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# The Effect of Glutathione Supplementation on Proline Levels and Skin Collagen Tissue Thickness in Cigarette Smoke-Induced Mice

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ABSTRACT

**Introduction:** Smoking is associated with various diseases and involves exposure to almost 7,000 dangerous chemicals in cigarette smoke. These chemicals are toxic and trigger the formation of free radicals. These free radicals are also known to damage cell structures, including the structure of amino acids such as proline. Cigarette smoke is also known to reduce collagen production and damage collagen through the inflammatory process. To date, there has been no previous research regarding the administration of glutathione to observe proline levels and collagen thickness in mice induced by cigarette smoke.
**Objective:** To determine the effect of administering glutathione to mice treated with cigarette smoke on skin proline and collagen levels.
**Methods:** This research used male *Rattus norvegicus* mice, which were given four treatments, namely the control and glutathione groups, which were divided into 3 doses (150 mg, 200 mg, and 250 mg). Each group received exposure to cigarette smoke for 14 days. Examination of proline levels uses ELISA with a proline kit, while examination of collagen thickness uses histology. The analysis test uses the ANOVA test, followed by the post-hoc least significant difference (LSD) test.
**Results:** From the results of the analysis, it was found that administration of glutathione at a dose of 250 mg was significantly better at increasing proline levels compared to the other two glutathione supplementation groups, namely 150 mg and 200 mg ($p$ = 0.001 and $p$ = 0.018, respectively) and control ($p$ = 0.007). However, there was no significant increase in proline levels in the two glutathione groups (150 mg and 200 mg) when compared to controls. In terms of collagen thickening, all doses of glutathione supplementation, and increasingly higher doses, had better collagen tissue thickness than controls ($p$ = 0.000).
**Conclusion:** Glutathione supplementation improves proline levels and collagen tissue thickness



## INTRODUCTION

Smoking is still a global health problem that cannot be ignored. Globally, in 2020, the prevalence of adults who smoke reached 32.6% (men) and 6.5% (women). About 1.18 (0.94 to 1.47) billion individuals consume tobacco regularly, which result in 7.0 (2.0 to 11.2) million fatalities by 2020. In males, the prevalence of smoking has decreased by 27.2%, and in women, by 37.9%, since 1990

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(Dai et al., 2022). In Indonesia, based on data from Riset Kesehatan Dasar 2018 (RISKESDAS 2018), the prevalence of people aged ≥ 10 years who smoke is 28.8% (Kemenkes RI, 2018).

Smoking is known to be associated with various diseases. This is because cigarettes contain almost 7,000 dangerous chemicals, and 69 of them are considered carcinogens. These chemicals are toxic and can trigger the formation of free radicals, causing inflammation and DNA damage (Balatif, 2020). The toxic effects of cigarettes are known to damage the skin structure, triggering premature aging. In previous research, the impact of smoking can affect physical skin parameters like density and thickness of the epidermis and dermis (Yazdanparast et al., 2019). However, the mechanism underlying this is still unknown.

Previous research was conducted in vitro on human skin cells to determine the impact of smoking on skin health. They found there was a decrease in the rate of collagen synthesis of types I, and III by 18-22% in smokers (Knuutinen et al., 2002). In addition, smokers are also known to have significantly lower plasma proline levels ($p$ = 0.0026) than non-smokers (Luykx et al., 2013). Proline contributes about 10% of the total collagen-forming amino acids. The presence of proline is very necessary for the biosynthesis of collagen and proteins containing proline. Therefore, regulation of proline availability is necessary to maintain tissue integrity, for example, during wound healing (Karna et al., 2020).

To overcome the toxic effects and free radicals caused by chemicals in cigarettes, the body needs antioxidants to neutralize them. Antioxidants can be formed by the body and obtained from intake, such as supplements. Glutathione supplements are one of the supplements that are often consumed to maintain healthy skin. A multitude of processes contribute to its anti-melanogenic qualities, including its antioxidant capabilities, the induction of pheomelanin production instead of darker eumelanin, and its disruption of the intracellular trafficking of melanogenic enzymes. Glutathione is classified as safe for consumption because the lethal dose 50 (LD50) effect in mice is around 5 g/kg, in other words, glutathione is non-toxic. Glutathione has anti-ageing and antioxidant effects (Weschawalit et al., 2017).

Research related to glutathione in maintaining proline and collagen levels is still very limited. This study aims to determine the effect of administering glutathione to mice treated with cigarette smoke on skin proline and collagen levels.

