Re: Re: Your manuscript is ready!

From: Veterinary World (editorveterinaryworld@gmail.com)

To: rma_fispro@yahoo.com

Cc: masudhariadi@yahoo.co.id

Date: Wednesday, June 19, 2019 at 11:19 AM GMT+7

Dear Dr. Erma Safitri,

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Dr. Anjum Sherasiya, Editor-in-Chief of Veterinary World is appointed as Crossref Ambassador.

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Crossref - Ambassador
Star, Gulshan Park, NH-8A,
Chandrapur Road, Wankaner - 363621,
Dist. Morbi (Gujarat), India.
Website: www.veterinaryworld.org, onehealthjournal.org
E-mail: editorveterinaryworld@gmail.com

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> > Dikirim dari Yahoo Mail di Android
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> > > Please review the edited manuscript and send me the manuscript as soon as possible so, we can produce the PDF.
> > >
> > > Editage suggested to remove author details but you have to keep that details and figures in the file as your article is accepted article.

Please inform us the line no. and corrections in separate word file if you do any corrections in the Edited file of Editage.

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On Mon, Jun 17, 2019 at 2:27 PM Editage Author Services <request@editage.com> wrote:

Dear Erma Safitri,

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For now, I am sure you're eager to go through your manuscript.

All the best! We wish the best for your manuscript. We work with the belief that our authors have great potential and wish your manuscript gets the visibility, fame, and glory that it deserves

Regards,
Customer Service Team, Editage

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Editage will send the re edited file to you by 17 June

From: Veterinary World (editorveterinaryworld@gmail.com)

To: rma_fispro@yahoo.com; masudhariadi@yahoo.co.id

Date: Wednesday, June 12, 2019 at 12:17 PM GMT+7

Dear Erma Safitri,

I received the email from Editage that they will send the re edited file to you by 17 June without any cost to you. Please inform Editage to put me in Cc for edited file.

Please, do the corrections in that re edited file and send to us. Please keep all the corrections in the track changes. There is no need to send any other files (previously Mas'ud Hariadi was sending 4 files to us).

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Website: www.veterinaryworld.org, onehealthjournal.org
E-mail: editorveterinaryworld@gmail.com

Re: Erma Safitri - Mas'ud Hariadi revised Manuscript Track 1-4.

From: Veterinary World (editorveterinaryworld@gmail.com)

To: rma_fispro@yahoo.com

Cc: masudhariadi@yahoo.co.id

Date: Tuesday, June 11, 2019 at 09:13 AM GMT+7

Dear Dr. Erma Safitri,

I asked each time to provide the file which was edited by Editage but each time Mas'ud Hariadi was sending four files instead of exact file we asked.

This is the first time we received the partial editing by Editage.

I hope that Editage will do the editing of whole manuscript without any cost to you.

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> On Mon, Jun 10, 2019 at 10:50 PM Safitri Erma <rma_fispro@yahoo.com> wrote:
>
> Thank you for the opportunity was given to me.
>
> To be honest, we did only receive one edited file from Editag Service (exact file), like the file we included below.
>
> I was also surprised, it turned out that the edits were not the whole text but only the sentences in the box that you gave and only suggest to replace the title (but with advanced edited costs)
>
> I also begged you to forgive me for the sentence: the dissatisfaction I sent to Editag.
>
> Please guide what steps I should take
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Pada Senin, 10 Juni 2019 21.43.33 WIB, Veterinary World <editorveterinaryworld@gmail.com> menulis:

Dear Dr. Erma Safitri,

Please do not re-edit your manuscript as I never told you that we are dissatisfied with the service of Editage.

You have never sent me the file which was edited by the editage. Mas'ud Hariadi sent me four files when I asked him to send the edited file only.

If you can send the file which was edited by Editage then send it otherwise we will remove your article from our system and return the half APC to you.

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> Dear Dr. Mas'id Hariadi,
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> I have informed you many a times in a simple English that you have to send/forward the email/file of Editage but you are sending four files each time.
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> We can not process your manuscript as you are not following the instructions and wasting time. So, please do not send any file now.
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Dear Dr. Mas'ud Hariadi,

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On Sat, Jun 8, 2019 at 8:44 AM Veterinary World - Publisher <veterinaryworldpublisher@gmail.com> wrote:

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1. Veterinary World is indexed in Emerging Sources Citation Index (ESCI) - Thomson Reuters. http://science.thomsonreuters.com/cgi-bin/jrnlst/jlresults.cgi?PC=EX&SC=ZC
2. Veterinary World is indexed in PubMed and PubMed Central.http://www.ncbi.nlm.nih.gov/nlmcatalog/101504872
3. A new, prestigious journal, International Journal of One Health (www.onehealthjournal.org) was launched in early 2015 by Veterinary World.

Best Regards,

Nazir
Editorial Assistant
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www.veterinaryworld.org
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---------- Forwarded message ---------
From: **Mas'ud Hariadi** <masudhariadi@yahoo.co.id>
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Subject: Erma Safitri - Mas'ud Hariadi revised Manuscript Track 1-4.
To: Veterinary World - Publisher <veterinaryworldpublisher@gmail.com>, Safitri Erma <rma_fispro@yahoo.com>, Mas'ud Hariadi <masudhariadi@yahoo.co.id>

Dear Sir,

> > > > Herewith I send a revised Manuscript track 1, 2, 3 and 4.
> > > > Best Regards,
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RESEARCH ARTICLE

Biotechnology of rat mesenchymal stem cell-conditioned hypoxia compared with conventional *in vitro* culture of rat mesenchymal stem cell-conditioned normoxia for testicular failure therapy with low libido

Erma Safitri[1,2] and Mas'ud Hariadi[1]

1. Department of Veterinary Reproduction, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya 60115, Indonesia; 2. Stem Cells Research Division, Institute Tropical Disease, Universitas Airlangga, Surabaya 60115, Indonesia.

Corresponding author: Mas'ud Hariadi, e-mail: **masudhariadi@yahoo.co.id**

Co-author: ES: erma-s@fkh.unair.ac.id

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Abstract

Aim: Biotechnology of rat mesenchymal stem cell-conditioned hypoxia (rMSC-CH) for testicular failure therapy with low libido has been shown to improve functional outcome of the testicle for producing spermatogenic cells and to repair Leydig cells of a rat (*Ratus norvegicus*).

Materials and Methods: The first group (T1): Rats with testicular failure and low libido were injected with rMSC-conditioned normoxia (CN) (oksigen 21%); the second group (T2): Rats with testicular failure and low libido were injected with rMSC-CH (oksigen 1%); the negative control group (T-): Rats with normal testis were injected with 0.1 mL phosphate-buffered saline (PBS); the sham group (TS): Rats with testicular failure and low libido were injected with 0.1 mL PBS.

Results: Vascular endothelial growth factor expression as a homing signal in group T2, T-, T1, and TS, respectively, was 2.00±0.5, 2.95±0.4, 0.33±0.48, and 0±0. The cell number of cluster differentiation (CD)34+ and CD45+ in the two groups (T- and TS) was <20% while in

T1 and T2 groups was >30% and >80%, respectively; it showed that there was a mobilization of hematopoietic stem cells. Number of spermatogenic cells (spermatogonia, spermatocyte primer, spermatocyte secondary, and spermatid) in TS was significantly decreased ($p<0.05$) when compared with T-, T1, and T2, while T2 did not show a significant ($p>0.05$) decrease as compared to T- group. The improvement of libido, based on number of Leydig cells as producing testosterone hormone for libido expression, in T1 was still not able to increase the number of Leydig cells while in T2 was able to maintain the number of Leydig cells significantly different between TS and T1.

Conclusion: rMSC-CH on testicular failure with low libido has shown improvement in functional outcome of the testicle and to repair Leydig cells.

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Keywords: low libido, rat mesenchymal stem cell-conditioned hypoxia, rat mesenchymal stem cell-conditioned normoxia, rat, testicular failure.

<H1>Introduction

One of the failures of the testicle such as oligospermia represents a major reproductive health problem in male causing a low fertility and libido [1,2]. Oligospermia is a condition which is a low concentration of spermatozoa produced by testicular seminiferous tubules and makes infertility in male and it means no offspring [3]. Oligospermia, for male, is the main cause of infertility with etiology varies such as genetic heredity, traumatic, tumor neoplastic, and degenerative disorders due to malnutrition [4].

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Transplantation therapy with mesenchymal stem cells (MSCs)-derived bone marrow gives very promising results in regeneration of spermatogenesis process in testicular tissue with infertility like as oligospermia [5]. The therapy efficacy was limited due to low viability of

stem cells after transplantation [6,7]. The low viability of the stem cells is due to the conventional *in vitro* culture with rat MSC-conditioned normoxia (rMSC-CN), with oksigen concentration >21%. The conventional culture causes cells senescence [8], cells apoptotic [9], and mutation of gene such as G:C to T:A [10]. Some researchers showed that >93% of stem cells die between 1st and 7th days after transplantation [11-15]. Therefore it is required in high doses and repeated several times (booster) for effectiveness of therapy, utilizing of stem cells with conventional culture (CN), so that the cost become very expensive [16]. To remain viable, *in vitro* cultured of stem cells must be adapted to a niche or microenvironment where the stem cell resides in bone marrow. In the normal condition the niche of stem cells in the bone marrow is in the low oksigen tension (conditioned-hypoxia [CH]) [5,8,10]. Therefore, it is needed a biotechnology rMSC-CH *in vitro* culture for homing signal and mobilization of stem cells to improve testicular for producing sperm. The homing signal of stem cells in the testicle tissue is based on the expression of vascular endothelial growth factor (VEGF), while the mobilization was based on the expression of cluster differentiation (CD) such as CD34+, CD45−, and CD105− [5-7].

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The objective of the study was utilization of biotechnology of rMSC-CH for testicular failure therapy with low libido. Finally the biotechnology of rMSC-CH has been able to improve functional outcome of testicle for producing of spermatogenic cells and repair Leydig cells of rat (*Ratus norvegicus*).

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<H1>Materials and Methods

<H2>Ethical approval

The animal studies were performed via a protocol approved by the Animal Care and Ethical Clearance Committee of Faculty of Veterinary Medicine, Universitas Airlangga, and conform

with the National Research Council's guidelines (239-KE) through the ethical seminar. The research was conducted at the laboratory in the Institute of Tropical Diseases and Faculty of Veterinary Medicine, Universitas Airlangga.

<*H2*>Isolation and culture method of stem cells

The MSCs was collected from the crista iliaca bone marrow of male rat (*R. norvegicus*) strain Wistar 3 months of age. The aspirate was put in heparinized tubes and stored at 4°C for transportation to the laboratory experimental in Institute of Tropical Diseases, Universitas Airlangga, for conducting *in vitro* culture [17].

