



The efficacy of captopril and 5-fluorouracil combination in the proliferation and collagen deposition of keloid fibroblast

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ABSTRACT Keloid is a benign fibroproliferative tissue growth that exceeds the initial wound margins. Captopril has been tested in vitro to reduce fibroblast proliferation and collagen deposition; thus, it has potential for use in the treatment of keloids. Meanwhile, 5-fluorouracil (5-FU) has already been used in keloid management. This study aimed to determine the efficacy of the combination of captopril and 5-FU in keloid fibroblast cultures. Keloid tissues were cultured up to passages 4–7. The study consisted of a control group, captopril in various concentrations (10^{-2} , 10^{-3} , 10^{-4} and 10^{-5} mol/L), 5-FU 1 mg/mL and a combination of captopril at various concentrations with 5-FU 1 mg/mL. After 144 hours of treatment, fibroblast proliferation and collagen deposition were measured. The study showed a significant decrease in the mean index of fibroblast proliferation and collagen deposition in the group receiving captopril in various concentrations (10^{-2} , 10^{-3} , 10^{-4} and 10^{-5} mol/L) and the 5-FU group against the control group ($p < 0.05$). In the combined-dose group, captopril at a concentration of 10^{-2} mol/L and 5-FU showed a significant reduction in fibroblast proliferation and collagen deposition compared to the 5-FU group and the captopril at the same dose ($p < 0.05$). In conclusion, the combination of captopril 10^{-2} mol/L and 5-FU 1 mg/mL is better at reducing fibroblast proliferation and collagen deposition in keloid fibroblast cultures than captopril or 5-FU as a single therapeutic agent.

KEYWORDS 5-fluorouracil; captopril; collagen deposition; fibroblast proliferation; keloid fibroblast

1. Introduction

Keloids are benign fibroproliferative tissue growths that exceed the initial wound margin without spontaneous regression (Mari et al. 2016; Shaheen 2018). The term keloid comes from the Greek word meaning crab claw (Mari et al. 2016; Akaa et al. 2017). Keloids appear as solid masses of pink to purplish color with a shiny surface, well demarcated, irregular edges, sometimes accompanied by pain and itch (Gauglitz and Korting 2011). Keloids can cause cosmetic and functional problems that interfere with a person's quality of life. Keloids can cause cosmetic problems that lead to embarrassment, low self-esteem, anxiety disorders, and depression. Keloids at the joints can also cause joint contractures, thereby limiting joint movement (Salati 2019).

Keloids occur due to disturbances in the wound healing phase (Gauglitz and Korting 2011). Keloid fibroblasts have a lower rate of apoptosis than normal fibroblasts (Huang et al. 2010). In addition, keloid fibroblasts show an increased number of growth factor receptors and an exaggerated response to growth factors, such as transforming growth factor- β 1 (TGF- β 1) and platelet derived

growth factor (PDGF) (Vijayakumar et al. 2015).

Until now, there is no most effective therapeutic modality for the management of keloids (Mari et al. 2016; Shaheen 2018). In recent years, various studies have shown that angiotensin-converting enzyme (ACE) plays a role in the pathogenesis of various fibrotic diseases. In skin fibrosis, ACE activity was found to be significantly higher in pathological scars than in normal wounds (Morihara et al. 2006; Fang et al. 2018a). Angiotensin-converting enzyme inhibitors (ACEI) can inhibit TGF- β 1 and PDGF signal transduction, thereby reducing scar tissue formation. This makes ACEI have potential to be used in the treatment of keloids (Fang et al. 2018b). Captopril is an ACEI that has been tested on keloid fibroblasts. A study by Chen et al. (2016) showed that captopril could inhibit proliferation and collagen deposition of keloid fibroblast cells.

5-fluorouracil is a pyrimidine analog that inhibits the synthesis of deoxyribonucleic acid (DNA) by inhibiting the enzyme thymidine synthase, thereby triggering fibroblast apoptosis and scar degradation (Shah et al. 2016). The use of intralesional 5-FU as a single agent or in combi-

nation has shown good effectiveness in the treatment of keloids (Huang et al. 2013). As a single therapeutic agent, the lowest concentration of 5-FU which can inhibit fibroblast proliferation and cause apoptosis of keloid fibroblasts is at a concentration of 1 mg/mL during an incubation period of 144 hours (h) (Huang et al. 2010). Huang et al. (2013) who studied the effect of the combination of triamcinolone acetonide (TA) and 5-FU on keloid fibroblasts showed that 5-FU was more dominant in inhibiting fibroblast proliferation and causing apoptosis of keloid fibroblasts than TA.

