

# Collagen synthesis on ultraviolet A irradiated human skin fibroblast treated with insulin

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## ABSTRACT

Ultraviolet (UV) irradiation from the sun can stimulate premature skin aging because UV irradiation inhibits collagen synthesis, promotes collagen degradation and inhibits fibroblast proliferation. Insulin is capable to stimulate fibroblast genes collagen expression, DNA synthesis, and collagen synthesis. The effect of insulin in reducing collagen synthesis among repeated-UVA irradiation on human skin fibroblast has never been studied. This study aims to investigate the effect of insulin in collagen synthesis among repeated-UVA irradiation on normal human skin fibroblast. To assess the collagen synthesis collagen degradation, collagen deposition and fibroblast proliferation were measured. Experimental study was performed among passage 3 of fibroblast which was isolated from a circumcised skin of a 6-year-old boy. Fibroblasts were irradiated with 3 repeated exposure with total cumulative dose 9000 mJ/cm<sup>2</sup> and treated with insulin 0.5; 1; 2 µg/mL and placebo. Cells were then incubated for 48 hours, collagen degradation, collagen deposition and fibroblast proliferation were read colorimetric by using Spectroscopy 550 nm. The effect of insulin 0.5; 1 and 2 µg/mL in collagen synthesis among repeated-UVA irradiation on normal human skin fibroblast with cumulative dose 9000 mJ/cm<sup>2</sup> was not capable to reduce collagen degradation, nor capable to increase collagen and fibroblast proliferation. Insulin dose 0,5 µg/ml-2 µg/ml among repeated-UVA irradiation on normal human skin fibroblast was not capable to increase collagen synthesis.

**Key words:** photoaging-DNA synthesis-proliferation-aging process-gene expression

## INTRODUCTION

Photoaging is a cutaneous aging caused by external environment influences especially by ultraviolet light (UV) irradiation<sup>1</sup> and marked with wrinkles, mottling, and brownish pigmentation spot with rough and thick surface.<sup>2</sup> A chronic UV-irradiation on human skin can stimulate aging process because UV irradiation can inhibits collagen synthesis and stimulate collagen degradation.<sup>3</sup> These reduction on collagen is not only due to reduced procollagen synthesis,<sup>4</sup> but also caused by the increased collagen degradation by matrix metallo-proteinases (MMPs).<sup>3,5</sup> It was reported that in photoaged skin, the total amount of collagen decreases as the amount of insoluble collagen increases.<sup>6</sup>

Based on the novel concepts of various gene silencing and oncogenes activation in aging, previous study revealed that there were changing in genes

expression in the aging process, especially silencing of insulin encoding gene/IGF-1 and tumor suppressor genes.<sup>7</sup> In human, normally, insulin sensitivity will be reduced along with the increased of human age and insulin resistance is considered to be related with several kinds of disease.<sup>8</sup> In addition, local injection of insulin is capable to stimulate wound healing of skin donor rabbits without affecting the blood sugar concentration<sup>9</sup> due to stimulation of DNA synthesis.<sup>10</sup> This mechanism is based on the binding of insulin and its fibroblast receptor and stimulate fibroblast collagen gene expression,<sup>11</sup> DNA synthesis<sup>9,10</sup> and collagen synthesis,<sup>12</sup> afterward. Other points showed that fibroblast activity in matrix extracellular collagen network arrangement is depend on the ability of collagen synthesis, collagen deposition and collagen degradation.<sup>13</sup> Collagen is synthesized as pro-collagen in soluble form, then it become insoluble collagen fibres due to activity of various enzymes, and it turns again to be soluble

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one when it is degraded.<sup>14</sup> Meanwhile, fibroblast's collagen synthesis is usually parallel with fibroblast proliferation.<sup>15</sup>

Based on those facts, insulin can be used as anti photoaging, theoretically. Here, we performed an experiment to evaluate the effect of insulin in UVA irradiated fibroblasts.

## MATERIALS AND METHODS

This experiment was an *in vitro* study using control group experimental design, whom research subjects were passages 3 of normal skin fibroblasts which was isolated from a circumcised skin of a 6-years old boy. This study was carried out in the Health Technology Laboratory of Department Dermato-Venereology, Faculty of Medicine, Gadjah Mada University, Yogyakarta.

Fibroblast was cultured with explants technique in 25 mL flask moisten with Dulbecco's Modified Eagle's Medium (DMEM) completed medium for 24 hours to allow explants attachment and then incubated at 37°C and CO<sub>2</sub>5%. Then, explants were soaked with complete medium which was changed for every 3-4 days or if the colour of the medium turns into yellowish. After fibroblast cells reached confluent up to 60–70 %, then secondary culture were performed and the third sub passage was used for this study. Fibroblast culture was divided into two groups, irradiated groups and non irradiated groups. Each group was divided into four sub groups consisted of placebo, insulin 0.5; 1 and 2 µg/mL.

