BIOLOGICAL ANALYSIS OF LEYDIG CELLS-CONDITIONED MEDIUM TO SUPPORT RAT BONE MARROW MESENCHYMAL STEM CELLS DIFFERENTIATION

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Abstract

The developed Leydig cells-conditioned medium (LCM) contains bioactive materials secreted by Leydig cells *in vitro*. LCM was used to evaluate the ability of bone marrow mesenchymal stem cells differentiation. Bone marrow mesenchymal stem cells (1x 10^6 cell/ml) were cultured in : 1) DMEM supplemented with 10% NBCS as a control (M), 2) M supplemented with 10 ng/ml testosterone; 3) M supplemented with 50% LCM ; 4) M supplemented with 50% LCM and 2.5 IU/ml hCG. Bone marrow mesenchymal stem cells that were cultured with LCM has a positive reaction (57.4%) to histochemistry staining 3β-HSD and produced 1.87 ng/ml testosterone. Supplementation of hCG to LCM increased the positive number of Leydig cells and testosterone production by 74.6% and 12.33 ng/ml ($P<0.05$). It can be concluded that Leydig cells-conditioned medium can support differentiation of bone marrow mesenchymal stem cells into Leydig cells.

Keywords: Conditioned medium, Leydig cells, mesenchymal stem cells, 3β-HSD, testoterone

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Introduction

Adult stem cells are undifferentiated cells found in tissues and it has multipotency. Function of these cells are maintaining and repairing tissue damage *in vivo* (Prentice, 2003). Since the report of several studies confirming that adult stem cells can do transdifferentiation, mesenchymal stem cells from bone marrow used as an alternative therapy for degenerative diseases (Fafian-Labora *et al.,* 2015). This is an alternative to overcome the ethical use of human embryonic stem cells.

Transdifferentiation is irreversible cell changes from one cell type into another (Eguizabal *et al.,* 2013). Transdifferentiation research suggests that mesenchymal stem cells differentiated into neural cells and hematopoietic stem cells differentiated into cardiac cells (Halim *et al.,* 2010). Mesenchymal stem cells induced transdifferentiation directly from one line to another mesenchymal type with the alteration of microenvironment (Song and Tuan, 2004). Mesenchymal stem cells also had the ability to differentiate into specific cells in combination with bioactive materials and specific stimuli of differentiation (Caplan and Bruder, 2001). Furthermore, transdifferentiation *in vivo* wasalso influenced by the microenvironment of tissue (Phinney and Prockop, 2007).

Conditioned medium (CM) has been used for cell culture in producing several bioactive compounds. Neuron cells CM were used to culture bone marrow mesenchymal stem cells *in vitro* and this stem cells differentiated into neuron cells (Djuwita *et al*., 2010). Leydig cell progenitors wereconsidered as mesenchymal cells because the morphology of cells was found in connective tissue derived from embryonic mesoderm (Hardy *et al*., 1991 in Robaire, 1991, Habert, 2001) and referred to mesenchymal stem cells (Ariyaratne *et al*., 2000). The main product of the Leydig cells is

testosterone, but the cells also secreted several bioactive compounds such as peptides, Interleukin - 1 (IL - 1) and Interleukin - 6 (IL - 6) (Chemes *et al*., 1992, Cudicini *et al*., 1997). Leydig cells also secrete another growth hormones such as Insulin - like Growth Factor I (IGF - I), Transforming Growth Factor - β (TGF - β), Epidermal Growth Factor (EGF), Fibroblast Growth Factor (FGF), Platelet - derived Growth Factor - A (PDGF - A) and the others (Mendis - Handagama and Ariyaratne, 2001; Saez, 1994; Avallet *et al*., 1991). Yazawa *et al.*,(2006) reported that bone marrow mesenchymal stem cells could differentiate into Leydig cells *in vivo* when the cells transplanted into testis. The results showed that rodentia mesenchymal stem cells have ability to differentiate into steroidogenic cells similar with Leydig cells .