Problems that arise due to keloids with no maximal keloid therapy encourage researchers to look for possible new therapeutic modalities that can be developed for the treatment of keloids. Previous studies have shown that combination therapy is better than single therapy for the treatment of keloids (Mari et al. 2016). In this study, we aimed to examine the efficacy of the combination of captopril and 5-FU on the proliferation and deposition of collagen in keloid fibroblast cultures. To the best of our knowledge, there have been no previous studies combining these two agents in cultured keloid fibroblasts.

2. Materials and Methods

2.1. Keloid fibroblast cell culture

Three human keloid samples identified by dermatologist were obtained from patients who gave informed consent for the use of their tissues for research purposes. None of the three patients had undergone treatment for their keloids. The research was approved by the Medical and Health Research Ethics Committee of the Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada.

The keloid fibroblast cells were harvested from the keloid tissue samples and tested separately. The keloid tissue samples were disinfected with 10% povidone iodine and washed with phosphate-buffered saline (PBS). Then the epidermis was cut and removed from the tissue. The dermal layer was minced into 0.5 cm×0.5 cm in size. These dermal tissues were placed in a culture flask, immersed in complete medium consisted of Dulbecco's Modified Eagle's Medium (DMEM, Gibco, USA) with 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin/streptomycin (Gibco, USA). The flask was incubated with 5% CO₂ at 37 °C continuously until the tissues adhered to the bottom of the flask. The medium was changed every 72 h until 80-90% of the fibroblast outgrowth could be observed under microscope. The cells then were sub-cultured using PBS containing 0.25% trypsin (Gibco, USA). After trypsinization, the fibroblast cells were incubated for 3-4 minutes (min) and were observed under microscope. The detached cells appeared rounded under microscope. Then, they were harvested and immersed in complete medium as mentioned above. Only cells of passages 4-7 were used in the present research. The fibroblast cells were cultured in the 96-well

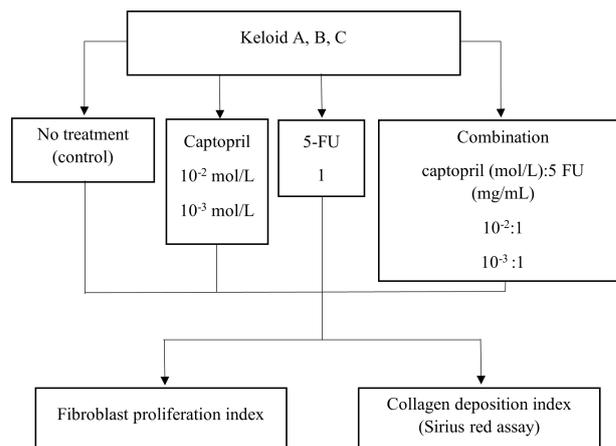


FIGURE 1 Summary of the experimental research design

plate (Iwaki, Japan) in concentration of 5,000 cells/plate and incubated for 48 h before receive any treatment.

2.2. The treatment of keloid fibroblast

This study consisted of a control group, captopril in various concentrations (10^{-2} , 10^{-3} , 10^{-4} , and 10^{-5} mol/L), 5-FU 1 mg/mL, and a combination of captopril in various concentrations with 5-FU 1 mg/mL. Captopril has high solubility and dissolves completely in water. Forty milligrams of powder captopril (Sigma, USA) were dissolved in 1.84 mL water to get a solution of 10^{-1} mol/L captopril. By using $M1.V1 = M2.V2$, the volume will be obtained for the manufacture of various concentration of captopril (10^{-2} , 10^{-3} , 10^{-4} , and 10^{-5} mol/L). 5-FU (Kalbe Farma, Indonesia) is available on liquid solution. Each of the 15 mL centrifuge tubes (Falcon, Mexico) was labeled. Each contained a 2,000 μ L solution that consisted of captopril, 5-FU, water, and complete medium according to the research design (Figure 1). The control was a mixture of water and complete medium. After the fibroblasts adhered to the bottom of the well, the supernatant was removed, and each well was filled with 200 μ L of various drug concentrations. Each group was duplicated three times.

