Original Article

Inhibition of ALA dehydratase activity in heme biosynthesis reduces cytoglobin expression which is related to the proliferation and viability of keloid fibroblasts

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The aim of this study was to analyze the effect of heme synthesis inhibition on cytoglobin expression and its correlation with keloid fibroblast viability and proliferation. The study was conducted on primary culture of keloid fibroblasts. Heme synthesis in keloid fibroblasts was inhibited using succinyl acetone. We measured amino levulinic acid dehydratase (ALAD) enzyme activity using a colorimetric method; cytoglobin mRNA expression using qRT-PCR, cytoglobin protein expression using ELISA and immunocytochemistry, fibroblast viability using the MTT test; and fibroblast proliferation using BrdU test. The results showed that the ALAD enzyme activity level was lower in the keloid fibroblasts treated with succinyl-acetone (SA, 1, 2.5, and 5 mM) than in the control. The cytoglobin mRNA and protein expressions level were significantly lower in the keloid fibroblasts cultured with 2.5 mM and 5 mM SA than in the control and 1 mM SA. The viability and proliferation of the keloid fibroblasts decreased when the SA concentration was increased. In conclusion, the use of succinyl acetone at a concentration of 1; 2.5; and 5 mM caused decrease ALAD enzyme activity which indicated the inhibition of the heme synthesis. Inhibition of heme synthesis can affect cytoglobin expression, which correlates with the viability and proliferation of keloid fibroblasts.

***Key Words*: keloid fibroblast, heme, succinyl acetone, cytoglobin, proliferation**

Keloid is a benign tumor caused by improper skin wound healing characterized by an excessive deposition of collagen, an extracellular matrix protein.[1,2] The collagen synthesis in keloid tissue is up to 20 times that in normal tissue.[3,4] This excessive collagen synthesis is induced by enhanced fibroblast activation and proliferation, with a lower rate of cell death than that in normal wound healing processes.[5]

Excessive fibroblast proliferation and collagen synthesis are undoubtedly related to increased energy needs. To meet these energy needs, cells require adenosine triphosphate (ATP), which is mostly produced by oxidative phosphorylation (OXPHOS) in mitochondria[6] and is related to biogenesis and mitochondrial function. A sufficient oxygen supply is needed to support mitochondrial biogenesis and optimal ATP production, and heme is known to play important roles in these processes. Heme also plays a significant role in supporting the energy formation process because it is a prosthetic group of hemoproteins involved in energy production in mitochondria and in oxygen transport.[6–8]

Heme is a complex of iron (Fe) atoms bound to protoporphyrin IX, which is synthesized through eight enzymatic steps in the mitochondria and cytosol.[6–8] Heme biosynthesis is a mechanism underlying the regulation of the availability of intracellular hemes and allows the formation of globin proteins. Heme levels can regulate the synthesis of globin proteins in hemoglobin, both at the transcriptional and translational levels.[9,10] Several studies have proven that the inhibition of heme biosynthesis using succinyl acetone (SA) can decrease lung cancer and HeLa cell proliferation and the expression levels of hemoproteins such as cytoglobin (CYGB).[11,12]

Wulandari's study (2016) showed that CYGB expression levels are higher in keloid tissue than in normal tissue.[13] CYGB is a hemoprotein that requires heme to perform functions related to intracellular oxygen transport.[14] Our previous study showed that inhibiting CYGB expression using siRNA reduces mitochondrial biogenesis in keloid fibroblasts, as shown by the lower expression levels of PGC1-mRNA and protein.[15] In addition, the inhibition of CYGB expression using siRNA tends to reduce the activity level of the enzyme succinate dehydrogenase (SDH), which plays a role in the energy formation process in the tricarboxylic acid (TCA) cycle.[15] Although inhibition of CYGB expression using siRNA tends to decrease mitochondrial biogenesis and SDH activity levels, its effect on keloid fibroblast proliferation is still unclear.[16] Hence, the aim of this study was to analyze the effect of heme synthesis inhibition on CYGB expression levels and its correlation with the proliferation and viability of fibroblast keloids.

