Effect of pirfenidone and its combination with 5-fluorouracil on keloid fibroblast proliferation and collagen deposition: in vitro study

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ABSTRACT

Background: Keloid is a fibroproliferative disease in which its etiology remains unknown. Various therapy modalities have been improved, but none of them gives a satisfying outcome. Pirfenidone (PFD) is a commonly used drug in pulmonary fibrotic diseases, which has an anti-fibrotic effect. 5-fluorouracil (5-FU) is one of the keloid therapy choices that has long been used. This study aimed to investigate PFD’s effect and its combination with 5-FU on fibroblast proliferation and collagen deposition.

Methods: This experimental study using post only control group design was conducted by exposing PFD in various doses (0.5, 1.0, 1.5, 2.0, 3.0 mg/ml) and its combination with 5-FU on keloid fibroblast proliferation and collagen deposition. We used MTT (3-[4,5-dimethylthiazol-2-yl]-2,5diphenyl tetrazolium bromide) and Sirius Red assays to measure the fibroblast proliferation and collagen deposition, respectively. The statistical analysis used one-way ANOVA with p-value p<0.05 was considered significant.

Results: The combination of PFD 3.0 mg/ml + 5-FU 1.0 mg/ml had more significant effect on inhibiting fibroblast proliferation as well as decreasing collagen deposition compared to 5-FU only (p<0.043). PFD 1.0 mg/ml + 5-FU 1 mg/ml as well as PFD 1.5 mg/ml+ 5-FU 1 mg/ml are more effective in inhibiting fibroblast proliferation and lowering collagen deposition than PFD only with p values of <0.001 and <0.000, respectively.

Conclusion: In general, PFD, as well as 5-FU, are effective in inhibiting fibroblast proliferation and decreasing collagen deposition in keloid. However, the combination of both has a better effect compared to PFD or 5-FU monotherapy.

Keywords: 5-Fluorouracil, collagen deposition, keloid fibroblast, pirfenidone, proliferation

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INTRODUCTION

Keloid is a fibroproliferative disease characterized by abnormal growth of connective tissue extending the original wound, commonly seen in people with a strong predisposition.1 In keloid, collagen deposition is excessive in the dermis, and the subcutaneous after trauma such as burn injury, surgery, vaccination, piercing, or spontaneously appears. It is also common in black skin people.2 Keloid is commonly encountered in daily practice. It causes cosmetic problems, itching, pain, and functional disturbance, which affects the quality of life. The keloid incidence varies among populations showing that keloid is affected by various factors. Ethnicity differences influence the prevalence of keloid around the globe.3,4

The pathophysiology of keloid remains unclear. However, it is presumed that it is related to wound healing abnormality process like in hemostasis phase, inflammation, proliferation, and remodeling. Previous studies suggested that the abnormality during the inflammation phase was the primary factor, but others reported a prolonged proliferation phase.5 There has been no single therapy that effectively treats keloid. Some common keloid therapies are intralesional triamcinolone acetonide (TAC), silicone gel, cryotherapy, excision, pressure therapy, laser, radiation, interferon, 5-FU, imiquimod, bleomycin, as well as combination therapy. Intralesional TAC remains the main choice for treating keloid.5,6

5-FU is a fluorinated pyrimidine analog that works as an anti-metabolite drug by inhibiting thymidylate synthase and interrupt RNA synthesis. In 1990, 5-FU was introduced as keloid therapy.7 Pirfenidone (5-methyl-1-phenyl-2-(1H)-pyridone) is a drug for idiopathic pulmonary fibrosis approved by FDA. It has an anti-inflammatory and anti-fibrotic effect on various fibrotic diseases.8 Based on in vivo and in vitro studies, PFD has an anti-inflammatory effect by decreasing TGF-β so that it inhibits fibroblast proliferation, myofibroblast differentiation and reduces extracellular matrix deposition.9 Therefore we conducted this experimental study to find out the effect of PFD and its combination with 5-FU on fibroblast proliferation and collagen deposition in keloid to develop effective keloid therapy.
METHODS

Three keloid patients were recruited in our study. The lesions were diagnosed as active stage keloid based on clinical appearance. The keloid specimens were obtained from patients without previous treatment. All keloid lesions were removed with surgical excision under local anesthesia. This study had been approved by the Committee of Ethics dr. Moewardi General Hospital Surakarta, Central Java, Indonesia. This study is in line with Helsinki's declaration based on human rights, and all subjects had received informed consent and signed an agreement document before data collection.

