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Keywords: Amniotic membrane MSCs; 3-dimensions (3D) micro-carrier; Non-human primate third-degree burn injury model; HGF; VEGF; Collagen

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burns wound using non-human primate (NHP), Macaca fascicularis, burn wound injury
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Methods. AM-MSCs was isolated by standard protocol in two-dimensional (2D)
cultures following the increasing process of cells in 3D micro-carrier cultures. These
were analyzed for its morphology, ability to proliferate, surface markers and multilineage
differentiation potential. The 3D AM-MSCs were then injected under the burns
wound lesion of the NHP burn wound injury model. Then, we observed the growth
factors, the speed of healing process and the scars formation of 3D AM-MSCs,
compared to the gold standard treatment of silver sulfadiazine (Burnazine) ointment.
Samples treated with the physiological sodium chloride (NaCl 0,9%) served as a
negative control.
Results. The surface antigen phenotype, morphology and differentiation characteristics
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significantly accelerated when 3D AM-MSCs were applied in NHP burn wound injury
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VEGF levels were increased, and there were no scars formation on the lesions treated
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growth in the lesion area found in 3D AM-MSCs-group.
Conclusion. 3D AM-MSCs transplantation effectively promotes rapid wound closure in
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Original Article

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Running title:

Allogeneic amniotic membrane-derived mesenchymal stem cells for therapy of burn injury

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Abstract

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Methods. AM-MSCs was isolated by standard protocol in two-dimensional (2D) cultures following the increasing process of cells in 3D micro-carrier cultures. These were analyzed for its morphology, ability to proliferate, surface markers and multi-lineage differentiation potential. The 3D AM-MSCs were then injected under the burns wound lesion of the NHP burn wound injury model. Then, we observed the growth factors, the speed of healing process and the scars formation of 3D AM-MSCs, compared to the gold standard treatment of silver sulfadiazine (Burnazine) ointment. Samples treated with the physiological sodium chloride (NaCl 0,9%) served as a negative control.

Results. The surface antigen phenotype, morphology and differentiation characteristics of 3D AM-MSCs exhibited properties of MSCs. The speed of wound closure was significantly accelerated when 3D AM-MSCs were applied in NHP burn wound injury model compared to the group with topical application of Burnazine ointment. HGF and VEGF levels were increased, and there were no scars formation on the lesions treated with 3D AM-MSCs. The healing speed were quickly restored, and we found new hair growth in the lesion area found in 3D AM-MSCs-group.

Conclusion. 3D AM-MSCs transplantation effectively promotes rapid wound closure in NHP burn wound model. The large amount of cells requirement are solved by 3D cultures that will facilitate the development and improve the burn treatment in clinical set-up.

Key words: Amniotic membrane MSCs, 3-dimensions (3D) micro-carrier, Non-human primate third-degree burn injury model, HGF, VEGF, Collagen.

Introduction

Burns are one of the most common and devastating forms of trauma worldwide. Most deaths in burn-injured patients are due to sepsis and its complications.[1] The vast majority of burn wound complications can be prevented by proper early management to prevent pain, discomfort and minimal scar formation.[2,3] Burn wound are classified based on its depth and severity:

1. First-degree (superficial) burns are burns that only affect the epidermis or the outer layer of skin. The burn site is red, painful, dry and with no blisters formation. Mild sunburn is an example. Long-term tissue damage is rare in this grade of burn and usually manifests of an increase or decrease in the skin color; wherein a white plaque and minor pain occur at the site of injury and repair takes place spontaneously. The burn wound resolves within 3 to 4 days without any complication.
2. Second-degree (partial thickness) burns involves all layers of epidermis, the upper part of the dermis (papillary dermis) and may involve the deep dermis (reticular). The burn site appears red (erythematous), with forming of superficial blisters, and might be swollen and painful. The burn wound has a longer healing process of about one month, with minimal cicatrix sequelae.
3. Third-degree (deep partial and full thickness) burns destroys the whole thickness of epidermis, dermis and might also affect the subcutaneous tissue. The burn site may appear white or charred.

4. Fourth degree burns also damage the underlying bones, a deeper muscles layer, tendons and ligament tissue. There is no sensory sensation since the nerve endings are also destroyed.

Of all the burn cases, most patients present with second-degree and third-degree burn injuries.[5] The healing process of burn wounds involves 3 phases: inflammation, proliferation and remodelling.

The mesenchymal stem cells (MSCs) are multipotent adult stem cells which are abundant in human tissue. MSCs are classified in accordance with their tissue of origin, such as amniotic membrane MSCs (AM-MSCs), bone marrow MSCs (BM-MSCs), adipose MSCs and umbilical cord MSCs (UC-MSCs). It has been proven that MSCs can be propagated *in vitro* and retain their multipotency through multiple passages. Moreover, MSCs can differentiate into bone, cartilage, adipose tissue, muscle, neurons, liver cells and cardiocytes, among other cell types.[6,7] Among these MSCs, AM-MSCs are thought to play a key role in wound healing.[8] AM-MSCs thus show tremendous potential to improve burn wound healing in many of rodent and rabbit models.[9,10]

Macaca fascicularis or a long-tailed macaque or crab-eating monkey; a laboratory animal, as known as Cynomolgus monkey, *Macaca irus* and *Simia aygula* (Raffles, 1821). *Macaca fascicularis* is a native animal of Southeast Asia, including Indonesia, Malaysia, Thailand, Indochina (see its distribution area in Fig. 1). The habitat of *Macaca fascicularis* includes tropical and mangrove forests. Usually, they inhabit coastal forests, forests along large rivers, near settlements, mixed gardens or plantations, in some places up to an altitude of 1.300 metre above sea level.[11] Study of an international consortium on the composition of the rhesus monkey genome found that the DNA similarity of non-human primate (NHP) species and humans was 98%. Currently, studies using

primate animals are increasing since many beneficial of primate animals themselves.[12]

The conventional method of culturing stem cells is on two-dimensional (2D) surfaces, which is not amenable for scale up to therapeutic quantities in bioreactors. We have developed a facile and robust methods for maintaining undifferentiated cells in novel three-dimensional (3D) suspension cultures on microcarriers achieving 2- to 4-fold higher cell densities than those in 2D AM-MSCs colony cultures for NHP, *Macaca fascicularis*. Micro-carrier cultures achieved even higher cell-concentrations in suspension spinner flasks, thus opening the prospect of propagation in controlled bioreactors.

In this study, we examined the feasibility and safety of 3D AM-MSCs injection to improve healing of burn wound in a NHP model. To the best of our knowledge, there are no reports on the use of AM-MSC that cultured (3D) to treat severely burn wound injuries in NHP, *Macaca fascicularis* burn wound model. Allogeneic AM-MSCs cultured 3D were injected beneath burn wounds in a NHP model and wound closure was evaluated by assessing wound surface closure efficiency, hepatocyte growth factor (HFG), vascular endothelial growth factor (VEGF), collagen and scars formation.

Materials and Methods

This study was approved by the Ethics Committee of the Institutional of Animal Care and Use Ethics Committee (IACUC) of PT Bio Farma (Persero) No. 01/IACUC-BF/III/2017 with a validity period of 2 years, April 14th, 2017−2019.

Harvest and preparation of Macaca fascicularis Amniotic Membrane

Macaca fascicularis placentas (n=3) from healthy donors (female, age 3-4 years old, 3000 ± 100 gram of body weight, spesific pathogen free for TB, SIV, SV40, Polio type 1, 2, 3, Foamy virus and Herpes B virus, were obtained from selected caesarean sections at the

Unit-3 Laboratory of Animal Test PT. Bio Farma (Persero), Bandung. Under stringent sterile conditions, the harvested placentae were placed in PBS medium containing 2% Antibiotic/Antimycotic. The amniotic membranes were then carefully separated from the chorion, which was then discarded, and the amnion was immediately washed three to five times with 0.9% NaCl solution to remove blood and mucus.

Isolation of MSCs from Amniotic Membrane (AM-MSCs).

Amnionic membranes (AM) were separately processed after the isolation according to the isolation protocols of Reinisch et al.,[13] Then, the AM was washed with PBS and digested with enzyme collagenase-1 for 1 hour (h) at 37°C, then filtered using cell strainer, then centrifugated and made into pellets which can be cultured on the plate by adding the appropriate medium. The mechanical method is carried out by washed the membrane with PBS and then chopped fine with scissors and cultured on plate by adding appropriate medium. The plated tissues are air-dried for 10 minutes (mins) before adding growth medium and then air-dried to remove as much PBS-KCL as possible so that tissues adhere to the plastic surface. Medium slowly and gently added. Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37ºC. Approximately 3-5 days after explantation, fibroblast-like adherent cells migrated from the tissue fragments. After one week of passage 0 (P0) growth with fresh growth medium replacements every 3 days, the sub-confluence (~80%) adherent cells were collected and replated on new dishes at a density of $2x10^3$ cells/cm^2 (P1).

Two-dimensional (2D) Culture of AM-MSCs Macaca fascicularis in a flask.

Defrosted cryopreserved MSC were plated with 2000 cells/cm^2 in αMEM proliferation medium. The medium was replaced every 3 to 4 days (d). At near confluency (80%) cells were subcultured until passage 3. The passage 3 harvested cells were used for the 3D

micro-carrier cultivation in spinner flasks.

