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Human platelet lysate-derived exosomes are superior to the lysate at increasing collagen deposition in a rat model of intrinsic aging

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ABSTRACT

The decreased production of collagen in intrinsic aging skin is most likely due to downregulation of connective tissue growth factor (GF) which is thought to be a regulator of collagen expression. Human platelet lysate (HPL) contains many nutrients needed for cell growth and proliferation. The GFs are contained not right in liquid lysate but in exosomes. Our study demonstrated the superiority of Exo-HPL compared to HPL in decreasing matrix metalloproteinase (MMP)-1 level and increasing collagen deposition in intrinsic aging rat models (IRMs) induced by injection of D-galactose. The highest deposition of collagen at 40× magnification is found in IRM + Exo-HPL treatment group (38.40%) which is close to collagen deposition in young rats group (42.90%). At 100× magnification the study demonstrated that there was higher collagen deposition in IRM + Exo-HPL treatment group (39.94%) than in IRM + HPL treatment group (34.53%) which was close to the young rat group's collagen deposition (43.26%). This study found a significant difference of MMP-1 level and collagen deposition among all groups. Both Exo-HPL and HPL treatment groups decreased MMP-1 level and increased collagen deposition compared to IRM group. Exo-HPL treatment group was more effective in decreasing MMP-1 level and increasing collagen deposition compared to HPL treatment group and IRM group significantly.

INTRODUCTION

Skin aging process is classified into intrinsic and extrinsic aging. Extrinsic aging is aging due to external exposure factors such as photoaging (Taylor *et al*., 2012). Intrinsic or physiological aging is caused by DNA damage that is influenced by hormonal disorders due to aging, genetic factors, and free radicals (Li *et al*., 2020; Zarei and Abbaszadeh, 2019). Intrinsic aging is a change in stem cells and their derivatives, including the expression of microRNA (miRNA), telomere length, cell cycle regulators, cellular secretomes, expression of apoptotic proteins, transmembrane receptors, and gene expression (Kammeyer and Luiten, 2015; Li *et al*., 2020).

The intrinsic aging rat models (IRMs) induced by injection of D-galactose (D-Gal) have been widely used in pharmacodynamic studies on anti-aging therapy because D-Gal has been shown to induce reactive oxygen species (ROS) and advanced glycation end products. The types of rats that are commonly used in the intrinsic aging acceleration model are Wistar (*Rattus novergicus*) and Sprague-Dawley (*Rattus novergicus* L). Induction of D-Gal in rats produces excessive ROS, decreases the activity of antioxidant enzymes in various organs, and forms more superoxide anions and various oxidation products, causing cell damage and functional decline of several organs and systems in rat and leading to intrinsic aging (Chogtu *et al*., 2018; Haider *et al*., 2015). D-Gal has been shown to successfully mimic the oxidative changes that occur in various tissues during the natural aging process (Yanar *et al*., 2019). Chronic D-Gal administration

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has been reported to cause memory deficits and cognitive and motor performance declines that mimic the symptoms of aging; thus it is viewed as an essential model of intrinsic aging in mice (Chogtu *et al*., 2018; Parameshwaran *et al*., 2010).

Intrinsic aging in skin is characterized by epidermal thinning, loss of rete ridges, and decreased collagen in the dermis. Type I collagen is the main component that provides tensile strength or strengthens the skin dermis so it plays a role in maintaining skin integrity (Widgerow *et al*., 2016). Decreased production of type I collagen in human skin occurs in intrinsic aging which most likely results from downregulation of tumor growth factor-beta (TGF-β) and connective tissue growth factor (GF) signaling. In the aging process, there are changes in the structure and organization of collagen, decreased extracellular matrix (ECM) protein synthesis, and an increase in metalloproteinases which degrade the matrix resulting in fragmentation of collagen fibrils which causes loss of mechanical strength (Cole *et al*., 2018; Poljšak *et al*., 2012).

The main enzyme responsible for the degradation of type 1 collagen is matrix metalloproteinase (MMP)-1; therefore treatments that play a role in regulating type I collagen production or regulating MMP-1 levels continue to be developed to prevent skin aging (Zhang and Duan, 2018). MMP-1 is the only MMP that can break down fibrillar collagen intact (Kammeyer and Luiten, 2015). A decrease in the mechanical strength of fibroblasts in aging skin can trigger an increase in MMP-1 levels in response to oxidative stress, resulting in increased collagen degradation (Holtz, 2009).

