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랫드 睾丸細胞의 培養條件 設定에 關한 硏究

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Establishment of Incubational Conditions for Rat Testicular Cells

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국문요약

화학물질의 개발사용 및 환경오염물질의 증가로 인하여 남성수태능력에 영향을 미치는 물질이 증가하고 있는 형편이다. 따라서 신속하고 경제적으로 남성 수태능력 영향물질을 평가하기 위하여 랫드의 고환세포를 이용한 남성 수태능력시험법을 개발하고자 본 시험을 실시하였다. 랫드의 고환세포로부터 주요한 기능을 가진 Sertoli cell과 Leydig cell을 효소처리, 필터 그리고 percoll gradient 과정을 거쳐 각각 분리하였다. 분리된 두 종류의 세포는 32℃에서 초기 배양한 후 37℃에서 계속 배양하였다. 본 배양조건에서 두 종류 세포는 양호한 발육을 보였으며 약 2주간의 배양이 가능하였다. Sertoli cell은 3주령의 랫드에서 분리가 잘되었고 Leydig cell은 8주령 이후의 랫드에서 분리가 잘 되었다. Sertoli cell은 89.0% 의 생존율을 보였고 Leydig cell은 85.7%의 생존율을 보였다. 이상의 결과와 같이 랫드의 고환세포중 Sertoli cell과 Leydig cell을 분리 배양하였으며, 앞으로 독성물질의 영향을 평가하기 위한 생화학적 및 구조적인 index 설정을 위한 연구가 필요하다고 사료된다.

I. Introduction

The testis have two important functions that are largely complicated, namely the production of spermatozoa and the production of hormone, principally testosterone. Any perturbations of either of these activities will result in reduced fertility. As a rule, damage to the overall hormonal controlling mechanisms at the hypothalamic-pituitary or testicular level, or to the direct action to the testicular cell level induce the testicular function. The toxic effects of chemicals can do their action via direct and/or indirect mechanisms.[1] Now we have conventional fertility test method tools that is the unique tool to assess the toxic effects on male reproduction.[2] However this one can provide restricted information of overall controlling mechanism, for example fertility profiles, sperm analysis and gross pathological findings. Therefore we need reliable and fast test tools which will provide more detailed information of events within the testis.[3] While many researches have been suggested for mammalian testicular cells, only recently have scientists been able to report evidence of functions or responses that may be specifically attributed to these cells. To determine the functions of the testicular cells especially with Sertoli and Leydig cells would appear ideal to have them in normal and isolated conditions-free of the other cells in the testis. The recent technique of culturing testicular cell types in vitro has potential to be an invaluable tool for assessing the mechanisms of testicular toxicity, especially those of intragonadal interactions and spermatogenesis. Moreover, Sertoli/germ cell cultures can provide additional essential information regarding damage to the early stages of spermatogenesis.[4,5] These tissue culture techniques are the only *in vitro*

methods available to assess testicular toxicity and they also fulfil the criteria for a test which is versatile and simple, while able to yield information quickly.[6]

In the first year of the study to establish in vitro testicular toxicity test method, we took efforts to establish the isolation and incubation method for testicular Sertoli and Leydig cells in rats.

Materials and Methods

1. Experimental animals

Rats were obtained from NISR SPF colony of Sprague-Dawley rats. They maintained on a 12 hour light, 12 hour dark photoperiod, at 22±4℃ and fed Shinchon Rat Chow and water ad libitum. Only males born on the same day were used for each preparation.

2. Cell isolation

Male rats are sacrificed by cervical dislocation always at the same time of day, in order to standardize for any possible effects of hormonal diurnal rhythms. The rat is laid on a clean surface on absorbent paper and the scrotum soaked with 70% ethanol. An incision is made with blunt end scissors through the scrotal wall and cremasteric fascia. A further incision is made through the tunica vaginalis, the testis located and removed using forceps, trimming away as much adherent fat as possible. It is then transferred rapidly to a 25 ml conical tube containing dissection medium at room temperature. The testis is transferred to a sterile petri dish containing a small amount of dissection scissors through the tunica vasculosa and albuginea at the appendix of the testis and a longitudinal section made, taking care not to damage the seminiferous tubules. The testicular capsule is teared off gently and discarded.