Macaca fascicularis placenta must be immediately processed to isolate MSCs after the birth process with a transfer medium containing DPBS + 2% Antibiotic/Antimycotic up to 125 ml. Isolation was carried out aseptically under BSC class II. The processing procedures were done as follows:

1. The amniotic membrane is released using sterile scissors and tweezers.
2. Processed with transfer medium (PBS+KCL+1% Antibiotic/Antimicotic with pH 7.4).
3. Washed membranes with transfer medium 8 times.
4. Isolation of MSCs from the amniotic membrane is done by a mechanical method by cutting or chopping amniotic membrane tissue with sterile scissors and tweezers (should not be done too smoothly since unwanted tissue can also be filtered).
5. Addition of collagenase 0.7 µl, collagenase is dissolved in the DPBS.
6. Samples are incubated for ± 1 h in a 37°C incubator, every 10 mins is vortex at the end of the incubation.
7. Samples are filtered with a ± 40 µm cell strainer (filtration is carried out and accommodated in the growth medium is done slowly so that the membrane network does not participate filtered (sample impurities).
8. Culture is incubated, then on the first day after isolation, the old medium is wtransferred to a new plate. In the old culture plate, a new medium was replaced. On the first day after isolation, there will be visible AM-MSCs that grow in length.
9. Make sure the growth of AM-MSCs is not over-confluent so that the character of AM-MSCs is maintained with no differentiation.
10. Growth medium is replaced every 3 days and we continually observe whether that the growth of AM-MSCs reaches its confluent > 80% (±5 days). Make sure the growth of

AM-MSCs is not over-confluent so that the character of AM-MSCs is maintained with no differentiation.

11. Subculture is carried out after the growth of MSCs reaches confluent > 80% (P0) and is planted into a new plate with a cell density of 2000 cells/cm^2. Calculate the number of cells/cm2 of explants and population doubling time AM-MSCs with the data obtained.

Subculture Procedure

1. TrypLE Select is inserted into a 37°C incubator for ± 20 min (depending on volume).
2. Culture is washed with 2x DPBS.
3. Discard DPBS add TrypLE Select as much as 1/3 of the total volume.
4. Culture is incubated for 8 minutes in the incubator.
5. Medium inactivation is added in the form of FBS 20% with a ratio of medium: TrypLE Select = 1 : 1, when using FBS 10%, a ratio of 2 : 1 is used.
6. If there are still cells that have not yet detached, then washing again with DPBS.
7. The sample is re-suspend so that the colony is single.
8. The cell count is calculated using a hemocytometer with the trypan blue: culture ratio = 1 : 1 (10µl)

Proliferation Assay

AM-MSCs were plated in 10 cm culture dish with seeding density of 2x10^3 cells/cm^2. The population doubling (PD) between each passage was calculated using the equation PD = [log10 (NH) – log10 (NI)/log10.[14] NH is the number of harvested cells and NI is the number of inoculums cells. PD time (PDT) calculated using the time interval between cell seeding and harvest divided by the PD for that passage, start from P0 to P2.

Freezing Cells

1. The culture was centrifuged to remove TrypLE Select, DPBS and medium at a speed

of 300 g for 4 mins,

2. Insert the pellets that have been mixed with FBS: DMSO = 9 : 1 in the volume of 1 ml into the cryo-tube. This procedure was done in cold conditions (using ice),
3. Store the cryo-tube into Mr. Frosty for 1 month (maximum), then transfer the cryo-tube into the liquid nitrogen.

Three-dimensional (3D) Micro-carrier cultures of AM-MSCs in agitated conditions

To passage AM-MSCs from 2D colony cultures to 3D micro-carrier, cells grown on a tissue culture flask 25 cm^2 were harvested by treatment with TrypLE Select, followed by pipetting to form clumps < 100 μm in diameter, typically ranging from 50-60 μm. Alternatively, a pipette was used to create clumps less than 100 μm.

Cytodex 1 microcarriers beads were used for AM-MSCs cultures. Micro-carriers were rehydrated in calcium- and magnesium-free PBS and sterilized by autoclaving at 121°C for 20 minutes. Then, these were micro-carrier washed three times using basal medium plus 1% antibiotic then adapted in the growth medium complete as much as the total volume of culture in the incubator and fermentor for 1 h. AM-MSCs were cultured at 37°C in 5% CO_2 with 50 rpm agitated for 2 h then increased to 70 rpm. Cells were seeded at 4×10^2 cells/ml for 2mg/ml concentration of cytodex 1 micro-carrier. The cultures were continuously run and cell confluence levels are observed every day. Cell counts were taken every day until harvest time and immunophenotyping was performed after harvesting. Harvesting of the cell and conditioned medium was carried out after the cells confluence on the cytodex 1 micro-carrier reached 80-90%. After cells on the surface of the micro-carrier confluent, agitation was stopped so that the cells and micro-carriers settle down and harvest the used growth medium. Then, the cells were washed with a growth medium without FBS and settled again, then the washing medium was removed.

Cells detached from the micro-carrier use TrypLE Select 10x solution which has warmed to the 37°C temperature with a volume ratio of 1:10 at low agitation of 20 rpm for 6-10 mins. After all the cells are released from the micro-carrier, filtering is carried out using a nylon 100 µm sterile filter bag (in-house filter). The cell was washed with growth medium and ready for use/storage.

Micro-carrier flask cultures

Mechanically dissociated AM-MSCs obtained from static MC were seeded at a density of 4×10^2 cells/ml into a presiliconized (Sigmacote, Sigma-Aldrich) 100 ml erlenmeyer dispossable flask (Corning) containing 2mg/ml of micro-carriers in 30 ml of growth medium (Lonza). The culture was incubated inside a controlled incubator at 37°C and 5% CO_2 at initial agitation speed of 20 rpm. After a day, the medium was topped up to 30 ml and agitation speed was increased to 70 rpm; 50% of the spent medium was removed every 2-3 days and replaced with fresh medium. Daily samples were taken for cell counts and FACS analysis was performed at harvest time (5 days on average).

Cell counts

AM-MSCs were sampled daily as a single cell suspension after detachment with TrypLE Select enzyme. Cells were counted at a confluence of 80-90% on cytodex 1 micro-carrier. Briefly, suspension cultured cells on cytodex 1 micro-carrier were detached by TrypLE Select 10x solution, then incubated 5-10 mins at 37^oC, completed medium consisting of 80% MEM-α, 20% FFP, 1% Antibiotic/Antimicotic were added to stop and centrifuged at 1600 rpm, 5 min at 24^oC. The pellet of cells was resuspended with trypan blue solution diluted 1:1. Cell number was determined with particle counter (Coulter-counter, Beckman Coulter). Cell viability was determined by trypan blue staining with 1:1 ratio (10 µL).

Immunophenotyping

According to a recent proposal of the International Society for Cellular Therapy (ISCT),[15] MSCs are multipotent nonhematopoietic progenitors that are phenotypically characterized by expression of several positive markers (e.g. CD73, CD90 and CD105) and the lack of expression of CD11b, CD19, CD34, CD45 and HLA-DR surface molecules.[16,17] Briefly, the MSCs (P3) were incubated for 15 mins at 4ºC dark condition with the following antibodies Anti Anti CD73-APC, Anti CD90-FITC, Anti CD105-perCP-CY5.5, Anti Linage-Negative-PE and HLA-DR-FITC (BD Biosciences kit). MSCs were acquired using a FACS Calibur (BD) and analyzed with Cell Quest Pro software.

Osteogenic, Chondrogenic and Adipogenic Differentiation

The MSCs (P3) were plated in six-well plate at $5x10^3$ cells/cm^2. The cells were incubated in MSCs growth medium in a humidified atmosphere with 5% CO_2 at 37ºC for 2 h-4 days. Replace media with pre-warmed complete osteogenesis, chondrogenesis and adipogenesis differentiation medium and continue incubation. MSCs will continue to expand as they differentiate under each conditions. The cultures were refeeded every 3-4 days. After 21 days or longer under differentiation medium, the medium was removed from the plate and rinsed once with PBS. Cell was fixed using 4% formaldehyde solution for 30 mins, then rinsed 2x with distilled water, and 60% isopropanol was added for 2-5 mins. Aspirate off ispropanol and stain with 2% Alizarin red S solution (pH 4.2) for 2-3 mins, 1% Alcian Blue solution for 30 mins, Oil Red O working solution for 5 mins, then rinsed 3x with distilled water and captured in microscope.

Non-human primate Burn Wound Injury Model

Male NHP, *Macaca fascicularis,* ages 3-4 years old; 3000 ± 100 gram of body weight, specific pathogen free for TB, SIV, SV40, Polio type 1, 2, 3, Foamy- and Herpes B-virus, were kept in cages with free access to food and water. Three placental amniotic

membranes from 3 female *Macaca fascicularis* were used for therapy in burn injury model. The full thickness burn sites (n=6) were made on each NHP. Burn wound induction was performed following a Mason-Walker burn model as guideline, with several modifications.[18] On burn induction day (day 0), *Macaca fascicularis* were generally anesthetized using atropine sulfas premedication 0.02-0.05 mg/kg, ketamine 5-10 mg/kg and xylazine 0.1-0.5 mg/kg body weight; before burn wound induction. A digital electrical appliance with a circular stainless steel plate was heated to 120°C, then applied for 20 seconds on a clean-shaved back skin area of the *Macaca fascicularis* to produce a grade 3 burn area of 4 cm diameter. Burns were made in 4 NHP, with 6 sites in total. After 24 h since the burns were made, the treatment was carried out in 4 groups: (i) physiological NaCl 0,9%, (ii) Silver Sulfadiazine (Burnazine), (iii) MSCs-1 dose of $5x10^5$ cells and (iv) MSCs-2 dose of $2x10^6$ cells. The observation of burn wound was carried out on days 0, 1, 7, 14, 21 and 28. On the 28^{th} day, the study was terminated and humane killing was carried out by using excessive doses of ketamine and perfusion through the heart using NaCl 0,9% and 40% formaldehyde according to the procedure. After the NHP is confirmed dead, the cadaver was then incinerated**.**

Assessment of Wound Healing.