Collagen is the primary component of the ECM (Watt and Fujiwara, 2011). It provides tensile strength which strengthens the skin dermis in order to maintain skin elasticity (Widgerow *et al*., 2016). The decreased production of collagen in intrinsic aging skin is most likely due to downregulation of TGF-β signaling and connective tissue GF which is thought to be a regulator of collagen expression (Cole *et al*., 2018; Poljšak *et al*., 2012).

Platelets are a reservoir of various GFs and important cytokines that can induce various tissue repairing, including skin tissue. Lysate is a liquid containing the contents of cells undergoing lysis. One type of lysate that is useful in medical therapy and research is human platelet lysate (HPL) derived from platelets (Bieback and Red, 2013). HPL contains many nutrients needed for cell growth and proliferation. Attachment factors (vitronectin and fibronectin) and some of the GFs present in platelets are released during their activation processes which are important in new tissue formation and tissue repair (Hemeda *et al*., 2014).

The GFs are contained not right in liquid lysate but in exosomes and vesicles. The content of exosomes includes biomolecules such as proteins, lipids, RNA, and DNA that play an important role in intercellular communication (Bang and Thum, 2012; Li *et al*., 2019). Active exosome molecules act directly on target cells through several mechanisms, stimulation of target cell ligands, activation of target cell receptors and epigenetic reprogramming through transfer of miRNA into cell nucleus, and regulating communication between cells through paracrine pathways. Exo-HPL has advantages such as high stability, high GF, no immune rejection, and direct stimulation to target cells so that it can contribute to a significant therapeutic effect on various target organs, including skin (Wang, 2021; Wang *et al*., 2019). These exosomes are also a promising alternative at a lower cost than other stem cell and progenitor cell therapies (Tao *et al*., 2017).

Exosomes derived from human umbilical vein endothelial cells (Exo-HUVEC) have been reported to be effective in ameliorating aging process in Ellistasari *et al*.'s study (2022). Another study by Oh *et al*. (2018) also stated that exosomes derived from induced pluripotent stem cells can ameliorate genotyping and phenotyping changes in photoaging skin. Exosomes derived from HPL (Exo-HPL) have been isolated previously. Exo-HPL, as has been reported by Torreggiani *et al*. (2014), can increase the deposit of mineral matrix in bone marrow stem cell, but there is no study yet that reported the usefulness of Exo-HPL in improving aging process. This study used young rats as animal model for the best outcome in intrinsic aging improvement after being treated with Exo-HPL and HPL. This study assessed the superiority of Exo-HPL compared to HPL in decreasing MMP-1 level and increasing collagen deposition on IRM.

MATERIALS AND METHODS

This research is an experimental post-test only group laboratory research conducted at the Experimental Animal Laboratory, Center for Food and Nutrition Studies, Gadjah Mada University, Yogyakarta and Dermama Biotechnology Laboratory of Surakarta and Anatomical Pathology Laboratory, Faculty of Medicine, Sebelas Maret University, Surakarta in the period April–July 2022. This study was approved by the Health Research Ethical Committee of Dr. Moewardi Hospital/Faculty of Medicine of Sebelas Maret University, Surakarta, Central Java, Indonesia (42/I/HREC/2022).

Preparation of HPL

Venous blood was taken from healthy donors with criteria for HPL samples as young age (12–25 years), normal platelet count (150,000–400,000 cells/mm^3), normal leukocyte count, no systemic disease, not taking any medicine, not smoking, not consuming alcohol, and having signed an informed consent. The method of making HPL followed the method of previous research on the manufacture of HPL. Twenty milliliters of venous blood was taken and then processed into platelet-rich plasma (PRP); after that the PRP was frozen at −80°C for 24 hours and then incubated at 37°C (one cycle); repeat up to two rounds. After two rounds of freezing and thawing, 4,000 × g was centrifuged for 15 minutes at 4°C to remove platelet particles and membrane fragments; then continue with separation between sediment and supernatant. The supernatant was filtered and sterilized and then stored at −20°C as HPL which would be used later (Schallmoser and Strunk, 2013).

Exo-HPL

Exo-HPL was isolated using the automatic fraction collector (AFC) izon qEV® (Izon Science, New Zealand) instrument with an assessment of 26 previously generated HPL fractions. The AFC device isolates Exo-HPL based on size exclusion chromatography with particle size 30–150 nm. Qualitative analysis and average particle size of Exo-HPL were obtained by using the nanoparticle tracking analysis (NTA) Viewsizer 3000® (Horiba, Japan) instrument. The results of NTA showed that the

number of Exo-HPL highest particles was 1.4 × 10^9 in the 11th exosomal fraction with an average particle size of 113 nm.