Mesenchymal stem cells transdifferentiation into Leydig cells was expected as an alternative source for supply Leydig cells transplantation and to replace synthetic testosterone therapy on hipogonadism. Sun *et al*., (2009) concluded that rat adult Leydig cells transplantation to hypogonadism at pre-puberty have potency as therapeutic way. The microenvironment of stem cells affects cells transdifferentiation. Leydig cells conditioned medium had several bioactive materials which support progenitor cells differentiation into Leydig cells. In this study, the ability of differentiation bone marrow mesenchymal stem cells into the Leydig cells was studied using LCM. The aim of this study was to know the ability of testosterone secretion and other proteins from Leydig cell culture and analyzed the effect of bioactive materials from LCM to bone marrow mesenchymal stem cells differentiation *in vitro*.

Materials and Methods

Leydig cells culture and collection of LCM

Testes were collected from the rats (Sprague Dawley) adult males aged 8-10 weeks after anesthetized and sacrificed by cervical dislocation.Testicular tissue was placed in a petri dish contains Dulbecco's Phosphate Buffer Saline (DPBS) without Ca and Mg (Gibco, 21600-010, Invitrogen, NY, USA). The tissue was washed three times using medium DPBS supplemented with 0.1% Newborn Calf Serum (NBCS, Gibco, 16010-159, Invitrogen, New Zealand) (DBPS). Testicular tissue were then put into tubes containing one ml of 0.04% collagenase type I (Sigma, C0130, St. Louis, MO, USA) and 10 ug/ml trypsin inhibitor (Sigma, T9003, St. Louis, MO, USA) in DPBS and put in a water bath at a temperature of 34° C for 40 min. Cells suspension was diluted four times to initial volume with DPBS then allowed to precipitate. Supernatant was collected and centrifuged at 200 g for three minutes. The pellet cells then were washed twice using DPBS and diluted with 0.5 ml DPBS. Isolation and purification of Leydig cells was conducted by using Nycodenz gradient. The cells suspension were put into Nycodenz gradient 4%, 8%, 10%, 12%, 15% and then were centrifuge using rotor swing (KOKUSAN H-26F) at 1500 g for 10 min. Cells layer formed were collected and washed four times with DPBS and Dulbecco's Modified Eagle's Medium(DMEM) (Sigma, D5532, St. Louis, MO, USA) supplemented 10% NBCS and centrifuged at 200 g for three minutes. Cells pellet was diluted with 0.5 ml DMEM and the cell concentration was calculated using Neubauer haemocytometer.

Leydig cells in $1x10^6$ cells/ml were placed in a petri dish (Corning, 430 165, NY USA) 35x10 mm in 4 treatment: 1)DMEM supplemented with 10% NBCS, 2)with 2.5 IU/ml human Chorionic Gonadotrophin, hCG (Chorulon, Intervet, EU), 3) with 5 µg/ml insulin, 10 µg/ml transferrin, 5 mg/ml Se (ITS, Sigma I3146, StLouis, MO, USA) and 4) with hCG and ITS then were cultured in a 5% CO_2 incubator (Sanyo, MCO-95, Japan) at temperature of 37ºC. After cultured for three days, the medium was replaced using DMEM without the addition of serum. On day 3, CM was then collected and sterilized with a 0.22µm syringe filter (MS ®CA).Data was collected from 5 replication of culture and presented in Table 1.

Testosterone was analyzed with Testosterone ELISA kit (DRG Diagnostics EIA 1559) at the Laboratory of Hormones, Reproduction Rehabilitation Unit, Department of Veterinary Clinic, Reproduction and Pathology, Faculty of Veterinary Medicine, Bogor Agriculture University (IPB). SDS PAGE (12% polyacrylamide gel) was used to analyze protein content. Chromatein Prestained Protein Ladder (PR - 0602

Vivantis) was used as standard marker protein (positive control) and protein visualization using Coomassie Brilliant Blue staining method (PHAST Gel^{TM} Blue - R , Pharmacia 17-0518-01). The analysis of the molecular weight differences from protein bands in each LCM was compared with the marker.

The concentration of protein in the LCM was measured by using a spectrophotometer (Shimadzu UV-1800) with UV Probe 2.42 software. Each sample (60µL) was added to 940µL Protein Assay Dye Reagent (Bradford reagent, BioRad 500-0006), and then put into the cuvettes. The absorbance readings was performed at a wavelength of 595 nm. Readings corrected by using a linear standard curves 0 µg/ml to 1500 µg/ml Bovine Serum Albumin (BSA) which was standardized earlier. The study was conducted at Laboratory of Animal Reproduction and Cell Culture, Research Center for Biotechnology LIPI.