2.3. Fibroblast proliferation

After 144 h of treatment, fibroblast proliferation was measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay. The cells were washed using PBS, then 200 μ L of DMEM containing 10% FBS and 50 μ L of MTT solution (Bio Basic, Canada) were added into each well. The well plate was then incubated with 5% CO₂ at 37 °C for 4 h. After incubation, 200 μ L of dimethyl sulfoxide (DMSO, Merck, Germany) was added into each well and the well plate was shaken in a shaker for 10 min. The formazan concentration was measured using a spectrophotometer at a wavelength of 570 nm. The fibroblast proliferation index of the control group was assumed to be 100%, while the fibroblast proliferation index in the treatment group was calculated as follows (1).

The fibroblast proliferation index in the treatment group

$$= \frac{\text{Cellular viabilities in the treated group}}{\text{Cellular viabilities in the control group}} \times 100\% \quad (1)$$

2.4. Collagen deposition

The deposition of collagen was measured using Sirius red assay after 144 h of treatment. After washed with PBS, the cells were fixated with Bouin's solution for 1 h. Bouin's solution is composed of 75 mL picric acid (Smart Lab, Indonesia), 25 mL of 40% formaldehyde (Smart Lab, Indonesia), and 5 mL glacial acetic acid (Smart Lab, Indonesia). After fixation, the well plates were washed with running water and dried at room temperature for 24 h. Then, 200 μ L of Sirius red solution was added into each well and incubated for 1 h. Sirius red solution was made by combining 0.2 gram of Direct Red (Sigma, USA) with 200 mL of picric acid (Smart Lab, Indonesia). Then, the well plates were washed with 0.1 N hydrochloric acid (Smart Lab, Indonesia) and 200 μ L of sodium hydroxide (Smart Lab, Indonesia) was added into each well. The optical density of deposited collagen was read with a spectrophotometer at a wavelength of 550 nm. The collagen deposition index of the control group was assumed to be 100% and for the treatment group it was calculated by formula (2).

Collagen deposition index in the treatment group

$$= \frac{\text{Collagen deposited in the treated group}}{\text{Collagen deposited in the control group}} \times 100\% \quad (2)$$

2.5. Statistical analysis

The data were analyzed using SPSS version 23.0 (IBM Corp., Armonk, NY). The Shapiro-Wilk test on the fibroblast proliferation index and collagen deposition showed the data were normally distributed, so the analysis used the ANOVA test and continued with Fisher's LSD tests. The results were statistically significant if the *p* value was <0.05.

3. Results and Discussion

3.1. Results

3.1.1 The proliferation of keloid fibroblast

Figure 2 shows the picture of blue formazan formation after MTT assay. The group that received captopril at a concentration of 10^{-2} – 10^{-5} mol/L and the group that received 5-FU showed a decrease in the proliferation of keloid fibroblasts. The combination of captopril in various concentrations and 5-FU showed a decrease in keloid fibroblast proliferation compared to captopril at the same concentration as a single therapeutic agent. The highest decrease in fibroblast proliferation was observed in the combination of captopril at a concentration of 10^{-2} mol/L and 5-FU.

Figure 3 shows the group that received 5-FU 1 mg/mL has a significant decreased in the mean fibroblast proliferation index after 144 h of treatment (34.43 ± 1.97). The mean index of keloid fibroblast proliferation was also

found to be significantly lower in captopril at a concentration of 10^{-2} – 10^{-5} mol/L after 144 h of treatment, with the highest decrease seen in the captopril at a concentration of 10^{-2} mol/L (43.59 ± 3.06). The group that received 5-FU showed a significantly lower mean fibroblast proliferation index than captopril in various concentrations as a single therapeutic agent. Captopril at a concentration of 10^{-2} mol/L combined with 5-FU decreased the proliferation of keloid fibroblasts significantly (22.48 ± 3.05) compared to the 5-FU group. Captopril at a concentration of 10^{-3} – 10^{-5} mol/L combined with 5-FU did not show a significant difference in the proliferation of keloid fibroblasts compared to the 5-FU group.