## METHODS & MATERIALS

This research is an experimental study. In the design, we used a post-test control group only. The research was conducted at the Pharmacology Laboratory, Faculty of Pharmacy, Universitas Sumatera Barat, for the maintenance and treatment of experimental animals. Examination of proline levels and collagen tissue histology was carried out at the Biomedical Laboratory of Universitas Andalas. The experimental animals used were male white mice (*Rattus norvegicus*) of the Wistar albino strain aged 8-10 weeks. Each group (explained below) was given the same amount of cigarette smoke (4 cigarettes/day) and for the same duration, namely 14 days.

This process is carried out using a device that can regulate air flow, so that cigarette smoke can be distributed consistently into the treatment room containing the experimental animals. The first step in this process is to prepare the air flow device and ensure that all components are functioning properly. This tool consists of a cigarette smoke generator, an airflow system, and an exposure chamber where the experimental animals are placed. Then, the cigarette is burned in a cigarette smoke generator. The resulting smoke is then channeled through a controlled airflow system. This device is designed in such a way that the airflow can be regulated with precision, allowing researchers to control the amount and concentration of smoke received by the

experimental animals (cigarette smoke flow speed = 900 $cm^3$/minute). Duration time for exposure to cigarette smoke for 30 minutes. An illustration can be seen in Figure 1.

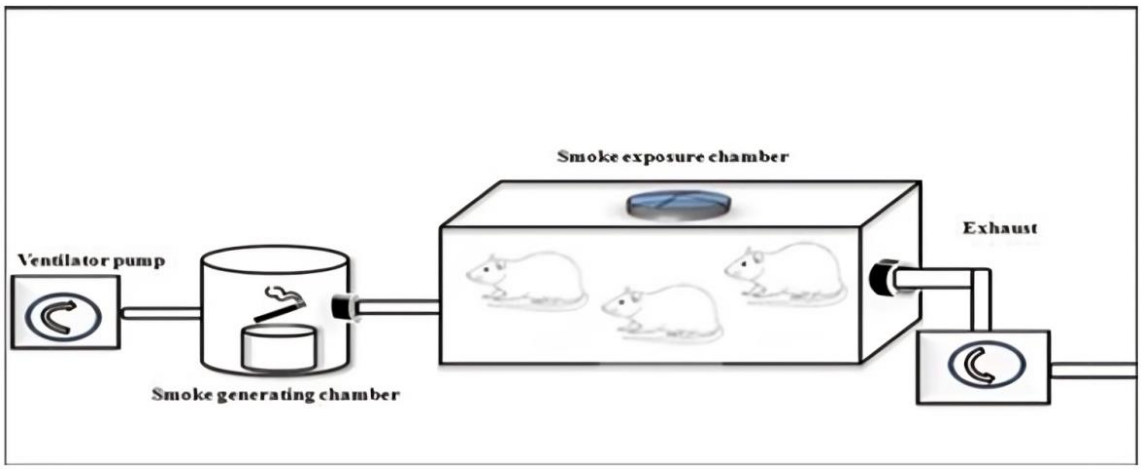



**Figure 1.** Illustration of exposure to cigarette smoke in mice (Santoso et al., 2021).

The smoking intervention was given at 08.00 (2 cigarettes) and 16.00 (2 cigarettes). The type of cigarette used in this study was unfiltered mild cigarettes which contain 39 mg tar and 2.3 mg nicotine purchased at the market (Santoso et al., 2021). Glutathione supplementation was given at 12.00. There are four interventions carried out, namely, control group (given only exposure to cigarette smoke), group P1 (cigarette smoke + glutathione supplementation 150 mg/day), group P2 (cigarette smoke + glutathione supplementation 200 mg/day), group P3 (cigarette smoke + glutathione supplementation 250 mg/day). The glutathione supplement (Glutanol) was provided from PT Immortal Pharmaceutical Laboratories and contained 500 mg pure glutathione powder. The number of mice used in the research was calculated using Federer formula, with four treatments groups. Therefore, this study used six mice for each group. To avoid sample shortages due to dropout, an addition of 20% was made to each group, so that the total sample was 32 samples (8 samples per group).