Methods

Study design. This study was an *in vitro* experiment using fibroblasts isolated from primary cultures of keloid tissues. The samples were cultured in heme-depleted serum (HDS) with (treatment groups) or without SA (control group) for 24 h. The concentrations of SA were 1, 2.5, and 5 mM. The research was conducted from September 2021 to June 2022 at the laboratories of the Biochemistry and Molecular Biology Department, Histology Department of the Faculty of Medicine of Universitas Indonesia, and Stem Cells and Tissue Engineering Cluster IMERI. The study was approved by the Faculty of Medicine Universitas Indonesia Research Committee (No. KET-543/UN2.F1/ETIK/PPM.00.02/2021).

Isolation of fibroblasts from primary cultures. Keloid tissue samples were obtained from two women who gave birth by

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cesarean section at Mitra Gading Serpong Hospital, Jakarta, and who had keloids from their previous caesarean section. All keloid samples were obtained from the patients with informed consent. The characteristics of the keloid samples were confirmed by hematoxylin-eosin and Masson's trichrome staining. Keloid fibroblast cells were isolated using the explant culture method, as in our previous study.[17,18] The keloid samples were cut into 3- × 3-mm tissue fragments. Then, each fragment was grown onto a 24-well plate in Dulbecco's Modified Eagle Medium (DMEM) (Cat No. 11885084; Gibco, New York, NY) with 10% fetal bovine serum (Cat No. A4766901; Gibco), 1% penicillin-streptomycin (Cat No. 15140122; Gibco), and 1% amphotericin (Cat No. 15290026; Gibco), and incubated at 37°C and 5% CO_2. With up to 80% confluence, the cells were harvested. The fibroblast population was analyzed using flow cytometry with CD73+, CD105+, and CD90+ marker antibodies (Cat No. 562245; BD Stemflow; Amersham Biosciences Corp., Piscataway, NJ). Fibroblasts from 4 to 5 passages were used in this research.

Inhibition of heme biosynthesis in keloid fibroblasts. Heme biosynthesis inhibition in fibroblast keloids was performed using SA (Cat No. D1415; Sigma-Aldrich, Champaign, IL) Initially, at a density of 3×10^5 cells per well, keloid fibroblasts were cultured in DMEM with 10% HDS, 1% penicillin-streptomycin, and 1% amphotericin, and incubated at 37°C with 5% CO_2 for 24 h. HDS was prepared as previously described by Yuan *et al.*[19] After 24 h, the cells were treated with SA at concentrations of 1, 2.5, and 5 mM. Then, the protein and RNA from each group of cells were extracted for further analysis.

Protein isolation for determination of amino levulinic acid dehydratase (ALAD) enzyme activity and CYGB protein. A radioimmunoprecipitation assay lysis extraction buffer (Cat No. 89900; Thermo Fisher Scientific, Rockford, IL) was used to isolate the protein from each cell group. The concentration of the total protein from each group was measured using the Bradford assay (Cat No. 5000006; Bio-Rad, Hercules, CA).

Activity level of ALAD. The activity level of the ALAD enzyme was measured using a colorimetric method based on Fujita's study.[20] This method measures the level of porphobilinogen, a product of ALAD enzyme activity, in the form of a porphobilinogen-Ehrlich salt at a 555-nm wavelength. First, the protein samples were added to 100 mM tris-acetic acid buffer at pH 7 and 100 mM ALA-HCl (Cat No. A3785; Sigma-Aldrich) and then incubated at 37°C for 3 h. After that, 10% TCA/0.1 M $HgCl_2$ was added and centrifuged at 1,000 × *g* for 5 min. Finally, the absorbance of the porphobilinogen-Ehrlich salt was read at 555 nm using a spectrophotometer.