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The aspirate of MSCs was put in 15 mL heparin tube (Z181099, Sigma Aldrich®, Burlington, Massachusetts, USA) which previously filled with 3 mL α-modified Eagle medium (α-MEM) (M0894, Sigma Aldrich®, Burlington, Massachusetts, USA). The aspirate was transferred to a 15-mL sterile blue cap tube and added with 1× phosphate-buffered saline (PBS) (MFCD00131855, Sigma Aldrich®, Burlington, Massachusetts, USA) sterile to a total volume of 10 mL. The tube with aspirate solution was then rinsed twice with 5 mL × PBS. The diluted sample was added over the same volume of Ficoll (F9378, Sigma Aldrich®, Burlington, Massachusetts, USA) at room temperature in a separate 15 mL tube. Furthermore, each aspirate was coated with Ficoll before being centrifuged (Sorvall™ MX Series Floor Model Micro-Ultracentrifuge, Thermo Fisher, Grand Island, USA) at 1600 rpm = 287 relative centrifugal force (rcf) for 15 min at room temperature. After centrifugation, mononucleated cells were collected in the form of "buffy coat" located on the surface of Ficoll-PBS by means of a sterile Pasteur pipette and placed in a 15 mL tube (Sigma Aldrich®, Burlington, Massachusetts, USA).

The sample was diluted with PBS to a total volume of 15 mL, with the tube being turned 3-5

times as a means of achieving an even mix. At the next stage, centrifugation was undertaken for 10 min at a speed of 1600 rpm = 287 rcf. Before heating, the supernatant was thrown away and the cells resuspended with 6 mL of α-MEM (M0894, Sigma Aldrich®, Burlington, Massachusetts USA). The suspension of cells had placed in 10 cm^2 of the plate (Falcon™, Thermo Fisher Scientific, Pittsburgh, PA, USA); they were incubated at 37°C in a humidified atmosphere containing at 5% CO_2 moisture, and this process has taken 24 hrs until they attached on the surface of plate. After 24 hrs, media and non-adherent cells were discarded. The cells that attach were rinsed twice using 5 mL of PBS and shaken before being heated in the culture. The supernatant was disposed and then the plate was washed again twice with PBS. 10 min later, 10 mL of fresh α-MEM (M0894, Sigma Aldrich®, Burlington, Massachusetts, USA) was added to the dish before it was returned to the incubator. The cells were incubated at 37°C and 5% CO_2 moisture with the culture being observed daily by means of an inverted microscope.

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Every 4 days, the media were removed and the cells rinsed with 5 mL or 10 mL of 1× PBS before heating. The PBS was subsequently discarded and the dish was filled with 10 mL of fresh α-MEM (M0894, Sigma Aldrich®, Burlington, Massachusetts, USA). The culture was continued until approximately 75-80% confluence was attained, and then cells were passed into several dishes for subculture [17].

Passage was conducted 3 times, and then cells were divided into two: rMSC-CH treatment with 1% O_2 in hypoxia chamber inside a 5% CO_2 incubator, another treatment was the use of rMSC-CN with 21% O_2 and booth were incubated for 4 days. The three gases needed for 1% low oxygen precondition are CO_2 gas 5% to flow in 37°C incubator, O_2 gas 100% to flow only for calibration and N_2 gas. N_2 gas was used to replace O_2 gas so that it can be in accordance with the desired concentration.

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<H2>Testicular failure animal model with low libido

Testicular failure in rats were obtained by fasting for 5 days but drinking water was available *ad libitum* [1]. The condition of 5 days fasting in the rats induced testicular failure in the rats because the work of adrenal cortex was less effective in producing dehydroepiandrosterone (DHEA) due to malnutrition. Low levels of DHEA in the blood can be a cause of fatigue and decreased sperm concentration. DHEA is the most potent precursor of steroid hormones such as testosterone which produced by the renal adrenal cortex [18] and Leydig cells of testis [3]. The low testosterone production can lead to decreased spermatogenesis process and libido for males.

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The animal model was used in this study was Wistar strain of male rats (*R. norvegicus*), 250-300 g body weight, 3 months age, and healthy condition. The rat was placed in a plastic cage individually in the Animal Laboratory Experimental, Faculty of Veterinary Medicine, Universitas Airlangga.

<*H2*>Biotechnology rMSC-CH compared with rMSC-CN

Biotechnology stem cells treatment was done by injecting MSC directly from either conditioned rMSC-CH or rMSC-CN in the medial part of both testes, after previously performed anesthesia in rat using ketamine and xylazine. Anesthesia for rat was given 87 mg ketamine/kg and 13 mg xylazine/kg of body weight, beginning 10-15 min after simultaneous injection and lasting 15-30 min, then followed by a relatively long period of immobility (mean, 3.8 h) and reduced responsiveness to external stimuli [19].

MSC that was injected into male rat (*R. norvegicus*) has been induced testicular failure such as oligospermia with low libido, compared with negative and the sham group. The first group (T1): Rats with testicular failure and low libido were injected with 200 million rMSC-CN/rat

from high oxygen culture (21% concentration of O_2); the second group (T2): Rats with testicular failure and low libido were injected with 200 million rMSC-CH/rat from hypoxia culture (1% concentration of O_2); the negative control group (T-): Rats with normal testicle were injected with 0.1 mL PBS; the sham group (TS): Rats with testicular failure and low libido were injected with 0.1 mL PBS.

After second cycles of spermatogenesis, male rats were operated surgically to collect testicle tissue performed. Immunohistochemical (IHC) observation was performed to determine the expression of VEGF as a homing signal of stem cells in the testicle tissue. Flow cytometric observation was performed to calculate the cell number of CD34+, CD45+, and CD105- as mobilization of stem cells to improve testicle disorders.

The improvement of testicle in producing sperm cells was observed by histopathological preparation with hematoxylin-eosin (HE) stain (B8438, Sigma Aldrich®, Burlington, Massachusetts, USA), then the number of spermatogenic cells (spermatogonia, spermatocyte primer, secondary spermatocyte, and spermatid) and Leydig cells were calculated.

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<H2>**IHC of VEGF**

The IHC observation was performed to determine the expression of VEGF. Histological preparation was made before IHC method was done. An incision of testicular tissue in the paraffin blocks was made transversely. Further examination by making outward expression of VEGF by IHC method using VEGF monoclonal antibody (A183C 13G8, Thermo Fisher Scientific, Pittsburgh, PA, USA) was done. The expression of VEGF was made by a regular light microscope with 200 times magnification and the expression of each variable was indicated by the number of cells with brownish discoloration chromogen in each incision [20].

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The observation was performed using regular luminescence microscope Nikon H600L which was equipped with digital camera DS Fi2 300 megapixels and image processing software and cell count Nikon Image System.

<*H2*>Flow cytometry observation

Flow cytometric (BD FACSCalibur™, BD Biosciences, San Jose, USA) observation of the stem cell mobilization was based on calculated cell number of CD34+, CD45+, and CD105-. After the treatment, whole blood collected from cardiac puncture and then placed in heparin tube to prevent coagulation. Flow cytometric observation reveals the expressions of CD34+, CD45+, and 105-. Flow cytometric method begins with whole blood centrifugation in 4°C temperature, 6000 rpm = 4032 rcf for 15 min. Cellular precipitation as a result of centrifugation was mixed with cytoperm/cytofix (554714, BD Cytofix/Cytoperm™, BD Biosciences, San Jose, USA) in the amount of 2 times of obtained cell number. This mixture then was centrifuged again and formed supernatant and pellet. BD wash was added to the pellet in the amount of 4 times of obtained cell number from the first centrifugation and then added lysis buffer in the amount of twice of the first obtained cell number. Subsequently labeled antibody was added to each sample, five tubes were arranged and processed parallelly: (1) single staining with CD34 PE (Rabbit Anti-CD34/HCAM/PGP1 Polyclonal Antibody, PE-conjugated Conjugated Primary Antibodies-bs-0521R-PE, Biossusa) was added to the wash tube; (2) double staining with CD34 PE and CD45 PerCP (PerCP-Cy5.5 anti-human cross anti-rabbit CD45–BD, Biosciences, USA); (3) double staining with CD34 PE ; (4) double staining with CD45 PerCP and (5) double staining with CD105 FITC (Rabbit Anti-Thy-1/CD105/Thy1.1 Polyclonal Antibody, -0778R-FITC, Bioss, USA) true count tube. All the samples were then stored in 4°C and dark room and were analyzed with flow cytometry for 1 h [21].

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<H2>Histopathology observation

The improvement of testicular tissue for producing sperm with low libido was observed by histopathology preparation with HE stained and and calculation of spermatogenic cells number (spermatogonia, spermatocyte primer, secondary spermatocyte, spermatid) and Leydig cells. Histopathological examination was done using a light microscope with a magnification of 200 times. The observation was done after one cycle of spermatogenesis process (35 days) and food was given normally; male rats were operated surgically to collect testicle tissue performed. The calculation.of spermatogenic cells number based on the total number of 4 cell types (cells of spermatogonia, spermatocyte primer, secondary spermatocyte and spermatid) located on the five lumens of the seminiferous tubules from five preparat glasses. Furthermore, the total number of the four cell types of the five tubules was divided by 5 [1,2].

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The testicular of the rat was fixated in 10% formalin, and 1 h later, mid-testis was injected with formalin 10%. Rats testes then were dehydrated in alcohol solution with a higher concentration gradually, i.e., start from 70%, 80%, 90%, and 96%. Then, the testes of rats were cleared with xylol solution. Furthermore, embedding was done using paraffin liquid and rat testes were put into molds containing paraffin liquid. Before stained and sectioning performed, an incision using a microtome and mounted on glass objects. Furthermore, the staining was done by removing of paraffin with xylol, then put into a solution of alcohol with decreased concentration, start from 96%, 90%, 80%, and 70%, and then put it into HE staining procedure. The last stage was done mounting by putting into alcohol with increasing concentration, start from 70%, 80%, 90%, and 96% to remove excess stain, and then put into xylol. The preparates then were covered with a cover glass and mounted with Canada balsam [22].

<*H2*>Statistical analysis

The expression of VEGF, cell number calculated of CD34+ and CD45+, and cell number of spermatogenic and Leydig cells were statistically analyzed using SPSS 17 for Windows XP with the confidence level 99% (α=0.01) and the level of significance 0.05 (p=0.05). The steps comparative of hypothesis testing were as follows: the normality data were tested with the Kolmogorov–Smirnov test, homogeneity of variance test with analysis of variants, and *post hoc* test using the Tukey honestly significantly different 5% as the least significant difference test.