(1). Leydig cell isolation

Two capsulated testis (rats 200-300g) are placed in a 25 ml conical tube containing 10 ml of Leydig cell dispersal media that has been filtered through a 0.22 μl filter, and incubated longitudinally in shaking water bath at 37℃ until the seminiferous tubules are separated (15~20 mins). 15 ml of dissection media is then added to the tube and inverted slowly 20 times, allowed to settle and the cell suspension decanted off using a sterile 50 ml syringe and filling attachment. The suspension is filtered through a 60 μm filter attached to the syringe and the centrifuged 5 minutes. The supernatant is removed, the cells resuspended and centrifuged as before. After again removing the supernatant, the cells are resuspended in 2 ml of dissection medium and pipetted onto the top of a 0~90% percoll gradient and centrifuged for 20 mins. The resultant cell bands are isolated using a Pasteur pipette and band (density 1.070 g/ml) collected into a separate 30 ml conical tube. The band cell suspension is then diluted three times with dissection media and the cells washed to remove all Percoll by centrifuging and resuspending three times as previously described. The cells are counted using a haemocytometer. Each cell suspension is drawn with culture medium and the cells incubated at 32℃, in atmosphere of 10% CO_2, initially for two hours before being used experimentally. At this time, the media is changed and replaced with culture medium.

(2). Sertoli cell isolation

During both collagenase and pancreatin treatments the tissue or cells were suspended in a calcium and magnesium-free phosphate buffered saline composed of NaCl, KCl, Na_2HPO_4, glucose, KH_2PO_4, penicillin, streptomycin sulfate and phenolred. The pH of solutions A during enzyme treatments was maintained at about pH 7.3 by addition of 0.1 M Ba_2HPO_4. Treatmemts were carried out at 28℃ in a constant temperature shaker bath. Two to 4 rats of the same age were ether anesthetized. Both testes were removed by abdominal incision in very young rats or by an incision in the scrotal sac in more mature animals. Each testis was placed in solution A on ice while the tunica albuginea was removed. The tissue was rinsed in solution A and placed on a conical tube where the tissue was cut into pieces about 2 mm on a slide using a stainless-steel razor blade. The minced tissue was washed three times for 5 min. each, in 20 ml of solution A in a glass stoppered 50 ml flask in the shaker bath. No more than 1 g of minced tissue was contained in one flask. The supernatants from the three washings of the minced whole testis were filtered through a silk

screen with a pore size of 0.08~0.10 mm. The tissue retained on the screen was returned to the flask containing the minced tissue. The filtrate consists primarily of germ cells. Fraction was treated with 20 m*l* of solution A containing 4 mg collagenase for 1 hr. in the shaker bath 28℃. At the end of this time a reaggregated mass of tissue could be removed leaving tubule pieces. The mass of reaggregated tissue composed primarily of the interstitial tissue, was washed twice in solution A to remove more tubule fragments. These fragments were returned to the flask. The tubule fragmemts of fraction 3 were allowed to settle and the collagenase solution was decanted. The tubules were washed twice in 20 m*l* of solution A containing 4 mg pancreatin. After 20 min. another mass of reaggregated tissue was removed from the remaining tubule fragments.