Total RNA isolation. For total RNA isolation from both the untreated and treated groups, initially, the cells were seeded in a 6-well plate at a density of 3×10^5 cells per well. Total RNA from each group of cells was isolated after 24 h of treatment. RNA was isolated using a Quick-RNA MiniPrep extraction kit (Cat No. R1057; Zymo Research, Irvine, CA) in accordance with the manufacturer's protocol.

Measurement of the relative gene expression of CYGB using quantitative real-time polymerase chain reaction (qRT-PCR). The SensiFast SYBR no-ROX kit (Cat No. BIO-72005; Bioline, UK) was used in accordance with the manufacturer's instructions to carry out reverse transcription and polymerase chain reaction (PCR) in a single step. A Bio-Gener Q3200 system was used to perform real-time PCR (RT-PCR). The primers for CYGB were: forward 5'-CAGTTCAAGCAC ATGGAGGA-3' and reverse: 5'-GTGGGAAGTCACTGGCAA AT-3'.[18] The primers for β-actin (reference gene) were: forward 5'-CACAGGGGAGGTGATAGCAT-3' and reverse 5'-CACGAA GGCTCATCATTCAA-3'.[11] RT-PCR amplification was performed for each sample in triplicate. The Livak formula was used to calculate the relative expression of CYGB.

Measurement of CYGB protein expression levels. Enzyme-linked immunosorbent assay (ELISA) and immunocytochemistry (ICC) methods were used to determine the expression levels of the CYGB protein. An ELISA kit (Cat No. E-EL-H2471; Elabscience, UK) was used in accordance with the manufacturer's protocol. The CYGB concentration was measured using a Hycultbiotech ELISA calculator with a four-parameter logistic regression analysis.

For the ICC assay, the keloid fibroblast cells from each group were seeded at a density of 1.5×10^3 cells/well in a cell culture slide chamber (Cat No. 07-2108; Biologix) in a HDS medium for 72 h at 37°C and 5% CO_2. After 72 h, the cells were treated with SA at different concentrations (1, 2.5, and 5 mM) for 24 h. Then, the medium was discarded, and the cells were washed three times using 0.1 M PBS at pH 7.2, followed by fixation using ice-cold methanol for 30 min. The cells were then washed again and blocked using 3% H_2O_2 solution for 30 min, followed by blocking using 5% BSA solution for 1 h. The cells were incubated with a CYGB primary antibody (1:100 dilution, sc-365246; Santa Cruz Biotechnology Inc., Dallas, TX) for 2 h, followed by incubation with IgG-HRP secondary antibody (1:500 dilution, Cat No. 31340; Thermo Fisher Scientific) for 1 h. The cells were then stained with 3,3'-diaminobenzidine (DAB) and counter-stained with hematoxylin. The results were documented using Optilab Viewer 3.0 and analyzed using ImageJ.

Measurements of cell viability and proliferation. To evaluate the effects of SA on keloid fibroblast viability and proliferation, the cells were seeded individually in a 96-well plate at a density of 1.5×10^3 cells per well in a HDS medium for 24 h. After 24 h, the cells were treated with 1, 2.5, and 5 mM SA. The viability of the keloid fibroblasts was measured using an MTT assay kit (Cat No. E-CK-A341; Elabscience), and their proliferation was measured using a BrdU assay kit (Cat No. ab126556; Abcam, Cambridge, MA).

Statistical analysis. Data from the triplicate experiment are presented as means with their SD. A one-way analysis of variance (ANOVA) followed by a least significant difference (LSD) post hoc test was used for normally distributed data. Meanwhile, for non-normally distributed data, the statistical analysis was performed using non-parametric tests, namely Kruskal–Wallis and Mann–Whitney post hoc tests. For the correlation analysis, we used the Spearman test. Statistical significance was set at a α value of 5% ($p<0.05$).

