

# EVALUATION OF RAT LEYDIG CELL CULTURE COLLECTED WITH NYCODENZ GRADIENT IN PRODUCING TESTOSTERONE IN VITRO

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## ABSTRACT

The aim of this study was to evaluate rat Leydig cells collected with Nycodenz gradient in producing testosterone in vitro. Leydig cells were collected using 5 column of Nycodenz gradient (4, 8, 10, 12, and 15%) and cells were evaluated regarding its concentration, viability, and purity of Leydig cells. Media used to culture Leydig cells were: Dulbecco's Modified Eagle's Medium (DMEM)+10% newborn calf serum (NBCS); DMEM+10% NBCS+2,5 IU/mL human chorionic gonadotrophin (hCG); DMEM+10% NBCS+ITS (5 µg/mL insulin, 10 µg/mL transferrin, and 5 µg/mL Se); DMEM+10% NBCS+hCG+ITS, and cultured in 5% CO<sub>2</sub> incubator with temperature of 37.5° C for 3 days. Culture medium was collected every day for testosterone analysis with enzyme linked immunosorbent assay (ELISA). By adding ITS to the medium, Leydig cells concentration was significantly increased (8.92x10<sup>6</sup> cells/mL) compared to medium with serum (7.74x10<sup>6</sup> cells/mL) or hCG (7.68x10<sup>6</sup> cells/mL) (P<0.05). ITS and hCG in medium significantly increased Leydig cells concentration (10.40x10<sup>6</sup> cells/mL) at day 3 of culture (P<0.05). The result of parallelism test showed that the assay obtained good validity to measure testosterone concentration in culture medium. Testosterone in medium was detected at 1.80-2.60 ng/mL at day 1 of culture. To conclude, Leydig cells collected with Nycodenz gradient had no effect on testosterone secretion from Leydig cells.

Key words: ELISA, hCG, in vitro culture, Leydig cells, Nycodenz, testosterone

## ABSTRAK

Penelitian ini bertujuan mengetahui kemampuan sel Leydig tikus hasil koleksi dengan gradien Nycodenz untuk menghasilkan testosteron secara in vitro di dalam kultur. Sel Leydig dikoleksi menggunakan gradien Nycodenz 4, 8, 10, 12, dan 15% (5 kolom), lapisan (fraksi) sel yang terbentuk kemudian dikoleksi dan diperiksa konsentrasi, viabilitas dan kemurniannya. Sel Leydig dikultur di dalam 4 perlakuan medium yaitu: Dulbecco's Modified Eagle's Medium (DMEM)+NBCS 10%; DMEM+newborn calf serum (NBCS) 10%+human chorionic gonadotrophin (hCG) 2,5 IU/ml; DMEM+NBCS 10%+ITS (insulin 5 µg/ml, transferrin 10 µg/ml, Se 5 µg/ml); DMEM+NBCS 10%+hCG+ITS, kemudian dikultur di dalam inkubator CO<sub>2</sub> 5% dengan suhu 37° C selama 3 hari. Medium kultur dikoleksi setiap hari untuk dianalisis kandungan testosteronnya menggunakan kit enzyme linked immunosorbent assay (ELISA). Hasil menunjukkan bahwa penambahan ITS ke dalam kultur meningkatkan konsentrasi sel Leydig (8,92x10<sup>6</sup> sel/ml) secara nyata (P<0,05) dibandingkan dengan perlakuan serum saja (7,74x10<sup>6</sup> sel/ml) atau penambahan hCG (7,68x10<sup>6</sup> sel/ml). Penambahan ITS bersama hCG meningkatkan secara nyata (P<0,05) konsentrasi sel Leydig pada kultur hari ketiga (10,40 x10<sup>6</sup> sel/ml). Hasil uji paralellism menunjukkan asai yang digunakan memiliki validitas yang baik untuk mengukur konsentrasi testosteron di dalam medium kultur. Konsentrasi testosteron di dalam medium kultur berkisar 1,80-2,60 ng/ml dan pada hari pertama kultur sudah terdeteksi adanya testosteron. Dapat disimpulkan bahwa koleksi sel Leydig dengan gradien Nycodenz tidak mengganggu kapasitas sel Leydig untuk memproduksi testosteron di dalam medium kultur in vitro.