Supplementation was given orally, and the food and drink of each group of mice were uniform. On the 14th day after the last exposure to cigarette smoke, the mice were given anesthesia in the form of ketamine, and then blood was drawn via retro-orbital. Blood was collected into a vacutainer with a yellow lid, and blood plasma was obtained after the blood was centrifuged at 3000 rpm (15 minutes). To check proline levels, use the ELISA method with a Rat Proline Rich Protein 3 (PRR3) ELISA kit (MyBioSource, USA). The assay was conducted in duplicate. To examine collagen thickness, a histological examination of skin tissue taken from the back with a tissue size of 2x1 cm and a thickness of 2 mm was performed. Histological tissue observations in the preparations were viewed using a microscope equipped with Indomicroview software. Collagen thickness was measured using Image J software.

Statistical analysis was conducted using SPSS version 17. The data was tested for normality using Shapiro-Wilk and continued with the ANOVA test. The significance of the ANOVA test was later analyzed using the post-hoc Least Significant Difference (LSD) test to determine the level of significance between research groups. This research has received approval from the Research Ethics Commission of the Faculty of Medicine, Universitas Andalas with No. 172/UN.16.2/KEP-FK/2023.

## RESULT

Of the 32 mice studied, 4 of them dropped out due to illness. The remaining 28 mice were included in the analysis for this study. The mean values for the control and treatment groups can be seen in Table 1. The normality test on the proline variable obtained a significance value of 0.155 and the collagen variable obtained 0.051. Thus, the proline and collagen variables were found to have a normal distribution.

**Table 1**. Differences in proline levels and collagen thickness between groups

| Groups | Mean ± SD | |
|---|---|---|
| | Proline level (µmol/mL) | Collagen thickness (µm) |
| **Control** | 25,58 ± 3,91 | 36,30 ± 1,00 |
| **P1** | 23,48 ± 5,34 | 44,21 ± 2,00 |
| **P2** | 26,85 ± 7,46 | 50,69 ± 2,02 |
| **P3** | 35,05 ± 6,78 | 61,18 ± 3,78 |

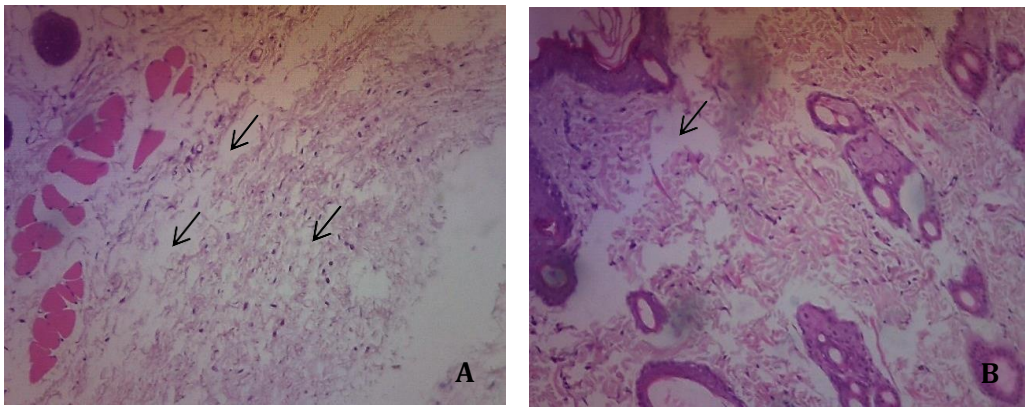
SD: Standard deviation

It was found that the proline level variable had a significance value of $p$ = 0.00 and collagen thickness $p$ = 0.00 (table 2). This indicates that there is a significant effect of administering graded doses of glutathione on proline and collagen levels in mice exposed to cigarette smoke.

**Table 2.** Hypothesis test of the effect of glutathione

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| **Proline** | Between Groups | 539.418 | 3 | 179.806 | 4.941 | **0.00** |
| | Within Groups | 873.391 | 24 | 36.391 | | |
| | Total | 1412.810 | 27 | | | |
| **Collagen** | Between Groups | 2326.161 | 3 | 775.387 | 132.198 | **0.00** |
| | Within Groups | 140.769 | 24 | 5.865 | | |
| | Total | 2466.930 | 27 | | | |

From the results of the LSD test, it was discovered that administering glutathione at a dose of ≥ 250 mg significantly increased proline levels compared to the control group ($p$ = 0.007) and the other two treatment groups (table 3). Based on collagen thickness, it was found that the higher the dose of glutathione given, the greater (significantly) the collagen thickness obtained (figure 2).



