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# *In Vitro* Culture of Primary Testicular Stromal Cells derived from Mouse with Different Genetic Background : Optimization of Culture Temperature

**Hye Jin Park[1], Jung Im Yun[2], Jung Hoon Choi[2], Eunsong Lee[2], Seung Pyo Gong[3] and Seung Tae Lee[1,†]**

*[1]Department of Animal Biotechnology, Kangwon National University, Chuncheon 200-701, Korea,*
*[2]College of Veterinary Medicine and Institute of Veterinary Science, Kangwon National University, Chuncheon 200-701, Korea,*
*[3]Department of Marine Biomaterials and Aquaculture, Pukyong National University, Busan 608-737, Korea*

## ABSTRACT

Spermatogonial stem cells (SSCs) developed into sperms through spermatogenesis have been utilized as a useful tool in the field of regenerative medicine and infertility. However, a small number of highly qualified SSCs are resided in the seminiferous tubule of testis, resulted in developing effective *in-vitro* culture system of SSCs for solving simultaneously quantitative and qualitative problems. Presently, SSCs can be enriched on testicular stromal cells (TSCs), but there are no systematic researches about TSC culture. Therefore, we tried to optimize culture condition of TSCs derived from mouse with different strains. For these, proliferation and viability were measured and compared by culturing ICR outbred or DBA/2 inbred mouse-derived TSCs at 35 or 37℃. In case of ICR strain, primary TSCs cultured at 37℃ showed significantly higher proliferation and viability than those at 35℃ and significant increase of proliferation and viability in sub-passaged TSCs was detected in the 35℃ culture condition. Moreover, sub-passage of primary TSCs at 35℃ induced no significant effects on proliferation and viability. In contrast, in case of DBA/2 strain, significantly improved proliferation were detected in the primary TSCs cultured at 35℃, which showed no significant difference in the viability, compared to those at 37℃. Furthermore, sub-passaged TSCs cultured at 37℃ showed no significant differences in proliferation and viability, compared to those at 35℃. However, with significant decrease of proliferation induced by sub-passage of primary TSCs at 35℃, no significant effects on proliferation and viability were resulted from sub-passage of primary TSCs at 37℃. From these results, culture temperature of primary TSCs derived from outbred and inbred strain of mouse could be separately optimized in primary culture and sub-culture.

(Key words: mouse, testicular stromal cell, culture temperature, proliferation, viability)

## INTRODUCTION

Spermatogonial stem cells (SSCs) self-renewed and differentiated into functional sperms throughout lifetime play an important role in eternally transferring the genetic information via spermatogenesis into offspring (Kubota *et al*., 2004; Kanatsu-Shinohara *et al*., 2008; Koruji *et al*., 2009). Moreover, they have a potential to differentiate into three germ layer lineages promised to a variety of tissue or organ consisting of our body (Guan *et al*., 2006; Guan *et al*., 2007; Kossack *et al*., 2009). Therefore, they have been considered as a useful tool treating fundamentally male-related infertility (Kanatsu-Shinohara *et al*., 2003; Sato *et al*., 2011; Tiptanavattana *et al*., 2013) as well as making it possible to develop patient-specific cell therapy without any immune responses (Daley *et al*., 2008). It is also believed that banking SSCs may contribute to developing future propagation and cell therapy through preserving a valuable genetic background.

Unfortunately, a population size of SSCs is extremely small within the testis (e.g., approximately 0.03% of the total number of cells in an adult testis) (Kostereva *et al*., 2008; Ko *et al*., 2009; Kanatsu-Shinohara *et al*., 2011) Therefore, requirement of effective techniques harvesting and culturing SSCs have been strongly emerged for progressing easily studies related with

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† Correspondence : E-mail : stlee76@kangwon.ac.kr

SSCs. Especially, in case of the culture of SSCs, they have been co-cultured together with feeder cells (Mohamadi *et al*., 2012; Nasiri *et al*., 2012; Baazm *et al*., 2013) and testicular stromal cells (TSCs) among diverse types of feeder cells have been used as candidate improving proliferation and maintenance of undifferentiated SSCs (Seandel *et al*., 2007; Kim J *et al*., 2008). However, there have been no systematic studies about TSC culture. Accordingly, we investigated proliferation and viability of mouse testicular stromal cells in the different culture temperatures and tried to optimize *In Vitro* culture condition of them according to different genetic background.

## MATERIALS AND METHODS

### 1. Animals

Three-week-old male ICR mice and DBA/2 mice as testicular cell donors were purchased from DBL (Eumseong, Korea). All animal housing, handling and experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Kangwon National University (IACUC approval No. KW-130307-1) and conducted according to the Animal Care and Use Guidelines of Kangwon National University.

