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Histology Study: Pre-Clinic Test of Nanogold in *Mus Musculus* Skin, at Fibroblast Proliferation and Collagen Biosynthesis

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Abstract
This study aimed to test the pre-clinical anti-aging activity of nanogold. The test performed in vivo using mice (*Mus musculus*) to assess cell proliferation and increasing the collagen quantity in the skin. The test result of anti-aging activity in vivo using mice showed that the quantity of collagen and fibroblasts increased in the treatment of mice with nanogold infiltration. Overall it can be concluded that the nanogold acts as anti-aging by promoting fibroblasts proliferation and increasing collagen biosynthesis.
Keywords: anti-aging, nanogold, activity, collagen, fibroblasts.

1. Introduction

Increased demand for anti-aging cosmetics trigger research to explore the sources of new anti-aging materials. Limitations of conventional anti-aging material in terms of quantity and quality are related to the safety of the wearer. Actually the development of anti-aging material based gold refers to the ancestral heritage in the form of "gold implants", which has been developed using the latest technology is the technology that uses nanomaterial synthesis from gold in nano size, called nanogold. The Potential of nanogold as material candidate of anti-aging in cosmetics has been studied in vitro, both physically and chemically (Sanjaya and Taufikurohmah, 2013). Test of the physical characters including texture, color and flavor have proven that nanogold qualify aesthetically as cosmetic materials. Chemically, Nanogold as well as sodium does not cause protein denaturation. Thus, nangold allowed to be used in cosmetics. This is in contrast to the heavy metals that is not feasible in the cosmetics, due to heavy metals generally causes protein denaturation (Taufikurohmah, *et al*, 2011). Test of the chemical activities showed that nanogold can reduce free radicals (Taufikurohmah, *et al*, 2012). Test of nanogold activity is also showed that nanogold can be used as sunscreen materials and supporting material for conventional sunscreen compounds (Sanjaya and Taufikurohmah, 2013).

The results of in vitro studies on the use nanogold as a cosmetic material primarily for sunscreen and anti-aging need support of the assay of in vivo. The studies was conducted in stages and begun with preclinical assays at animal test *Mus musculus*. Infiltration of nanogold on the surface of animal skin is done for 14 days, refers to previous work on animals test with the infiltration of drug compounds (Triyono, 2005). Histology of the skin of both the treated animals and the control animals that did not receive nanogold infiltration then compared.

Based on this study, it can be seen the difference in the quality of cross-sectional slices of skin, in the quantity of fibroblast proliferation, and in the quantity of collagen in the skin of the animal treatment and the control. The purpose of this study was to obtain supporting data for determining the effect of nanogold treatment on the repair of the skin tissue of the animals test *Mus musculus*. The benefit of this study is to prepare nanogold as the safe and important material in modern cosmetics.

2. Method

2.1 Nanogold Synthesis

Starting materials in this research are yellow solution of $HAuCl_4$ 1000 ppm and reducing agent of sodium citrate 2 g. A total of (1000-x) ml of distilled water is heated in the 1000 ml beaker glass to boiling. Sodium citrate 2 g is added and stirred until homogeneous. Then, add a solution of $HAuCl_4$ x ml (x = 5, 10, 15, 20, 25, 30, 35 and 40) while stirring. The solution seems to change into colorless. Continue stirring until the mixture changes color from colorless to dark blue, red and burgundy. After that, stirring and heating is stopped. Thus it is generated nanogold colloids 5, 10, 15, 20, 25, 30, 35 and 40 ppm(Taufikurohmah, et al, 2011). Then the nanogold colloids are used in the activity test.

2.2 Animals

Test animal *Mus musculus* is obtained from farmers that assisted Brawijaya University in Malang. Total of test animals were 20. They were divided into 2 groups, each group consisting of 10 mices. They were randomly

selected into the control group and the treatment group using nanogold. Adaptation period for each group is 2 weeks. After the adaptation period is completed, the test animals get the treatment period.

2.3 Infiltration of Nanogold

Ten test animals in the treatment group get nanogold infiltration by smearing a cream containing nanogold. The control group receive the same treatment, but smeared with cream that does not contain nanogold. The treatment by smearing the cream is done every morning for 14 days.