Animal models and D-Gal induction

We used Sprague-Dawley male rats aged 8 weeks with a body weight of 160–200 g. Sprague-Dawley strain rats were obtained from the Central Laboratory of Food and Nutrition Studies, Gadjah Mada University, Yogyakarta. Twenty-eight rats met the inclusion criteria, and they were randomly assigned into four groups, namely the young rats, IRM, IRM + HPL subcutaneous injection treatment, and IRM + Exo-HPL subcutaneous injection treatment groups. Intrinsic aging models were achieved by induction of D-Gal C6H12O6® (Pudak Scientific, Indonesia) performed intraperitoneally with the dose of 150 mg/kgBW/day for 8 weeks. On physical examination, the rats which had been induced with D-Gal showed dry, rough, shedding, and brownish-yellow hair, while the young rats had soft, thick, and pure white fur. The eye lenses of rats induced by D-Gal showed slight cloudiness, whereas in young rats the lenses of the eyes were clear and dark red in color.

HPL and Exo-HPL injection

HPL and Exo-HPL injections were performed after D-Gal induction for 8 weeks. HPL and Exo-HPL injections were given by subcutaneous injection on the back skin area once weekly on the same day. After 4 weeks, a 2 × 2 cm skin biopsy was performed at the injection site and then was separated into two parts for tissue homogenates calculation of MMP-1 level and histological preparation of Masson's Trichrome staining for calculation of collagen deposition. Termination of animal models was carried out by euthanasia using chloroform.

Calculation of MMP-1 level

We measured MMP-1 level using tissue homogenates samples taken from the back skin area after 4 weeks of treatment. The calculation of the MMP-1 level was carried out using the enzyme-linked immunosorbent assay (ELISA) method and measured by ELISA reader (Thermo Fisher Scientific, USA).

Preparation of MMP-1 tissue homogenates was carried out by washing the tissue with phosphate-buffered saline (PBS) once to remove adhering blood. Then it was homogenized with 20 ml of PBS liquid and stored overnight at −20°C. After two cycles of freezing and thawing to destroy the cell membrane, the tissue homogenates were centrifuged for 5 minutes at a speed of 5,000 × g. Then remove the supernatant and read using an ELISA reader.

Calculation of collagen deposition

Masson's Trichrome staining was performed to see collagen deposition by following Masson's Trichrome staining protocol. Collagen deposition in the dermis was observed with light microscope with 40× and 100× magnification in five fields of view using Optilab Viewer® (Optilab, USA). The interpretation of the results was carried out by an anatomical pathologist with repeated readings. Quantitative data were obtained using ImageJ software (%) in the bluish-green area of observation from Masson's Trichrome staining slides.

Statistical analysis

Data analysis was done using Statistical Package for the Social Sciences version 21. The normality test was performed using Shapiro–Wilk test. When a normal data distribution ($p > 0.05$) was obtained, one-way analysis of variance (ANOVA) data analysis was used to assess collagen deposition on study days and its MMP-1 levels and to determine significant differences for each group based on study results followed by the post-hoc least significant difference (LSD) test. The data were considered statistically significant if the p-value <0.05.

RESULTS AND DISCUSSION

The highest mean MMP-1 level was found in the IRM group. The lowest level of MMP-1 is located in the IRM + Exo-HPL treatment group (14.02 ng/ml) which was close to MMP-1 level in young rats group (10.26 ng/ml) (Fig. 1). ANOVA statistical analysis test revealed that there was a significant difference in MMP-1 levels among all the four groups (p-value <0.001); then post-hoc LSD test obtained significant differences in MMP-1 levels between IRM + Exo-HPL treatment group and IRM + HPL treatment group, respectively ($p < 0.001$). Our study found that the lowest MMP-1 level was in the young rats group, while the highest MMP-1 level was in the IRM group. MMP-1 had its lowest level among all of the treatment groups in IRM + Exo-HPL group; therefore Exo-HPL is more effective in decreasing MMP-1 level than HPL. The highest mean of skin collagen deposition in 40× magnification is found in the young rat group. Between treatment groups, the highest deposition of collagen is found in IRM + Exo-HPL treatment group (38.40%) which was close to collagen deposition on young rats group (42.90%) (Fig. 2). ANOVA statistical analysis test revealed that there was a significant difference of collagen deposition levels among all the four groups (p-value <0.001). Post-hoc test analysis showed more collagen deposition in IRM + Exo-HPL treatment group than in IRM + HPL treatment group observed on 40× magnification. Features of collagen deposition in 40× magnification can be seen in Figure 3. At 100× magnification the study demonstrated that there was higher collagen deposition in IRM + Exo-HPL treatment group (39.94%) than in IRM + HPL treatment group (34.53%), which was close to the young rats group's collagen deposition