Wound area was diameter were measured every 7 days, started from day 1 until day 28 post-burn wound induction. Wound size was measured by imprinting the wound area on a transparent paper, then the diameter of wound is measured using a caliper.

Then, percentage of wound closure (%) was calculated by formula below:

$$\frac{\text{(Wound Initial Area – Wound End Area)}}{\text{Wound Initial Area}} \text{ x } 100\%$$

Initial wound closure (in day 0) was defined as 0% wound closure. The percentage of wound closure is considered as observation data in this study for the next analysis.

ELISA for measure check HGF, VEGF and Collagen levels after treatment

Macaca fascicularis's burn wound injury model serum sample was checked for its HGF, VEGF and Collagen levels. Growth factors assay measured using ELISA kit at room temperature according to the manufacturer's instructions (*Novex Lifesciences*). Serum was measured in day 1, 7, 14, 21 and 28 according to a standard curve constructed for each assay. The colorimetric absorbance for HGF-concentrations (pg/ml), VEGF- (pg/ml) and Collagen- (μg/ml) were analyzed at a wavelength of 450 nm using a microplate reader.

Statistical Analysis

Six replicates for each experiment were performed. The percentage of wound area and the number of growth factors for each group are presented as mean ± standard deviation and were analyzed by analysis of variance followed by Bonferroni's test. Correlation test was done by Pearson correlation test. The observation data were analyzed in order to assess the normality of the data distribution by using Shapiro-Wilk. ANOVA test was used for data that are normally distributed and followed by multiple comparisons post-hoc *Turkey* test, whereas Kruskall Wallis test was used for abnormal distribution data. All the statistical analyses were performed with SPSS software version 25.0 for Windows, with a p-values of <0.001 and <0.05 considered as statistically significant.

Result

Isolation of MSCs from Amniotic Membrane (AM-MSCs)

The average number of processed 2D AM-MSCs isolated was $9{,}6x10^5$-$1{,}2x10^6$ cells (n=3) in 75 cm^2 tissue culture flask (TCF) with mean viability 97,3%; 2D AM-MSCs expanded easily in vitro and exhibited a fibroblast-like morphology (Fig. 2). In flow cytometry,

characteristic expressions of surface markers were confirmed. 2D AM-MSCs expressed CD73, CD90, CD105 and lacking of negative markers; osteogenic, chondrogenic and adipogenic differentiation was also confirmed by conventional method (data not shown).

Population Doubling Time (PDT) 2-dimensional (2D) vs. 3-dimensional (3D)

The PDT for each 2D AM-MSCs vs. 3D AM-MSCs isolated cells were shown in Table 1. Statistical analysis was performed to observed the significant different of 2D PDT vs 3D, using independent t-test followed by one tailed of analysis of variance (ANOVA).

Growth of 3-dimensional (3D) culture of MSC

In the process of cell culture, the risk of contamination is closely related to the stages in handling culture, such as opening and closing bottles or flasks on a 2D monolayer culture repeatedly. By using micro-carrier in the bioreactor the risk of contamination can be avoided because culture takes place in a closed system and automatically. The sampling process can be carried out without risk of contamination.[19] Cell in 3D micro-carrier is a simulation of cell growth conditions in vivo because cells can grow freely, and were not constrained in the form of platting following the shape of the base as on the surface of a bottle or flask. Therefore, the cells can be rounded (spheroid) or elliptical (ellipsoid), and were able to join (aggregate), communicate and release proper secretions in vivo conditions.[20] In this study it was found that the morphological characteristics can be seen in Fig. 3, it appears that the growing cell adheres to the surface of the cytodex 1 micro-carrier and reached a 90-95% confluence on day 5, spindle-shaped cells like rounded fibroblasts of smaller size than 2D culture in the flask. In this study, it was found that the MSCs size averaged 14 μm.

Three-dimensional MSC culture using cytodex 1 micro-carrier suspension showed that the MSCs growth rate measured as the MSCs proliferation rate with the population

doubling time (PDT) was obtained 1.2 days (29 hours) on average. It can be said that in the 3D environment, the cells grew better because it resembles the actual conditions of cell life in the body (mimicking in vivo conditions), therefore there will be better results in many ways including potential differentiation, morphology, proliferation, cell function general for example against drug response, expression (genes, proteins), stimulating responses and also viability.[21] In other words, cell excellence is seen from its function. Cells that are not natural in an unnatural environment cannot get natural functions.[22] From the results of this study (Fig. 3) it was found that MSCs from *Macaca fascicularis* amniotic membrane included CD clusters that were in accordance with MSCs in humans, namely positive CD clusters (Fig. 4A) for CD73 (98.97%), CD90 (98.80%), CD105 (78.40%) and negative CD clusters (Fig. 4b) for negative coctail (CD34/CD45/CD11b/CD19/HLA-DR) (0.48%) and HLA-DR (0.37%). In Fig. 5, we can see that the MSC from the amnion membrane of *Macaca fascicularis* which is cultured in 3D using a cytodex 1 micro-carrier suspension in the bioreactor can differentiate into osteocytes by red alizarine staining, chondrocytes with alcian blue and adipocyte with red oil staining.

Macaca fascicularis Burn Wound Injury Model

The development of the percentage of wound closure in our NHP model among negative control (NaCl 0,9%) group, gold standard (Burnazine) group, 3D AM-MSCs $5x10^5$ and 3D AM-MSCs $2x10^6$ cells groups. The development of the percentage of wound closure among those four groups on day 7 to 28 was shown in the Fig. 6. There was a significant different of 3D AM-MSCs $5x10^5$ and $2x10^6$ cells groups to accelerate wound closure and the hair began to grow on the 14^{th} day vs. Burnazine as the gold standard (p=0,001). There no significant different between 3D AM-MSCs $5x10^5$ group vs. 3D AM-MSCs

$2x10^6$ group (p=0,887). Furthermore, the representative of the wound closure progress, with or without keloid formation, of four groups was presented in the Fig. 7.

HGF, VEGF and Collagen Measurement Results using ELISA

HGF, VEGF and Collagen level was measured using ELISA kit using blood sample taken from treated NHP femoral vein. There are significant differences between 3D AM-MSCs $5x10^5$ group and $2x10^6$ group compared to the negative control (NaCl 0,9%) along with gold standard Burnazine in terms of HGF level both on daily basis from day 7 to 28 (p=0.000) and on the mean value of HGF (p=0.000), Fig. 8A and B respectively. HGF in NaCl 0,9% was too low and not detected. There were significant differences between 3D AM-MSCs $5x10^5$ group and $2x10^6$ group compared to negative control (NaCl 0,9%) and gold standard Burnazine in VEGF level both on day 7 to 28 (p=0.000) and on the mean value of VEGF (p=0.000), Fig. 9 and 8B respectively. VEGF level was decreasing in time dependent manner also VEGF in NaCl 0,9% was too low and not detected. There are significant difference between 3D AM-MSCs $5x10^5$ and $2x10^6$ group very low compared to negative control (NaCl 0,9%) and gold standard Burnazine in collagen level both on day 1 to 28 (p=0.000) and on the mean value of collagen (p=0.000), Fig. 10A and B respectively.

Effect of HGF and VEGF serum levels, on healing burn wound is greater than the collagen. Wound healing of burns treated with 3D AM-MSCs did not cause scarring since the collagen concentration was very low compared to the effects obtained from Burnazine (p=0.786), while for MSCs (p=0.001 and 0.000). It appeared that the wound healing effect of burns are strongly influenced by levels of HGF and VEGF concentrations (p=0.001) compared to the effect of collagen concentration (p> 0.001).

Discussion

In the past 30 years, there has been a clear evolution of the conceptual strategy in the field of burns treatment. The each one of the three Rs, "Replace, Reconstruction, Regenerate" has clinical applications in transplantation, tissue engineering and now stem cell therapy.[23,24] MSC is a cell that can renew itself and can differentiate into several other types of cells. MSCs has been isolated from various sources such as placenta, bone marrow, adipose tissue, peripheral blood and other adult tissues. The interesting aspects about MSC lies in its ability to cure degenerative diseases, and MSC is currently in the phase of clinical studies. Three remarkable things about stem cells are their role in the field of cell therapy, regenerative therapy and the discovery of new drugs in animal and human models.[25]

Animal models have greatly improved our understanding of the cause and progression of many human diseases and have proven to be a useful tool for discovering therapeutic drugs. Previous reports found that DNA sequence similarities between NHPs and humans can reach up to 98.77%[26] and the concordances rates of gene transcription levels of spleen, peripheral blood monocyte cells (PBMCs) and liver between NHPs and humans can reach as high 91.41%, 84.36% and 74.29%, respectively.[27] The data garnered from such NHP experiments is invaluable as a starting point, because it ensures greater efficiency and surety of future clinical application, making NHPs the “gold standard” for preclinical studies.[28] For burn wound studies, in vitro models are limited in their ability to capture all aspects of burn pathophysiology and the complex clinical features of human burn wound injury. NHPs are phylogenetically close to humans, with many similarities in terms of physiology, anatomy, immunology, as well as neurology, all of which make them excellent experimental models for biomedical research such as burn wound injury.