2.4 Skin Tissue Preparation

The animals prepared for the treatment of tissue fixative. The tissue used in this process is a part of skin, which each has 1 x 2 cm^2 in the size. This tissue soaked in 4% formalin for the fixative process. The tissue formalin fixed was removed and immersed in graded alcohol concentration of 10%, 20%, 30%, 50%, 70%, 90% and absolute alcohol. Furthermore dipped in Xylol and embedded in liquid paraffin which will be solid at room temperature. Paraffin blocks were made to prepared thin slicing. Thin slices made with a thickness of 4 μm with microtome. This slice used at staining preparation of tissue. The Staining used Hematoxcylin-eosin (HE) to get a lot of information of fibroblast. The Staining used Van Geison's stain to get information of collagen quantity.

2.5 Analysis of Skin Tissue

Qualitative analysis of the skin tissue is performed to distinguish the skin tissue in the treated group and the control group related to the characteristics of the epidermis, dermis and tissue. The quantitative analysis of the tissue is conducted on the quantity of the existing fibroblast and collagen in the skin tissue. Fibroblast cells are very numerous and small in size so that for the purposes of calculating the quantity should be limited on a more narrow area. It is computed at view field of microscope that taken. While the number of fibroblast cells is calculated by making five cropping with a particular area in the HE staining. The quantity of collagen is calculated as a percent of the total field area of collagen by cropping the red area in the Van Geison staining.

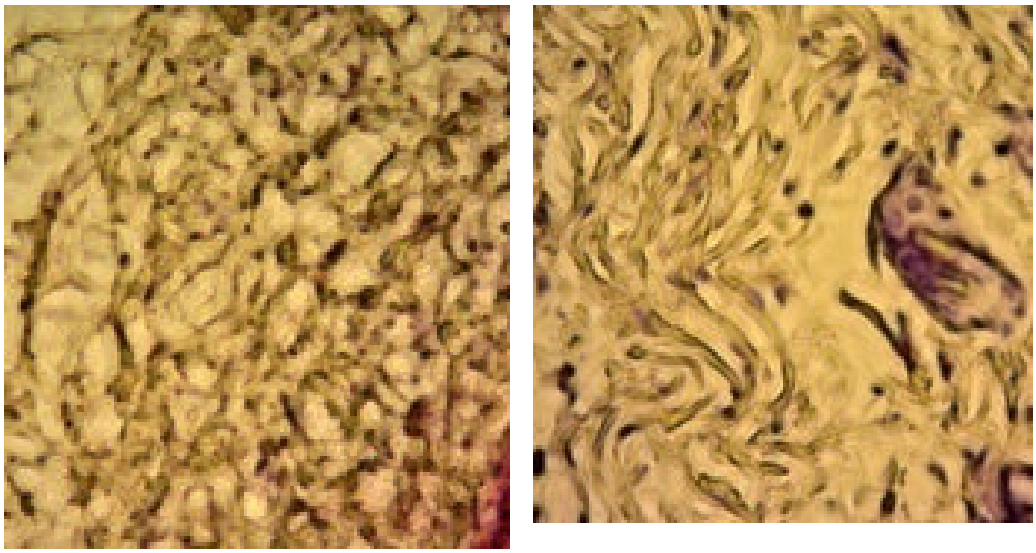
3. Result and Discussion

3.1 Analysis of Skin Tissue

The color of skin tissue obtained, showed differences though both are using HE. This suggests differences in the chemical environment of skin tissue. More acidic tissue will absorb the bases dye i.e. haematoxylin, so it tends to be purple colored. Alkaline tissue will absorb the acid dye eosin so inclined reddish. The tissue infiltrated with nanogold shows colors which tend to be more purples than normal tissue. Such conditions were more favorable for the skin tissues. The potential of nanogold to increase the quantities of muscle tissue in the skin of animals. The tissue color in nanogold tretment is more red than normal group. This suggests that tissue with infiltration of nanogold absorbs acid dyes because the tissue is alkaline. Chemical conditions in such away increases the growth of the muscle tissue and other tissue.