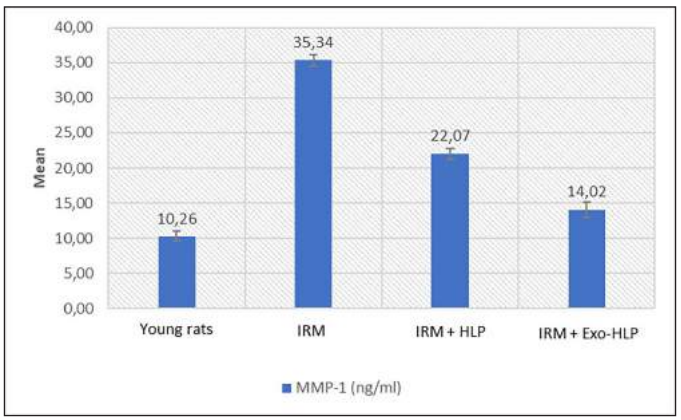

Figure 1. Mean value of MMP-1.

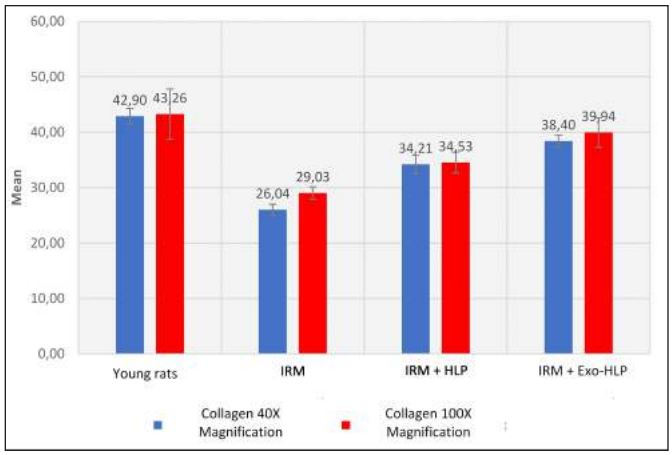

Figure 2. Mean value of collagen deposition at 40× and 100× magnification.

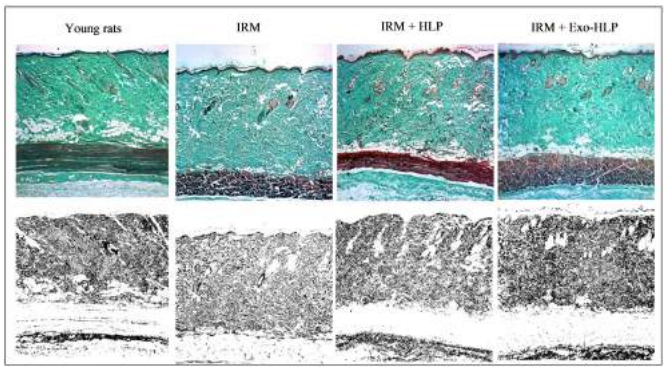

Figure 3. Collagen deposition using Masson's Trichrome staining (top row); collagen deposition using ImageJ software (bottom row) at 40× magnification.

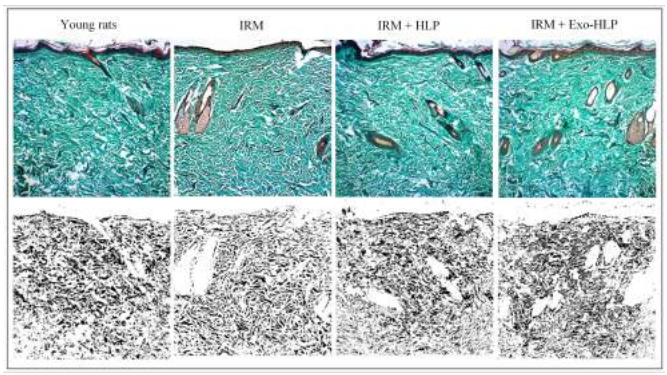

Figure 4. Collagen deposition using Masson's Trichrome staining (top row); collagen deposition using image J software (bottom row) at 100× magnification.

(43.26%) (Fig. 2). p-value < 0.001 in ANOVA test means that there is a significant difference in all groups. A post-hoc test followed the analysis. There is a significant difference ($p < 0.001$) of collagen deposition in 100× magnification between IRM + Exo-HPL treatment group and IRM + HPL treatment group, which means Exo-HPL is superior to HPL in increasing skin collagen deposition. Features of collagen deposition in 100× magnification can be seen in Figure 4.