The current 2D tissue culture platform can be used when low doses are needed and it becomes impractical when doses above $50x10^6$ are needed; especially considering the high risk of cells contamination along its productions for in-and-out incubator repeatedly. We were interested in the usage of the micro-carrier platform to generate stem cells in a scalable 3D manner. MSCs originally grow as monolayer, as we developed method to culturing MSCs to a micro-carrier based expansion platform. To this date, the usage of micro-carrier has been limited mainly to vaccine production. However, in terms of bioprocessing unit that demanding a large scale of cells productions and the low risk of contamination, 3D micro-carrier platform were able to provide an alternative solution for its application, since the system are closed and automatic. In this present study, we also compared 2D AM-MSCs with 3D AM-MSCs, and our results indicated that there were no differences in terms of differentiation *in vitro*, in specific cell marker expression but only in cell population doubling time, suggesting that 3D AM-MSCs grow much faster in term of PDT. From the study on the expansion of the 3D ex-vivo method using a suspension of micro-carrier in a bioreactor, obtain morphological and immunophenotyping results that are in accordance with human MSC characteristics. Likewise, it is proven that *Macaca fascicularis* amniotic membranes derived-MSC is multipotent; can differentiate into 3 main strains (osteocytes, chondrocytes and adipocytes).

In this present study, 3D AM-MSCs were superior to the gold standard therapy for burn wound (Burnazine) in improving burn wound injury healing in our NHP model, Implantation of 3D AM-MSCs significantly accelerated the wound closure rate; the wound closure rate were significantly accelerated in day 7, followed by an increase of the rates of HGF concentrations for increases migration, reflecting higher differentiation of many cells, especially epithelialization[29] and VEGF for angiogenesis that decreases in

time dependent manner along remodelling phase. These advancements, accompanied with the dry wound conditions, prevented excessive cicatrisation-scars and remodelling of hair growth while the body-weight of NHP treated with 3D AM-MSCs was restored more quickly and they also displayed much better mobility compared to the negative control group and gold standard group (Table 2).

Furthermore, HGFs play a role in the metabolism of collagen fibrils, which are stable under the physiological conditions of the extracellular environment.[30] Besides this direct modification of the collagen, HGF has been shown to influence transforming growth factor A1, which, in turn, modifies collagen-synthesis and –degradation.[31] During the wound healing process, the imbalance of collagen synthesis and degradation resulting in excess accumulation of dermal collagen can lead to the scar complications.[32] The production of collagen can be a double-edged sword: on the one hand, it is necessary for wound healing; on the other hand, excess deposition of collagen can result in scarring;[33] as in our study shown that collagen in negative control (NaCl 0,9%) 20x higher and the gold standard (Burnazine) 10x higher compared 3D AM-MSCs groups, which made scars formation. Therefore, the appropriate expression of collagen is required for ideal wound healing such as shown in our 3D AM-MSCs treatment. There were no significant different in burn wound closure rate and measurement of growth factors between both dose of $5x10^5$ and $2x10^6$ 3D AM-MSCs. Therefore, we suggests to use a minimum therapeutical dose to reach optimal burn wound closure and healing.

Conclusion

In conclusion, we demonstrate the potential treatment of 3D AM-MSCs for burn wound injuries in NHPs that effectively promoted rapid wound closure. Our results shows that

3D AM-MSCs potentially facilitate regenerative medicine in industrial scale productions and promote stem cell therapy in the future, especially burn wound injuries in clinical set-up.

Declarations

Ethics Approval and Consent to Participate

This study was approved by the Ethics Committee of the Institutional of Animal Care and Use Ethics Commission (IACUC) of PT Bio Farma (Persero) No. 01/IACUC-BF/III/2017 with a validity period of 2 years, April 14th , 2017−2019.

Consent to Publish

N/A

Availability of data and materials section

Authors declare that the data will not be shared, unless requested.

Competing Interests

Authors have declared that no competing interests exist.

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Figure Legends

Figure 1. *Macaca fascicularis* distribution areas (red) as a native of Southeast Asia's animal, including Indonesia, Malaysia, Thailand and Indochina.

Figure 2. Culture of 2D AM-MSCs. The first hours the cells were mostly round or oval in shape; after the third day most of the cells were adherent to the plate, exhibiting spindle-shape/fibroblast-like cell morphology with swirling in pattern, has reached confluence (inverted microscope, 100× magnification).

Figure 3. AM-MSCs *Macaca fascicularis* in 3D micro-carrier Citodex-1 as empty (upper) and confluent (lower) (A), cells stained blue in DAPI (B) and stained red in propidium iodide (PI) (C).

Figure 4. Flow cytometric histograms of 3D AM-MSCs *Macaca fascicularis*. The green line represents control and the solid purple line represent the specific positive markers (CD73, CD90 and CD105) indicated (A). The green line represents control and the solid purple line represent the specific negative-panel markers (CD34/45/11b/19/HLA-DR) and FITC-HLA-DR as indicated (B).

Figure 5. Osteogenic differentiation was determined by the presence of calcium deposits characteristic of osteoblasts detected by alizarin red stain AM-MSCs were also tested for osteogenic differentiation (left). Chondrogenic differentiation was determined by the presence of chondrocytes detected using alcian blue stain (middle). Adipogenic differentiation was determined by the presence of adipocytes with lipid drops detected using Oil-red O (OR-O) stain (right).

Figure 6. The development of the percentage of wound closure among negative control (NaCl 0,9%), gold standard (Burnazine) and 3D AM-MSCs treated groups on day 7, 14, 21 and 28 after wound induction.

Figure 7. The representative pictures of wound closure progress of four groups in time dependent manner.

Figure 8. Effects of 3D AM-MSCs activated growth factors, such as on HGF level on daily basis from day 7 to 28 (A); on the mean value of HGF and VEGF (B).

Figure 9. Effects of 3D AM-MSCs activated growth factors, such as on VEGF level on daily basis from day 7 to 28.

Figure 10. Effects of 3D AM-MSCs activated growth factors, such as on collagen level on daily basis from day 7 to 28 (A) and on the mean value of collagen (B).

TABLES

Table 1. The PDT 2D AM-MSCs vs. 3D AM-MSCs isolated *Macaca fascicularis* cells

Culture Methods	**Population Doubling Time (PDT) in days**						**p-value**
	P1-0	P1-1	P1-2	P2-0	P2-1	P2-2	
2D	4,0	3,4	4,2	3,6	4,0	3,8	0,000*
3D	2,7	1,2	1,1	1,2	1,1	1,15	

Note: *Significant

Table 2. Weight of *Macaca fascicularis* measured after each treatments from day 7 up to 28

Treatment	***Macaca fascicularis* body weight (gram) in time dependent manner**			
	Day 7	**Day 14**	**Day 21**	**Day 28**
NaCl 0,9%	2870	2590	2560	2450
Burnazine	2830	2780	2680	2680
$5x10^5$ MSCs	2880	2900	2920	3050
$2x10^6$ MSCs	2860	2880	2910	3090

Fig 1. 3D AM-MSCs NHP_mapR0.jpg
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Fig 2. 3D AM-MSCs NHP_2DR0.jpg
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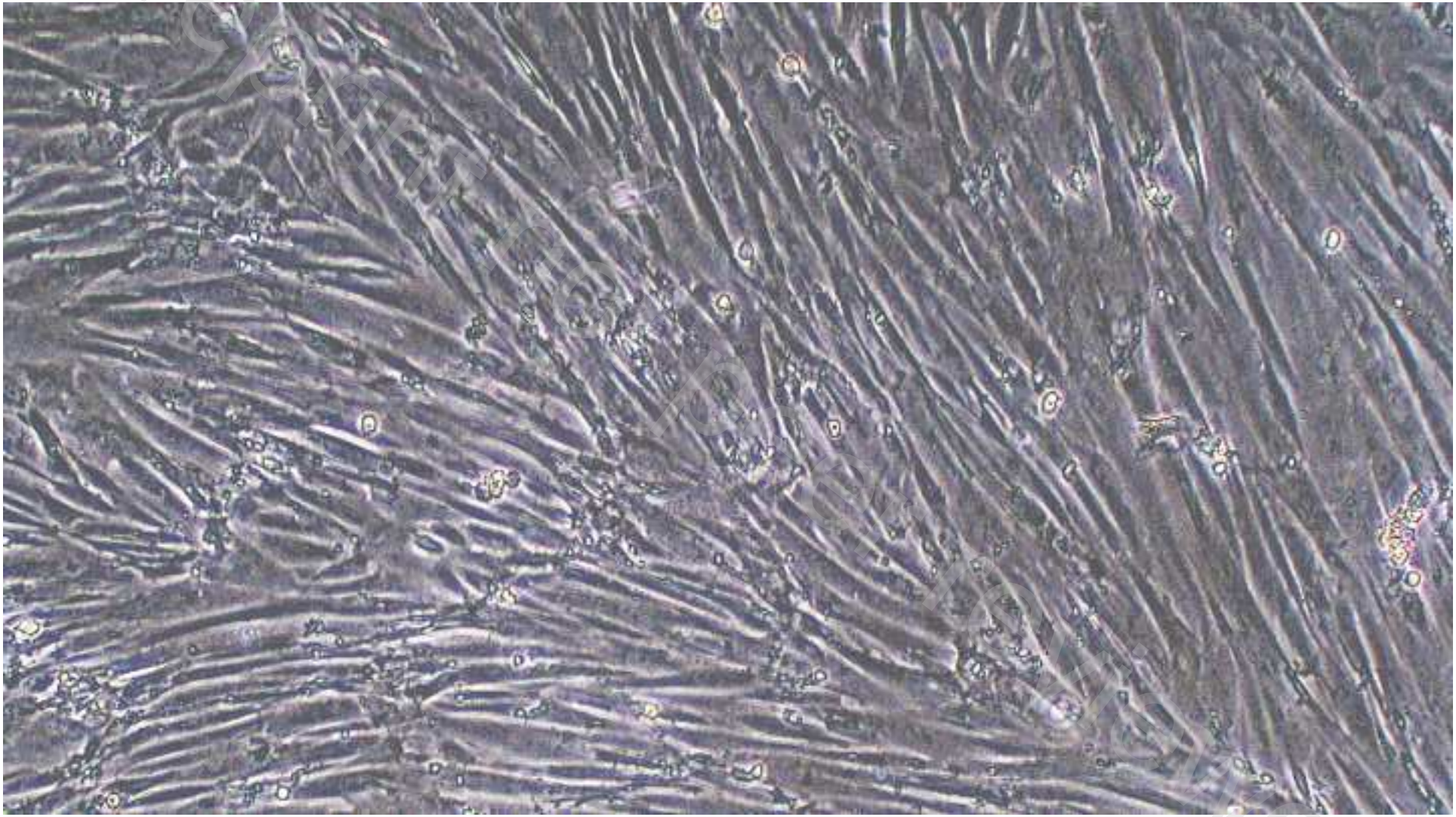