<*H1*>Results

The purity of MSCs was done through the identification of CD105+ and CD45- by immunofluorescence [17]. Immunofluorescence identification for CD105 was positive expression and CD45 was negative expression. CD105+ and CD45- were specific cell surface markers of MSCs using monoclonal antibody FITC anti-rabbit CD105 (Biolegend) and FITC anti-rabbit CD45 (Biolegend). Immunofluorescence results show that cultured cells were true MSCs (Figures-1 and 2).

<H2>IHC observation

The IHC observation was performed by scoring 0 to 5 as follows: score 0 means no chromogen colored, scores 1, 2, 3, 4 mean 1 – 25%, 26 – 25%, 51 – 75%, 76 – 100% chromogen color expression of VEGF respectively.. The average expression of VEGF in T2 group was 2.00^{b}±0.5 (25% and 50%) (Figure-3d), although the percentage was lower than negative control (T-) group which was found to be 2.95^{c}±0.4 (>50%) (Figure-3a), while the percentage was still higher than T1 group with score being 0.33^{a}±0.48 (≤ 5%) (Figure-3c), and the sham group (TS) was not expressed at all 0^{a}±0 (0%) (Figure-3b). The all score of

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VEGF is summarized in Table-1.

<*H2*>Flow cytometric observation

Flow cytometric observation of stem cell mobilization was based on calculated cell number of CD34+, CD45+, and CD105-. The average of cell number calculated of CD34+ and CD45+ in the two groups (T- and TS) was <20%. On the other hand, those percentages in T1 were more than 30% and T2 were more than 80%; it indicated hematopoietic stem cells (HSCs) mobilization. Statistically, there was a significantly difference ($p<0.05$) between T2 and the other three treatments (T-, TS, and T1) and no difference ($p>0.05$) between those two groups (T- and TS), although both groups were different with T1 being observed (Figure-4 and Table-1).

<H2>Histopathology and libido observation

The improvement of testicular tissue with oligospermia condition was observed by histopathology based on production of spermatogenic (spermatogonia, spermatocyte primer, secondary spermatocyte, and spermatid). Assessment for libido of male rat based on number of Leydig cells (Table-2)., in which the function of these cells are producing testosterone hormone, to stimulate libido [21,22]. Analysis of the results showed that although spermatogenic production in T2 group showed decreased significantly ($p>0.05$) compared to control negative group (T-) , , the decreased still showed different significantly ($p<0.05$) when compared with the sham group (TS) and (T1) (Table-2). Based on Table-2, it was found that T1 still showed different significantly ($p<0.05$) when compared with the control negative T- and T2.

<*H1*>Discussion

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It is known that MSCs can differentiate into a variety of cell types such as osteoblasts, chondrocytes, myocytes, and adipocytes. Nowadays, however, MSCs can differentiate into germ cell lineage or MSCs that assist in the regeneration of germ cell lineage. There is a phenomenon of transdifferentiation on MSCs, so it has multipotent characteristic more than before. It means that MACs can differentiate into various cells that a group (mesoderm) can also cross the class such as the ectoderm class, neuronal cells [23] or endoderm groups [24] and germline cells (spermatogonial, sertoli, and Leydig) and tissues (seminiferous tubules) [25].

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The efficacy of rMSC-CH biotechnology for testicular failure therapy with low libido was based on: (a) Expression of VEGF as the homing signal, (b) increase of cell number calculated of CD34+ and CD45+ as the mobilization of stem cells, (c) the improvement of testis for producing spermatogenic cells, and (d) the improvement of libido, based on the number of Leydig cells.

The expression of protein VEGF as a marker of homing signal was done through IHC methods. VEGF is a component of extracellular matrix from stem cells. VEGF has a role in supporting a conducive microenvironment for stem cells to remain viable [26]. The low oxygen culture for stem cells in this study provides supportive niche and through trigger process of VEGF-1 which is homing signal. Furthermore, VEGF-1 binds to VEGF receptor-1 and then will be passing a series of signaling that activates of stem cell factor (SCF). SCF is a mechanic in the niche of protein signaling that is physiologically will be happened further communication [27]. The presence of SCF will be recognized SCF receptor complex and toward into the cell nucleus so that expression of nuclear β1-integrine for activating octamer 4 (OCT 4). OCT 4 is a member of the POU family from transcription factors. OCT 4 has a major role in the proliferation, self-renewal, and differentiation of the stem cells. The

existence of the proliferation causes HSCs change of shape from quiescent of stem cells into a cycling state so that the HSCs located in the central endosteum area toward bone marrow. This suggests that cycling HSCs occurred outside of their niche and mobilized to the peripheral circulation [28].

Stem cells mobilization could occur because induction of stem cells mobilized toward defect place, in this research, based on calculated cell number of CD34+, CD45+. The mobilization process can occur in several ways: (a) Proteolytic induction of pharmacological agents such as granulocyte-colony stimulating factor or cyclophosphamide from bone marrow inside microenvironment; (b) blockade from specific locking molecules like as AMD3100 or BIO4860CXCR4 or VLA-4; (c) effect of dopamine and receptors b2-adrenergic as neural mediators; (d) elements modulation from cascade coagulation; (e) induction of inflammatory signals such as cytokines, Nuclear Factor-kappaB (NFkB), β catenin through Wnt due to the tissue damage; (f) homing signals such as stromal-cell-derived factor-1, (C-X-C Motif Chemokine Ligand 12 (CXCL12), VEGF, hepatocyte growth factor, platelet-derived growth factor, and integrin that appeared and to be act as recruitment of stem cells [17]. In this research, in “f” point, mobilization can occur caused expression of homing signal like as VEGF.

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Assessment of libido test from male rats was based on number of Leydig cells. Decreased libido on the sham group (TS) was occurred because the adrenal cortex work becomes ineffective in producing DHEA due to malnutrition (rat experience 5-day fasting). Low levels of DHEA in the blood can be a cause of fatigue and decreased body stamina. DHEA is the most potent precursor of steroid hormones such as testosterone produced by the renal adrenal cortex [18] and Leydig cells or interstitial cells between tubulus seminiferus of testis [3]. In this result, second group (T2): Rats with oligospermia and low libido were injected with 200

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million stem cells/rat from low oxygen culture (1% concentration of O_2). This suggests that a 200 million stem cells/rat from low oxygen culture (1% concentration of O_2) has maintened. libido as same as libido in the negative control group (T-). Increased DHEA aside as a precursor of steroid hormones, such as the hormone testosterone, is also responsible for fat metabolism and acts as an enzyme inhibitor of glucose 6-phosphate dehydrogenase, which has a role as a biocatalyst of changes in glucose to fat. Thus, the increase in DHEA allows for an increase in the amount of free adenosine triphosphate (ATP) in the body and reproduction organ, thus increasing the stamina of the body [29] and health of reproduction [3]. Further, increases in free ATP in the body and reproduction organ in addition to increasing stamina will also support an increase in libido. It can be explained that libido can be said good if the body is in a state of excellent stamina and then allows the process of reproduction such as spermatogenesis.

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In the first group (T1): Rats with testcular failure and low libido were injected with 200 million stem cells/rat from high oxygen culture (21% concentration of O_2). This suggests that a 200 million stem cells/rat from high oxygen culture (21% concentration of O_2) has not able maintained.libido as in the negative control group (T-). It means that the decrease of Leydig cells could reduce testosterone production and lead to decreased libido in males.

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The libido process begins with stimulation of the central nervous system in hypothalamus, where dopamine is produced as neurotransmitters and neurohormones that affect sexual behavior and activity in individuals. The stimuli were received by the sensory nerve trigger acetaminophen in stimulating endothelial cells to secrete nitric oxide to activate cyclic guanosine monophosphate (cGMP), cGMP. This process caused the muscles of corpus cavernosum of the penis becomes swollen, so that causes the occurrence of constriction of the penis arteriole so that the bloodflow heavyly. It causes the erectile tissue of penis becomes

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erection by full of blood flow will cause the veins depressed and inhibit the release of blood flow so that there is an increase of turgor from the reproduction organs and the erection penis was occurred [30].

The lack of glucose in both the sham group (TS) and the first treatment group (T1) causes no or less fuel and energy sources in this case glucose that is universal for all cells including spermatogenic and sperm cells. It causes no carbon source due to unavailability of glucose for synthesis of several compounds such as fatty acids, cholesterol, amino acids, nucleic acids, and steroid hormones such as testosterone. Glucose is also needed for a precursor to a variety of other glucoses, such as lactose, nucleotides, and glycosaminoglycans [31].

Glucose in the cytoplasm of spermatozoa cells will experience a glycolysis process, whereby glucose is broken into pyruvate and produce 2 ATP by phosphorylation at the substrate level. In this process, Nicotinamida adenine dinukleotida (NAD+) will be changed to nikotinamida adenine dinukelotida hidrogen (NADH) and then the NADH will be transferred to the mitochondrial electron chain to form pyruvic acid in citric acid cycle and the oxidation completely to CO_2. Cycle of citric acid, known as the cycle of tricarboxylic, is the catabolism reaction path, which occurs in the matrix of mitochondria. As the process of citric acid cycle and glycolysis proceeds on the mitochondria inner membrane, mitochondria will cause oxidative phosphorylation process that producing a lot of ATP [32]. The processes series is known as the aerobic glucose oxidation that will produce 38 ATP [31].

In this study, the group T1 suggests that rats injected with 200 million rMSC-CN/rat from normoxia culture (21% concentration of O_2) were still not able to stimulate libido. However, rats injected with 200 million rMSC-CH/rats from hypoxia culture (1% concentration of O_2) increased the number of Leydig cells significantly which different from the sham group (TS) and group T1. However, the increase in Leydig cells in this group T2 was different from the

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negative control group (T-) (Table-2).

<H1>Conclusion

The efficacy of rMSC-CH biotechnology for testicular failure therapy with low libido was based on the expression of VEGF, the increase of cell number of CD34+ and CD45+, the improvement of the testis to produce of spermatogenic cells, and then the improvement of libido due to the number of Leydig cells.

<H1>Authors' Contributions

ES: Research project leader, research and ethical clearance preparation, observation of IHC and flow cytometric method, stem cells isolation from rats bone marrow, rMSC-CH and rMSC-CN procedure, stem cells transplantation, statistical procedure, and draft for manuscript preparation (wrote of the paper). MH: Rat testicular failure and low libido model, observation of improvement of testicular tissue based on calculated number of spermatogenic and sperm cells, observation of libido, based on number of Leydig cells, designed study, analyzed statistical data, proofreading, and corresponding author. All the authors have read and approved the final version of the manuscript.