3.2 Quantitative Analysis of Skin Tissue

Furthermore, the same coloring, which is still using dye hematoxylin-eosin (HE), a histochemical can clarify the fibroblast cells which is the cell that secrete extra cellular products, one of which is collagen. To find the presence of fibroblast cells in the skin tissue, it is necessary to do the search sequence of the exact location. Fibroblast cells in the skin tissue with infiltration nanogold (Fig. 1a) have a higher cell density than in the normal tissue (Fig. 1b). The size of fibroblast cells in Figure 1a is smaller than the cells size in Figure 1b. This shows nanogold infiltration causes increased proliferation of skin cells.



a b

Figure 1 Fibroblast of *Mus Muscullus*: a. treatment group and b. control group

The quantity of fibroblast cells have increased from the normal condition due to nanogold infiltration. The density of fibroblast cell can be observed in Figure 1. The quantity of fibroblast cells are more numerous and with smaller size (young cells) shown in Figure 1 parts a compare to parts b.

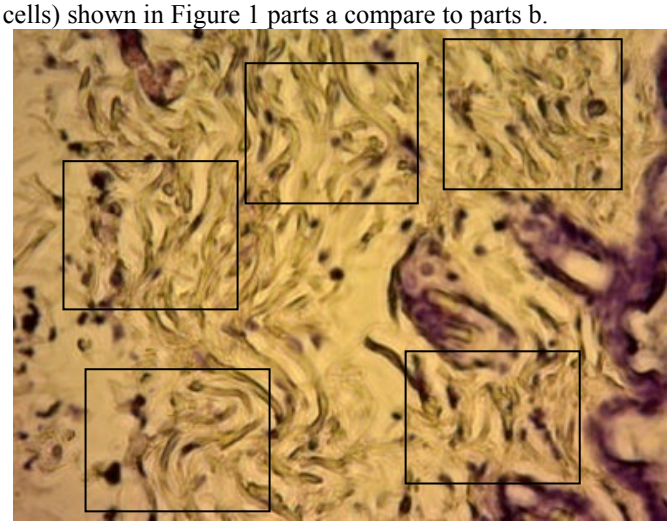


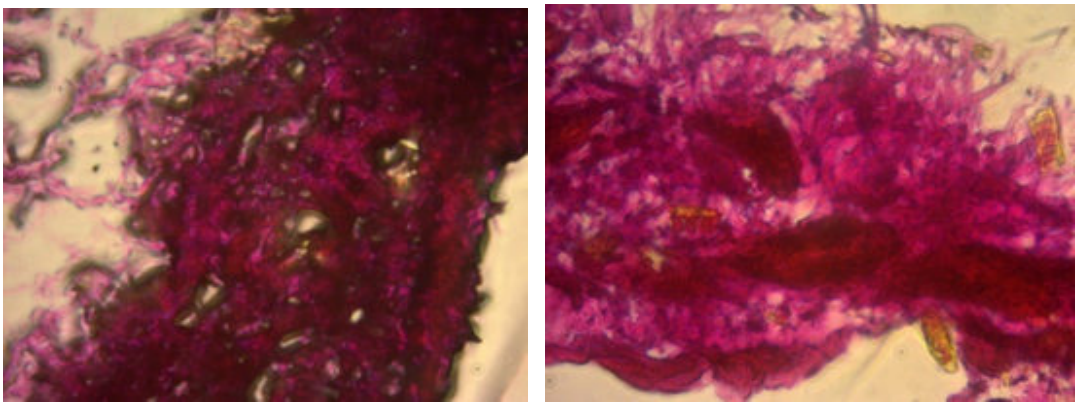
Figure 2 Calculation of fibroblast cells number.

The calculation result of the number of fibroblast cells is obtained by calculating each 80.000 μm^2 field area of the microscope. In detail, from each field of view of the microscope is made 5 cropping, as shown in figure 2. In each of those areas, the number of fibroblast cells is counted. The average number of cells per field of view of the microscope is a quantitative data.

Table 1. Fibroblast Cell Number of *Mus Musculus*

No	*Mus Musculus* group	Cell number in 80.000μm^2 area	The rate of number
1	nanogold (rep1)	45 47 39 50 54 46 43 45 52 50	47,1 ---> 46,775
	nanogold (rep2)	46 43 45 52 50 45 44 48 51 48	47,2
	nanogold (rep3)	45 46 48 41 52 52 44 43 47 50	46,8
	nanogold (rep4)	47 40 51 53 41 39 51 43 46 49	46,0
2	normal (rep1)	30 31 26 33 36 32 29 30 34 31	31,2 ---> 31,550
	normal (rep2)	32 28 29 35 34 31 29 32 33 32	31,5
	normal (rep3)	31 29 28 34 33 32 30 31 35 36	31,9
	normal (rep4)	28 34 31 29 30 34 35 36 29 30	31,6

Figure 3 Cropping collagen area to calculate collagen quantity.



a b

Figure 4 Collagen of *Mus Musculus*: a. nanogold treatment, b. normal group.