Cell culture was performed after epidermis, and fat tissue was removed; keloid specimens were cut into 2 × 2 mm in size. All specimens were then cultured in 100mm Petri dishes with Dulbecco's minimal essential medium (DMEM). The culture dishes were incubated at 37°C in humidified (95%) incubator under 5% CO₂. (THERMO SCIENTIFIC, 8000-DH). The culture medium was changed every three days. After outgrowing of spindle cells reached 70% of confluence, the cells were trypsinized with 0.25% trypsin/EDTA solution in phosphate buffered saline (PBS) and sub-cultured in Petri dishes. In passage five, the fibroblasts were seeded on 96-well plates with a density of 5 × 10⁴ cells per well and incubated for 48 h. Afterwards, the cells were treated with PFD (Octagon Chemical Limited, Cas. 53179-13-8) 0.5 mg/ml (PFD-0.5), 1.0 mg/ml (PFD-1.0), 1.5 mg/ml (PFD-1.5), 2.0 mg/ml (PFD-2.0), 3.0 mg/ml (PFD-3.0) in combination with 5-FU 1.0 mg/ml (5-FU) (Curasil, Kalbe Farma) and incubated for 72 h. All fibroblasts were triplicated each treatment.

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay (Biobasic, CAS 1098-93-1) were used to determined fibroblast proliferation. After 72 h of treatment, the supernatant was removed and washed with 10% PBS. All wells were checked under the microscope to determine cell viability. MTT 50 µL was added into each well then incubated for 4 hours under dark condition. After incubation, the supernatant was removed, and 200 µl DMSO was added to each well. The optical density (OD) was immediately measured using a Microplate reader (iMark™, BIO-RAD) at 570 nm to determine cell viability. Viable cells will convert MTT into a purple-colored formazan product with an absorbance maximum of nearly 570 nm. Meanwhile, the dead cells will lose the ability to convert MTT to formazan. Every 96 multi-well plates were divided into a) control wells containing untreated cells b) blank wells containing medium only c) Test wells containing treated cells.

The proliferation rate (%) was calculated using the formula of:

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\text{Proliferation rate} (%) = \frac{\text{OD of treatment} - \text{OD of control}}{\text{OD of control}} \times 100
\]

To see the therapy effect on insoluble collagen deposition, we used Sirius Red assay.10,11 After 72 h of intervention, all cells were washed with PBS and fixed with Bouin solution. An hour later, the well plate was rinsed indirectly under the running water until Bouin solution resolved and dried by turning over the well plate overnight. Sirius Red (Direct Red 80, Sigma Aldrich) 200 µL was added into each well and washed away an hour later. Washed the well with 0.1 N HCL and added 200 µL 0.5N NaOH then left it for 30 minutes then read the well plates under microplate reader. This experimental study used a post-test only control group design. Significance tests of different treatments were conducted using one-way ANOVA. All statistical analyses were performed with SPSS software. Statistical significance was considered with the p-value of < 0.05.

RESULTS

Our study showed that all the intervention groups inhibited fibroblast proliferation and collagen deposition significantly compared to untreated cells (control cell) (p<0.05) (Figures 1 and 2). All the combinations of PFD with 5-FU groups demonstrated anti-proliferation and collagen deposition inhibition effects greater than PFD only. However, of all the combinations, PFD-1.0/5-FU and PFD-1.5/5-FU were statistically significant (p <0.05) (Figures 1 and 2).

This current study also demonstrated that based on the number of cell proliferation PFD 2.0 (92.76%), 3.0 (96.52%), as well as the combinations of PFD-1.0/5-FU (90.53%), PFD-1.5/5-FU (96.67%), PFD-2.0/5-FU (97.32%), PFD-3.0/5-FU (98.90%) had anti-proliferation effect more remarkable than that of 5-FU (89.66%), but only PFD-3.0/5-FU was statistically significant (Figure 1). All treatment groups inhibited collagen deposition in keloid fibroblast significantly (p<0.05) except for PFD-0.5, PFD-1.0 dan PFD-1.5 (Figure 2).