Fig 3. 3D AM-MSCs NHP_3DR0.jpg
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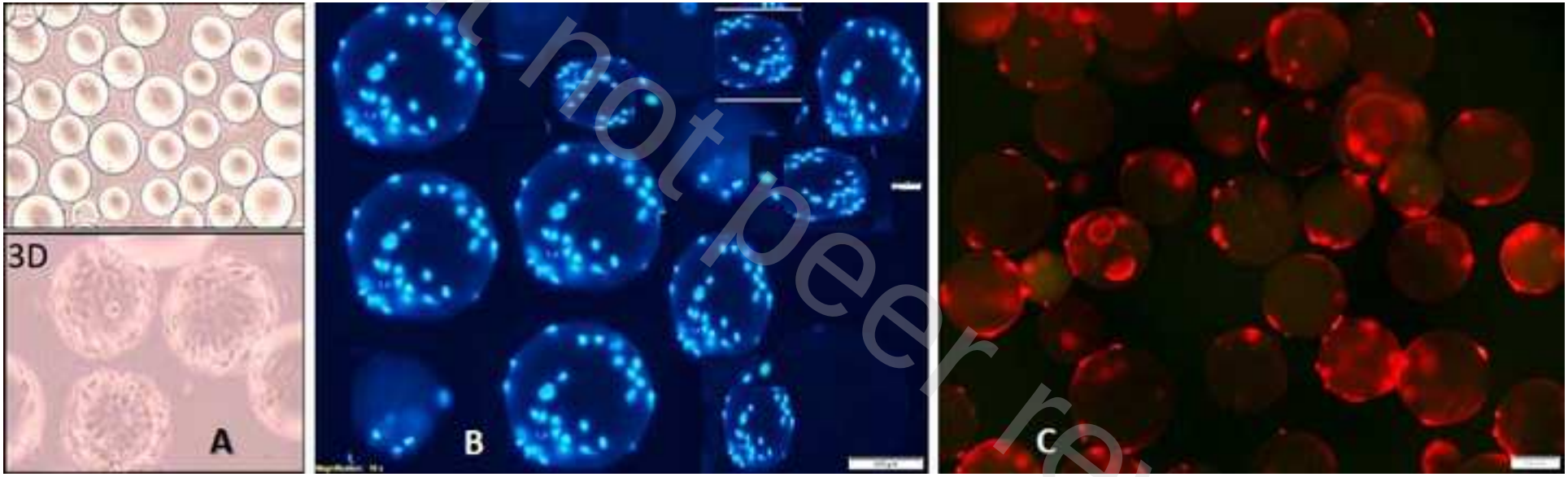

Fig 4A. 3D AM-MSCs NHP_FACs posR0.jpg
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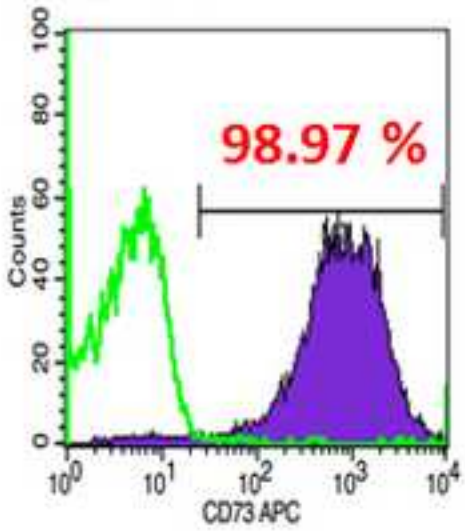
CD 73



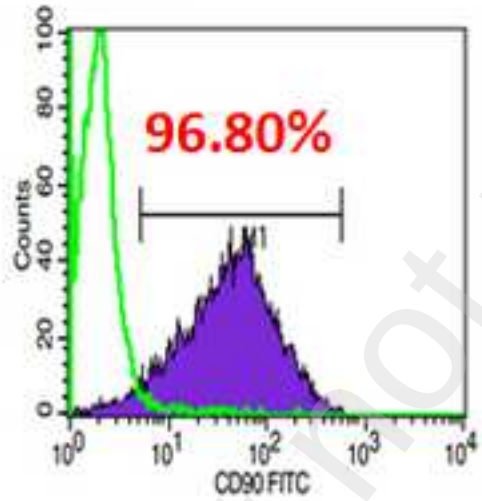
Histogram Statistics

* Numerator Histogram *
File: MIX 3.017 Log Data Units: Linear Values
Sample ID: MIX 3 Patient ID:

* Denominator Histogram *
File: ISOTYPE 3.016 Log Data Units: Linear Values
Sample ID: ISOTYPE 3 Patient ID:

Marker	Events	% Gated	% Total
All	10000	100.00	100.00
M1	9897	98.97	98.97

CD90



Histogram Statistics

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Sample ID: MIX 3 Patient ID:

* Denominator Histogram *
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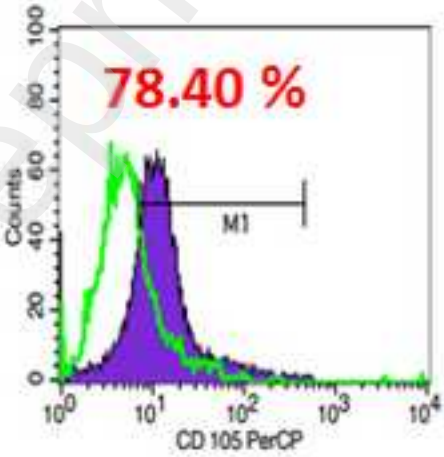
Sample ID: ISOTYPE 3 Patient ID:

Marker	Events	% Gated	% Total
All	8670	99.38	86.70
M1	8445	96.80	84.45

CD105



Histogram Statistics

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Sample ID: MIX2 Patient ID:

* Denominator Histogram *
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Sample ID: ISOTYPE 2 Patient ID:

Marker	Events	% Gated	% Total
All	7539	97.15	75.39
M1	6084	78.40	60.84

Fig 4B. 3D AM-MSCs NHP_FACs negR0.jpg
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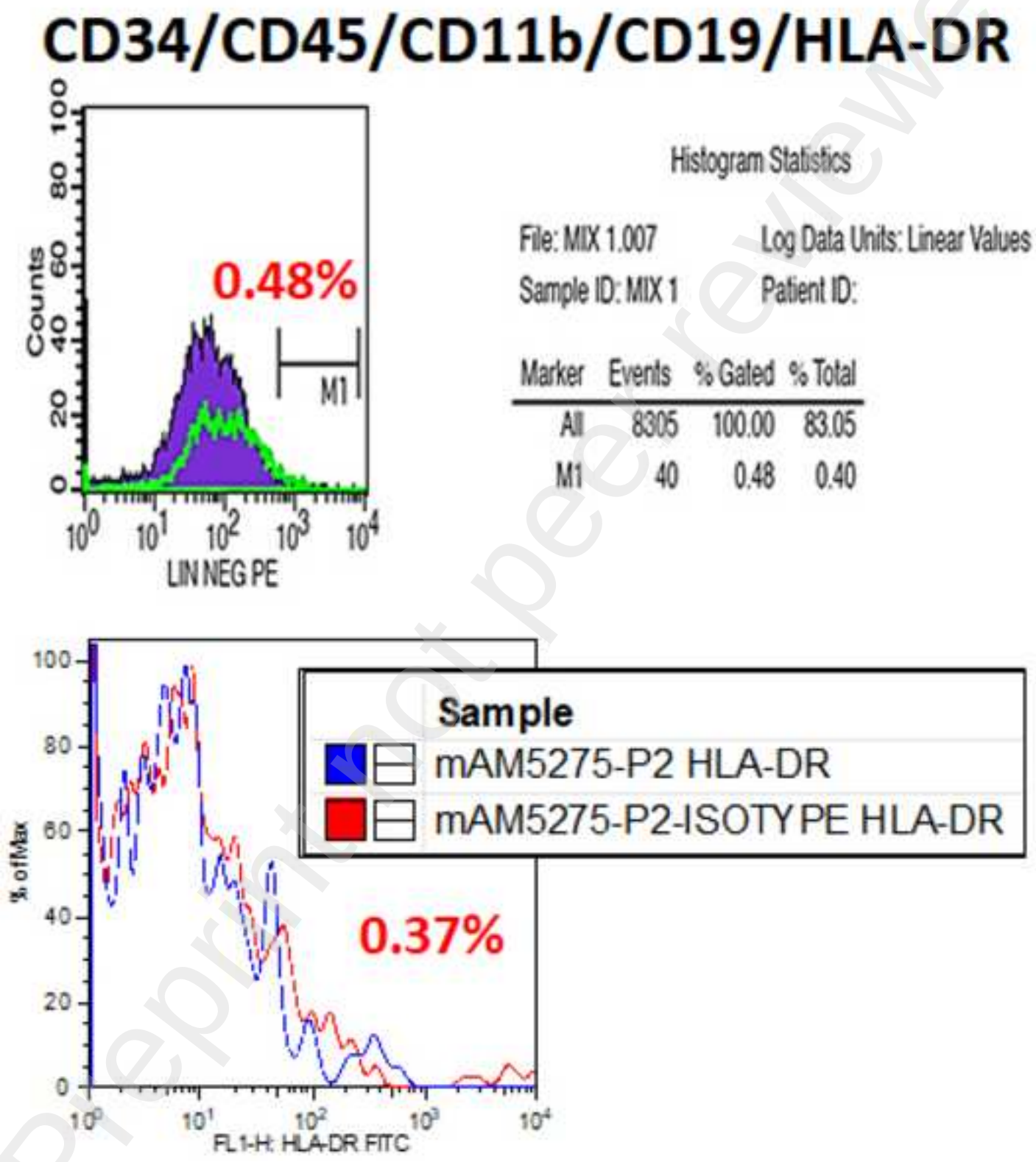