Isolation and culture of bone marrow mesenchymal cells

Bone marrow was isolated from the femur of adult male Sprague Dawley rats aged 2-3 months. Tibia or femur was cleaned and cut at the end of the cartilage. The bone stromal was flushed with 3ml of DPBS using 1ml syringe with 26G needle, collected in a sterile glass petri dishes and were homogenized with 1000 µL micropipette. After homogenized, the suspension put in 15 ml tube and centrifuged at 200g for 10 min. Supernatant was discarded and the pellet was resuspended with 3 ml of medium DPBS and centrifuged again. Washing the suspension was done twice using DPBS and once with DMEM. After that the cell pellet were resuspended to 1000µL with DMEM.

In vitro culture of bone marrow mesenchymal stem cells with LCM

Bone marrow mesenchymal stem cells approximately 1 x 10^6 cells / ml were cultured in a petri dish containing DMEM plus 10 % NBCS. After 24 hours, the medium changed to: 1) DMEM with 10 % NBCS; 2) with 10 ng/ml testosterone (Nacalay Tesque 32811-61 Kyoto , Japan); 3) with 50 % LCM; 4) with 50 % LCM and 2.5 IU/ml hCG then culture in 5% CO_2 incubator at 37^0C of temperature. LCM used were obtained from the treatment DMEM with ITS and hCG (presented in Table 1). Medium replacement every 48 hours until day 10. The cell morphology and histochemical staining 3β-HSD was observed to detect Leydig cells.

Analysis the culture medium of bone marrow mesenchymal stem cells

Testosterone ELISA kit (DRG Diagnostics EIA 1559) was used to analyzed testosterone. Measurement of protein concentration from the medium used spectrophotometer (Shimadzu UV-1800) with Probe 2.42 software.

Statistical analysis

Bone marrow mesenchymal stem cells culture were replicated at least three times in each treatment. Data were analyzed using one way ANOVA followed by Duncan and all cases of significance was set at $p<0.05$.

Results

Leydig cells conditioned medium (LCM) from 4 treatment resulted in 0.65 to 0.94ng/ml of testosterone. The combination of hCG and ITS produced more testosterone (0.94 ng/ml) compared with other treatments. Results showed that protein concentration in treatment with combination of hCG and ITS (1.083,40 µg/ml) were higher ($P<0.05$) than of others treatment (ITS 572.04 µg/ml; hCG 643.55 µg/ml; control 455.60 µg/ml) (Table 1). The protein analysis from Leydig cells conditioned medium with SDS PAGE exhibited protein bands with molecular weight (MW) closed to 29 kDa, 62-70 kDa and 95-130 kDa (Figure 1.)

Table 1. Analysis of testosterone and protein concentration from LCM

LCM from the treatment :	Testoterone (ng/ml)	Protein (µg/ml)
DMEM+ 10% NBCS	0.65±0.05	455.60± 0.14^a
DMEM+ 2.5 IU/ml hCG	0.74±0.01	643.55± 0.14^b
DMEM+ insulin 5 µg/ml, transferrin, 10 µg/ml, Se 5 µg/ml (ITS)	0.79± 0.02	572.04±0.34^c
DMEM+hCG+ITS	0.94± 0.03	1.083.40±0.07^d

Note: Different superscript letters on the same column indicate a significant differences ($p<0.05$) by Duncan

Ability of LCM to induce bone marrow mesenchymal stem cells transdifferentiation showed that the cells reacted positively to specific staining 3β-HSD (57.4%) and produced testosterone (1.87 ng/ml) (Table 2 and Figure 2c). Supplementation of hCG to medium culture significantly ($p < 0.05$) increased the testosterone concentration (74.69%). The cells from treatment with testosterone also showedpositive reaction to 3β-HSD (Fig. 2b).