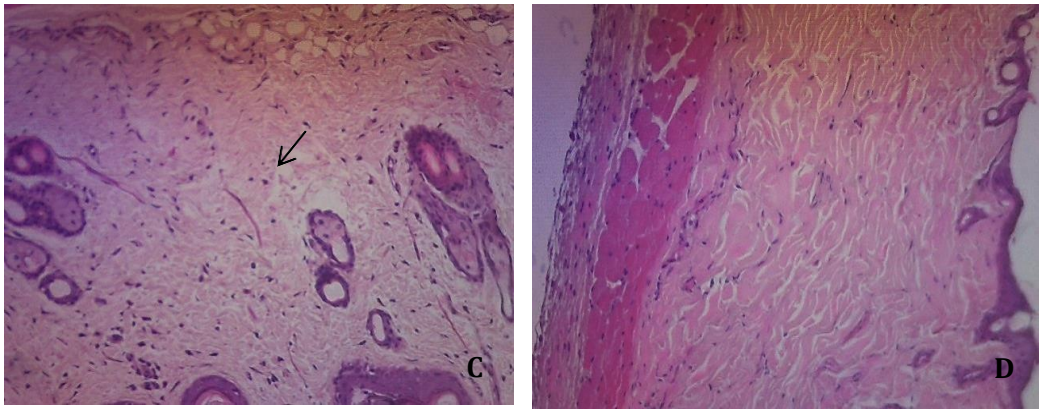



**Figure 2.** Histology of skin tissue with 400x magnification, using hematoxylin-eosin staining. Arrows indicate the degradation of collagen tissue. A) histology of skin tissue in the Control group; B) in the group that received 150 mg glutathione; C) in the group that received 200 mg glutathione; D) in the group that received 250 mg glutathione

**Table 3.** Results of analysis of the effect of glutathione for each group

| Dependent Variable | Group | Group | Mean Difference | Std. Error | Sig. |
|---|---|---|---|---|---|
| Proline Level | Control | P1 | 2.10000 | 3.22452 | 0.521 |
| | | P2 | -1.27143 | 3.22452 | 0.697 |
| | | P3 | -9.47143* | 3.22452 | 0.007 |
| | P1 | Control | -2.10000 | 3.22452 | 0.521 |
| | | P2 | -3.37143 | 3.22452 | 0.306 |
| | | P3 | -11.57143* | 3.22452 | 0.001 |
| | P2 | Control | 1.27143 | 3.22452 | 0.697 |
| | | P1 | 3.37143 | 3.22452 | 0.306 |
| | | P3 | -8.20000* | 3.22452 | 0.018 |
| | P3 | Control | 9.47143* | 3.22452 | 0.007 |
| | | P1 | 11.57143* | 3.22452 | 0.001 |
| | | P2 | 8.20000* | 3.22452 | 0.018 |
| Collagen Thickness | Control | P1 | -7.91571* | 1.29453 | 0.000 |
| | | P2 | -14.39714* | 1.29453 | 0.000 |
| | | P3 | -24.88571* | 1.29453 | 0.000 |
| | P1 | Control | 7.91571* | 1.29453 | 0.000 |
| | | P2 | -6.48143* | 1.29453 | 0.000 |
| | | P3 | -16.97000* | 1.29453 | 0.000 |
| | P2 | Control | 14.39714* | 1.29453 | 0.000 |
| | | P1 | 6.48143* | 1.29453 | 0.000 |
| | | P3 | -10.48857* | 1.29453 | 0.000 |
| | P3 | Control | 24.88571* | 1.29453 | 0.000 |
| | | P1 | 16.97000* | 1.29453 | 0.000 |
| | | P2 | 10.48857* | 1.29453 | 0.000 |

*. The mean difference is significant at the 0.05 level

## DISCUSSION

The results of this study showed that there was a significant increase in proline levels in mice that received 250 mg of glutathione supplementation. Previous research regarding the effect of administering exogenous glutathione on preventing the formation of free radicals due to exposure to cigarette smoke has never been carried out. The underlying mechanism is thought to originate from free radicals. Free radicals are unstable molecules and very reactive towards sub-cellular targets because they contain unpaired electrons. These free radicals can come from the body's metabolism or be obtained from outside sources, such as cigarette smoke. Free radicals can damage cellular structures such as cell membranes and macromolecules such as proteins, lipids, and nucleic acids (Caliri et al., 2021). Reactive oxygen species (ROS) can mediate the damage of various amino acids and turn these amino acids into oxidation products such as proline to 2-pyrrolidone, and glutamic semialdehyde (Phaniendra et al., 2015).