### 2. Preparation of Testicular Cells from Testis

The tunica albuginea and epididymis removed from the testes were washed with Dulbecco's phosphate-buffered saline (DPBS; Welgene Inc., Daegu, Korea) and the seminiferous tubules were incubated at 37℃ for 20 minutes in Dulbecco's modified Eagle's Medium (DMEM; HyClone, Logan, UT) supplemented with 0.5 mg/ml type IV collagenase (Sigma-Aldrich, St. Louis, MO). Fragmented seminiferous tubules were washed with DMEM (HyClone) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS; HyClone) and dissociated with 0.25% trypsin-EDTA (Gibco Invitrogen, Grand Island, NY) at 37℃ for 5 minutes. Then, the dissociated seminiferous tubules were filtered through a 70 μm nylon mesh (SPL, Pocheon, Korea) and the retrieved testicular cells were enumerated using a hemocytometer.

### 3. Isolation and Culture of TSCs from Testicular Cells

The dissociated testicular cells of mice were incubated for 1 hour on 0.1% (wt/v) gelatin-coated culture dish in SSC growth medium described previously (Guan *et al*., 2009) and buoyant cells that didn't attach to the bottom of the dishes were collected and discarded. Subsequently, TSCs that attached quickly to the bottom of the dishes were trypsinized by 0.25% trypsin-EDTA (Gibco Invitrogen) and $1 \times 10^6$ TSCs were cultured on 0.1% (wt/v) gelatin-coated 60 mm culture dish in DMEM (HyClone) supplemented with 20% (v/v) heat-inactivated FBS (HyClone), 1% (v/v) antibiotic-antimycotic (Gibco Invitrogen), 1% (v/v) glutamax$^{TM}$ (Gibco Invitrogen), 1% (v/v) non-essential amino acids (NEAA; Gibco Invitrogen), 0.1 mM β-mercaptoethanol (Gibco Invitrogen) and 10 ng/ml basic fibroblast growth factor (bFGF; PeproTech, Inc., Rocky Hill, NJ) at 35 or 37℃ under 5% $CO_2$ in a humidified air atmosphere. Culture medium was exchanged at 2 day intervals. After 7 days of culture, confluent TSCs were dissociated by 0.25% trypsin-EDTA (Gibco Invitrogen) and the number of the cells was determined using a hematocytometer. Subsequently, they were reseeded and cultured continuously at the same cell density under the same conditions.

### 4. Measurement of Cell Viability

For measuring cell viability, trypan blue exclusion assay was used. Briefly, 10 μl cell suspensions in culture medium was mixed with 10 μl of 0.4% (wt/v) trypan blue solution (Sigma-Aldrich) and gently mixed suspension was adjusted to a hematocytometer. Cells stained positively due to failing to exclude dyes were regarded as dead cells and transparent cells stained negatively due to succeeding in excluding dyes as viable cells. Moreover, the percentage of viable cells was calculated on the basis of total number of cells (viable plus dead).

### 5. Statistical Analysis

All numerical data about cell number and viability were analyzed by the Statistical Analysis System (SAS) program. Comparisons among treatment groups were performed using the least-square difference or Duncan's method and the significant effects were decided by analysis of variance (ANOVA) in the SAS package. A value of $p<0.05$ was taken to indicate a significant difference among treatments.

## RESULTS

In order to determine culture temperature optimized to the *in vitro* culture of ICR or DBA/2 mouse-derived TSCs, primary TSCs isolated from testes were cultured and sub-passaged at 35 or 37℃. Moreover, proliferation and viability were mea-

sured at each passage and compared according to different culture temperature and sub-passage or not.

1. Optimization of Culture Temperature Stimulating Proliferation and Viability of TSCs Derived from ICR Outbred Mouse

In the primary culture of TSCs, significant increase of proliferation (Fig. 1A) and viability (Fig. 1B) were observed in the TSCs cultured at 37℃, compared to those at 35℃. However, sub-passaged TSCs showed significantly higher proliferation (Fig. 1C) and viability (Fig. 1D) in the 35℃ culture condition than in the 37℃ culture condition. In addition, sub-passage of primary TSCs cultured at 35℃ did not induce any significant difference in the proliferation (Fig. 2A) and viability (Fig. 2B) of TSCs, whereas significant decrease in proliferation (Fig. 2C) and viability (Fig. 2D) of TSCs was induced by sub-passage of primary TSCs maintained in 37℃ culture condition. These results demonstrated that *in vitro* culture of primary TSCs derived from ICR outbred mouse testis should be conducted at 37℃ and TSCs should subsequently be sub-passaged and cultured at 35℃.