<H1>Acknowledgments

The study was supported by funding from The Directorate General of Higher Education of The National Education Ministry, Republic of Indonesia, Grant number: 004/ADD/SP2H/LT/DRPM/VIII/2017.

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<H1>Competing Interests

The authors declare that they have no competing interests.

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Tables

Table-1: Score of VEGF expression with IHC method and number cells calculated of CD34+ and CD45+ with flow cytometry in rat testicle tissue on several treatments (mean% ± SD).

Treatments	**Average VEGF expression score ± SD**	**Average number cells calculated CD34+ and CD45+ (%) ± SD**
The negative control group (T-): Rats with normal testicle were injected with 0.1 cc PBS	2.95^{c}±0.40	18.25^{a}±0.50
The sham group (TS): Rats with testicular failure and low libido were injected with 0.1 cc PBS	0^{a}±0	19.45^{a}±0.35
The first group (T1): Rats with testicular failure and low libido were	0.33^{a}±0.48	32.15^{b}±1.65

injected with 200 million rMSC-CN/rat		
The second group (T2): Rats with testicular failure and low libido were injected with 200 million rMSC-CHcells/rat	2.00^{b}±0.50	83.65^{c}±1.50
^{a,b,c}**Values in the same column with different superscripts indicate significant difference at p<0.05 (n=10). SD=Standard deviation**, VEGF=Vascular endothelial growth factor, IHC=Immunohistochemically, CD=Cluster differentiation, rMSC-CH=Rat mesenchymal stem cell-conditioned hypoxia, rMSC-CN=Rat mesenchymal stem cell-conditioned normoxia		

Table-2: Number of spermatogenic (spermatogonia, spermatocyte primer, spermatocyte secondary, and spermatid) and Leydig cells on several treatments.

Treatments	**Average spermatogenic cells (spermatogonia, spermatocyte primer, secondary spermatocyte, spermatid) ± SD**	**Average Leydig cells ± SD**
The negative control group (T-): Rats with normal testicle were injected with	252.20^{d}±2.55	12.50^{d}±0.50

0.1 cc PBS		
The sham group (TS): Rats with testicular failure and low libido were injected with 0.1 cc PBS	63.35^{a}±2.40	2.10^{a}±0.40
The first group (T1): Rats with testicular failure and low libido were injected with 200 million rMSC-CN/rat	97.35^{b}±1.35	5.75^{b}±0.30
The second group (T2): Rats with testicular failure and low libido were injected with 200 million rMSC-CH/rat	199.75^{c}±1.53	9.25^{c}±0.50
^{a,b,c,d}Different superscripts in the same column was significantly different ($p<0.05$). SD=Standard deviation, VEGF=Vascular endothelial growth factor, PBS=Phosphate-buffered saline, rMSC-CH=Rat mesenchymal stem cell-conditioned hypoxia, rMSC-CN=Rat mesenchymal stem cell-conditioned-normoxia		

Figure Legends

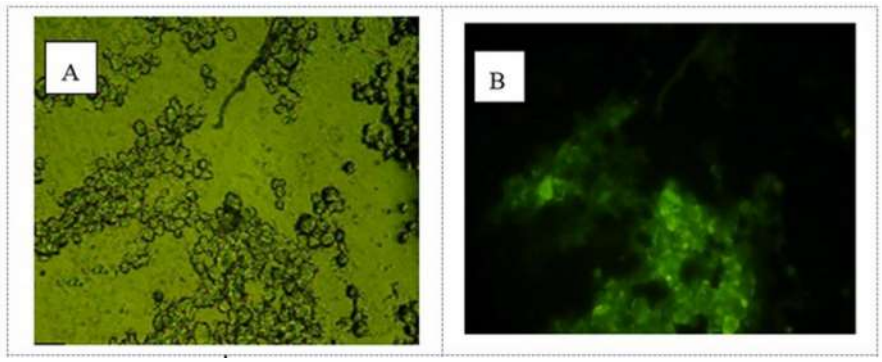

Figure-1: Positive expression cluster differentiation (CD)105 of mesenchymal stem cells (MSCs) with microscope fluorescent 400×. (a) Without filter, MSCs; (b) with green filter, MSCs (CD105) fluorescent.

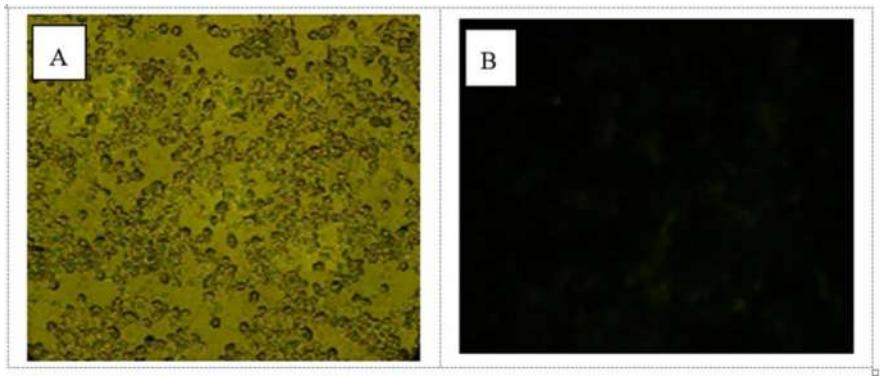

Figure-2: Negative expression from cluster differentiation (CD)45 of mesenchymal stem cells (MSCs) with microscope fluorescent 400×. (a) Without filter, MSCs; (b) with green filter, MSCs (CD45) not fluorescent.

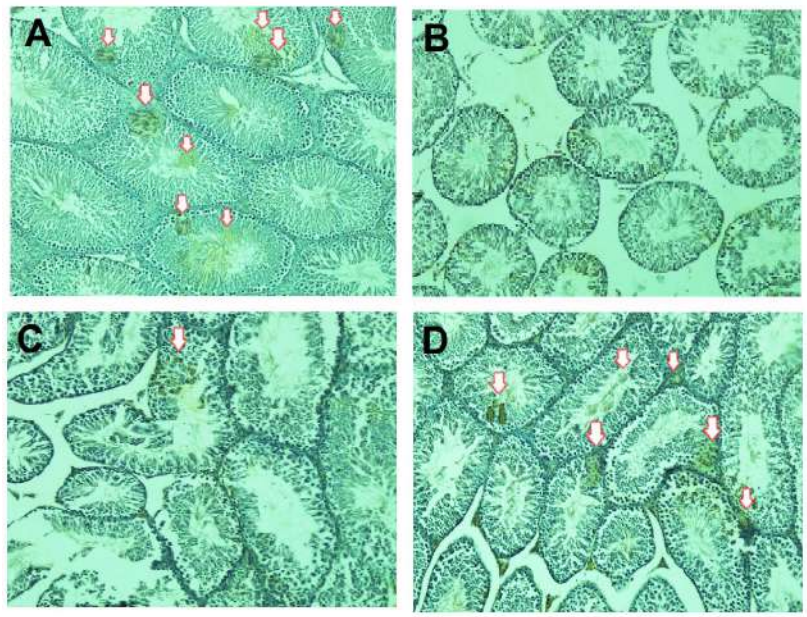

Figure-3: Immunohistochemical analysis from homing signal of stem cells based on vascular endothelial growth factor (VEGF) expression (brown chromogen) on several treatment 200× magnification (NikkonH600L Microscope; digital camera DS Fi2 300megapixel). The different superscripts indicate significant difference at $p<0.05$. (a) The negative control group (T-): Rats with normal testicle injected with 0.1 cc phosphate-buffered saline (PBS) showed expression of VEGF = $2.95^{c}\pm0.40$; (b) the sham group (TS): Rats with testicular failure and low libido injected with 0.1 cc PBS showed expression of VEGF = $0^{a}\pm0$; (c) the first group (T1): Rats with testicular failure and low libido injected with 200 million rat mesenchymal stem cells (rMSC)-conditioned normoxia/rat showed expression of VEGF = $0.33^{a}\pm0.48$; (d) the second group (T2): Rats with testicular failure and low libido injected with 200 million rMSC-conditioned hypoxia/rat showed expression of VEGF = $2.00^{b}\pm0.50$.

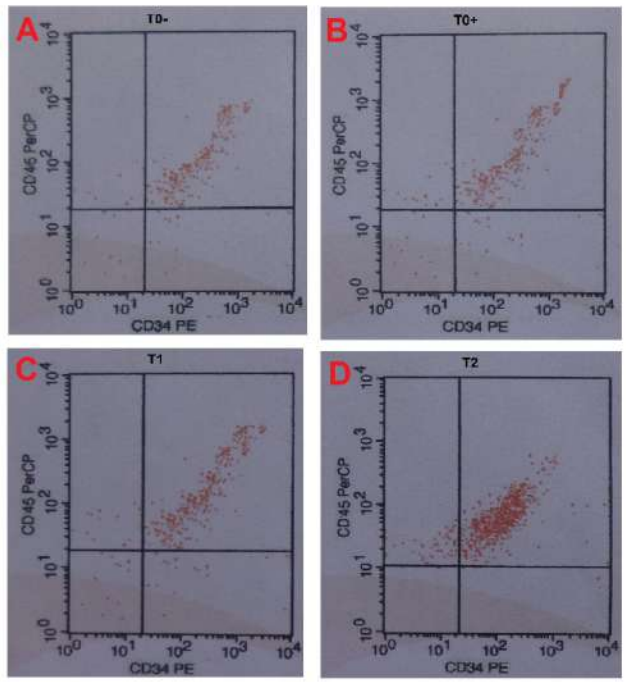

Figure-4: Flow cytometric analysis from hematopoietic stem cells mobilization based on cluster differentiation (CD)34+ and CD45+ expression on several treatment. The different superscripts indicate significant difference at $p<0.05$. (a) The negative control group (T-): Rats with normal testis injected with 0.1 cc phosphate-buffered saline (PBS) showed expression of CD34+ and CD45+ = $18.25^{a}\pm0.50$; (b) the sham group (TS): Rats with oligospermia and low libido injected with 0.1 cc PBS showed expression of CD34+ and CD45+ = $19.45^{a}\pm0.35$; (c) the first group (T1): Rats with oligospermia and low libido injected with 200 million stem cells/rat from high oxygen culture (21% concentration of O_2) showed expression of CD34+ and CD45+ = $32.15^{b}\pm1.65$; (d) the second group (T2): Rats with oligospermia and low libido injected with 200 million stem cells/rat from low oxygen culture (1% concentration of O_2) showed expression of CD34+ and CD45+ = $83.65^{c}\pm1.50$.

Re: Erma Safitri - Mas'ud Hariadi revised Manuscript Track 1-4.