Fractions, composed of Sertoli and germ cells, was washed twice in 15 m*l* of solution A and once with 15 m*l* solution A containing 5% fetal calf serum. Sertoli cell fraction was passed through a syringe needle to break the tubular fragments into groups of Sertoli cells containing embedded spermatogonia and spermatocytes. The cell suspension was shaken moderately on a vortex mixer for 15 sec. to loosen and detach embedded germ cells from the Sertoli cell clusters. The cell suspension was immediately layered over a plate. The pellet was washed in solution A and recentrifuged. The purity of the pelleted Sertoli-germ cell fraction was estimated by aceto-orcein smear preparations and the viability, by nitro bluetetrazolium. Final cell fraction was resuspended in 5 m*l* of solution A. Cultures were started in 50 m*l* sterile petri dishes with 0.5 m*l* of Sertoli cell suspension and 10 m*l* of cell culture medium containing 5% fetal calf serum. The cultures were maintained in an incubator with humidified air atmosphere. The cultures were maintained at 32℃ for 22 hr., then the temperature was raised to 37℃ for 40 hr. after which time the temperature was lowered to and maintained at 32℃.

Results

A summary of parameters from the control Sertoli and Leydig cell cultures are presented in Table 1 and Table 2.

Table 1. Summary of test parameters from primary Sertoli cell cultures

Rat ages (days)	Cell counted Total	Sertoli	Percent of Sertoli cell	Percent Sertoli in whole tubule[1]
21	200	128	64.0	32
30	200	115	57.5	20
56	200	121	60.5	–

1): Welsh M. J., *Endocrinology* **96**, 618, 1975.[6]

Table 2. Summary of test parameters from primary Leydig cell cultures

Rat ages (days)	Cell counting Total cells	Sertoli cells	Percent of Sertoli cell
56	200	133	66.5
70	200	147	73.5

Table 3. Cell viability of Sertoli and Leydig cells using NBT (unit: percents)

Number	Sertoli cells	Leydig cells
1	85.0	80.0
2	88.0	87.0
3	94.0	90.0
Total	89.0	85.7

Differential count and identification of cells, Sertoli cells comprised from 57.5% to 64.0% of final fractions. Sertoli cell isolation rate from the testicular tissue was different from the rat ages, because of the percent Sertoli cells in whole tubule is higher in the young male rats than the old one's. Viability of greater than 85.0% was indicated by nitro blue tetrazolium (Table 3). The contaminants were spermatogonia and primary spermatocytes, and to a much lesser degree, peritubular cells.

In the case of Leydig cells composed of the final fractions from 14.0% to 17.5%. Leydig cell isolation rate from the testicular tissue was different from the rat ages, because of the percent of active Leydig cells in whole tubule is much higher in the mature male rats than the young one's. Viability of greater than 80.0% was indicated (Table 3).

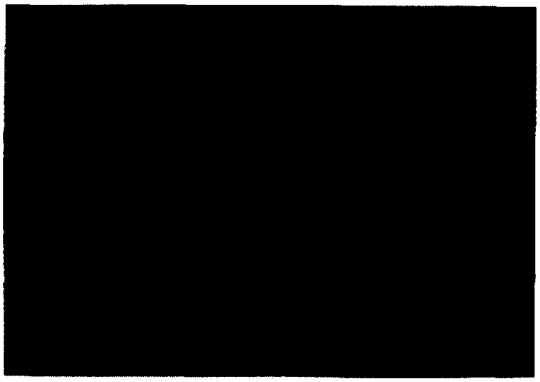

Fig. 1. Leydig cells from 70-day-old rat after days in culture (×200).

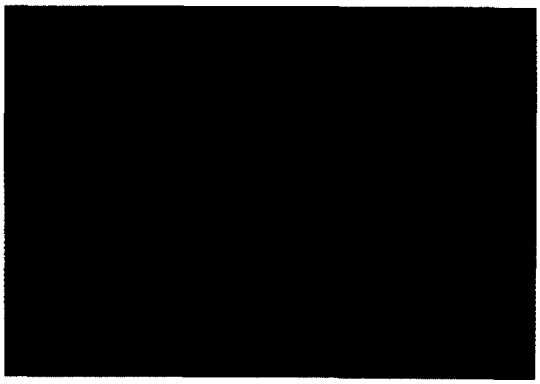

Fig. 2. Leydig cells from 70-day-old rat after 5 days in culture (×200).

Fig. 3. Leydig cells from 70-day-old rat after 10 days in culture (×200).