Fibroblasts were removed based on trypsinized enzymatic technique and 200 µL cells suspension consisted of  $2 \times 10^4$  cell were coated onto each of 96 multi well plate disk, and each sub group was replicated 6 times. After the cell firmly attached to the shaft bottom, and before UVA-irradiation, medium was removed, washed and replaced with phosphate buffer saline (PBS). UVA past sun LIPI™ was used as a source of UVA and 3000 mJ/cm<sup>2</sup> dose of irradiation were given onto irradiated groups while the non irradiated groups were placed in the laminar flow hood.

After irradiation, PBS was removed and replaced into multi-wells plate with medium containing insulin 0.5; 1 and 2 µg/mL and placebo onto irradiated groups and non irradiated groups and then incubated for 48 hours. Plate disk was removed

from incubator and from each well, the collagen degradation, collagen deposition were measured with Sirius Red binding assay method; fibroblast proliferation was measured with MTT technique and all were read colorimetric by using 550 nm ELISA reader. The fibroblast was irradiated with UV light for three times, until the total dosage was 9000 mJ/cm<sup>2</sup>.

The data were analysed by using SPSS program. The average differentiation of optic density collagen and fibroblast proliferation was tested using paired t – test analysis. If the data distribution was not in normal distribution, thus used Wilcoxon signed Rank Test. A probability of less than 0.05 was considered significant.

## RESULTS

In the UVA irradiated group with cumulative dose 9000 mJ/cm<sup>2</sup>, the collagen degradation increased significantly compared to non irradiated group ( $p < 0.05$ ) as shown in FIGURE 1.

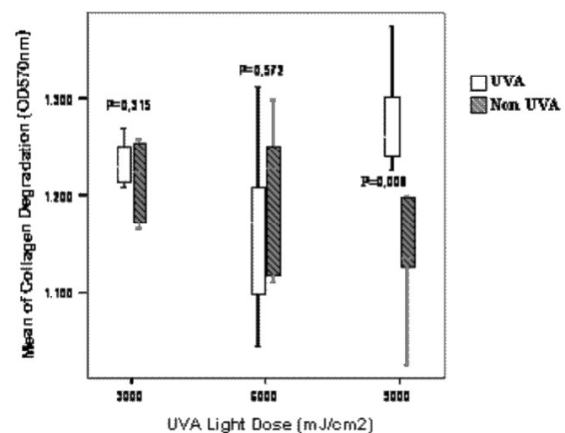


FIGURE 1. Effects of UVA irradiation on collagen degradation

FIGURE 2 demonstrated the effects of UVA irradiation on the collagen deposition. UVA exposure was started at 3000 mJ/cm<sup>2</sup> and the cumulative dose 9000 mJ/cm<sup>2</sup> demonstrated a reduce in the collagen deposition compared to non-irradiated group ( $p < 0.05$ ). While at UVA dose 6000 mJ/cm<sup>2</sup> showed reduced collagen deposition on irradiated group although it was not significantly different ( $p > 0.05$ ).

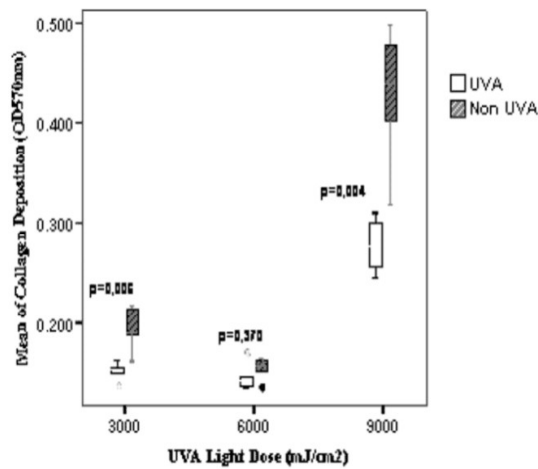


FIGURE 2. Effects of UVA irradiation on collagen depositions

FIGURE 2 demonstrated the effect of UVA irradiation on the fibroblasts proliferation. It can be observed that UVA dose at 3000, 6000 mJ/cm<sup>2</sup> and cumulative dose 9000 mJ/cm<sup>2</sup> UVA were able to suppress fibroblast proliferation ( $p < 0.05$ ).