Table 2. The quantities of collagen of *Mus Musculus*

No	Mus Muscullus group	Percent area of collagen	The rate (%) area
1	*nanogold* (rep1)	81,25; 81,00; 79,85; 79,95; 79,50 80,75; 80,25; 81,05; 79,75; 79,95	80,33
	nanogold (rep2)	81,15; 80,43; 79,90; 78,45; 80,05 81,00; 80,63; 78,93; 79,68; 78,05	79,83 79,99
	nanogold (rep3)	68,90; 83,67; 83,01; 78,58; 79,90 79,87; 83,01; 75,89; 80,05; 78,80	79,17
	nanogold (rep4)	79,16; 80,34; 78,78; 84,45; 78,80 80,23; 79,65; 84,23; 80,23; 80,20	80,61
2	normal (rep1)	40,63; 40.50; 39,93; 39,75; 39,96 34,45; 41,84; 41,51; 39,29; 39,95	39,78
	normal (rep2)	40,38; 40,13; 40,53; 39,88; 39,98 39,94; 41,51; 37,95; 40,03; 39,40	39,97 40,00
	normal (rep3)	40,56; 40,23; 39,95; 39,23; 40,03 39,58; 40,17; 39,39; 42,23; 39,40	40,08
	normal (rep4)	40,50; 40,32; 39,47; 39,84; 39,03 40,12; 39,83; 42,12; 40,12; 40,10	40,15

Analysis of the data in Table 1 and 2 performed using the statistical Manova to examine the effect of type of test group (nanogold concentration) on cell proliferation and an increase in the quantity of collagen. The multivariate statistical analysis performed gives the following explanation.

Significance values in the table is 0.000 for both intercept and test group. Because this value is less than 0.05 then the Ho is rejected and H1 accepted. This means that there is the influence of the test to the depending variables, ie the quantity of collagen and fibroblast cells. There is a very strong relationship between the parameters of the test group and the dependent variables, especially the quantity of collagen and fibroblast cell number.

Glycopeptide is a protein located in the muscle tissue. Because it does not cause denaturation of proteins, the presence of nanogold in the tissue is generally not harmful to the health. This is evident from the test in vivo using the test animals, where nanogold shows its ability to increase the quantity of the tissue (Mi, et al, 2010).

Bonding that occurs between nanogold with thiol group and the amine group that is always present in the amino acids which is a monomer of the polypeptides or proteins, would be implications for how nanogold take part in the process of accelerating the synthesis of collagen. This is a catalytic mechanism of nanogold. This all began with the ability of nanogold to attract electrons, thus forming a bridge electronics that generate a strong enough bond between the molecules in polymerization processes (Jane, *et al,* 2007).

Nanogold is also capable to make a bond between the beta sheet in the tertiary structure of proteins. It supports the stability of the tertiary bond, because the interaction between the beta sheets is often cause protein aggregation. When folding occurs, it will be followed by destruction orders of these proteins by proteases. In the existance of nanogold, process of the protein destruction can be prevented. Metabolism takes place continuously without being interrupted by process of the protein destruction. The quantity of tissue that composed of proteins continues to grow (Michael, *et al,* 1994).

Nanogold ability to prevent the detruction of proteins is highly supported by the test results of its activity in vivo studies in animal tests. It is evident from the process of improvement in both collagen and fibroblast cells. A process which is closely related to the polypeptide chains that have the chemical bonds, both secondary and tertiary, to form a larger tissue (Jane, et al, 2007).

Nanogold can perform activities of antiaging such as the activities of antiaging that can be done by the antiaging conventional. Nanogold is able to replace all the antiaging activities, even with higher activity and more durable because nanogold not easily damaged. The reasearch proves that nanogold can reduce the artificial free radical DPPH with greater activity than vitamin C at the same concentration. Nanogold was proven be able to increase the activity of proliferation of fibroblasts cell, which is producing collagen. It is identified quantitatively by the increase in cells number. Nanogold is also increasing the quantity of collagen, expressed as a percent of area (Jane, *et al,* 2007).