The cigarettes used in this study were mild unfiltered cigarettes. In previous studies, mainstream smoke from regular and light cigarettes had total ROS levels reaching 120-150 nmol and 90-110 nmol, respectively. In side stream smoke, ROS quantity levels reach 60-90 nmol (regular) and 30-70 nmol (light cigarette) (Zhao & Hopke, 2012). Apart from being able to damage various amino acids, ROS can also increase the activity of metalloproteinase enzymes, enzymes that degrade collagen, elastic fibers, and proteoglycans. Smoking is also involved in the aging process, and this is directly correlated with the number of pack-years. It is known that this happens to smokers who consume more than 40 packs of cigarettes per year (Lipa et al., 2021). Even in an experiment conducted by (Okada et al., 2013)on two twins who smoked and who did not smoke, it showed that the twins who smoked had significantly worse facial skin health, especially in the upper eyelids, malar bags, nasolabial crease, lower lid bags, and lower lip vermillion lines. A similar thing was also found in the twin who smoked > 5 years earlier than the other smoking twin.

Glutathione, which is sometimes referred to as "the mother of all antioxidants", is the main endogenous antioxidant produced by cells and plays a direct role in neutralizing free radicals (Adeoye et al., 2018). Glutathione can also increase the work of other antioxidants, such as vitamins C and E. Glutathione homeostasis disorders are often associated with various diseases such as premature aging, cardiovascular disease, cancer, and diabetes (Al-Temimi et al., 2023). The body's ability to synthesize glutathione decreases with age (Sharma & Sharma, 2022). This results in the body needing exogenous glutathione intake. Giving exogenous glutathione will increase the production of endogenous glutathione in cells (Al-Temimi et al., 2023). Thus, this is the basis for this research to use glutathione supplements rather than using other antioxidants.

Glutathione is a thiol-containing tripeptide compound consisting of glycine, L-cysteine, and L-glutamic acid, which is often studied as an antioxidant agent. This antioxidant defense effect of glutathione has a role in cell proliferation and death via the main redox regulatory signaling pathways in cells. Glutathione can prevent cell death due to oxidative stress (Kwon et al., 2019). It also has a role in the detoxification of toxins, xenobiotics, and as a medicine. Decreased glutathione levels are observed in various conditions, such as smokers, alcoholics, and patients with chronic liver disease. Administration of glutathione or its precursors (e.g. N-acetylcysteine) can reduce oxidative stress and the production of inflammatory mediators (Hristov, 2022).

In this study, smoking can trigger the degradation of the collagen layer in the skin. Smoking can cause damage to the skin by reducing capillary blood flow to the skin. This causes a decrease in oxygen and nutrient supply to skin tissue. Smokers also have a thinner layer of collagen and elastin in the dermis, which causes the skin to sag and lack elasticity (Farage et al., 2008). This

collagen damage occurs not only in the skin but also in other tissues, such as the lungs. This collagen damage is thought to be caused by an inflammatory response that triggers neutrophils to degrade collagen (Overbeek et al., 2013). Glutathione supplementation increases collagen production by increasing collagen contraction in dermal fibroblast cells in humans and protecting keratinocytes from apoptosis under unfavorable conditions (Watanabe et al., 2014).

This research is the first study conducted to examine the proline levels and collagen thickness in mice induced by cigarette smoke accompanied by glutathione supplementation. Until now, we have not found previous research data regarding normal levels of proline and glutathione in mice. Although theoretically the body can produce endogenous glutathione, from the results of our research increasing the dose of glutathione will provide benefits in the form of increasing proline and collagen thickness. The control group (without glutathione supplementation) had lower proline levels and collagen thickness than the group receiving 200-250 mg glutathione supplementation. In future research, it is hoped that we can first measure endogenous proline and glutathione levels before smoking exposure and compare other antioxidants in increasing proline levels and collagen thickness in the skin.

## CONCLUSION

Supplementing with 250 mg of glutathione significantly increases proline levels compared to other groups. The higher dose also effectively repairs collagen tissue damaged by cigarette smoke exposure. Considering that smoking can also reduce collagen production and proline levels, it is possible that glutathione supplementation at this dose can increase collagen production and proline levels in the body. This study has limitations in that it did not measure endogenous glutathione levels before and after exposure in the control and intervention groups. Future research should address this gap and compare glutathione's effects with other antioxidants commonly used for skin health, such as vitamin E.

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