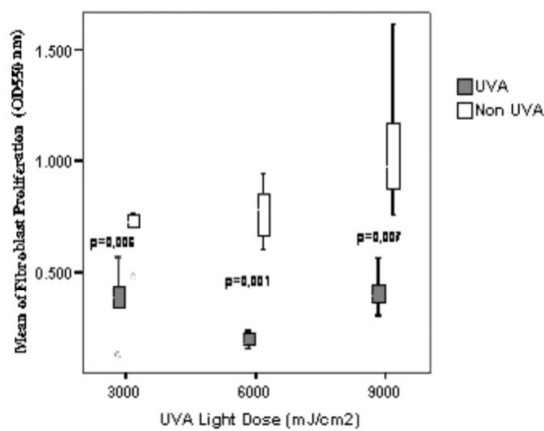


FIGURE 3. Effects of UVA irradiation on fibroblast proliferations

The effect of various insulin dosages in collagen degradation, collagen deposition, and fibroblast proliferation can be observed in the FIGURE 4. Various dosages of insulin that were given after 9000 mJ/cm<sup>2</sup> UVA irradiation were not able to inhibit collagen degradation significantly ( $p > 0.05$ ).

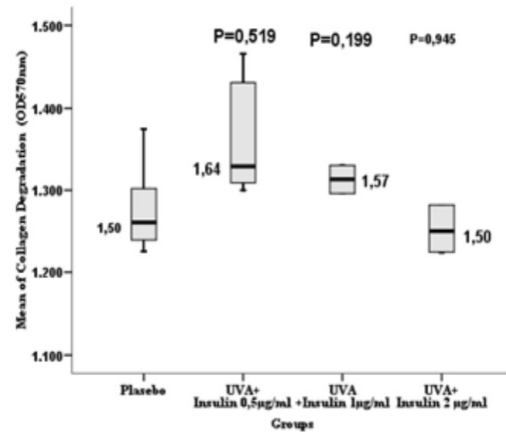


FIGURE 4. Effect of insulin on collagen degradation of UVA irradiated fibroblasts group with 9000mJ/cm<sup>2</sup> cumulative dosage

FIGURE 5 demonstrated the effect of insulin on collagen deposition of UVA irradiated fibroblast. Insulin addition was not able to restore collagen deposition as shown by the significantly lower level of collagen deposition among insulin treated group ( $p < 0.05$ ).

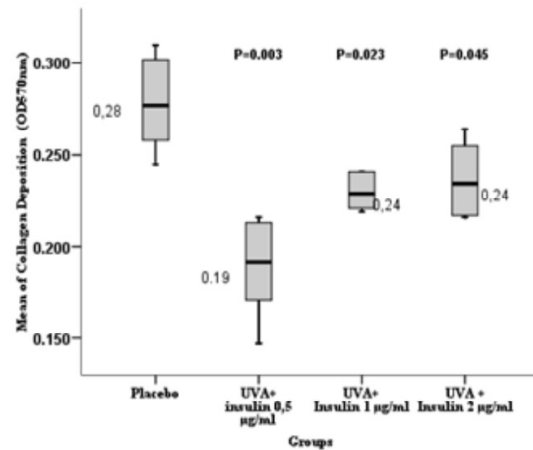


FIGURE 5. Effect of insulin on collagen deposition of UVA irradiated fibroblasts group with 9000mJ/cm<sup>2</sup> cumulative dosage

FIGURE 6 demonstrated the effect of insulin on proliferation of UVA irradiated fibroblast. It can be observed that addition of insulin was not able to restore fibroblast proliferation ( $p > 0.05$ ).

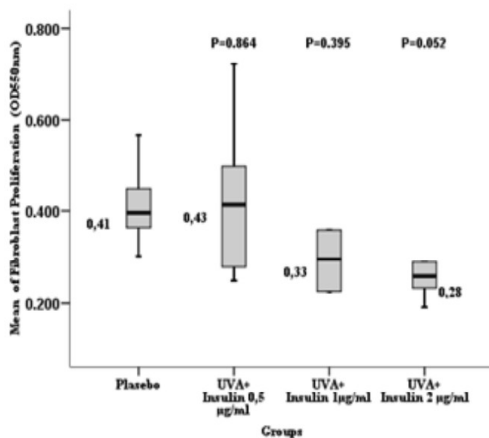


FIGURE 6. Effect of insulin on UVA irradiated fibroblast proliferation with 9000mJ/cm<sup>2</sup> cumulative dosage

## DISCUSSION

Cultured fibroblasts were accepted and considered as a good model for *in vitro* aging study which was firstly introduced by Haflick and Moorehead at 1961. In our study, we used the fibroblast culture from passage 3 of normal human skin fibroblast. Based on reference, the use of fibroblast passage 3 on *in vitro* study are more stable, and more resistant against microorganism contamination.<sup>16</sup>

It was stated that UV exposure on fibroblast could damage the collagen by increasing elaboration of collagen-degrading matrix metalloproteinases and reducing collagen synthesis and increasing MMPs. Thus, the increase of the collagen degradation will contribute to connective tissue damage leading to photoaging.<sup>17</sup> FIGURE 1, 2 and 3 demonstrated the effects of repeated-UVA irradiation among fibroblast in cumulative dose 9000 mJ/cm<sup>2</sup> in collagen degradation, collagen deposition and fibroblast proliferation significantly different ( $p < 0.05$ ) compared to placebo.