3.1.2 Collagen deposition

Figure 4 shows the picture of collagen deposition after Sirius red staining. The group that received captopril at a concentration of 10^{-2} – 10^{-5} mol/L and the group that received 5-FU as a single therapeutic agent showed a decrease in collagen deposition. The group that received the combination of captopril in various concentrations and 5-FU showed a decrease in collagen deposition compared to the captopril group at the same concentration as a single therapeutic agent. The highest decrease in collagen deposition was observed in the combination group of captopril 10^{-2} mol/L and 5-FU.

As can be seen in Figure 5, the 5-FU group showed a significant decrease in the mean collagen deposition index after 144 h of treatment (60.85 ± 0.58). The mean index of collagen deposition was found to be significantly lower in captopril at a concentration of 10^{-2} – 10^{-5} mol/L after 144 h of treatment with the highest decrease seen in captopril concentration of 10^{-2} mol/L (63.01 ± 0.68). Collagen deposition was found to be significantly lower in the group receiving 5-FU compared to the captopril group at a concentration of 10^{-2} – 10^{-5} mol/L as a single therapeutic agent. The results of this study showed that captopril at a concentration of 10^{-2} mol/L combined with 5-FU decreased collagen deposition significantly compared to the 5-FU (50.37 ± 0.66). Captopril at a concentration of 10^{-3} – 10^{-5} mol/L combined with 5-FU but did not show a significant difference to 5-FU group.

3.2. Discussion

3.2.1 The proliferation of keloid fibroblast

In this study, the group that received captopril at a concentration of 10^{-2} – 10^{-5} mol/L for 144 h showed a significant reduction in keloid fibroblast proliferation (*p*<0.05). This is in accordance with a study by Chen et al. (2016) who found that captopril at a concentration of 10^{-2} – 10^{-7} mol/L could significantly reduce the proliferation of keloid fibroblasts. Captopril dramatically decreased the expression of TGF- β 1 and PDGF, two major growth factors that increase keloid fibroblast proliferation. Although no statistically significant difference was observed in the 10^{-2} – 10^{-7} mol/L group after 48 h of treatment, some indications of a dose-dependent relationship between fibroblast prolifera-

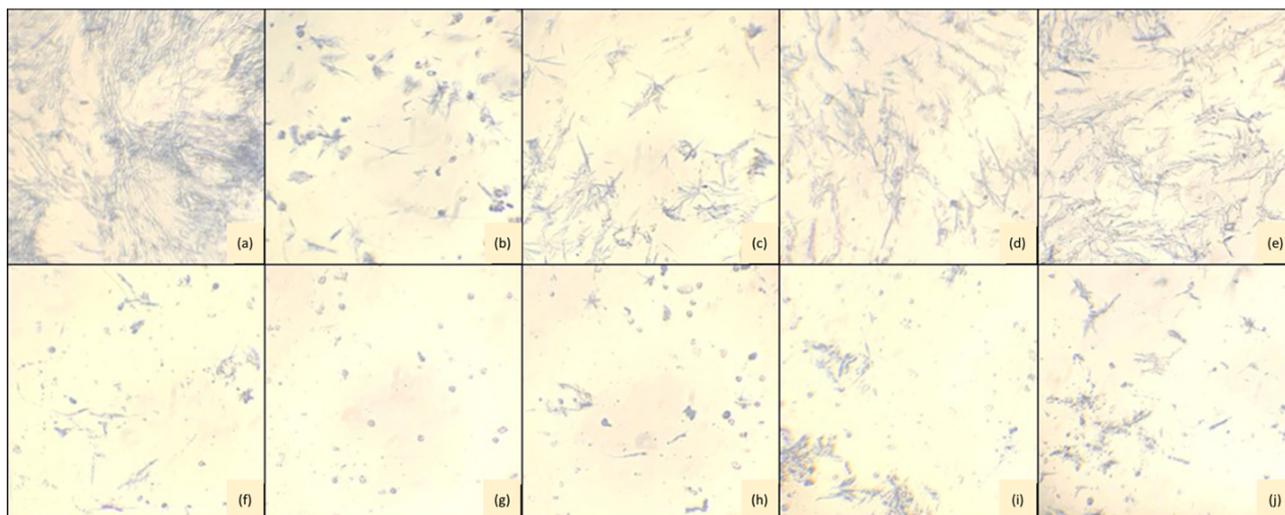


FIGURE 2 Blue formazan formation after MTT assay in various groups (under microscope, $\times 10$). (a) Control. (b) Captopril 10^{-2} mol/L. (c) Captopril 10^{-3} mol/L. (d) Captopril 10^{-4} mol/L. (e) Captopril 10^{-5} mol/L. (f) 5-FU 1 mg/mL. (g) Captopril 10^{-2} mol/L + 5-FU 1 mg/mL. (h) Captopril 10^{-3} mol/L + 5-FU 1 mg/mL. (i) Captopril 10^{-4} mol/L + 5-FU 1 mg/mL. (j) Captopril 10^{-5} mol/L + 5-FU 1 mg/mL.