From: Veterinary World (editorveterinaryworld@gmail.com)

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> Date: Thu, May 30, 2019 at 9:50 AM

Subject: Mas'ud Hariadi - Erma Safitri Manuscript, Revised paper
To: Veterinary World - Publisher <veterinaryworldpublisher@gmail.com>, Safitri Erma <rma_fispro@yahoo.com>

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Thank you for the good cooperation and help.
Yours sincerely,
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Cc: rma_fispro@yahoo.com; veterinaryworldpublisher@gmail.com

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> Date: Sat, May 25, 2019 at 9:25 AM
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Thank you very much for the good cooperation and help.

Yours Sincerely,

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RESEARCH ARTICLE

Biotechnology of rat mesenchymal stem cell-conditioned hypoxia compared with conventional *in vitro* culture of rat mesenchymal stem cell-conditioned normoxia for testicular failure therapy with low libido

Erma Safitri[1,2] and Mas'ud Hariadi[1]

1. Department of Veterinary Reproduction, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya 60115, Indonesia; 2. Stem Cells Research Division, Institute Tropical Disease, Universitas Airlangga, Surabaya 60115, Indonesia.

Corresponding author: Mas'ud Hariadi, e-mail: **masudhariadi@yahoo.co.id**

Co-author: ES: erma-s@fkh.unair.ac.id

Received: 27-12-2018**, Accepted:** 15-04-2019**, Published online: *****

doi: * How to cite this article:** Safitri E, Hariadi M (2019) Biotechnology of rat mesenchymal stem cell-conditioned hypoxia compared with conventional *in vitro* culture of rat mesenchymal stem cell-conditioned normoxia for testicular failure therapy with low libido, *Veterinary World*, 12(5): 0-0.

Abstract

Aim: Biotechnology of rat mesenchymal stem cell-conditioned hypoxia (rMSC-CH) for testicular failure therapy with low libido has been shown to improve functional outcome of the testicle for producing spermatogenic cells and to repair Leydig cells of a rat (*Ratus norvegicus*).

Materials and Methods: The first group (T1): Rats with testicular failure and low libido were injected with rMSC-conditioned normoxia (CN) (oksigen 21%); the second group (T2): Rats with testicular failure and low libido were injected with rMSC-CH (oksigen 1%); the negative control group (T-): Rats with normal testis were injected with 0.1 mL phosphate-buffered saline (PBS); the sham group (TS): Rats with testicular failure and low libido were injected with 0.1 mL PBS.

Results: Vascular endothelial growth factor expression as a homing signal in group T2, T-, T1, and TS, respectively, was 2.00±0.5, 2.95±0.4, 0.33±0.48, and 0±0. The cell number of cluster differentiation (CD)34+ and CD45+ in the two groups (T- and TS) was <20% while in

T1 and T2 groups was >30% and >80%, respectively; it showed that there was a mobilization of hematopoietic stem cells. Number of spermatogenic cells (spermatogonia, spermatocyte primer, spermatocyte secondary, and spermatid) in TS was significantly decreased ($p<0.05$) when compared with T-, T1, and T2, while T2 did not show a significant ($p>0.05$) decrease as compared to T- group. The improvement of libido, based on number of Leydig cells as producing testosterone hormone for libido expression, in T1 was still not able to increase the number of Leydig cells while in T2 was able to maintain the number of Leydig cells significantly different between TS and T1.

Conclusion: rMSC-CH on testicular failure with low libido has shown improvement in functional outcome of the testicle and to repair Leydig cells.

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Keywords: low libido, rat mesenchymal stem cell-conditioned hypoxia, rat mesenchymal stem cell-conditioned normoxia, rat, testicular failure.

<H1>Introduction

One of the failures of the testicle such as oligospermia represents a major reproductive health problem in male causing a low fertility and libido [1,2]. Oligospermia is a condition which is a low concentration of spermatozoa produced by testicular seminiferous tubules and makes infertility in male and it means no offspring [3]. Oligospermia, for male, is the main cause of infertility with etiology varies such as genetic heredity, traumatic, tumor neoplastic, and degenerative disorders due to malnutrition [4].

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Transplantation therapy with mesenchymal stem cells (MSCs)-derived bone marrow gives very promising results in regeneration of spermatogenesis process in testicular tissue with infertility like as oligospermia [5]. The therapy efficacy was limited due to low viability of

stem cells after transplantation [6,7]. The low viability of the stem cells is due to the conventional *in vitro* culture with rat MSC-conditioned normoxia (rMSC-CN), with oksigen concentration >21%. The conventional culture causes cells senescence [8], cells apoptotic [9], and mutation of gene such as G:C to T:A [10]. Some researchers showed that >93% of stem cells die between 1st and 7th days after transplantation [11-15]. Therefore it is required in high doses and repeated several times (booster) for effectiveness of therapy, utilizing of stem cells with conventional culture (CN), so that the cost become very expensive [16]. To remain viable, *in vitro* cultured of stem cells must be adapted to a niche or microenvironment where the stem cell resides in bone marrow. In the normal condition the niche of stem cells in the bone marrow is in the low oksigen tension (conditioned-hypoxia [CH]) [5,8,10]. Therefore, it is needed a biotechnology rMSC-CH *in vitro* culture for homing signal and mobilization of stem cells to improve testicular for producing sperm. The homing signal of stem cells in the testicle tissue is based on the expression of vascular endothelial growth factor (VEGF), while the mobilization was based on the expression of cluster differentiation (CD) such as CD34+, CD45−, and CD105− [5-7].

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The objective of the study was utilization of biotechnology of rMSC-CH for testicular failure therapy with low libido. Finally the biotechnology of rMSC-CH has been able to improve functional outcome of testicle for producing of spermatogenic cells and repair Leydig cells of rat (*Ratus norvegicus*).

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<H1>Materials and Methods

<H2>Ethical approval

The animal studies were performed via a protocol approved by the Animal Care and Ethical Clearance Committee of Faculty of Veterinary Medicine, Universitas Airlangga, and conform

with the National Research Council's guidelines (239-KE) through the ethical seminar. The research was conducted at the laboratory in the Institute of Tropical Diseases and Faculty of Veterinary Medicine, Universitas Airlangga.

<*H2*>Isolation and culture method of stem cells

The MSCs was collected from the crista iliaca bone marrow of male rat (*R. norvegicus*) strain Wistar 3 months of age. The aspirate was put in heparinized tubes and stored at 4°C for transportation to the laboratory experimental in Institute of Tropical Diseases, Universitas Airlangga, for conducting *in vitro* culture [17].

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The aspirate of MSCs was put in 15 mL heparin tube (Z181099, Sigma Aldrich®, Burlington, Massachusetts, USA) which previously filled with 3 mL α-modified Eagle medium (α-MEM) (M0894, Sigma Aldrich®, Burlington, Massachusetts, USA). The aspirate was transferred to a 15-mL sterile blue cap tube and added with 1× phosphate-buffered saline (PBS) (MFCD00131855, Sigma Aldrich®, Burlington, Massachusetts, USA) sterile to a total volume of 10 mL. The tube with aspirate solution was then rinsed twice with 5 mL × PBS. The diluted sample was added over the same volume of Ficoll (F9378, Sigma Aldrich®, Burlington, Massachusetts, USA) at room temperature in a separate 15 mL tube. Furthermore, each aspirate was coated with Ficoll before being centrifuged (Sorvall™ MX Series Floor Model Micro-Ultracentrifuge, Thermo Fisher, Grand Island, USA) at 1600 rpm = 287 relative centrifugal force (rcf) for 15 min at room temperature. After centrifugation, mononucleated cells were collected in the form of "buffy coat" located on the surface of Ficoll-PBS by means of a sterile Pasteur pipette and placed in a 15 mL tube (Sigma Aldrich®, Burlington, Massachusetts, USA).

The sample was diluted with PBS to a total volume of 15 mL, with the tube being turned 3-5

times as a means of achieving an even mix. At the next stage, centrifugation was undertaken for 10 min at a speed of 1600 rpm = 287 rcf. Before heating, the supernatant was thrown away and the cells resuspended with 6 mL of α-MEM (M0894, Sigma Aldrich®, Burlington, Massachusetts USA). The suspension of cells had placed in 10 cm^2 of the plate (Falcon™, Thermo Fisher Scientific, Pittsburgh, PA, USA); they were incubated at 37°C in a humidified atmosphere containing at 5% CO_2 moisture, and this process has taken 24 hrs until they attached on the surface of plate. After 24 hrs, media and non-adherent cells were discarded. The cells that attach were rinsed twice using 5 mL of PBS and shaken before being heated in the culture. The supernatant was disposed and then the plate was washed again twice with PBS. 10 min later, 10 mL of fresh α-MEM (M0894, Sigma Aldrich®, Burlington, Massachusetts, USA) was added to the dish before it was returned to the incubator. The cells were incubated at 37°C and 5% CO_2 moisture with the culture being observed daily by means of an inverted microscope.

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Every 4 days, the media were removed and the cells rinsed with 5 mL or 10 mL of 1× PBS before heating. The PBS was subsequently discarded and the dish was filled with 10 mL of fresh α-MEM (M0894, Sigma Aldrich®, Burlington, Massachusetts, USA). The culture was continued until approximately 75-80% confluence was attained, and then cells were passed into several dishes for subculture [17].

Passage was conducted 3 times, and then cells were divided into two: rMSC-CH treatment with 1% O_2 in hypoxia chamber inside a 5% CO_2 incubator, another treatment was the use of rMSC-CN with 21% O_2 and booth were incubated for 4 days. The three gases needed for 1% low oxygen precondition are CO_2 gas 5% to flow in 37°C incubator, O_2 gas 100% to flow only for calibration and N_2 gas. N_2 gas was used to replace O_2 gas so that it can be in accordance with the desired concentration.

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<H2>Testicular failure animal model with low libido

Testicular failure in rats were obtained by fasting for 5 days but drinking water was available *ad libitum* [1]. The condition of 5 days fasting in the rats induced testicular failure in the rats because the work of adrenal cortex was less effective in producing dehydroepiandrosterone (DHEA) due to malnutrition. Low levels of DHEA in the blood can be a cause of fatigue and decreased sperm concentration. DHEA is the most potent precursor of steroid hormones such as testosterone which produced by the renal adrenal cortex [18] and Leydig cells of testis [3]. The low testosterone production can lead to decreased spermatogenesis process and libido for males.

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The animal model was used in this study was Wistar strain of male rats (*R. norvegicus*), 250-300 g body weight, 3 months age, and healthy condition. The rat was placed in a plastic cage individually in the Animal Laboratory Experimental, Faculty of Veterinary Medicine, Universitas Airlangga.