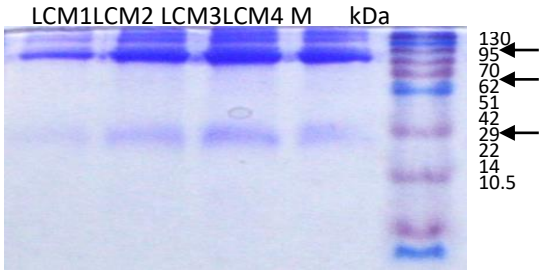

Figure 1. The results of SDS PAGE analysis from LCM

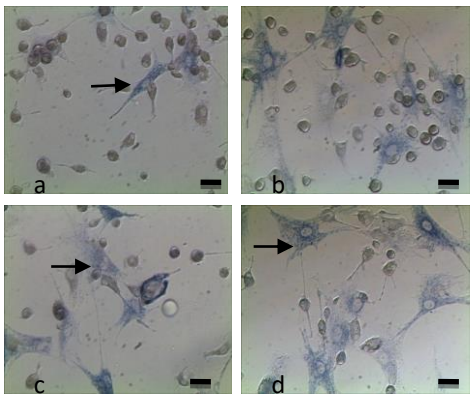

Figure 2. Rat bone marrow mesenchymal stem cells with: a. DMEM+10% NBCS, b. DMEM+ testosterone 10 ng/ml, c. DMEM+50% LCM, d. DMEM+50% LCM+2.5 IUhCG. Staining with 3β-HSD. Scale= 20 µm

Mesenchymal stem cells were cultured with LCM produced different morphology compared to that of controls. Fibroblast like cells (→Figure 2a) was found at mesenchymal stem cells culture with supplementation with NBCS, but when cultured with LCM, the cells have different morphology (→, Fig 2c & d).

Table 2. *In vitro* culture of bone marrow mesenchymal stem cells with LCM and the analysis of testosterone concentrations and other proteins in the culture medium.

Treatments	3 β-HSD	Leydig Cells (%)	Testoterone (ng/ml)	Protein (µg/ml)
DMEM + 10% NBCS	+/-	3.9[a]	1.97±0.15 [a]	1804.66±2.59[a]
DMEM+ 10 ng/ml testosteron	+	22.3[b]	4.65±1.91[a]	1246.28±0.18[b]
DMEM+ 50% LCM	+++	57.4[c]	1.87±0.04[a]	1298.94±0.12[c]
DMEM+5 0% LCM + 2.5 IU/ml hCG	+++	74.6[d]	12.33±0.02[b]	823.45±0.11[d]

Note: Different superscript letters on the same column indicate a significant differences ($p < 0.05$) by Duncan. +/-: 0-5 cells, + : 6- 20 cells, + +: 21-40 cells, +++ : >40 cells

Discussion

Leydig cells play an important role in the production of testosterone in a male. Based on previous studied, treatment rat Leydig cell culture with a combination of ITS and hCG in medium produces the highest testosterone concentration at 5.25 ng/ml (Kaiin *et al.*, 2013). In this study, testosterone content from LCM which was treated with hCG and ITS showed higher concentrations of testosterone compared with other treatments. Bilinska *et al.* (2009) suggested that human Leydig cell testosterone production *in vitro* after purified with Percoll was equal to 5.76 ng /10^6 cells within 24 hours and hCG supplementation increased testosterone production into 9.83 ng/10^6 cells. Leydig cells culture *in vitro* produced testosterone about 3.5 ng/10^6 cells, while supplementation of hCG into medium, increased testosterone to 4.5 ng/10^6 cells (Risbridger *et al*., 1981). Molecular weight (MW) of protein was about 29 kDa found in LCM and was expected as Interleukin 1 (IL - 1). According to Saez (1994), IL - 1 has a molecular weight that varies between 10-30 kDa. Other proteins were detected by SDS PAGE has considerable MW between 62-70 kDa and 95-130 kDa. Lakshmanan *et al.* (1990) found that EGF detected in the adult mice has molecular weight of 66 and 56 kDa , whereas pro - EGF is 165 kDa. TGF-β is a family of 25 kDa polypeptide distributed and synthesized by different cells (Kropf *et al*., 1997). Other

growth factor, EGF, which stimulates androgen synthesis, and influenced *in vitro* production of testosterone by Leydig cells (Suarez-Quian dan Niklinski, 1990). PDGF- A is an important factor in the process of Leydig cell differentiation, but the mechanism has not been known (Mendis-Handagama dan Ariyaratne, 2001). There are many other factors secreted by the Leydig cells such as corticotropin-releasing factor (CRF), arginine-vasopressin (AVP), oxytocin (OT), angiotensin-II (A - II) and other peptides (Saez, 1994). Further research is needed to determine the protein in LCM.