Fig 5. 3D AM-MSCs NHP_diffR0.jpg
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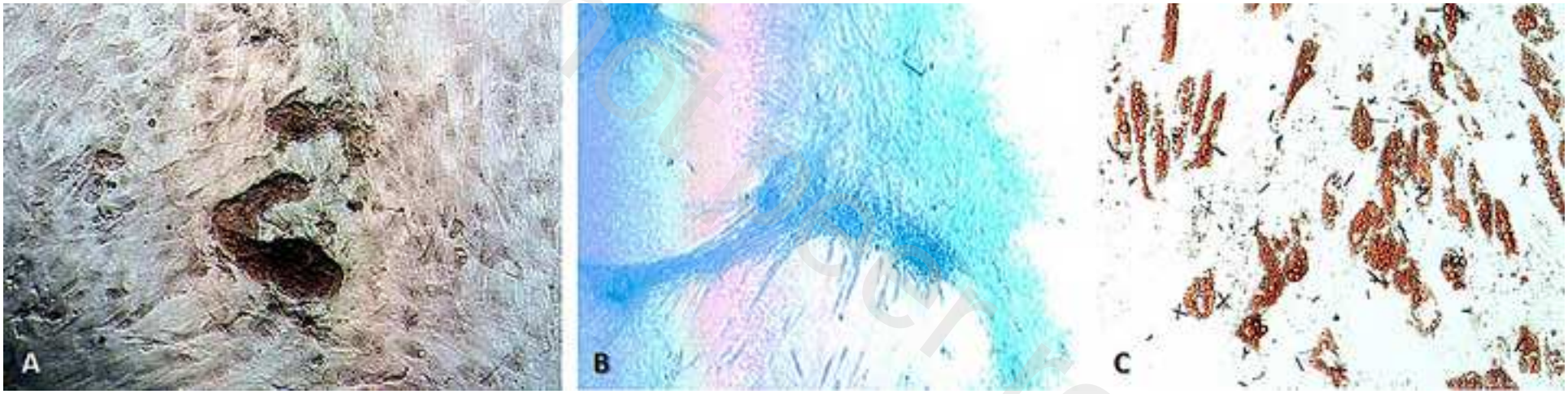

Fig 6. 3D AM-MSCs NHP_wound closeR0.jpg
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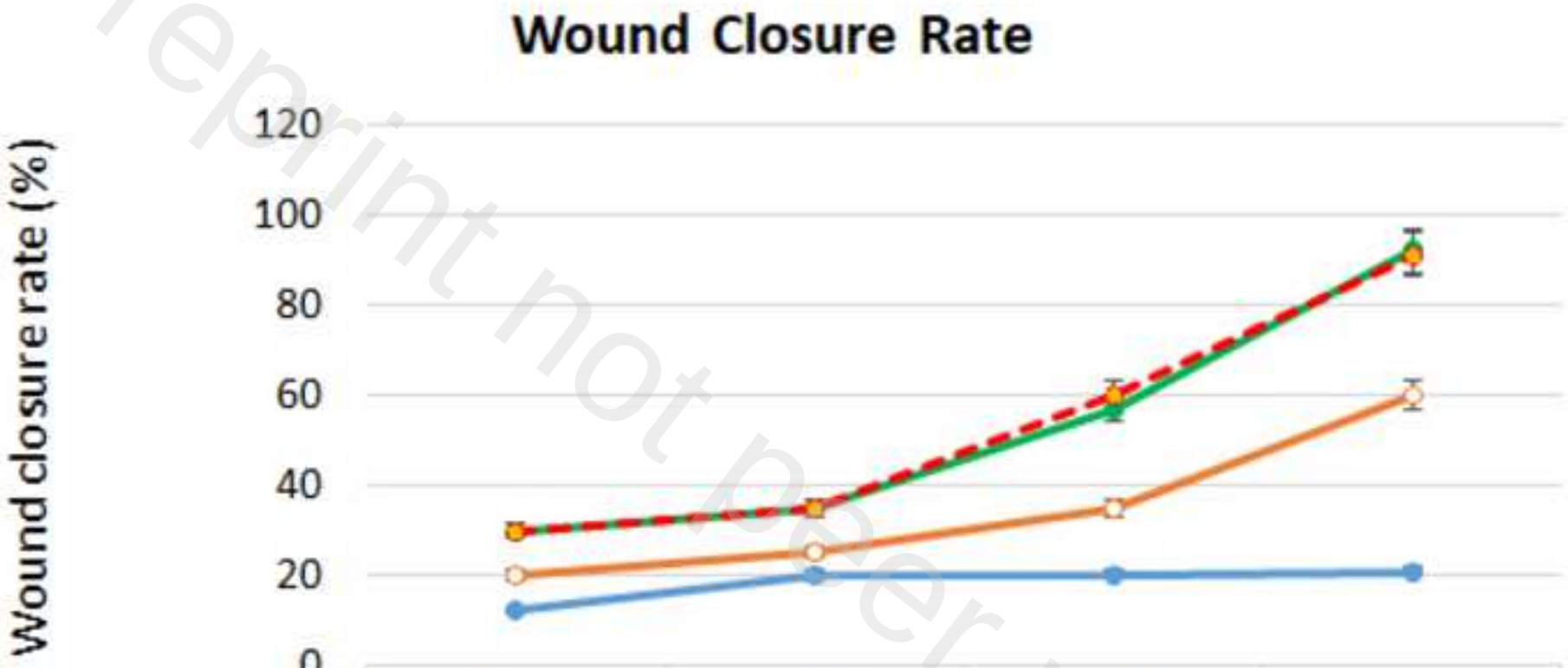

	(Days) 7	14	21	28
NaCl 0,9%	12.5 ± 2.7	20.0 ± 0.0	20.9 ± 2.0	20.1 ± 2.1
Burnazine	20.5 ± 0.0	25.0 ± 0.0	35.0 ± 0.0	60.0 ± 7.1
$5x10^5$ MSCs	30.0 ± 0.0	35.0 ± 0.0	57.0 ± 2.4	92.1 ± 2.7
$2x10^6$ MSCs	30.0 ± 4.5	35.0 ± 0.0	60.0 ± 4.1	91.2 ± 2.4