<*H2*>Biotechnology rMSC-CH compared with rMSC-CN

Biotechnology stem cells treatment was done by injecting MSC directly from either conditioned rMSC-CH or rMSC-CN in the medial part of both testes, after previously performed anesthesia in rat using ketamine and xylazine. Anesthesia for rat was given 87 mg ketamine/kg and 13 mg xylazine/kg of body weight, beginning 10-15 min after simultaneous injection and lasting 15-30 min, then followed by a relatively long period of immobility (mean, 3.8 h) and reduced responsiveness to external stimuli [19].

MSC that was injected into male rat (*R. norvegicus*) has been induced testicular failure such as oligospermia with low libido, compared with negative and the sham group. The first group (T1): Rats with testicular failure and low libido were injected with 200 million rMSC-CN/rat

from high oxygen culture (21% concentration of O_2); the second group (T2): Rats with testicular failure and low libido were injected with 200 million rMSC-CH/rat from hypoxia culture (1% concentration of O_2); the negative control group (T-): Rats with normal testicle were injected with 0.1 mL PBS; the sham group (TS): Rats with testicular failure and low libido were injected with 0.1 mL PBS.

After second cycles of spermatogenesis, male rats were operated surgically to collect testicle tissue performed. Immunohistochemical (IHC) observation was performed to determine the expression of VEGF as a homing signal of stem cells in the testicle tissue. Flow cytometric observation was performed to calculate the cell number of CD34+, CD45+, and CD105- as mobilization of stem cells to improve testicle disorders.

The improvement of testicle in producing sperm cells was observed by histopathological preparation with hematoxylin-eosin (HE) stain (B8438, Sigma Aldrich®, Burlington, Massachusetts, USA), then the number of spermatogenic cells (spermatogonia, spermatocyte primer, secondary spermatocyte, and spermatid) and Leydig cells were calculated.

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<H2>**IHC of VEGF**

The IHC observation was performed to determine the expression of VEGF. Histological preparation was made before IHC method was done. An incision of testicular tissue in the paraffin blocks was made transversely. Further examination by making outward expression of VEGF by IHC method using VEGF monoclonal antibody (A183C 13G8, Thermo Fisher Scientific, Pittsburgh, PA, USA) was done. The expression of VEGF was made by a regular light microscope with 200 times magnification and the expression of each variable was indicated by the number of cells with brownish discoloration chromogen in each incision [20].

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The observation was performed using regular luminescence microscope Nikon H600L which was equipped with digital camera DS Fi2 300 megapixels and image processing software and cell count Nikon Image System.

<*H2*>Flow cytometry observation

Flow cytometric (BD FACSCalibur™, BD Biosciences, San Jose, USA) observation of the stem cell mobilization was based on calculated cell number of CD34+, CD45+, and CD105-. After the treatment, whole blood collected from cardiac puncture and then placed in heparin tube to prevent coagulation. Flow cytometric observation reveals the expressions of CD34+, CD45+, and 105-. Flow cytometric method begins with whole blood centrifugation in 4°C temperature, 6000 rpm = 4032 rcf for 15 min. Cellular precipitation as a result of centrifugation was mixed with cytoperm/cytofix (554714, BD Cytofix/Cytoperm™, BD Biosciences, San Jose, USA) in the amount of 2 times of obtained cell number. This mixture then was centrifuged again and formed supernatant and pellet. BD wash was added to the pellet in the amount of 4 times of obtained cell number from the first centrifugation and then added lysis buffer in the amount of twice of the first obtained cell number. Subsequently labeled antibody was added to each sample, five tubes were arranged and processed parallelly: (1) single staining with CD34 PE (Rabbit Anti-CD34/HCAM/PGP1 Polyclonal Antibody, PE-conjugated Conjugated Primary Antibodies-bs-0521R-PE, Biossusa) was added to the wash tube; (2) double staining with CD34 PE and CD45 PerCP (PerCP-Cy5.5 anti-human cross anti-rabbit CD45–BD, Biosciences, USA); (3) double staining with CD34 PE ; (4) double staining with CD45 PerCP and (5) double staining with CD105 FITC (Rabbit Anti-Thy-1/CD105/Thy1.1 Polyclonal Antibody, -0778R-FITC, Bioss, USA) true count tube. All the samples were then stored in 4°C and dark room and were analyzed with flow cytometry for 1 h [21].

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<H2>Histopathology observation

The improvement of testicular tissue for producing sperm with low libido was observed by histopathology preparation with HE stained and and calculation of spermatogenic cells number (spermatogonia, spermatocyte primer, secondary spermatocyte, spermatid) and Leydig cells. Histopathological examination was done using a light microscope with a magnification of 200 times. The observation was done after one cycle of spermatogenesis process (35 days) and food was given normally; male rats were operated surgically to collect testicle tissue performed. The calculation.of spermatogenic cells number based on the total number of 4 cell types (cells of spermatogonia, spermatocyte primer, secondary spermatocyte and spermatid) located on the five lumens of the seminiferous tubules from five preparat glasses. Furthermore, the total number of the four cell types of the five tubules was divided by 5 [1,2].

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The testicular of the rat was fixated in 10% formalin, and 1 h later, mid-testis was injected with formalin 10%. Rats testes then were dehydrated in alcohol solution with a higher concentration gradually, i.e., start from 70%, 80%, 90%, and 96%. Then, the testes of rats were cleared with xylol solution. Furthermore, embedding was done using paraffin liquid and rat testes were put into molds containing paraffin liquid. Before stained and sectioning performed, an incision using a microtome and mounted on glass objects. Furthermore, the staining was done by removing of paraffin with xylol, then put into a solution of alcohol with decreased concentration, start from 96%, 90%, 80%, and 70%, and then put it into HE staining procedure. The last stage was done mounting by putting into alcohol with increasing concentration, start from 70%, 80%, 90%, and 96% to remove excess stain, and then put into xylol. The preparates then were covered with a cover glass and mounted with Canada balsam [22].

<*H2*>Statistical analysis

The expression of VEGF, cell number calculated of CD34+ and CD45+, and cell number of spermatogenic and Leydig cells were statistically analyzed using SPSS 17 for Windows XP with the confidence level 99% (α=0.01) and the level of significance 0.05 (p=0.05). The steps comparative of hypothesis testing were as follows: the normality data were tested with the Kolmogorov–Smirnov test, homogeneity of variance test with analysis of variants, and *post hoc* test using the Tukey honestly significantly different 5% as the least significant difference test.

<*H1*>Results

The purity of MSCs was done through the identification of CD105+ and CD45- by immunofluorescence [17]. Immunofluorescence identification for CD105 was positive expression and CD45 was negative expression. CD105+ and CD45- were specific cell surface markers of MSCs using monoclonal antibody FITC anti-rabbit CD105 (Biolegend) and FITC anti-rabbit CD45 (Biolegend). Immunofluorescence results show that cultured cells were true MSCs (Figures-1 and 2).

<H2>IHC observation

The IHC observation was performed by scoring 0 to 5 as follows: score 0 means no chromogen colored, scores 1, 2, 3, 4 mean 1 – 25%, 26 – 25%, 51 – 75%, 76 – 100% chromogen color expression of VEGF respectively.. The average expression of VEGF in T2 group was 2.00^{b}±0.5 (25% and 50%) (Figure-3d), although the percentage was lower than negative control (T-) group which was found to be 2.95^{c}±0.4 (>50%) (Figure-3a), while the percentage was still higher than T1 group with score being 0.33^{a}±0.48 (≤ 5%) (Figure-3c), and the sham group (TS) was not expressed at all 0^{a}±0 (0%) (Figure-3b). The all score of

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VEGF is summarized in Table-1.

<*H2*>Flow cytometric observation

Flow cytometric observation of stem cell mobilization was based on calculated cell number of CD34+, CD45+, and CD105-. The average of cell number calculated of CD34+ and CD45+ in the two groups (T- and TS) was <20%. On the other hand, those percentages in T1 were more than 30% and T2 were more than 80%; it indicated hematopoietic stem cells (HSCs) mobilization. Statistically, there was a significantly difference ($p<0.05$) between T2 and the other three treatments (T-, TS, and T1) and no difference ($p>0.05$) between those two groups (T- and TS), although both groups were different with T1 being observed (Figure-4 and Table-1).

<H2>Histopathology and libido observation

The improvement of testicular tissue with oligospermia condition was observed by histopathology based on production of spermatogenic (spermatogonia, spermatocyte primer, secondary spermatocyte, and spermatid). Assessment for libido of male rat based on number of Leydig cells (Table-2)., in which the function of these cells are producing testosterone hormone, to stimulate libido [21,22]. Analysis of the results showed that although spermatogenic production in T2 group showed decreased significantly ($p>0.05$) compared to control negative group (T-) , , the decreased still showed different significantly ($p<0.05$) when compared with the sham group (TS) and (T1) (Table-2). Based on Table-2, it was found that T1 still showed different significantly ($p<0.05$) when compared with the control negative T- and T2.

<*H1*>Discussion

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It is known that MSCs can differentiate into a variety of cell types such as osteoblasts, chondrocytes, myocytes, and adipocytes. Nowadays, however, MSCs can differentiate into germ cell lineage or MSCs that assist in the regeneration of germ cell lineage. There is a phenomenon of transdifferentiation on MSCs, so it has multipotent characteristic more than before. It means that MACs can differentiate into various cells that a group (mesoderm) can also cross the class such as the ectoderm class, neuronal cells [23] or endoderm groups [24] and germline cells (spermatogonial, sertoli, and Leydig) and tissues (seminiferous tubules) [25].

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The efficacy of rMSC-CH biotechnology for testicular failure therapy with low libido was based on: (a) Expression of VEGF as the homing signal, (b) increase of cell number calculated of CD34+ and CD45+ as the mobilization of stem cells, (c) the improvement of testis for producing spermatogenic cells, and (d) the improvement of libido, based on the number of Leydig cells.

The expression of protein VEGF as a marker of homing signal was done through IHC methods. VEGF is a component of extracellular matrix from stem cells. VEGF has a role in supporting a conducive microenvironment for stem cells to remain viable [26]. The low oxygen culture for stem cells in this study provides supportive niche and through trigger process of VEGF-1 which is homing signal. Furthermore, VEGF-1 binds to VEGF receptor-1 and then will be passing a series of signaling that activates of stem cell factor (SCF). SCF is a mechanic in the niche of protein signaling that is physiologically will be happened further communication [27]. The presence of SCF will be recognized SCF receptor complex and toward into the cell nucleus so that expression of nuclear β1-integrine for activating octamer 4 (OCT 4). OCT 4 is a member of the POU family from transcription factors. OCT 4 has a major role in the proliferation, self-renewal, and differentiation of the stem cells. The

existence of the proliferation causes HSCs change of shape from quiescent of stem cells into a cycling state so that the HSCs located in the central endosteum area toward bone marrow. This suggests that cycling HSCs occurred outside of their niche and mobilized to the peripheral circulation [28].