2. Optimization of Culture Temperature Stimulating Proliferation and Viability of TSCs Derived from DBA/2 Inbred Mouse

The culture of primary TSCs at 37℃ showed significantly

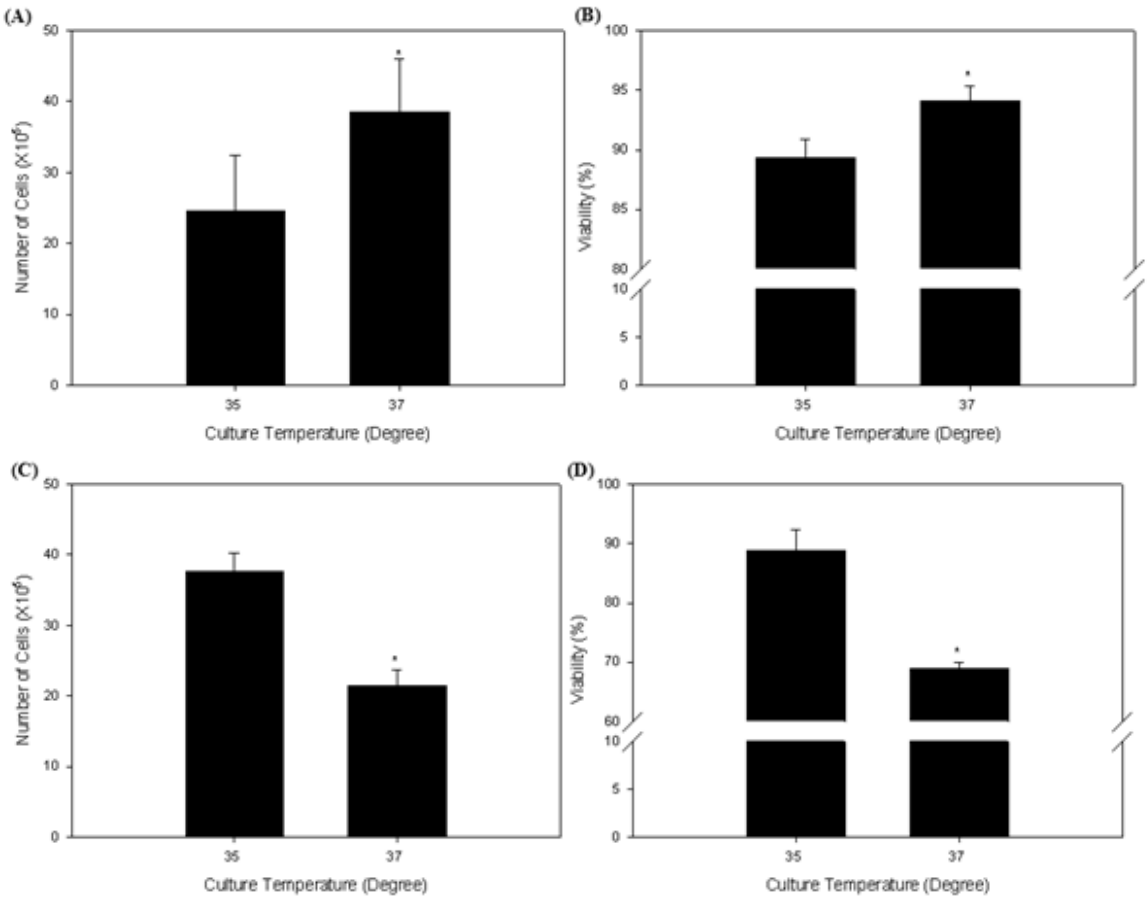


Fig. 1. Effects of different culture temperature on the proliferation and viability of testicular stromal cells (TSCs) derived from outbred ICR male mouse. Primary culture of TSCs retrieved enzymatically from testis was conducted by culturing for 7 days in TSC culture medium at 35 and 37℃, respectively. Subsequently, in order to conduct sub-passage, confluent TSCs cultured at each temperature were trypsinized and the dissociated TSCs continued to be cultured at the same temperature as primary culture. Per sub-passage, numbers and viability of TSCs were respectively investigated by hemocytometry and trypan blue assay. As the results, primary TSCs cultured at 37℃ showed significantly higher proliferation (A) and viability (B) than those at 35℃. However, post-sub-passage, proliferation (C) and viability (D) potentials of TSCs cultured at 37°C were decreased significantly, compared to those at 35°C. Error bars represent standard deviation (S.D). n=3. * $p<0.05$.