Results

ALAD enzyme activity in keloid fibroblasts. The ALAD enzyme activity in keloid fibroblasts cultured with a medium containing SA is shown in Fig. 1. The used of SA cause a decrease of ALAD enzyme activity in keloid fibroblast. The ALAD enzyme activity was significantly decreased in the keloid fibroblasts cultured in media containing 2.5 mM SA compared with the control and 1 mM SA. The ALAD enzyme activity in the keloid fibroblast cultured in media containing 5 mM SA also significantly decreased compared to control, 1 mM SA, and 2.5 mM SA. These results indicate the inhibition of heme biosynthesis in keloid fibroblasts.

CYGB mRNA expression in keloid fibroblasts. The effects of SA on the CYGB mRNA expression in keloid fibroblasts are shown in Fig. 2. The results showed that the CYGB mRNA expression decreased with the increases in the SA concentrations. The CYGB mRNA expression was significantly lower in the keloid fibroblasts cultured with 2.5 mM SA than in the control and 1 mM SA. The CYGB mRNA expression is also significantly lower in keloid fibroblast cultured in medium containing 5 mM SA compared to control and 1 mM SA.

CYGB protein expression in keloid fibroblasts. The CYGB protein expression in the keloid fibroblasts cultured in

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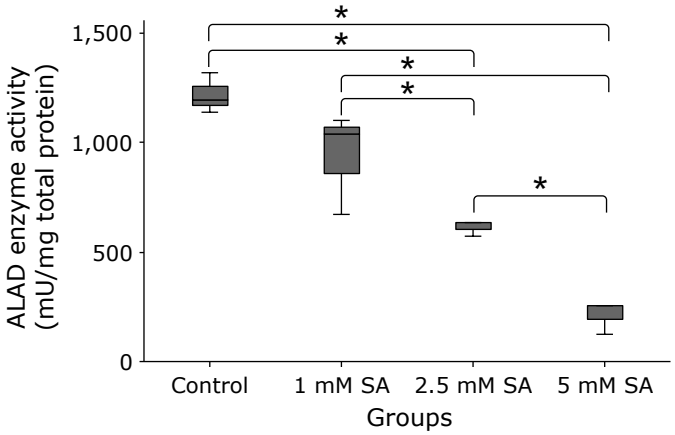

Fig. 1. ALAD enzyme activity of keloid fibroblast cultured in medium containing succinyl acetone (SA). ALAD enzyme activity was lower in keloid fibroblast cultured with SA (1, 2.5, and 5 mM) compared to control. A significant decrease in ALAD activity was found between the group with SA (Kruskal–Wallis, Mann–Whitney *p<0.05, n = 3).

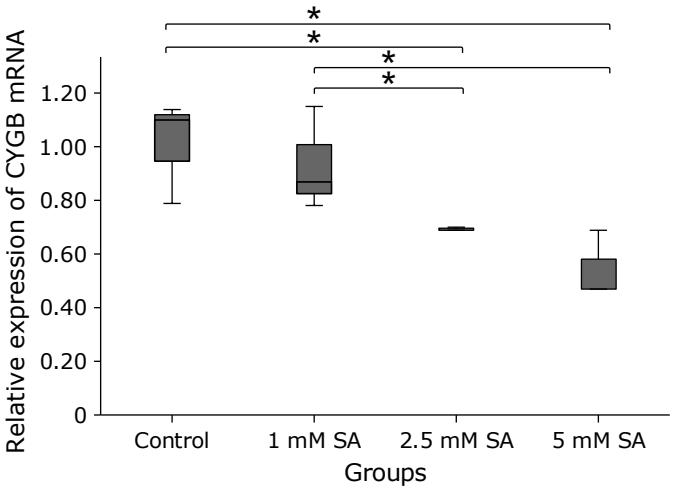

Fig. 2. CYGB mRNA expression of keloid fibroblast cultured in medium containing succinyl acetone (SA). The expression of CYGB mRNA was lower in keloid fibroblast cultured with SA (1, 2.5, and 5 mM) compared to control. The CYGB mRNA expression was significantly lower in keloid fibroblast cultured with 2.5 mM SA and 5 mM SA compared to control and 1 mM SA. (Kruskal–Wallis, Mann–Whitney *p<0.05, n = 3). The CYGB mRNA relative expression was measured using Livak method ($2^{-\Delta\Delta CT}$).