Adult Leydig cells are derived from mesenchymal stem in the testes. These cells can express several specific markers such as 3β - HSD, and LH receptor androgen production (Ariyaratne *et al*., 2000). Specific cell surface marker protein CD90 (Thy1) was found to be unique in Leydig cells (Li *et al*., 2016). Djuwita *et al*. (2010) found that bone marrow mesenchymal stem cells were culture for 24 hours had two kind of morphology of cell, polygonal-shaped cell and fibroblast-like cell. The fibroblast-like cells morphology was found at the control group (Figure 1a).Lotfi *et al*. (2014) also found the morphology of rat bone marrow was spindle-shaped cells. Bone marrow mesenchymal stem cells culture with LCM showed different cell morphology and had a positive reaction to specific staining 3β - HSD (Fig. 1 c, d). These morphological changes could be a process of transdifferentiation. Transdifferentiation is greatly influenced by the microenvironment and LCM used as a medium culture of bone marrow mesenchymal stem cells. It was creating environmental condition that was suitable for the Leydig cell differentiation. LCM contain bioactive materials such as testosterone, growth factors and other proteins that are secreted by Leydig cells in the culture. Mesenchymal stem cell has multipotency and was influenced by microenvironment which supporting the process of transdifferentiation. Wu *et al*. (2012) reported that human bone marrow stem cells cultured with medium supplemented with PDGF-α, LH, hCG and IL-1, they produced steroidogenic cells or Leydig cells *in vitro*. LCM also inducing human umbilical cord MSC differentiation into steroidogenic cells (Xing *et al*., 2016) and can promote recovery of serum testosterone levels in the rat via differentiation into normally functioning Leydig-like cells (Zhang et al., 2017). These results also supported to our study because the LCM could contain similar factors to support Leydig cells transdifferentiation.

Androgens play a role in differentiation of rodentia Leydig cells (Habert *et al*., 2001). Differentiation of Leydig cell progenitors into mature Leydig cells *in vitro* was also influenced by LH and dihydrotestosterone (Mendis - Handagama dan Ariyaratne, 2001). It supported by the study that supplementation of testosterone at mesenchymal stem cells medium culture resulted in a positive reaction to 3β-HSD. Chemes *et al*. (1992) suggest that mesenchymal cells from patients who are not sensitive to androgen and culture with hCG in medium, then cells could differentiate and produce testosterone. The activity of 3 β - HSD and testosterone production increased when hCG was added to the culture medium. Supplementation of LH or hCG is needed in process of multiplication and differentiation of Leydig cells (Saez, 1994).The addition of hCG into the culture medium of bone marrow mesenchymal stem cells, which were treated with LCM, could produce testosterone at the highest concentration but it decreasedthe protein concentration. The low of protein concentrationwas thought that induction of hCG is used by Leydig cells to increase testosterone production instead of total protein??) synthesis (Table 2). In this study, mesenchymal stem cells were cultured in medium containing testosterone also reacted positively to the 3 β - HSD staining. Serum was added as nutrients such as essential fatty acids and cholesterol as well as a number of growth factors and proteins. Seedelar and Isaacs (2009) reported that in bovine serum could contain testosterone between 1.2 to 7.5 ng /ml when collected after birth until the age of 1 year. The discovery of some cells that reacted positively to the 3 β-HSD in bone marrow mesenchymal stem cells from control, probably caused by the testosterone in the serum. It can be concluded that supplementation of hCG to LCM can increase the number of cells reacted positively to the 3 β-HSD and testosterone production from mesenchymal stem cells culture medium *in vitro*. Mesenchymal stem cells transdifferentiation into steroidogenic cell/Leydig cells is complex, so that further

research using cell surface marker and molecular marker is needed to know the process.

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