Stem cells mobilization could occur because induction of stem cells mobilized toward defect place, in this research, based on calculated cell number of CD34+, CD45+. The mobilization process can occur in several ways: (a) Proteolytic induction of pharmacological agents such as granulocyte-colony stimulating factor or cyclophosphamide from bone marrow inside microenvironment; (b) blockade from specific locking molecules like as AMD3100 or BIO4860CXCR4 or VLA-4; (c) effect of dopamine and receptors b2-adrenergic as neural mediators; (d) elements modulation from cascade coagulation; (e) induction of inflammatory signals such as cytokines, Nuclear Factor-kappaB (NFkB), β catenin through Wnt due to the tissue damage; (f) homing signals such as stromal-cell-derived factor-1, (C-X-C Motif Chemokine Ligand 12 (CXCL12), VEGF, hepatocyte growth factor, platelet-derived growth factor, and integrin that appeared and to be act as recruitment of stem cells [17]. In this research, in “f” point, mobilization can occur caused expression of homing signal like as VEGF.

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Assessment of libido test from male rats was based on number of Leydig cells. Decreased libido on the sham group (TS) was occurred because the adrenal cortex work becomes ineffective in producing DHEA due to malnutrition (rat experience 5-day fasting). Low levels of DHEA in the blood can be a cause of fatigue and decreased body stamina. DHEA is the most potent precursor of steroid hormones such as testosterone produced by the renal adrenal cortex [18] and Leydig cells or interstitial cells between tubulus seminiferus of testis [3]. In this result, second group (T2): Rats with oligospermia and low libido were injected with 200

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million stem cells/rat from low oxygen culture (1% concentration of O_2). This suggests that a 200 million stem cells/rat from low oxygen culture (1% concentration of O_2) has maintened. libido as same as libido in the negative control group (T-). Increased DHEA aside as a precursor of steroid hormones, such as the hormone testosterone, is also responsible for fat metabolism and acts as an enzyme inhibitor of glucose 6-phosphate dehydrogenase, which has a role as a biocatalyst of changes in glucose to fat. Thus, the increase in DHEA allows for an increase in the amount of free adenosine triphosphate (ATP) in the body and reproduction organ, thus increasing the stamina of the body [29] and health of reproduction [3]. Further, increases in free ATP in the body and reproduction organ in addition to increasing stamina will also support an increase in libido. It can be explained that libido can be said good if the body is in a state of excellent stamina and then allows the process of reproduction such as spermatogenesis.

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In the first group (T1): Rats with testcular failure and low libido were injected with 200 million stem cells/rat from high oxygen culture (21% concentration of O_2). This suggests that a 200 million stem cells/rat from high oxygen culture (21% concentration of O_2) has not able maintained.libido as in the negative control group (T-). It means that the decrease of Leydig cells could reduce testosterone production and lead to decreased libido in males.

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The libido process begins with stimulation of the central nervous system in hypothalamus, where dopamine is produced as neurotransmitters and neurohormones that affect sexual behavior and activity in individuals. The stimuli were received by the sensory nerve trigger acetaminophen in stimulating endothelial cells to secrete nitric oxide to activate cyclic guanosine monophosphate (cGMP), cGMP. This process caused the muscles of corpus cavernosum of the penis becomes swollen, so that causes the occurrence of constriction of the penis arteriole so that the bloodflow heavyly. It causes the erectile tissue of penis becomes

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erection by full of blood flow will cause the veins depressed and inhibit the release of blood flow so that there is an increase of turgor from the reproduction organs and the erection penis was occurred [30].

The lack of glucose in both the sham group (TS) and the first treatment group (T1) causes no or less fuel and energy sources in this case glucose that is universal for all cells including spermatogenic and sperm cells. It causes no carbon source due to unavailability of glucose for synthesis of several compounds such as fatty acids, cholesterol, amino acids, nucleic acids, and steroid hormones such as testosterone. Glucose is also needed for a precursor to a variety of other glucoses, such as lactose, nucleotides, and glycosaminoglycans [31].

Glucose in the cytoplasm of spermatozoa cells will experience a glycolysis process, whereby glucose is broken into pyruvate and produce 2 ATP by phosphorylation at the substrate level. In this process, Nicotinamida adenine dinukleotida (NAD+) will be changed to nikotinamida adenine dinukelotida hidrogen (NADH) and then the NADH will be transferred to the mitochondrial electron chain to form pyruvic acid in citric acid cycle and the oxidation completely to CO_2. Cycle of citric acid, known as the cycle of tricarboxylic, is the catabolism reaction path, which occurs in the matrix of mitochondria. As the process of citric acid cycle and glycolysis proceeds on the mitochondria inner membrane, mitochondria will cause oxidative phosphorylation process that producing a lot of ATP [32]. The processes series is known as the aerobic glucose oxidation that will produce 38 ATP [31].

In this study, the group T1 suggests that rats injected with 200 million rMSC-CN/rat from normoxia culture (21% concentration of O_2) were still not able to stimulate libido. However, rats injected with 200 million rMSC-CH/rats from hypoxia culture (1% concentration of O_2) increased the number of Leydig cells significantly which different from the sham group (TS) and group T1. However, the increase in Leydig cells in this group T2 was different from the

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negative control group (T-) (Table-2).

<H1>Conclusion

The efficacy of rMSC-CH biotechnology for testicular failure therapy with low libido was based on the expression of VEGF, the increase of cell number of CD34+ and CD45+, the improvement of the testis to produce of spermatogenic cells, and then the improvement of libido due to the number of Leydig cells.

<H1>Authors' Contributions

ES: Research project leader, research and ethical clearance preparation, observation of IHC and flow cytometric method, stem cells isolation from rats bone marrow, rMSC-CH and rMSC-CN procedure, stem cells transplantation, statistical procedure, and draft for manuscript preparation (wrote of the paper). MH: Rat testicular failure and low libido model, observation of improvement of testicular tissue based on calculated number of spermatogenic and sperm cells, observation of libido, based on number of Leydig cells, designed study, analyzed statistical data, proofreading, and corresponding author. All the authors have read and approved the final version of the manuscript.

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<H1>Competing Interests

The authors declare that they have no competing interests.

<H1>Publisher's Note

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Tables

Table-1: Score of VEGF expression with IHC method and number cells calculated of CD34+ and CD45+ with flow cytometry in rat testicle tissue on several treatments (mean% ± SD).

Treatments	**Average VEGF expression score ± SD**	**Average number cells calculated CD34+ and CD45+ (%) ± SD**
The negative control group (T-): Rats with normal testicle were injected with 0.1 cc PBS	2.95^{c}±0.40	18.25^{a}±0.50
The sham group (TS): Rats with testicular failure and low libido were injected with 0.1 cc PBS	0^{a}±0	19.45^{a}±0.35
The first group (T1): Rats with testicular failure and low libido were	0.33^{a}±0.48	32.15^{b}±1.65

injected with 200 million rMSC-CN/rat		
The second group (T2): Rats with testicular failure and low libido were injected with 200 million rMSC-CHcells/rat	2.00^{b}±0.50	83.65^{c}±1.50
^{a,b,c}**Values in the same column with different superscripts indicate significant difference at p<0.05 (n=10). SD=Standard deviation**, VEGF=Vascular endothelial growth factor, IHC=Immunohistochemically, CD=Cluster differentiation, rMSC-CH=Rat mesenchymal stem cell-conditioned hypoxia, rMSC-CN=Rat mesenchymal stem cell-conditioned normoxia		

Table-2: Number of spermatogenic (spermatogonia, spermatocyte primer, spermatocyte secondary, and spermatid) and Leydig cells on several treatments.

Treatments	**Average spermatogenic cells (spermatogonia, spermatocyte primer, secondary spermatocyte, spermatid) ± SD**	**Average Leydig cells ± SD**
The negative control group (T-): Rats with normal testicle were injected with	252.20^{d}±2.55	12.50^{d}±0.50

0.1 cc PBS		
The sham group (TS): Rats with testicular failure and low libido were injected with 0.1 cc PBS	63.35^{a}±2.40	2.10^{a}±0.40
The first group (T1): Rats with testicular failure and low libido were injected with 200 million rMSC-CN/rat	97.35^{b}±1.35	5.75^{b}±0.30
The second group (T2): Rats with testicular failure and low libido were injected with 200 million rMSC-CH/rat	199.75^{c}±1.53	9.25^{c}±0.50
^{a,b,c,d}Different superscripts in the same column was significantly different ($p<0.05$). SD=Standard deviation, VEGF=Vascular endothelial growth factor, PBS=Phosphate-buffered saline, rMSC-CH=Rat mesenchymal stem cell-conditioned hypoxia, rMSC-CN=Rat mesenchymal stem cell-conditioned-normoxia		

Figure Legends

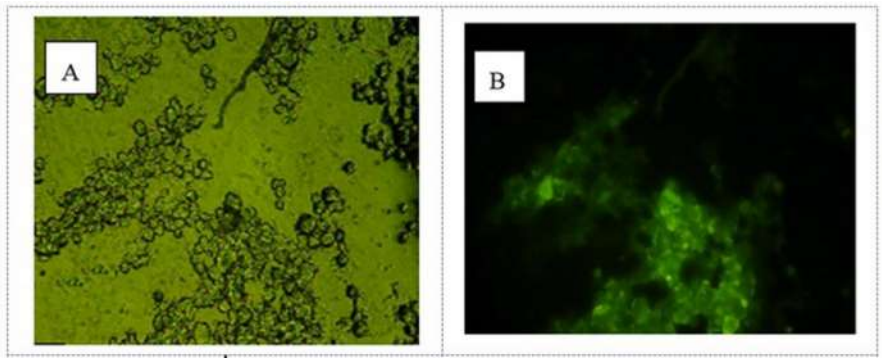

Figure-1: Positive expression cluster differentiation (CD)105 of mesenchymal stem cells (MSCs) with microscope fluorescent 400×. (a) Without filter, MSCs; (b) with green filter, MSCs (CD105) fluorescent.