Kata kunci: ELISA, hCG, sel Leydig, kultur in vitro, Nycodenz, testosteron

## INTRODUCTION

The process of spermatogenesis in males is greatly influenced by testosterone. Deficiency of this hormone causes infertility where hormonal therapy usually takes place. Hormonal therapy as a treatment of testosterone deficiency is performed using synthetic testosterone. But in humans, long term administration of these therapies lead to some disturbances, such as increased risk of coronary heart disease, fluid retention, prostate cancer, and others (Gruenewald and Matsumoto, 2003).

Some researchers provide therapeutic use of Leydig cells as a source of natural testosterone-producing cells. Leydig cells transplanted into the testes produce testosterone naturally. Mature Leydig cells is derived from mesenchymal stem cells progenitor Leydig cell found in the testes. In conditions of Leydig cells shortage in the testes, Leydig cell biopsy results will be multiplied in number by performing in vitro cell culture, before the cells are transplanted. Cells that are cultured in vitro were able to secrete a variety of bioactive materials, such as growth factors that are useful for cell growth. Cultured Leydig cells are suspected to secrete various

bioactive ingredients such as peptides, growth factors, and testosterone into the culture medium (Chemes *et al.*, 1992; Cudicini *et al.*, 1997; Hu *et al.*, 1998).

The main function of Leydig cells in the testes of adult animals is to produce testosterone in spermatogenesis. The secretion of testosterone is regulated by luteotropic hormone (LH) from the pituitary and is part of the hypothalamic-pituitary-testes axis (Yang *et al.* 2003; Senger, 2005). Testosterone production is determined by the number of Leydig cells in the testes, so lesser number of cells leads to less production of testosterone and the disruption of spermatogenesis can causes infertility (Hardy *et al.*, 1990). In this study, we evaluate the cultured Leydig cell collected by a Nycodenz gradient and its effect on the cell's ability to produce testosterone in vitro.

## MATERIALS AND METHODS

### Testicular Cells Suspension Preparation

The testicular tissue was collected from the testes of adult male rats (Sprague Dawley) aged 8-10 weeks. Tunica albuginea and other connective tissue were

removed, then, the testicular tissue was placed in a petri dish containing Dulbecco's phosphate buffer saline (DPBS) medium without Ca and Mg. The tissue was then washed 3 times with the DPBS medium supplemented with serum (newborn calf serum, NBCS) 0.1%. The testicular tissue sampling was carried out in aseptic procedure then inserted into tubes containing 1 mL of 0.04% collagenase and 10 ug/mL trypsin inhibitor in DPBS, and incubated in a water bath at a temperature of 34° C for 40 minutes. The cell suspension was diluted 4 times the initial volume with DPBS serum medium, and then it was allowed to stand for 2 minutes to let the cells settle. The supernatant fluid was collected and centrifuged at 200 g for 3 minutes. The cell pellet was washed 2 times using DPBS serum medium in the same way. Finally, the cell pellet was diluted with 500 µL of DPBS serum medium.

**Leydig Cells Collection**

Leydig cell was collected using a modified Nycodenz gradient method of Nakayama *et al.* (1999). The cells were placed into a 5 column Nycodenz solution with a gradient of 4%, 8%, 10%, 12%, and 15%. Thereafter, the Nycodenz gradient column was centrifuged using a centrifuge rotor swing at a speed of 1,500 g for 10 minutes at room temperature. Layer of cells formed was collected and washed with DPBS+0.1% NBCS by centrifuged at 200 g for 3 minutes (3 times) and with DMEM+10% NBCS (2 times). The pellet cells were diluted with 500 µL of DMEM+10% NBCS.