Fig. 4. Sertoli/germ cells from 21-day-old rat after isolated from testis (×200).

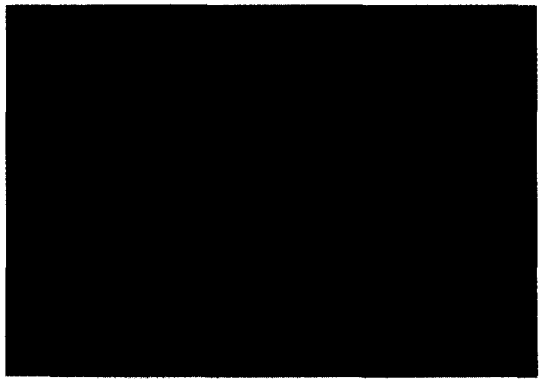

Fig. 5. Sertoli/germ cells from 21-day-old rat after 2 days in culture (×200).

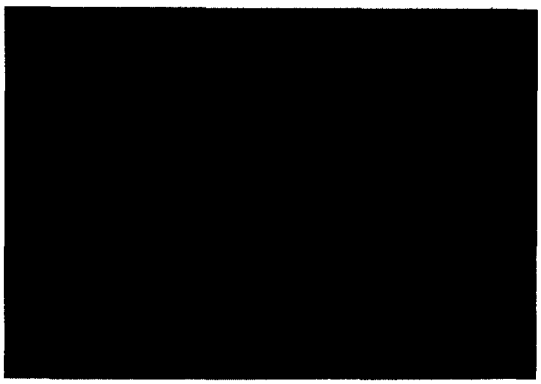

Fig. 6. Sertoli/germ cells from 21-day-old rat after 10 days in culture (×200).

The Sertoli cells isolated from the testis of the SD rats grew into a monolayer on about the 2nd~3rd day of cultures, an appreciable cell increment being observed between the 4th~5th day (figure not shown). On the 9th~10th day, the amount of Sertoli cells slightly decreased (Fig. 4, 5 and

6). The Leydig cells isolated from the testis of the SD rats grew into a monolayer on about the 3rd-4th day of culture, an appreciable cell increment being observed between the 5th and 7th day. On the 9th-10th day, the amount of Leydig cells slightly decreased (Fig. 1, 2, and 3).

Discussion

Testicular cell cultures are particularly useful in facilitating the understanding of the mechanisms of action involved in testicular toxicity. The Sertoli/germ cell cultures provide a sensitive *in vitro* model for investigating toxic effects on spermatogenesis. Testicular toxicity *in vivo* is often characterised by an early detachment of spermatocytes and spermatids, suggesting that there is disruption of the normal interactions between germ cells and Sertoli cells. The cultures of Sertoli/germ cells show a similar shedding of germ cells when treated with known testicular toxicants which correlate well with the *in vivo* studies. The Leydig cell cultures are a valuable tool for investigating the action of testicular toxicants on testosterone production and *in vitro* receptor assays could provide a basis for indicating the potential of drugs and chemicals to produce direct hormonal disturbances. Leyding cells provide comprehensive and detailed information on the action of testicular toxicants at the level of the testis.[7] However, Sertoli and Leydig cell cultures are limited in their ability to take account metabolic and pharmacokinetic factors which influence toxicity *in vivo*, such an absorption from the gut and distribution to the testis. After the testis tissue is minced, the germ cells which are not deeply embedded in the Sertoli cells escape from the tubules.[8] The germ cells appear to be expelled from the tubule as if by an internal pressure, possibly a result of contractile tension produced by the peritubular cells. The tubules are freed from the interstitial tissue matrix by collagenase treatment and agitation. The interstitial fragments exhibit an adhesive quality which the tubule fragments lack. The interstitial fragments tend to reaggregate into one mass that may be removed using forceps. Using a suspension of individual Sertoli cells amid pachytene spermatocytes, we have not found out possible to separate the two cell types. Separation was not accomplished by either velocity sedimentation or density gradient centrifugation. Maintenance of the cells *in vitro*, temporarily at an elevated temperature, destroys almost all of the germ cells which are then phagocytized by the Sertoli cells. The Sertoli cells appear to be unharmed by the temporary temperature increase and form a monolayer which has been maintained for 14 days.[9] Chawdhury and Steinberger have indicated that higher temperature (43℃) for a shorter period of time, although injurious to germ cells, apparently is not harmful to Sertoli cells *in vivo*.[10] After centrifuged the Leydig cells, the gradient is divided into a lighter than 1.068 g/m*l* fraction which contains germ cells, macrophages, and damaged Leydig cells: and a heavier than 1.068 g/m*l* fraction containing intact, steroidogenically active Leydig cells. Many laboratories have reported obtaining highly purified (>90%) Leydig cells utilizing a combination of enzymatic dissociation and density gradient separation. In recent years however, other laboratories have begun to include centrifugal elutriation to obtain a higher (>95%) purity.[11] The multistep procedure we describe herein for the isolation of well purified adult rat Leydig cells (70.0%) involves the use of testicular perfusion, and enzymatic dissociation, and density gradient centrifugation. Recent study reported that the addition of Leydig cells in an impure preparation to Leydig cells in a purified preparation decreased the LH responsiveness of Leydig cell by 75%. Thus, it seems that both testicular perfusion and elutriation are worth the extra work and expense in one's endeavor to obtain highly purified adult rat Leydig cells.