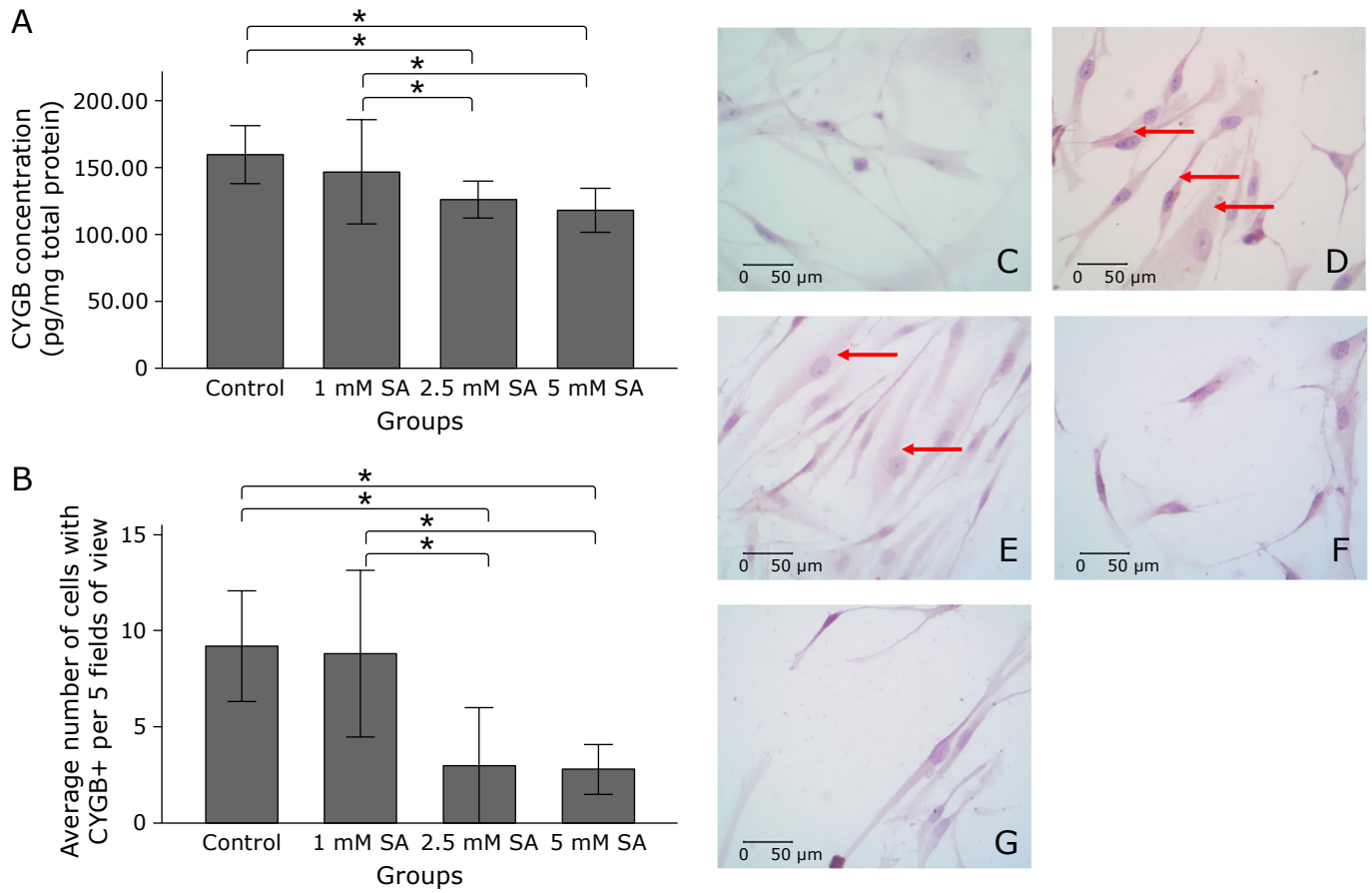

Fig. 3. The effect of succinyl acetone (SA) to CYGB protein expression in keloid fibroblast. (A) The concentration of CYGB protein (pg/mg total protein), (B) The number of keloid fibroblast with CYGB positive per 5 field of views, (C) Unstained keloid fibroblast (negative control), (D) Keloid fibroblast density in untreated medium, (E) Keloid fibroblast density in treated medium with 1 mM SA, (F) 2.5 mM SA, and (G) 5 mM SA. Red arrow showed the CYGB positive cells, with the cytosolic areas stained with brown DAB. Statistical analysis was done using One-way Anova with LSD as post-hoc test, *p<0.005. See color figure in the on-line version.

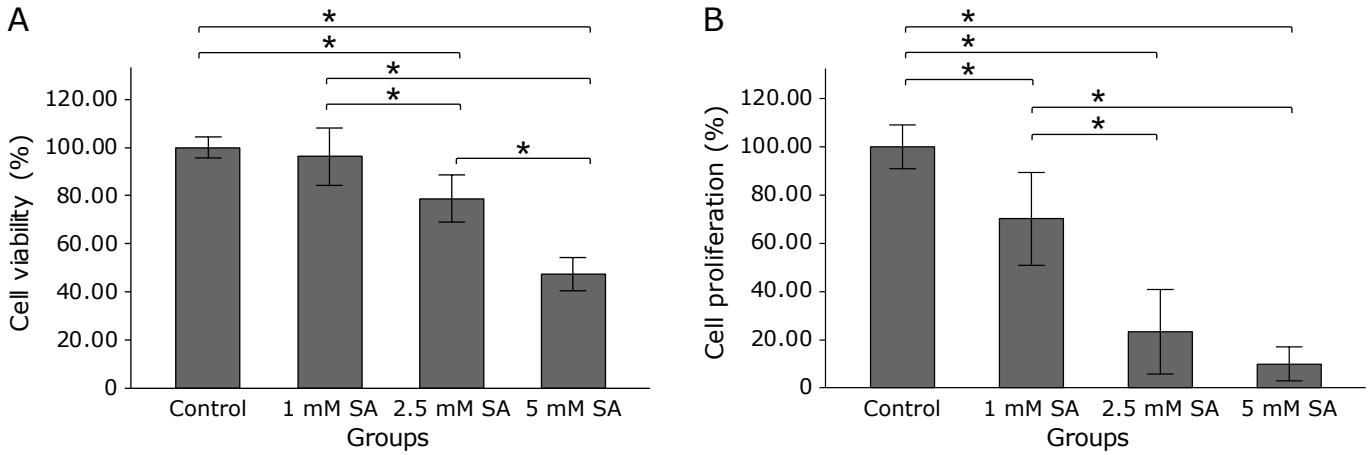

Fig. 4. Viability and proliferation of keloid fibroblast cultured in medium containing succinyl acetone (SA). The viability (A) and proliferation (B) of keloid fibroblast were lower when cultured in medium with 1, 2.5, and 5 mM SA compared to control. Data was normalized to control (One-way ANOVA, LSD, *p<0.005; n = 3).

media containing SA are shown in Fig. 3. The results showed that the CYGB protein expression level decreased with the increases in SA concentrations (Fig. 3A and B). The CYGB protein expression was significantly lower in the keloid fibroblasts cultured with 2.5 SA compared to control and 1 mM SA. The CYGB protein expression also significantly lower in the keloid fibroblasts cultured in medium with 5 mM SA compared to control and 1 mM SA. These results are consistent with the CYGB mRNA expression. The CYGB-positive cells showed a brownish cytosolic area and are indicated by the red arrow in Fig. 3D.