**In Vitro Culture**

A total of 1x10<sup>6</sup> cells/mL Leydig cells suspension were cultured in medium with treatment of: 1) DMEM +10% NBCS; 2) DMEM+10% NBCS+2.5 IU/mL human chorionic gonadotrophin (hCG); 3) DMEM+10% NBCS+5 µg/mL insulin, 10 µg/mL transferrin, 5 µg/mL Se (ITS); 4) DMEM+10% NBCS+hCG+ITS. The cells was then cultured in 5% CO<sub>2</sub> incubator at 37° C for 3 days. Media culture were collected every 24 hours until day 3, and then frozen and stored at -20° C.

**Testosterone Concentration Measurement**

Testosterone concentration in culture medium was analyzed by enzyme linked immunosorbent assay

(ELISA) using a commercial ELISA Testosterone kit (EIA 1559, DRG Diagnostic, Germany). Before the hormone analysis was performed, a parallelism assay was conducted to assess the validity of the assay used, as described by Gholib *et al.* (2016) and Pettit *et al.* (2007). Testosteron absorbance analysis reading was done using the ELISA reader (Biotek EL 808) that comes with Gen5 software.

**Evaluation and Data Analysis**

Evaluation was carried out by calculating the concentration of cells using Neubauer hemocytometer (room count). The purity of Leydig cells was determined with 3β-HSD staining which specifically stained the Leydig cells to purple-blue. Cell viability was tested using 4% trypan blue dye. Cells that died will become blue because of the damaged membrane. Each treatment was repeated 3 times. Data in Table 2 and Table 4 were statistically tested by analysis of variance (ANOVA) with 95% confidence level.

**RESULTS AND DISCUSSION**

**The Quality of Leydig Cells with Nycodenz Gradient Collection Results**

The quality of Leydig cells cultured in this study is shown in Table 1. Leydig cells with Nycodenz gradient collection results were qualified for culture because it has sufficient viability and high purity. Examination was performed to ensure the Leydig cells in the culture have good quality. Leydig cells collection can affect the ability of the Leydig cells to respond to stimulation factor on in vitro conditions (Hedger and Eddy, 1990).

Medium supplemented by ITS showed a significant increase in concentration of cells cultured for 3 days (8.92x10<sup>6</sup> cells/mL) (P<0.05) compared to serum supplemented (7.74x10<sup>6</sup> cells/mL) or hCG supplemented medium (7.68x10<sup>6</sup> cells/mL). The hCG and ITS combined treatment increased Leydig cells concentration significantly at the end of culture (10.40x10<sup>6</sup> cells/mL) (P<0.05). ITS components were insulin, transferrin, and selenium which were a growth factor used as supplement to increase cell growth in culture. Insulin was a growth factor that played a role in the use of glucose and amino acids by cells, whereas transferrin had role in the binding of Fe<sup>2+</sup> to increase absorption of cell nutrient. Selenium was needed to

**Table 1.** Isolated Leydig cells with Nycodenz gradient quality

Parameters	Test result
Concentration (x10 <sup>6</sup> cells/mL)	375
Viability (%)	97.7
Purity (%)	91.2

**Table 2.** Results of Leydig cell cultures for 3 days

Culture medium treatment	Parameters at H3 culture		
	Concentration (x10 <sup>6</sup> cells/mL)	Viability (%)	Purity (%)
DMEM+10%NBCS	7.74 <sup>a</sup>	92.0 <sup>a</sup>	88.8 <sup>a</sup>
DMEM+10%NBCS+hCG	7.68 <sup>a</sup>	91.3 <sup>a</sup>	88.3 <sup>a</sup>
DMEM+10%NBCS+ITS	8.92 <sup>b</sup>	92.0 <sup>a</sup>	88.0 <sup>a</sup>
DMEM+10% NBCS+hCG+ITS	10.40 <sup>c</sup>	92.6 <sup>a</sup>	88.5 <sup>a</sup>

<sup>a, b, c</sup>Different superscripts within the same column indicates significant differences (P<0.05)