Our study demonstrated the superiority of Exo-HPL compared to HPL in decreasing MMP-1 level and increasing collagen deposition in rat model of intrinsic aging. Exosomes are metabolite microvesicles that carry messenger ribonucleic acid (mRNA) and miRNA. HPL is rich with a variety of GFs and cell growth-promoting molecules, including a new range of blood products used for regenerative therapy, cell therapy, and tissue engineering (Delila *et al*., 2020; Wang, 2021). Exo-HPL becomes a critical paracrine secretion which acts as an information transfer mediator and cell communication through bioactive protein, mRNA, and miRNA inside of it. Torreggiani *et al*.'s (2014) study which analyzed the protein content of Exo-HPL revealed amounts of basic fibroblast GF, vascular endothelial GF, platelet-derived GF, and transforming GF-β (TGF-β) up to fourfold higher compared to HPL. These results suggest that exosomes consistently contribute to HPL activity and are suitable for cell-free regeneration therapy. Since Exo-HPL contains nano-sized particles that can reach all cells rapidly, repair cells, and improve cell-to-cell communication, Exo-HPL may be useful in future physiological and pathological aging studies. In addition, exosomes are non-immunogenic in nature, and therefore they do not cause any rejection (Torreggiani *et al*., 2014; Zhao *et al*., 2020).

Induction of intrinsic aging with D-Gal has been widely used for anti-aging treatment study. IRM in this study was obtained by D-Gal induction intraperitoneally for 8 weeks. This induction was based on a prior study by De Almeide Rezende *et al*. (2021), which successfully induces aging in rat with intraperitoneal injection of 150 mg/kgBW/day for 8 weeks. The results of this study on MMP-1 level in Figure 1 showed that there was a significant difference in MMP-1 level among all groups. The ANOVA test showed that both Exo-HPL and HPL are effective in decreasing MMP-1 level in IRM. Exo-HPL is superior to HPL in decreasing MMP-1 level confirmed with post-hoc LSD test. This result was in line with an *in vitro* study by Umbayev *et al*. (2020), suggesting that in skin aging MMP-1 is activated by nuclear factor-kappa beta and activator protein-1, which further degrade collagen in aged skin. MMP-1 is the only MMP that can break down intact fibrillar collagen. It recognizes substrates via a hemopexin-like domain and is capable of degrading types I and III fibrillar collagen (Kammeyer and Luiten, 2015; Umbayev *et al*., 2020).

This study showed that both Exo-HPL and HPL were effective in increasing skin collagen deposition in IRM observed on 40× and 100× magnifications. Our statistical analysis found that Exo-HPL is superior to and more effective than HPL in increasing skin collagen deposition. This finding supports Ellistasari *et al*.'s study (2022) which reported that Exo-HUVECs significantly decrease MMP-1 level and increase collagen level in aged skin. A study by Hu *et al*. (2019) also found that the use of exosomes derived from three-dimensional cultures of human dermal fibroblasts has anti-aging properties resulting in significant increase in dermal collagen deposition and a decrease in MMP-1 level. This may also occur via the downregulation of tumor necrosis factor-α and upregulation of TGF-β (Hu *et al*., 2019). The limitation of this study is that we only examined MMP-1 level and skin collagen deposition locally on the back area of the rats and did not examine other parts of the skin so the systemic effects of decreased MMP-1 level and increased skin collagen deposition could not be assessed. This study did not

examine the types of miRNAs present in Exo-HPL. Assessment of molecules such as p16, p21, p53, and lamin should further improve the results of this study.

CONCLUSION

Exo-HPL and HPL are effective in lowering MMP-1 levels and increasing collagen deposition in IRM. Since Exo-HPL is superior to and more effective than HPL in reducing MMP-1 level and increasing collagen deposition, then Exo-HPL is recommended as a therapeutic modality for intrinsic aging.

AUTHORS' CONTRIBUTIONS

E. R., E. Y. E., A. K., and I. J. contributed to the concept and design of the work; E. R., E. T. E., A. K., I. J., and N. A. S. contributed to data acquisition; E. R., E. Y. E., A. K., S. W., and N. A. S. contributed to data analysis/interpretation; E. R., E. Y. E., A. K., and F. Y. contributed to drafting the manuscript; E. R., E. Y. E., and A. K. contributed to critical revision of manuscript; E. R., A. K., S. W., and F. Y. contributed to statistical analysis; E. Y. E., A. K., I. J., and W. S. contributed to supervision; All authors approved the final manuscript.

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CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

This study was approved by the Health Research Ethical Committee of Dr. Moewardi Hospital/Faculty of Medicine of Sebelas Maret University, Surakarta, Central Java, Indonesia (42/I/HREC/2022).

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

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