Viability and proliferation of keloid fibroblasts. The viability and proliferation of the keloid fibroblasts are shown in Fig. 4 and decreased when cultured in media with SA at different concentrations (1, 2.5, and 5 mM). The results (Fig. 4A) showed that the viability of the keloid fibroblasts was significantly decreased in the media with 2.5 and 5 mM SA compared with the control. Meanwhile, the proliferation of the keloid fibroblasts was significantly decreased in all the treatment media compared with the control (Fig. 4B). Significant decreases in keloid fibroblast viability and proliferation were found between the treatment groups. Overall, the viability and proliferation of the keloid fibroblasts decreased when the SA concentration was increased.

Correlations of the CYGB mRNA and protein expressions with the viability and proliferation of the keloid fibroblasts. The correlations of the CYGB mRNA and protein expression with the viability and proliferation of the keloid fibroblasts are presented in Table 1. The results showed that the CYGB mRNA expression positively correlated with the viability (r = 0.714, p = 0.009) and proliferation (r = 0.765, p = 0.004) of the keloid fibroblasts. The CYGB protein expression also positively correlated with the viability (r = 0.851, p = 0.000) and proliferation (r = 0.643, p = 0.024) of the keloid fibroblasts.

Table 1. The correlation between CYGB mRNA and protein expression with viability and proliferation of keloid fibroblast

Correlation (Spearman, n = 12)	CYGB mRNA expression		CYGB protein expression	
	r	p	r	p
Viability	0.714	0.009*	0.851	0.000*
Proliferation	0.765	0.004*	0.643	0.024*

r = Spearman correlation value. *p<0.05, statistically significant.

These results indicate that the suppression of CYGB mRNA and protein expressions tended to decrease the viability and proliferation of keloid fibroblasts.

Discussion

The study showed that the ALAD enzyme activity in the keloid fibroblasts were decreased when cultured in media containing SA (Fig. 1). These results indicate that heme biosynthesis was inhibited. SA is known as an inhibitor of ALAD enzyme, which plays a role in producing porphobilinogen in heme biosynthesis.[6,20,21] SA has a structure like ALA, the substrate of ALAD enzyme. The ketone group in SA is known to have the same position as ALA, so it can bind to the active site of the ALAD enzyme.[21] After SA binds to the active site, the enzyme will undergo a conformational change and can no longer process two ALA molecules. As a result, porphobilinogen is not produced.[20] The absence of porphobilinogen then disrupts the process of heme biosynthesis. Heme biosynthesis has been inhibited using SA in non-small cell lung cancer (HCC4017), HeLa, and colorectal cancer cells (HCT116).[11,12,22]

Our study showed that the CYGB mRNA (Fig. 2) and protein (Fig. 3) expressions of the keloid fibroblast treated with SA were lower than those of the control group. The CYGB mRNA and protein expressions decreased with the increasing SA concentrations used. Our results are consistent with those of Hooda's study, which found that the expressions of hemoproteins decreased when intracellular heme was inhibited by SA and increased again when the extracellular heme levels increased with the addition of hemin.[11] Intracellular heme levels are known to regulate globin synthesis at the transcriptional and translational levels.[10] The results (Fig. 3D–G) indicate a difference in keloid fibroblast cell density between the treatment groups. This is because SA also affects the viability of keloid fibroblasts, which leads to the wasting of dead cells during the washing process in ICC.

The results (Fig. 4A) showed that the viability of the keloid fibroblasts decreased when cultured in media containing SA. This decrease can be due to the toxicity from the accumulation of ALA. Inhibition of heme biosynthesis using SA caused intracellular accumulation of ALA.[23] Low intracellular heme levels can trigger the activity amino levulinic acid synthase 1 (ALAS1) enzyme, which plays a role in ALA production.[23] Research by Hooda *et al.* (2013) has proven that inhibition of heme synthesis using SA causes an increase in ALAS1 expression and a decrease

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of non-small cell lung cancer cell viability.[11] Accumulation of intracellular ALA is known to cause oxidative stress, DNA modification, fragmentation, and damage to organelle membranes such as mitochondria, which in turn causes a decrease in cell viability.[24]