Fig 7. 3D AM-MSCs NHP_WC picR0.jpg
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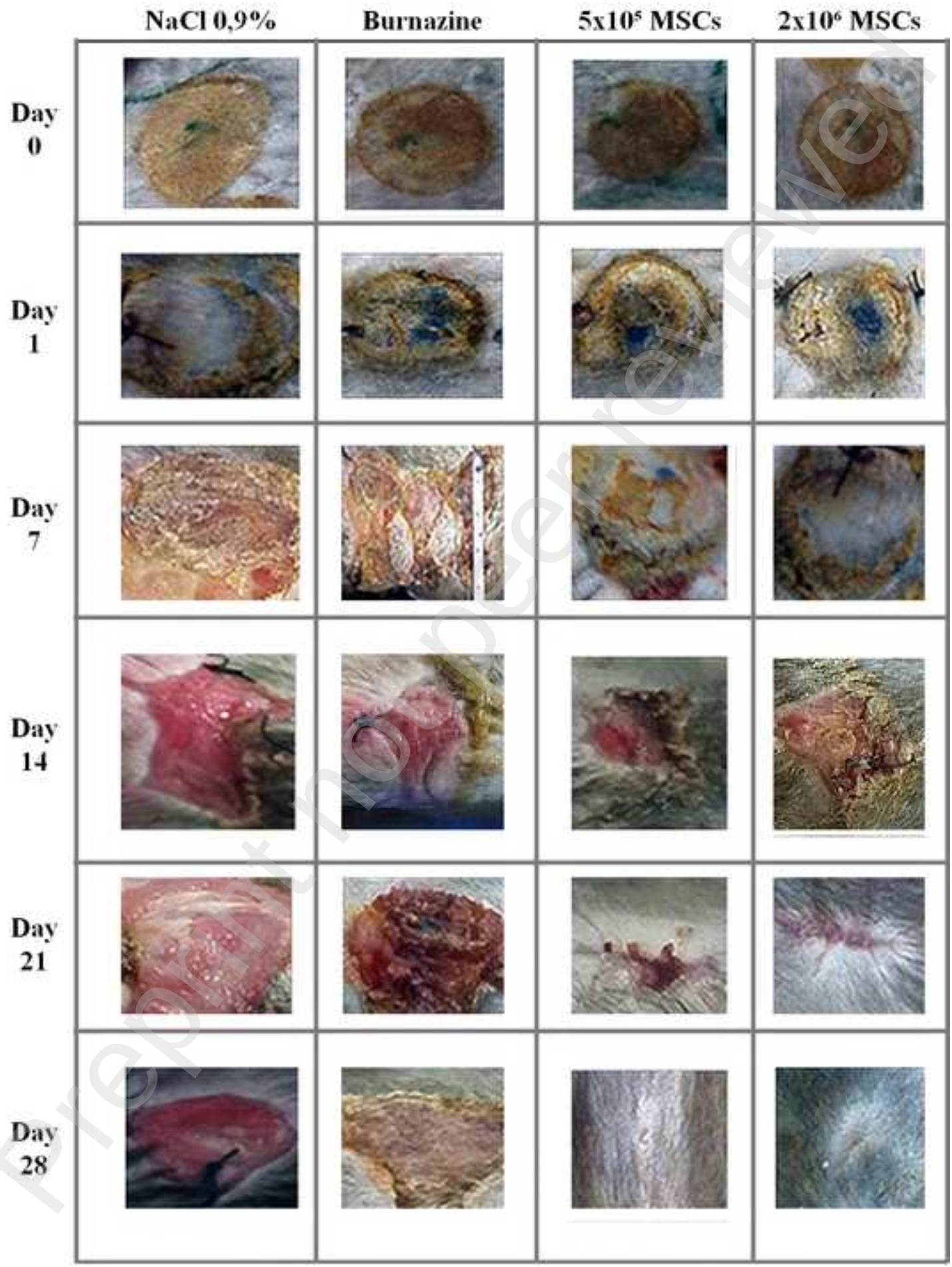

Fig 8A. 3D AM-MSCs NHP_dHEGFR0.jpg
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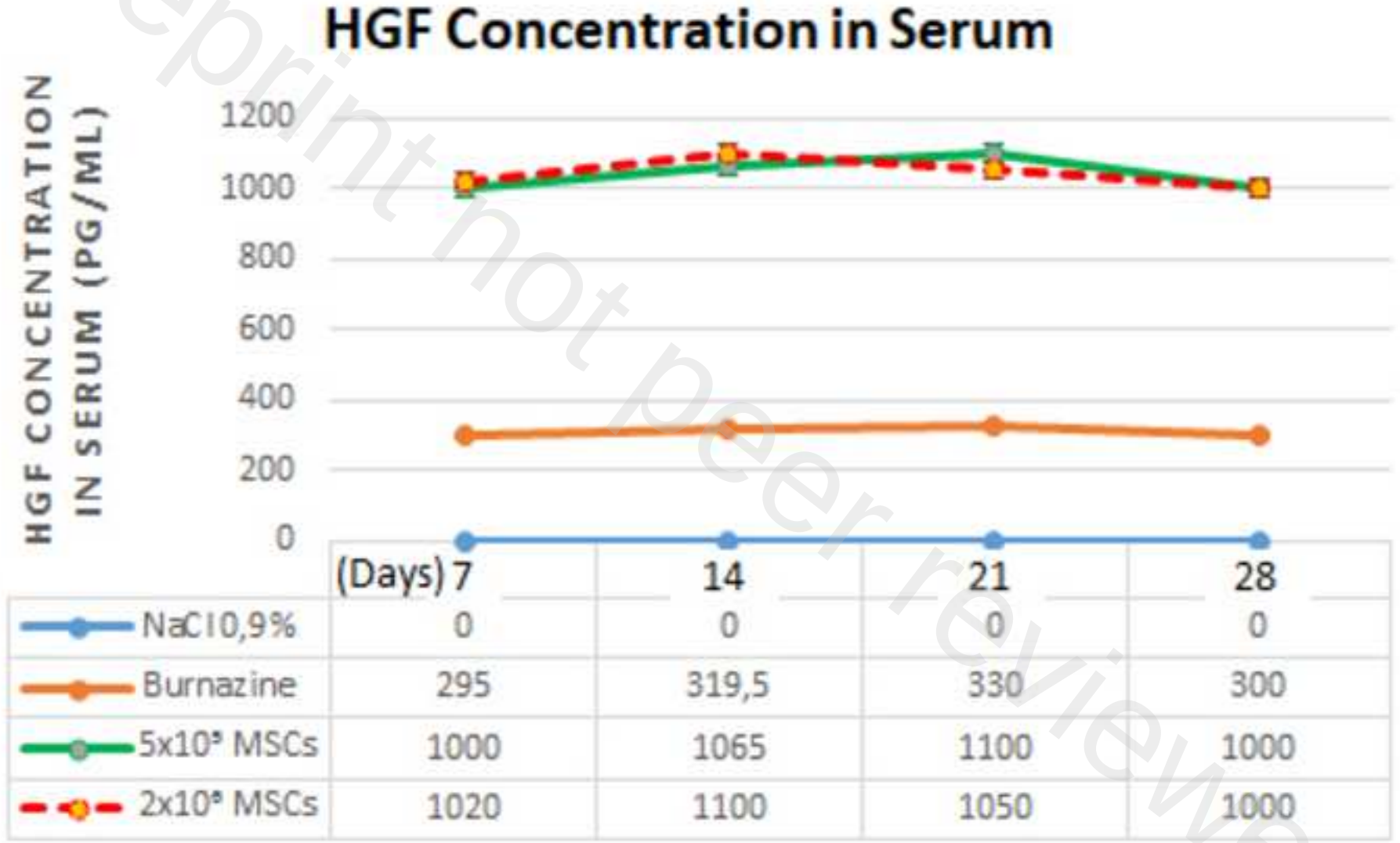

(Days)	7	14	21	28
NaCl 0,9%	0	0	0	0
Burnazine	295	319,5	330	300
5x10⁵ MSCs	1000	1065	1100	1000
2x10⁶ MSCs	1020	1100	1050	1000

Fig 8B. 3D AM-MSCs NHP_mHGF VEGFR0.jpg
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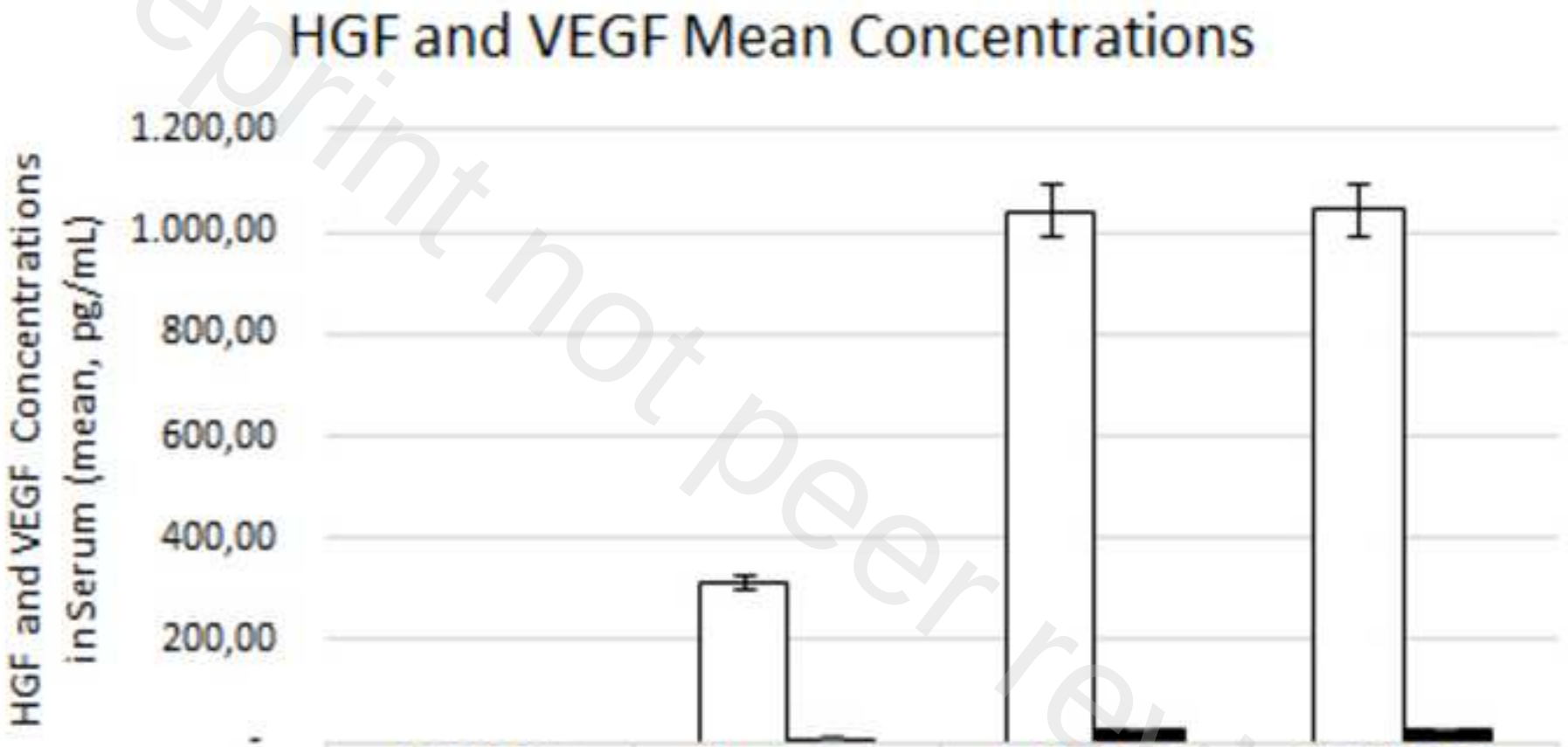