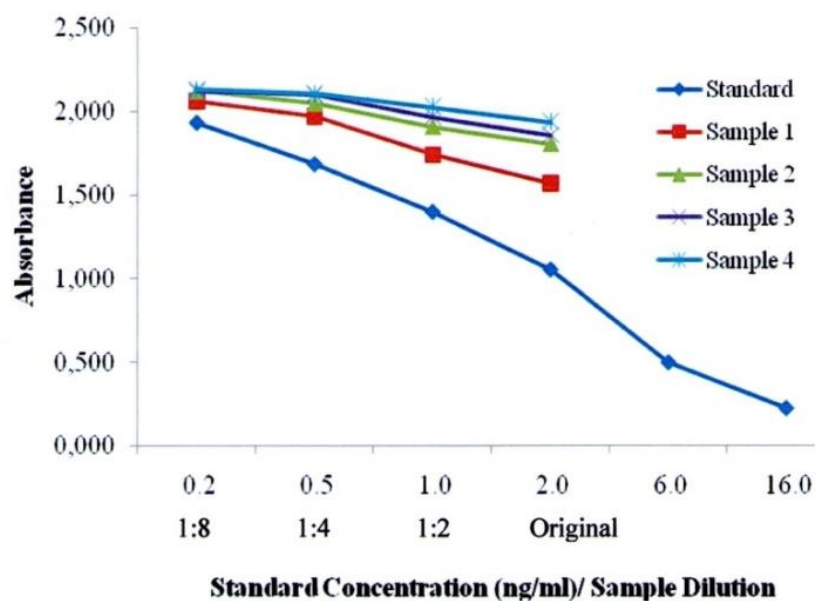


Figure 1. The sample standard concentration curve (ng/mL)

Table 4. The concentration of testosterone in the Leydig cells culture medium

Culture medium treatments	Testosterone concentrations (ng/mL)		
	H1 culture	H2 culture	H3 culture
DMEM+10% NBCS	1.912	1.865	2.159
DMEM+10% NBCS+hCG	2.48	2.098	2.509
DMEM+10% NBCS+ITS	2.628	2.423	2.442
DMEM+10% NBCS+hCG+ITS	1.801	1.844	1.935

activate certain essential enzyme in the detoxification process, and in addition, selenium also plays a role in disabling free radicals (Maurer, 2005 as cited in Freshney, 2005). Those were the underlying reasons suspected to cause higher concentration of Leydig cell cultured in the ITS medium. In terms of its viability and purity, Leydig cell cultured from the four different medium showed no difference in percentage. The results showed that the quality of cultured Leydig cells were similar and were not influenced by hCG and ITS. The average purity of Leydig cells was 88%, this was because the cultured cells were the primary cultures isolated from the testis seminiferous tubule tissue, so the presence of any type of cell other than the Leydig cells were possible. Habert *et al.* (2001) stated that the proliferation of Leydig cell precursors were stimulated by growth factors such as insulin-like growth factor (IGF-I) and transforming growth factor (TGF- $\alpha$ ).

#### Testosterone Concentration in Leydig Cells Culture Medium

Measurement of circulating testosterone concentrations using ELISA kits as used in this study was generally performed by sampling blood or plasma-derived component as samples. However, since the sample being tested were Leydig cell culture medium, we needed to do a parallelism test before the measurement to determine the appropriate standard curves to be used in this study. Parallelism test was performed to determine the standard concentration of testosterone in sample (culture medium) compared to a standard curve of testosterone that was available within the kit.

The standard value of absorbance and recovery of testosterone were shown in Table 3. Analysis results on a standard curve showed that the recovery rate of the standard measurement of testosterone was 99.7%. Parallelism test results were shown in Figure 1. Leydig cell culture medium sample dilution showed a parallel result to the standard curve of testosterone. The hormone concentrations measured were around 50% (30-70%) lower than the standard, so in the next measurement, no dilution of Leydig cells culture medium. Testosterone (17  $\beta$ -hydroxy-4-androstene-3-one) was a steroid hormone secreted into the blood with a molecular weight (MW) of 288.47. The low measurement result might be caused by unavailability of Sertoli cells, other hormone and growth factor in vitro, since testosterone secretion in vivo was a series of processes involving those components. Those were the suspected reason of why the testosterone secretion by the Leydig cells was less than optimum than in vivo.