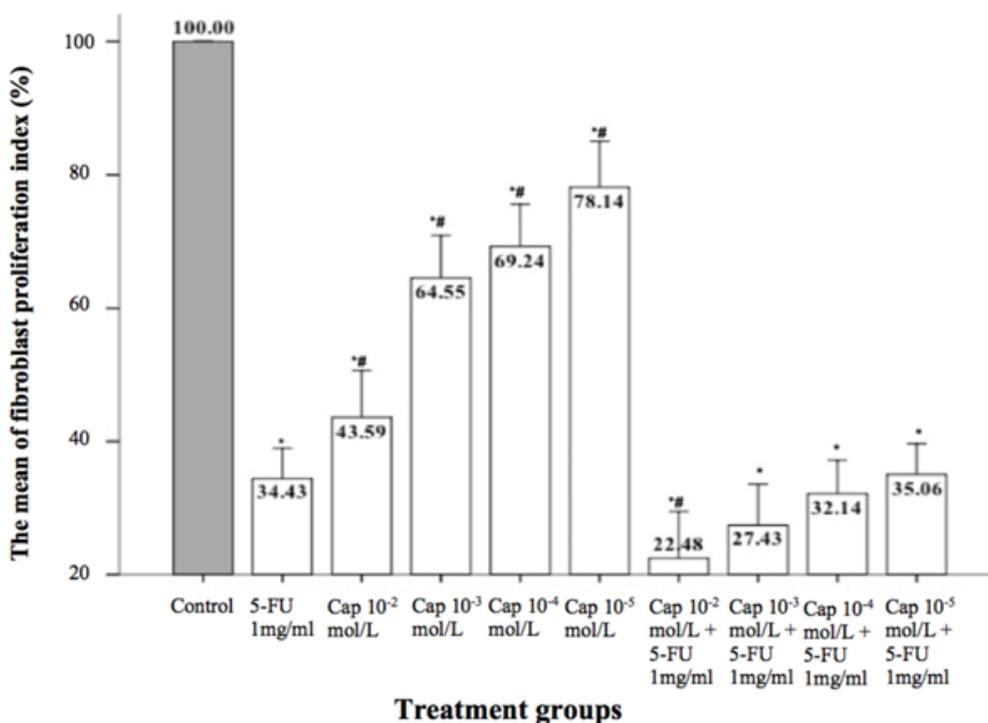


FIGURE 3 Comparison of the mean fibroblast proliferative index in various treatment groups. Graph indicates means value from triplicate \pm standard error. *, significant to control ($p < 0.05$); #, significant to 5-FU ($p < 0.05$).

tion and the concentration of captopril could be observed (Chen et al. 2016). This study found a significant decrease in keloid fibroblast proliferation between various concentrations of captopril after treatment for 144 h. This may be caused by the longer duration of treatment whereby the effect of inhibition of fibroblast proliferation can be observed more clearly. Huang et al. (2010) suggested that drugs with smaller doses generally require a longer incubation time to show the effect of reducing fibroblast pro-

liferation (Huang et al. 2010).

The 5-FU 1 mg/mL group showed a significant reduction in fibroblast proliferation ($p < 0.05$). This is in accordance with research by Huang et al. (2010) who stated that the lowest concentration of 5-FU which can inhibit fibroblast proliferation and cause apoptosis of keloid fibroblasts is at a concentration of 1 mg/mL during an incubation period of 144 h. Administration of 5-FU 1 mg/mL can inhibit fibroblast proliferation, stop the cell cycle in the G2/M