Based on the result of our study, it was suggested that the use of fibroblast passage 3 culture, procedure of exposure and dose of UVA irradiation were correct and appropriate. Thus using of fibroblast culture which was isolated from preputium in our study showed there was no an extraordinary to others previous *in vitro* study among irradiated UVA exposure. With this result, discussion will be focused on the effect of insulin among irradiated

fibroblast culture in cumulative dose 9000 mJ/cm<sup>2</sup>. From the literature which explained that UVA-irradiation on human skin fibroblast more than 8000 mJ/cm<sup>2</sup> can stimulate production of collagenase mRNA in human fibroblast culture that cause decreasing of collagen synthesis.<sup>18</sup> Previous *in vitro* study also showed that degraded collagen due to UVA as well as UVB in hairless mouse skin<sup>19</sup> and equivalent fibroblast dermis culture,<sup>20</sup> was able to make collagen more sensitive against collagenase, thus easier to be degraded. Collagen degradation induced by UVA and UVB irradiation can inhibit collagen synthesis.<sup>21</sup>

This study suggested that increasing the dose of additional insulin among irradiated group would increase the average of collagen degradation average (FIGURE 4), collagen deposition (FIGURE 5) and fibroblast proliferation (FIGURE 6). These findings showed that UVA-irradiation has an effect to inhibits collagen glycosylation, thus inhibits collagen deposition. Collagen deposition needs glycosylation process, and the binding of collagen with sugar which was happened in matrix extracellular after the collagen synthesis. The UVA irradiation was capable to make an oxidative stress, which inhibits collagen glycosylation.<sup>22</sup> Our data also demonstrated that even being treated with insulin, UVA irradiation still performed an anti proliferation effect. Study by Fisher *et al.*<sup>23</sup> suggested that a single UVA irradiation exposure can inhibit pro-collagen synthesis and inhibit fibroblast break down while decreased fibroblast proliferation indicated cell death.<sup>15</sup>

The concentration of insulin that used in this study based on the research of the effect of insulin in human skin wound healing by Neago *et al.*<sup>24</sup> The result of the study showed that insulin 1 µg/mL was the optimum dose to accelerates skin wound healing. It was estimated that addition of insulin 2 µg/mL in the culture medium leads to increases collagen synthesis. It was stated that IGF-1 and insulin have very similar molecular structures, and high concentrations of insulin will activate the IGF-1 receptor (IGF-1R). However, it was demonstrated that the effect of insulin-treated irradiated fibroblast culture in dose-dependent manner has been proven cannot increased collagen synthesis and fibroblast proliferation. This might caused by in chronic UVA exposure among fibroblast, the expression of insulin/IGF-1R will decrease or inactive or even death.

Insulin action begins with its binding to its receptor, and activation of insulin receptors which cause the mechanisms of insulin action. Based on the previous study by Coffey *et al.*<sup>25</sup> suggested that in fibroblast which received UV irradiation the expression of insulin /IGF-1 receptor will decrease and even silence.<sup>26</sup> These fact also proved by Lewis *et al.*<sup>27</sup> that showed UVB irradiation in old skin will inactivate the IGF – 1 receptor, thus the reparation of DNA and cell proliferation become imperfect.<sup>27</sup>

The effect of insulin in skin wound healing can stimulate the collagen synthesis, while in our study, the addition of insulin in irradiated fibroblast cultur has been proven cannot increase collagen synthesis and fibroblast proliferation. From this result, it was suggested that insulin can not be used as an alternative therapy of photoaging. The irradiation of UV light can lead to decreasing amount of collagen by decreasing its synthesis and also increasing collagen degradation. Collagen synthesis type 1 started to decrease significantly in fibroblast culture of normal human skin<sup>28</sup> and diminished parallel with increasing of UVA radiation dose.<sup>18</sup>

## CONCLUSION

The effect of insulin 0.5 – 2 µg/mL doses had not been proven to increase collagen synthesis, decrease collagen degradation and increase fibroblast proliferation, thus insulin can not be used as a modality to treat photoaging. Continued research is really needed to know the expression of insulin/IGF-1 receptor among UVA-irradiated fibroblast culture.

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