Results of the testosterone concentration measurement in the Leydig cells culture medium is shown in Table 4. Testosterone concentration in the culture medium had been detected from the first day of culture. Testosterone concentrations that existed in the medium containing hCG or ITS seemed to show a higher concentration than other treatments. On the second day, there appeared to be a decrease in testosterone levels, which was then followed by an increase on the third day of culture. The culture results indicated that hCG or ITS supplemented medium tended to produce more testosterone than other treatments. Leydig cells were generally collected using

**Table 3.** The standard value of testosteron absorbance and recovery

Standard	Concentration (ng/mL)	Absorbance measurement result	Measurement correction result (ng/mL)	Recovery (%)
1	0.1	2.129	0.1	100
2	0.2	2.0085	0.2	100
3	0.5	1.7565	0.5	100
4	1	1,4775	1.0	100
5	2	1.105	2.1	105
6	4	0.82	3.7	92.5
7	8	0.472	8.2	102.5
8	16	0.261	15.6	97.5
Average recovery				99.7

Percoll gradient (Chemes *et al.*, 1992; Bilinska *et al.*, 2009) and resulted in Leydig cells purity of about 80-90%. However, the discovery of Wakefield *et al.* (1982) reported that the Percoll gradient was cytotoxic to the cell and could cause a decrease in cell viability. Therefore, we used Nycodenz gradient as the isolation gradient of Leydig cell and the cell collection results were tested by their in vitro testosterone production capability in this study. In the short culture period of 3 hours, adult rat Leydig cells had been able to increase testosterone secretion stimulated by hCG (Hedger and Eddy, 1990).

Browning *et al.* (1982) stated that the Leydig cells in the culture were able to secrete testosterone for up to 72 hours. LH or hCG hormone was required for proliferation and differentiation of Leydig cells (Saez, 1994). LH stimulation and additions of growth factor such as IGF-1 was required to set the number of Leydig cells and increase the effect of LH on Leydig cells (Ge and Hardy, 1997). Increased concentration of testosterone in human Leydig cell culture medium occurred after the addition of hCG 1 IU/mL (Chemes *et al.*, 1992; Bilinska *et al.*, 1997). El-Sherbiny *et al.* (1994) made the addition of 5 IU hCG on Leydig cell culture medium of young rabbits and resulted in production of large amounts of testosterone (150 ng/10<sup>6</sup> Leydig cells). The maximum dose of the hCG addition to stimulate testosterone secretion in vitro was 10 IU/mL (Hedger and Eddy, 1990). Leydig cell proliferation in culture could be improved by the addition of bioactive ingredients, such as ITS. Addition of 10 ug/mL transferrin and insulin could increase the in vitro testosterone production of fetal mice Leydig cells in culture medium for 24 and 48 hours (Pointis *et al.*, 1984). Bernier *et al.* (1983) gave additional insulin (5 ug/mL) and hCG (1 IU/mL) into the culture of piglets Leydig cells and resulted in an increase of the testosterone synthesis compared to previous treatments where there is no addition of hCG. Increased testosterone concentrations in the ITS supplemented culture medium was probably due to higher number of Leydig cells in culture (Table 1). However, the lower testosterone concentration in case of hCG and ITS addition treatment, although the Leydig cells concentration was higher, indicated the need of further investigation since there might be other factors that could affect the result when the two materials were administered simultaneously in culture medium. Hedger and Eddy (1990) stated that there were several mechanisms that could affect the stimulation of Leydig

cells, namely: the specific number of Leydig cells in order to optimize the function of Leydig cells, the secretion of essential intermediate form factors that was required on the steroidogenesis pathway, as well as the secretion of stimulating factor by Leydig cells that regulated steroidogenesis. Moreover, the process required coordination with other factors secreted in vivo by other cells in the testes.

## CONCLUSION

In conclusion, Leydig cell collection with Nycodenz gradient did not affect testosterone production by the Leydig cells in culture from day one.

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