷ In mouse colitis models, exposure to IL-4 enhanced the IFN- γ expression.²⁸

Differentiation of fibroblasts into myofibroblasts during the fibroproliferative phase is very important for fibrosis formation, and is influenced by TGF- β 1. TGF- β 1 mediates the fibroblast differentiation through the phosphorylation of Smad2 and Smad3 signal transducer proteins, resulting in the formation of their complexes with Smad4 and then translocation into the cell nucleus. This Smad protein complex increases the myofibroblast target gene regulation.²⁹ Myofibroblasts are the fibroblast phenotypes that actively synthesize extracellular matrix proteins (metalloproteinases and type I collagen). Myofibroblasts exhibit high α -SMA expression.^{30,31} Observations in skin cell cultures that were exposed with TGF- β 1 revealed α -smooth muscle actin expression, unlike the control. In the present study, increasing the goat milk kefir doses was followed by a slight decrease in the α -SMA expression, although the decrease was not statistically significant. This suggested that increasing the doses of goat milk kefir up to 4.5 mL/200 g BW was able to decrease the concentration of TGF- β 1 in the blood plasma, although it was not sufficient to lessen the expression of α -SMA in the bronchioles, therefore, not being sufficient to inhibit the progression of fibrosis due to bleomycin induction.

The findings of the present study propose an important implication for fibrosis therapy, suggesting that during the treatment of fibrosis, the patient should not consume goat milk kefir as it may worsen the fibrosis progression.

CONCLUSIONS

The data presented clearly demonstrated that goat kefir decreased the concentration of TGF- β 1, significantly increased the IL-4 levels, and only slightly raised the levels of IFN- γ in bleomycin-induced sub chronic pulmonary fibrosis rat model. Nevertheless, since the expression of α -SMA in the bronchioles is still high, therefore fail to inhibit to the progression of fibrosis.

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CONFLICTS OF INTEREST

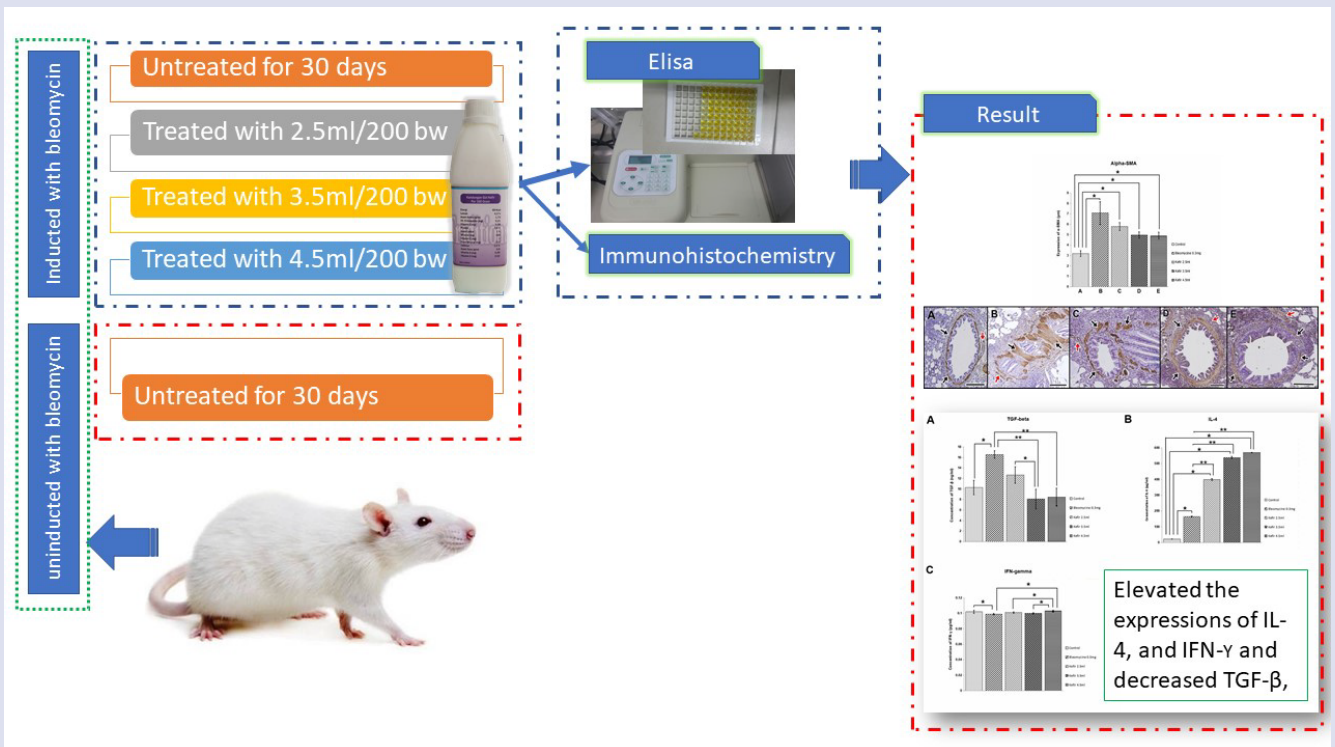
We declare that we have no conflicts of interest.

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GRAPHICAL ABSTRACT



ABOUT AUTHORS



Tias Pramesti Griana is the Head of Laboratory of Anatomy, Faculty of Medicine and Health Science, State Islamic University Maulana Malik Ibrahim Malang, Indonesia. She received her master degree in Master Program on Biomedical Science, Faculty of Medicine, Brawijaya University. Her research interest includes immunology of fibrosis. After completing kefir research on pulmonary fibrosis, she is currently working on research on the effect of *Schleichera oleosa* leaves on animal models of liver fibrosis.



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Karyono Mintaroem is a lecturer in Department of Anatomical Pathology, Faculty of Medicine, Brawijaya University. He completed his Doctoral degree in Doctoral Degree of Faculty of Medicine, Airlangga University, Indonesia. His research interest is Anatomic Pathology. He was involved in research on the effect of T Regulator CD4 CD25 Foxp3 cells and TGF β on airway remodeling of lung bronchioles on the animal model of asthma.



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