	NaCl 0,9%	Burnazine	5x10⁵ MSCs	2x10⁶ MSCs
HGF	-	**311,1 ± 16**	**1.041 ± 49**	**1.042 ± 44**
VEGF	-	**7,1 ± 2.1**	**22,8 ± 7.0**	**23,6 ± 7.0**

Fig 9. 3D AM-MSCs NHP_dVEGFR0.jpg
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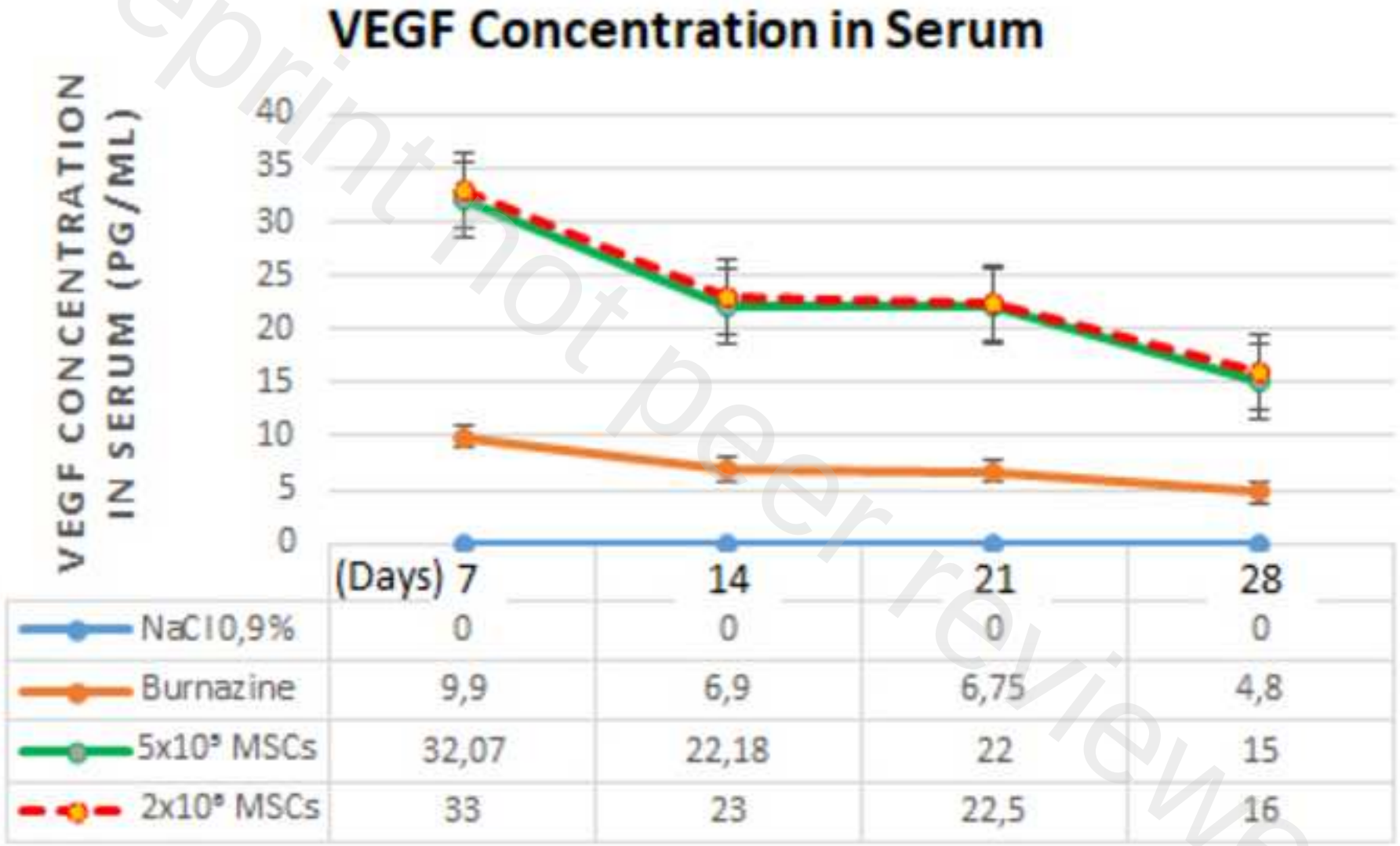

(Days)	7	14	21	28
NaCl 0,9%	0	0	0	0
Burnazine	9,9	6,9	6,75	4,8
5x10⁵ MSCs	32,07	22,18	22	15
2x10⁸ MSCs	33	23	22,5	16

Fig 10A. 3D AM-MSCs NHP_dColR0.jpg
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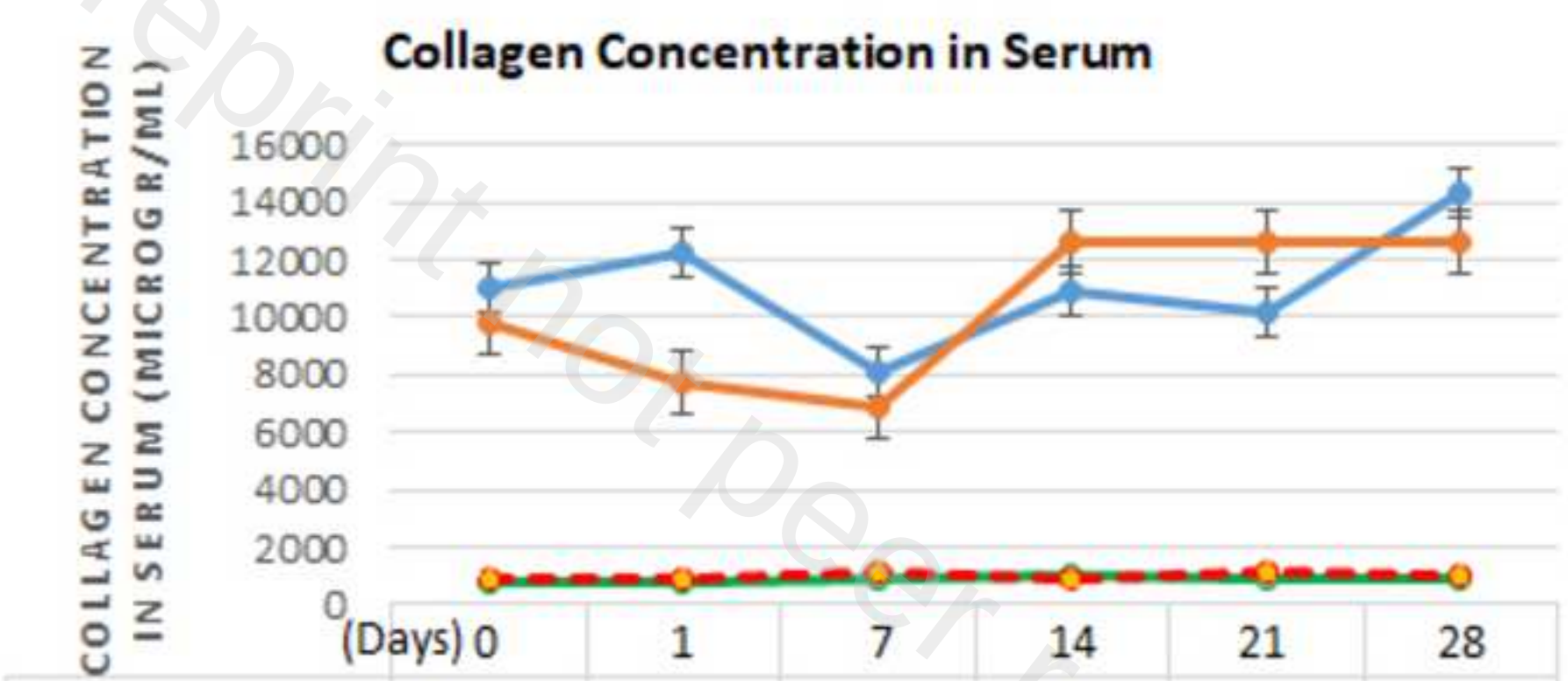

(Days)	0	1	7	14	21	28
NaCl 0,9%	10993	12210	8133	10934	10128	14336
Burnazine	9805	7752	6901	12591	12620	12605
$5x10^5$ MSCs	843,5	844,4	854,6	990,7	935,7	918,3
$2x10^6$ MSCs	900,5	862,9	1194	913,5	1161	1040

Fig 10B. 3D AM-MSCs NHP_mColR0.jpg
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