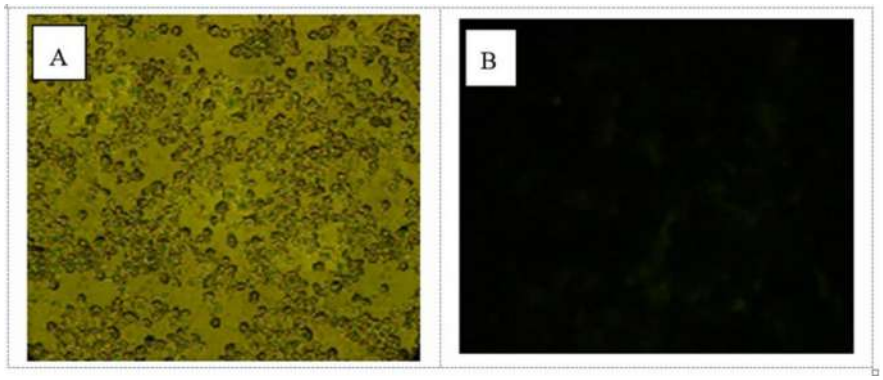

Figure-2: Negative expression from cluster differentiation (CD)45 of mesenchymal stem cells (MSCs) with microscope fluorescent 400×. (a) Without filter, MSCs; (b) with green filter, MSCs (CD45) not fluorescent.

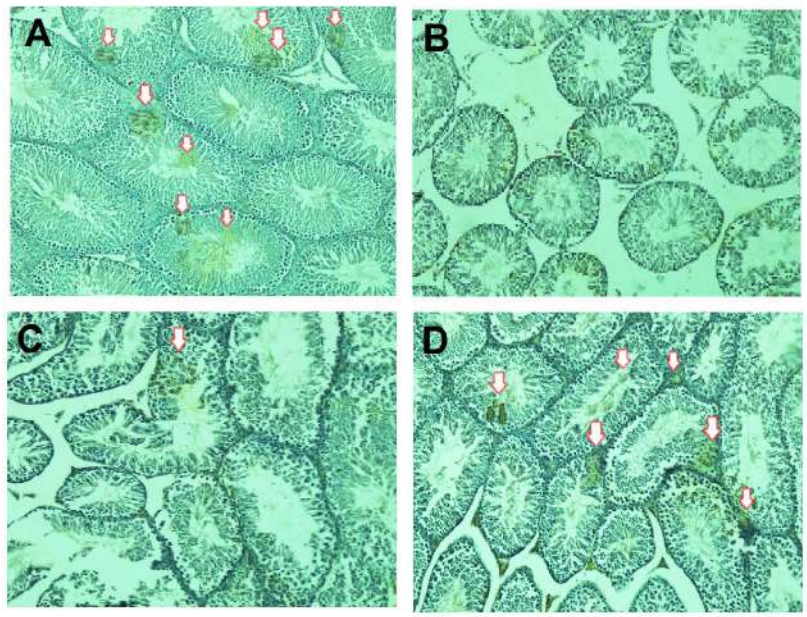

Figure-3: Immunohistochemical analysis from homing signal of stem cells based on vascular endothelial growth factor (VEGF) expression (brown chromogen) on several treatment 200× magnification (NikkonH600L Microscope; digital camera DS Fi2 300megapixel). The different superscripts indicate significant difference at $p<0.05$. (a) The negative control group (T-): Rats with normal testicle injected with 0.1 cc phosphate-buffered saline (PBS) showed expression of VEGF = $2.95^{c}\pm0.40$; (b) the sham group (TS): Rats with testicular failure and low libido injected with 0.1 cc PBS showed expression of VEGF = $0^{a}\pm0$; (c) the first group (T1): Rats with testicular failure and low libido injected with 200 million rat mesenchymal stem cells (rMSC)-conditioned normoxia/rat showed expression of VEGF = $0.33^{a}\pm0.48$; (d) the second group (T2): Rats with testicular failure and low libido injected with 200 million rMSC-conditioned hypoxia/rat showed expression of VEGF = $2.00^{b}\pm0.50$.

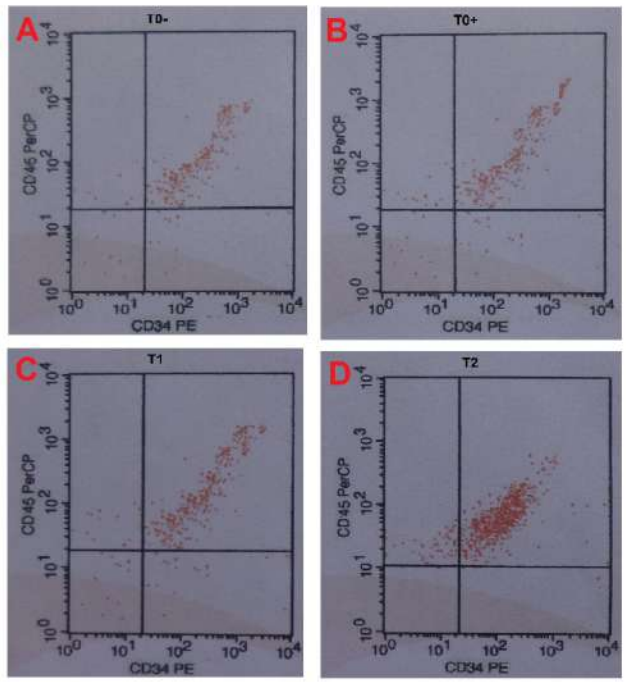

Figure-4: Flow cytometric analysis from hematopoietic stem cells mobilization based on cluster differentiation (CD)34+ and CD45+ expression on several treatment. The different superscripts indicate significant difference at $p<0.05$. (a) The negative control group (T-): Rats with normal testis injected with 0.1 cc phosphate-buffered saline (PBS) showed expression of CD34+ and CD45+ = $18.25^{a}\pm0.50$; (b) the sham group (TS): Rats with oligospermia and low libido injected with 0.1 cc PBS showed expression of CD34+ and CD45+ = $19.45^{a}\pm0.35$; (c) the first group (T1): Rats with oligospermia and low libido injected with 200 million stem cells/rat from high oxygen culture (21% concentration of O_2) showed expression of CD34+ and CD45+ = $32.15^{b}\pm1.65$; (d) the second group (T2): Rats with oligospermia and low libido injected with 200 million stem cells/rat from low oxygen culture (1% concentration of O_2) showed expression of CD34+ and CD45+ = $83.65^{c}\pm1.50$.

Re: Fw: Author declaration form

From: Veterinary World (editorveterinaryworld@gmail.com)

To: masudhariadi@yahoo.co.id; rma_fispro@yahoo.com

Date: Monday, April 15, 2019 at 04:22 PM GMT+7

Dear Dr. Mas'ud Hariadi,

I am in receipt of updated author declaration certificate.

NEWS:
Dr. Anjum Sherasiya, Editor-in-Chief of Veterinary World is appointed as Crossref Ambassador.

Best Regards,

Dr. Anjum Sherasiya
Editor-in-Chief, Veterinary World
Crossref - Ambassador
Star, Gulshan Park, NH-8A,
Chandrapur Road, Wankaner - 363621,
Dist. Morbi (Gujarat), India.
Website: www.veterinaryworld.org, onehealthjournal.org
E-mail: editorveterinaryworld@gmail.com

On Mon, Apr 15, 2019 at 11:20 AM Mas'ud Hariadi <masudhariadi@yahoo.co.id> wrote:

> ----- Pesan yang Diteruskan -----
> **Dari:** Safitri Erma <rma_fispro@yahoo.com>
> **Kepada:** Mas'ud Hariadi <masudhariadi@yahoo.co.id>
> **Terkirim:** Senin, 15 April 2019 10.46.34 WIB
> **Judul:** Author declaration form

Author declaration form: to be signed by all authors

Veterinary World

I/We the undersigned author(s) of the manuscript entitled: Biotechnology of Rat Mesenchymal Stem Cells conditioned Hypoxia (rMSC-CH) Compared with conventional in Vitro of Rat MSC-CN for testicular Failure Therapy with low libido,

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- **I/we declare that permission of animal ethics committee has been taken and included a statement in the manuscript.**
- I/we have read the final version of the manuscript and am/are responsible for what is said in it.
- The work described in the manuscript is my/our own and my/our individual contribution to this work is significant enough to qualify for authorship.
- No one who has contributed significantly to the work has been denied authorship and those who helped have been duly acknowledged.
- Full name and address of funding institute (it is compulsory to include for research article): Directorate General of Higher Education (DIKTI 2017), Grant No.: 1004/AMD/SP2H/LT/DRPM/VIII/2017

I/we also agree to the authorship of the article in the following sequence: (please add or delete extra nos. as per requitement)

	Author's name	Signature	email
1.	Erma Safitri		erma-s@fkh.unair.ac.id
2.	Mas'ud Hariadi		masudhariadi@yahoo.co.id
3.			
4.			
5.			
6.			
7.			

Date: March, 13 2019

Corresponding author address and email

Department of Veterinary Reproduction
Faculty of Veterinary Medicine
Universitas Airlangga
masudhariadi@yahoo.co.id

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Dikirim dari Yahoo Mail di Android

Include name and address of funding institute in the author declaration.

From: Veterinary World (editorveterinaryworld@gmail.com)

To: masudhariadi@yahoo.co.id; rma_fispro@yahoo.com

Cc: veterinaryworldpublisher@gmail.com

Date: Monday, April 15, 2019 at 10:26 AM GMT+7

Dear Dr. Mas'ud Hariadi,

We have asked you to provide updated author declaration certificate with name and address of funding institute (please refer notification for status email dated 12-04-2019) but still, we have not received it. So, send it on urgent basis.

We will provide the signed acceptance letter to you only after receipt of the updated author declaration form.

Best Regards,

Dr. Anjum Sherasiya
Editor-in-Chief, Veterinary World
Crossref - Ambassador
Star, Gulshan Park, NH-8A,
Chandrapur Road, Wankaner - 363621,
Dist. Morbi (Gujarat), India.
Website: www.veterinaryworld.org, onehealthjournal.org
E-mail: editorveterinaryworld@gmail.com

Receipt of the payment

From: Veterinary World (editorveterinaryworld@gmail.com)

To: masudhariadi@yahoo.co.id; rma_fispro@yahoo.com

Date: Saturday, April 13, 2019 at 10:00 AM GMT+7

Dear Dr. Mas'ud Hariadi,

We have received the payment into our PayPal account. We will issue the signed acceptance letter once the payment is received in our bank account. This is an automated transfer process and it may take up to 3-5 days.

NEWS:
Dr. Anjum Sherasiya, Editor-in-Chief of Veterinary World is appointed as Crossref Ambassador.

Best Regards,

Dr. Anjum Sherasiya
Editor-in-Chief, Veterinary World
Crossref - Ambassador
Star, Gulshan Park, NH-8A,
Chandrapur Road, Wankaner - 363621,
Dist. Morbi (Gujarat), India.
Website: www.veterinaryworld.org, onehealthjournal.org
E-mail: editorveterinaryworld@gmail.com