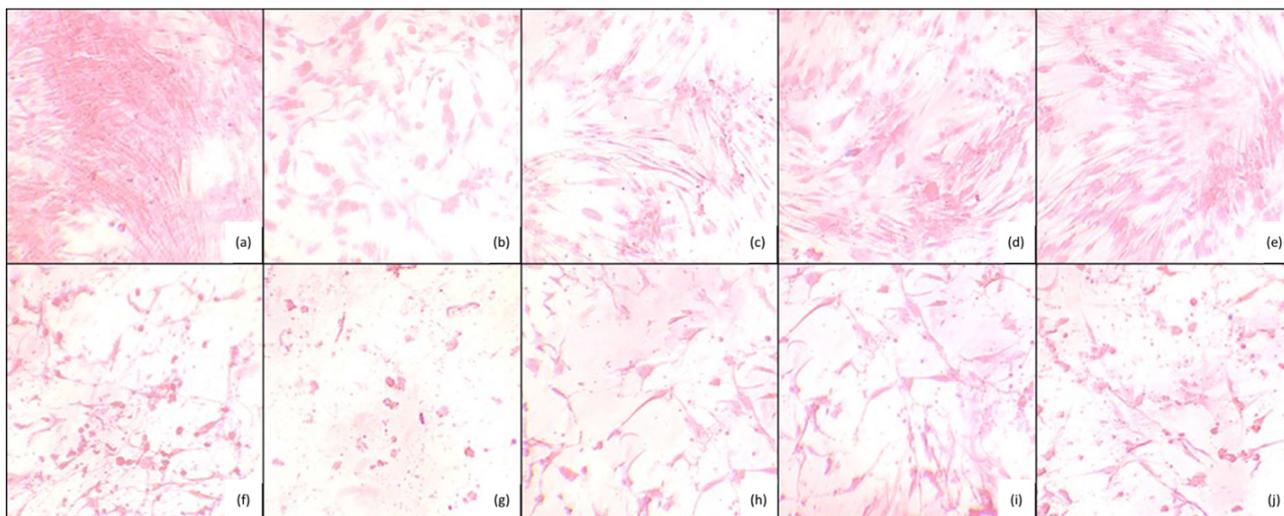


FIGURE 4 The collagen deposition shown by Sirius red staining in various groups (under microscope, ×10). (a) Control. (b) Captopril 10⁻² mol/L. (c) Captopril 10⁻³ mol/L. (d) Captopril 10⁻⁴ mol/L. (e) Captopril 10⁻⁵ mol/L. (f) 5-FU 1 mg/mL. (g) Captopril 10⁻² mol/L + 5-FU 1 mg/mL. (h) Captopril 10⁻³ mol/L + 5-FU 1 mg/mL. (i) Captopril 10⁻⁴ mol/L + 5-FU 1 mg/mL. (j) Captopril 10⁻⁵ mol/L + 5-FU 1 mg/mL.