Most of the skin tissue is composed of the proteins, mainly collagen and fibroblast cells as one kinds of cells that produce collagen. In this study, it has been proven that the nanogold can increase the number of fibroblast cells. This research could also show an increase in the quantity of collagen, which is calculated as a percent of the field area.

Nanogold is able to bind to glutathione, where nanogold as a center of the chemical bonding and glutathione as a ligand that surround it. This is supported by the ability of nanogold attract electrons, to form chemical bonding, and stabilize the bond and the resonance in the molecule. This chemical bonds establish the existence of nanogold in cells, because nanogold has no charge and can be easily pushed out or enter the body's secretion system. When bound to glutathione, the nanogold become increasingly difficult regardless (Ji-Ae*, et al,* 2008).

Glutathione is an antioxidant which served as supporting the immune system of cells and tissues. Glutathione can also serve as an antidote to the poison that entered the body and also natural antidote to overdose conditions. Glutathione concentrations increase when the body got poisoning, free radical attack, overdose and other abnormal conditions. In this condition, the glutathione in the liver tissue may reach 5 mM (Ji-Ae, *et al*, 2008).

Glutathione protects proteins containing thiol groups and generally protects also other tissues from oxidative stress either by the attack of free radicals and heavy metals such as mercury. Glutathione is the main guard of the body's defense layers of oxidative stress. Bonding between glutathione and nanogold will increase defense system of cells and tissues by attacks of free radicals and heavy metals because nanogold itself also counteract free radicals. This combination of glutathione and nanogold is multiply their capabilities as antioxidant and free-radical prophylactic (Jane*, et al*, 2007).

5. Conclusion

- There is a difference in texture of the cross-sectional of skin slices between the test animals which treated with infiltration nanogold and the control.
- There is a qualitative difference between muscle tissue, epidermis and dermis of the test animals with infiltration nanogold and the control.

- There is an increase in muscle tissue as well as the quantities of fibroblasts and collagen in the test animals compared to the control.

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References

Jane, T., Olaf, M., & Jacques, M. (2007), "Stimulation of Collagen Production in Human Fibroblasts", *Cosmetic Science Technology*, 15-17.

Ji-Ae, P., Pattubala, A. R., Hee-Kyung, K., In-sung, K., Gab-Chul, K., Yongmin, C., et al. (2008). "Gold nanoparticles fungtionalized by GD-complex of DTPA-bis(amide) conjugate of glutathione an MRI contrast agent", *Bioorganic & Medicinal Chemistry Letters*, **18**(23), 6135-6137.

Mi, H. K., Soo, S. L., Sang J, C., Hyun, H. J., Sujung, Y., Sudeok, K., et al. (2010), "Real-time colorimetric screening of endopeptidase inhibitors using adenosine triphosphate (ATP)-stabilized gold nanoparticles", *Tetrahedron Letter*, 51(17), 2228-2231.

Michael, J.P., Franke, W., & Kleinschmidt, J. (1994), "Distinct 19S and 20S subcomplexes of the 26 S proteasome and their distribution in the nucleus and the cytoplasm", *The Journal of Biological Chemistry*, 269(10), 7709-7718.

Sanjaya, I.G.M. & Taufikurohmah, T. (2013), "Nanogold as Supporting Activities of Conventional Sunscreen of Octyl-p-Methoxycinnamate to Inhibit Photoaging", *Chemistry and Materials Research*, 3(3), 73-80.

Taufikurohmah, T., Sanjaya, I.G.M., Baktir, A., & Syahrani, A. (2012), "Activity Test of Nanogold for Reduction of Free Radicals, a Pre-Assessment Utilization Nanogold in Pharmaceutical as Medicines and Cosmetics", *Journal of Materials Science and Engineering*, B2 (12), 611-617.

Taufikurohmah, T., Sanjaya, I.G.M., & Syahrani, A. (2011), "Nanogold Synthesis Using Matrix Mono Glyceryl Stearate as Antiaging Compounds in Modern Cosmetics", *Journal of Materials Science and Engineering*, A1, 857-864.

Triyono, B. (2005), The Difference of Collagen Appearance Around Wound Incision Between Infiltrated and Non Infiltrated Levobupivacaine Pain-Relief on Wistar Rats Histochemistry Study. Semarang: Universitas Diponegoro Press.

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