DISCUSSION

In comparison to normal skin or hypertrophic scars, keloid has higher fibroblast proliferation as well as apoptotic process abnormality. This shows that keloid appears due to abnormal wound healing process with prolonged proliferation phase resulting from apoptotic failure thus triggers excessive collagen production.12 Collagen type I is more dominant in keloid, while collagen type III is more prominent in the hypertrophic scar.
In keloid, the ratio of collagen type I: type III is higher, it is 17:1.\textsuperscript{13,14} 5-FU inhibits DNA synthesis during S-phase in cell cycles by limiting thymidylate availability. This drug inhibits thymidylate synthase, an important metabolic enzyme, via its metabolite that is 5-fluorodeoxyuridine monophosphate (FdUMP).\textsuperscript{15,16} RNA synthesis and thymidylate synthase are also inhibited by other 5-FU metabolites; fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP).\textsuperscript{17} The expression of Thymidylate synthase is low in some human body tissue, but in keloid and other tumors, it is expressed excessively.\textsuperscript{15} Huang et al. ’s study reposted that 5-FU inhibited fibroblast proliferation, G2/M cell cycle, and cell apoptosis.\textsuperscript{18} 5-FU inhibits fibroblast proliferation and collagen synthesis.\textsuperscript{19} A review study analyzing the efficacy of 5-FU and its combination in treating keloid revealed intraleralional 5-FU monotherapy successfully treated keloid by 45-78%. However, adding TAC in combination, its efficacy increased into 50-96%.\textsuperscript{7} Nevertheless, TAC has various adverse events, so clinicians keep developing other combination therapies to seek the most effective keloid therapy.

PFD has an anti-inflammatory effect by inhibiting inflammatory mediators production such as TGF-β, which can inhibit fibroblast proliferation, myofibroblast differentiation, and extracellular matrix accumulation.\textsuperscript{9} This drug can regulate growth factor and cytokines which play a role in fibrotic diseases whereas collagen deposition is abnormal.\textsuperscript{20} PFD is commonly used for idiopathic pulmonary fibrosis, but it is also reported that it can inhibit fibroblast proliferation in patients with Crohn’s disease,\textsuperscript{21} human Tenon’s fibroblast,\textsuperscript{22} epidural scar fibroblast,\textsuperscript{9} human pterygium fibroblasts,\textsuperscript{23} and human dermal fibroblast.\textsuperscript{8} PFD inhibits fibroblast proliferation with its ability to inhibit TGF-ß1 via both Smad-dependent (phosphorylation of Smad2 and Smad3) and -independent (Akt and p38) pathways.\textsuperscript{9} TGF-ß1 is one of the important growth factors in both inhibiting pro-apoptotic protein (Bad) and inducing anti-apoptotic protein (Bcl-2). These two proteins are changed their action by PFD in which Bad will be induced while Bcl-2 will be inhibited. In this way, PFD inhibits fibroblast proliferation and induces apoptosis.\textsuperscript{24} However, the PFD proliferation inhibition effect is reversible because fibroblasts will proliferate again after stopping PFD administration.\textsuperscript{21} Zou study suggested that PFD not only inhibits proliferation but also induces apoptosis in hepatocellular carcinoma.\textsuperscript{25}

In our study, either PFD and 5-FU showed anti-proliferative and inhibition of collagen deposition effects. The combination of both will result in a greater anti-proliferation effect than monotherapy. The combination of PFD-3/5-FU showed the most significant effect on the inhibition of keloid fibroblast proliferation. Single-dose PFD-2.0 and PFD-3.0, as well as the combination with 5-FU, can inhibit collagen more vigorously then 5-FU only. However, only treatment groups using PFD-1.0/5-FU and PFD-1.5/50FU, which gave collagen deposition more significantly than PFD alone.

Lin Huang’s study of a combination of 5-FU 1 mg/ml with TAC 20 µM in fibroblast keloid revealed that 5-FU alone had a greater anti-proliferative effect than TAC, and its effect increased significantly when both of them were combined. The combination of 5-FU/TAC...
resulted in significant outcome statistically in inhibiting collagen type I expression. Kun Shi, who performed a study in human epidural scar fibroblast using PFD (0.01, 0.1, 0.5 dan 1.5 mg/ml) reported this drug could inhibit collagen type I in a dose-dependent manner. Nevertheless, PFD has no significant apoptotic and cytotoxic effect so that it can be inferred that the proliferation inhibition effect of PFD is greater than its apoptotic and cytotoxic. This finding is in line with the study by Caroline L. Hall which investigated the effect of PFD on human dermal myofibroblast whereas this drug can inhibit fibroblast proliferation by blocking myofibroblast differentiation and inhibiting collagen production through regulating its biosynthesis.

CONCLUSION

Either PFD or 5-FU has an effect on inhibiting proliferation and decreasing collagen deposition. The combination of PFD with 5-FU results in a significant effect compared to monotherapy. Therefore, this study can be used as the basis of further study in seeking effective therapy for keloid.

CONFLICT OF INTEREST

The authors declare that there is no competing interest regarding the manuscript.

ETHICAL CONSIDERATION

The ethical clearance was obtained from the ethical committee of dr. Moewardi Hospital.

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AUTHOR CONTRIBUTION

All of the authors are equally contributed to the study from the conceptual framework, data gathering, data analysis, until interpreting the results of the study on publication.

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