Inhibition of heme biosynthesis can affect hemoproteins expression in the electron transport chain (ETC), which is also related to energy production.[25,26] A study on human retinal endothelial cells showed that inhibition of heme biosynthesis using SA cause decreased expression of cytochrome c protein and the hemoprotein of complex III in ETC.[27] Dysfunction in the ETC protein complex triggers a disruption of energy production in cells and depolarization of mitochondrial membranes, causing an increase in reactive oxygen species in cells.[27]

In addition to measuring viability, our study also measured the proliferation of keloid fibroblasts cultured with or without SA. Our study (Fig. 4B) showed that the proliferation of the keloid fibroblasts cultured in media with SA was lower than control. Reduced cell proliferation due to the inhibition of heme synthesis using SA was also observed in lung cancer, HeLa, and colorectal cancer cells.[11,12,22] Heme is known to bind directly and regulate the activity of various components of cellular regulators, such as p53 and p21 a cyclin-dependent kinase (CDK) inhibitor, which can generate apoptotic conditions in cells.[12]

In addition, the decreased viability and proliferation of keloid fibroblasts may also be related to the role of CYGB. Our study showed statistically significant strong positive correlations between CYGB mRNA and protein expressions with keloid fibroblast viability and proliferation (Table 1). The decrease in CYGB expression tended to reduce the viability and proliferation of the keloid fibroblasts. This may be due to CYGB, a hemoprotein involved in oxygen transport in cells.[28] An adequate oxygen supply is needed to support the function and biogenesis of mitochondria that enables efficient ATP formation through OXPHOS in cells.[6] A decrease in CYGB expression can disrupt the oxygen supply to mitochondria and mitochondrial biogenesis mechanisms. Our previous study showed that the inhibition of HIF-1α protein using ibuprofen suppresses CYGB (mRNA and protein) expressions and proliferation of keloid fibroblast.[16] Furthermore, it showed that inhibition of CYGB mRNA expression using siRNA tends to reduce the expressions of PGC1-α mRNA and protein, which are markers of mitochondrial biogenesis, and the activity of the SDH enzyme, which plays a role in the process of energy formation in the TCA cycle.[15] Inhibition of CYGB expression using siRNA tends to decrease mitochondrial function and biogenesis, which can interfere with the production of the energy needed to support the viability and proliferation of keloid fibroblasts. Our present study supports our previous studies regarding the effect of CYGB on the viability and proliferation of keloid fibroblasts.

There is limitation in our study regarding the number of collected sample due to Covid-19 pandemic, but our study revealed significant results of the effect of heme synthesis inhibition on the viability and proliferation of keloid fibroblasts.

Conclusion

We conclude that heme inhibition using SA can reduce CYGB mRNA and protein expression levels, where it also suppresses the viability and proliferation of keloid fibroblasts. Targeting heme function can reduce energy production, mitochondrial function, and many other cellular processes related to the regulation of cell function. Hence, it is expected to be a therapeutic approach for preventing excessive cell proliferation.

Author Contributions

RN, conducting data collection & analysis, writing, editing, and completion of the manuscript; SIW, contributing to the completion of the manuscript; MS, contributing to the completion the manuscript; RDA, supervising the data collection; SWAJ, conceptualizing and design the research, supervising, writing, data analyzing, reviewing, editing, completion of the manuscript, obtaining the funding.

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Abbreviations

ALA	amino levulinic acid
ALAD	amino levulinic acid dehydratase
ALAS1	amino levulinic acid synthase 1
ATP	adenosine triphosphate
CYGB	cytoglobin
DMEM	Dulbecco's Modified Eagle Medium
ELISA	enzyme-linked immunosorbent assay
ETC	electron transport chain
HDS	heme-depleted serum
ICC	immunocytochemistry
OXPHOS	oxidative phosphorylation
qRT-PCR	quantitative real-time polymerase chain reaction
SA	succinyl acetone
SDH	succinate dehydrogenase
TCA	tricarboxylic acid

Conflict of Interest

No potential conflicts of interest were disclosed.

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