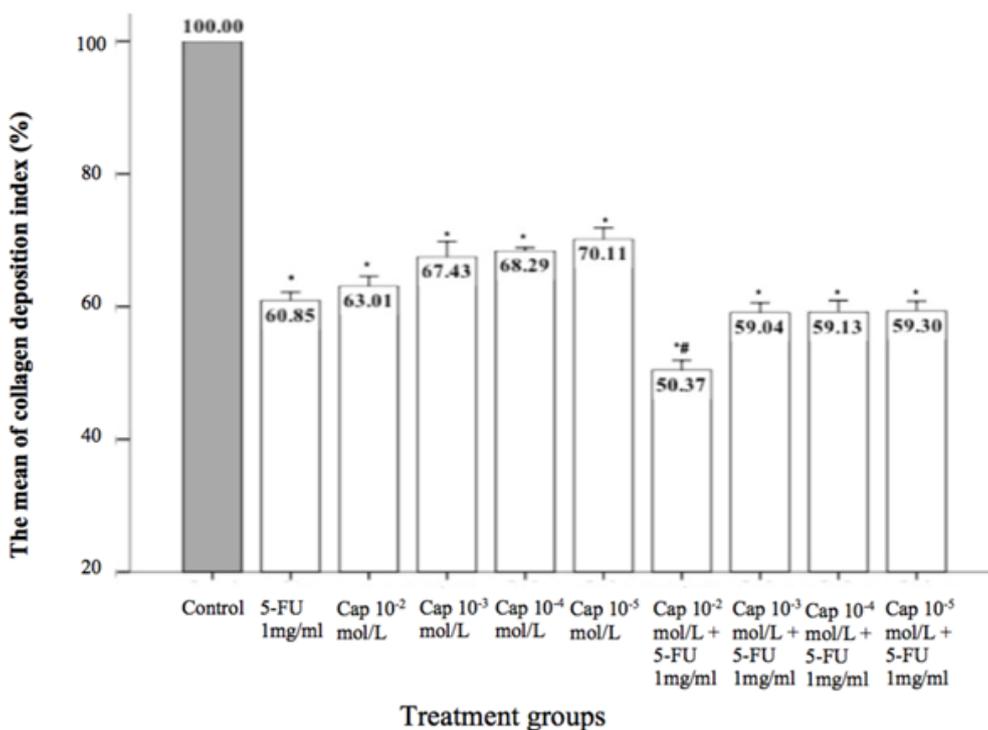


FIGURE 5 Comparison of the mean collagen deposition index in various treatment groups. Graph indicates means value from triplicate ± standard error. *, significant to control ($p < 0.05$); #, significant to 5-FU ($p < 0.05$)

phase, and cause apoptosis of keloid fibroblasts after 144 h of exposure (Huang et al. 2010).

In this study, the decrease in keloid fibroblast proliferation was greater in the group receiving 5-FU than the group receiving captopril at a concentration of 10⁻²–10⁻⁵ mol/L as a single therapeutic agent. 5-FU can inhibit DNA synthesis in keloid fibroblasts and causing fibroblast apoptosis (Huang et al. 2013). The ability of 5-FU to cause apoptosis of keloid fibroblasts may cause a greater reduction in keloid fibroblast proliferation in the group receiving

5-FU than the group receiving captopril as a single therapeutic agent.

3.2.2 Collagen deposition

Captopril at a concentration of 10⁻²–10⁻⁵ mol/L showed a significant decrease in the average collagen deposition index ($p < 0.05$). This is in accordance with Chen et al. (2016) who stated that captopril at a concentration of 10⁻²–10⁻⁵ mol/L was able to reduce the expression of type I colla-

gen. Captopril is also known to decrease the expression of HSP47, a protein that plays a role in collagen synthesis. Although no statistically significant difference was observed in the 10^{-2} – 10^{-7} mol/L group after 48 h of treatment, some indications of a dose-dependent relationship between the expression of type I collagen and the concentration of captopril could be observed (Chen et al. 2016).

The group that received 5-FU showed a significant decrease in collagen deposition after 144 h of treatment ($p < 0.05$). The mechanism of action of 5-FU in reducing collagen deposition is by inhibiting the production of type I collagen (Mauviel et al. 2003). The results in this study are different from previous study by Kurniaty et al. (2020) which showed that there was no significant reduction in collagen deposition after administration of 5-FU 1 mg/mL after 72 h of treatment (Kurniaty et al. 2020). The difference in results occurred because the administration of 5-FU 1 mg/mL for 72 h had not shown a significant inhibitory effect of type I collagen so that collagen deposition was not found to have a significant decrease (Huang et al. 2013).

A study by Huang et al. (2013) showed that the combination of TA 20 M and 5-FU 1 mg/mL significantly inhibited type I collagen production in keloid fibroblast cultures after 72 h of treatment, but the inhibitory effect was found to be not significant with TA or 5-FU as a single therapeutic. In this study, a significant reduction in collagen deposition in the control group was seen in the group receiving the combination of captopril in various concentrations and 5-FU 1 mg/mL as well as the group receiving either captopril or 5-FU as a single therapeutic agent. This may be due to the longer treatment duration so that the effect of decreasing collagen deposition can be observed more clearly (Huang et al. 2010).

4. Conclusions

The combination of captopril 10^{-2} mol/L and 5-FU 1 mg/mL was better in reducing the proliferation and deposition of collagen in keloid fibroblasts than the administration of captopril or 5-FU as a single therapeutic agent. This study is the first study to determine the efficacy of the combination of captopril and 5-FU in cultured keloid fibroblasts.

Authors' contributions

JA designed the study, conducted the laboratory work, analyzed the data, and wrote the manuscript. ASS and YWW gave the suggestion to the manuscript written and to the statistical analysis of the data. All authors read and approved the final version of the manuscript

Competing interests

All of the authors declare that there is no competing financial, professional, or personal interest that might have

influenced this manuscript.

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