Conclusions

We studied the isolation and culture condition of testicular cells including Leydig and Sertoli cells, in order to establish the in vitro screening method of male fertility test and to examine the action mechanism of toxic substances on the testis. The first year of this projects, we found out the isolation, culture conditions and viability of Leydig and Sertoli cells from the rat's testicular tissue. We concluded as follows:

1. Differential count and identification of cells, Sertoli cells comprised from 57.5% to 64.0% of

final fractions. Sertoli cell isolation rate from the testicular tissue was different from the rat ages. Younger male rats were higher yields of Sertoli cell than the old one's. Viability of greater than 85.0% was indicated by nitro blue tetrazolium.

2. In the case of Leydig cells composed of the final fractions · from 14.0% to 17.5%. Leydig cell isolation rate from the testicular tissue was different from the rat ages. The percent of active Leydig cells in whole tubule is much higher in the mature male rats. Viability of greater than 80.0% was indicated.

3. The Sertoli cells isolated from the testis of the SD rats grew into a monolayer on about the 2nd~3rd day of cultures, an appreciable cell increment being observed between the 4th~5th day. On the 9th~10th day, the amount of Sertoli cells slightly decreased.

4. The Leydig cells isolated from the testis of the SD rats grew into a monolayer on about the 3rd~4th day of culture, an appreciable cell increment being observed between the 5th and 7th day. On the 9th-10th day, the amount of Leydig cells slightly decreased.

ABSTRACT

This study of culturing testicular cell types in vitro has potential to be an invaluable tool for assessing the mechanisms of testicular toxicity, especially those of intragonadal interaction and spermatogenesis. Combined with the Sertoli/germ cell cultures, Leydig cells provide comprehensive and detailed information on the action of testicular toxicants at the level of the testis. Sertoli/germ cell were isolated and incubated well in vitro from 20~30 g rats and Leydig cells from 250~300 g rats. The Sertoli cells isolated from the testis of the SD rats grew into monolayer on about the 2nd~3rd day of culture, an appreciable cell increment being observed between the 4th~5th day. The Leydig cells isolated from the testis of the SD rats grew into a monolayer on about the 3rd-4th day of culture, an appreciable cell increment being observed between the 5th-7th day. These results suggest that Sertoli and Leydig cells can be cultured as a male fertility evaluation method alternative to the *in vivo*/conventional fertility test method and further study for the physio-chemical determination is needed.

Keywords : testitcular cell, Leydig cell, Sertoli cell, culture, male fertility.

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