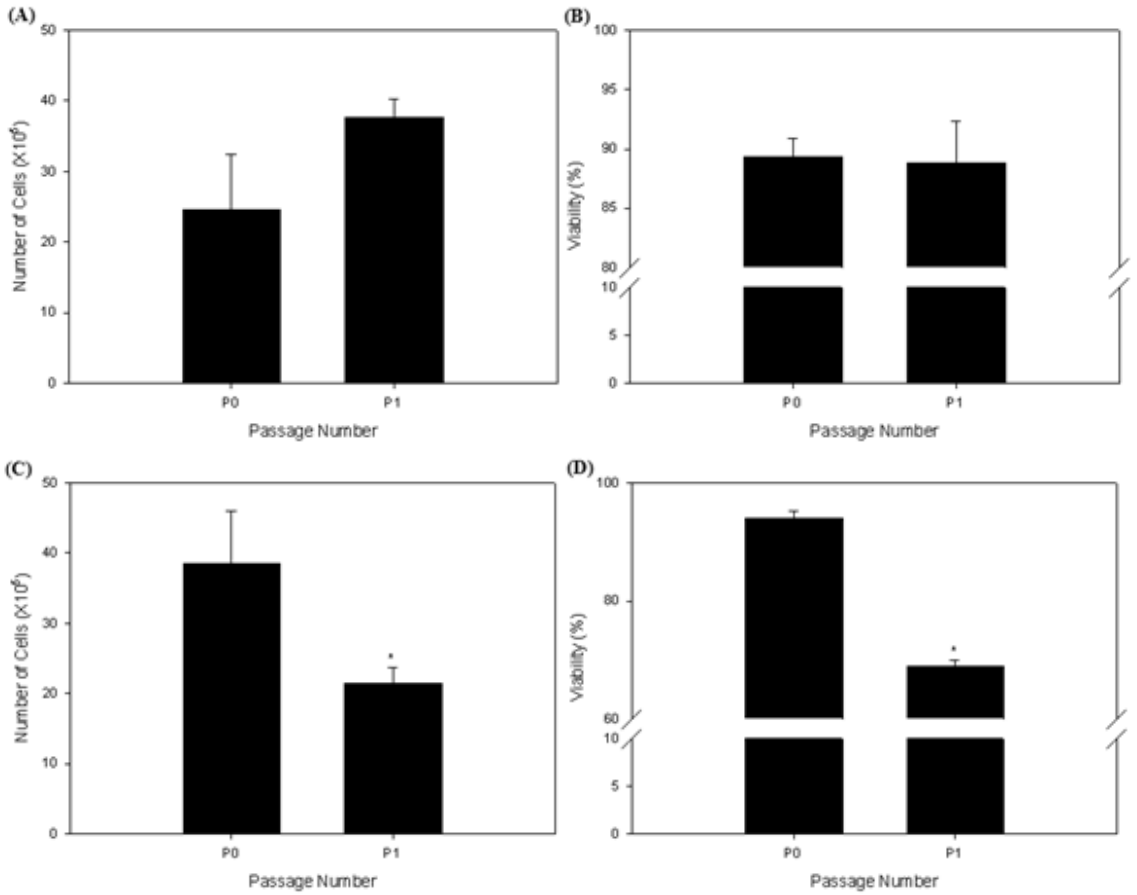


Fig. 2. Comparison of the proliferation and viability of outbred ICR mouse-derived primary testicular stromal cells (TSCs) before or after sub-passage at the specific culture temerature. Primary culture of testis-retrieved TSCs in TSC culture medium was performed for 7 days at 35 or 37℃ and sub-passage was conducted by culturing confluent TSCs dissociated by trypsinization at the same temperature as primary culture. Hematocytometry and trypan blue assay were respectively used for analyzing numbers and viability of testicular stromal cells per sub-passage. Sub-passage procedure of TSCs maintained at 35℃ induced no significant decrease in proliferation (A) and viability (B) of TSCs, whereas significant decrease in proliferation (C) and viability (D) of TSCs by sub-passage procedure was observed at 37℃. Error bars represent standard deviation (SD). n=3. *$p$<0.05.

lower proliferation than those at 35℃ (Fig. 3A) and any significant effects on viability of the primary TSCs were not induced by the difference of culture temperature (Fig. 3B), which caused no significant differences in proliferation (Fig. 3C) and viability (Fig. 3D) of sub-passaged TSCs. Sub-passage of primary TSCs cultured at 35℃ decreased significantly proliferation (Fig. 4A) and any significant increase or decrease in viability was not induced by sub-passage (Fig. 4B). Moreover, sub-passage of primary TSCs cultured at 37℃ showed no significant effects on proliferation (Fig. 4C) and viability (Fig. 4D) of sub-passaged TSCs. These results demonstrated that in-vitro culture of primary TSCs derived from DBA/2 inbred mouse testis should be conducted at 35℃ and TSCs should subsequently be sub-passaged and cultured at 37℃.

## DISCUSSION

In this study, culture temperature optimized to *in vitro* culture of TSCs used as one of feeder cells requested essentially in the SSC culture was determined in ICR outbred and DBA/2 inbred strains, respectively. In case of ICR strain, the best of primary TSC culture was observed at 37℃ and the culture of primary TSCs post-sub-passage was the best at 35℃. However, TSCs derived from DBA/2 strain showed the opposite results as following: the culture of primary TSCs was the best at 35℃ and the best culture of sub-passaged primary TSCs was detec-

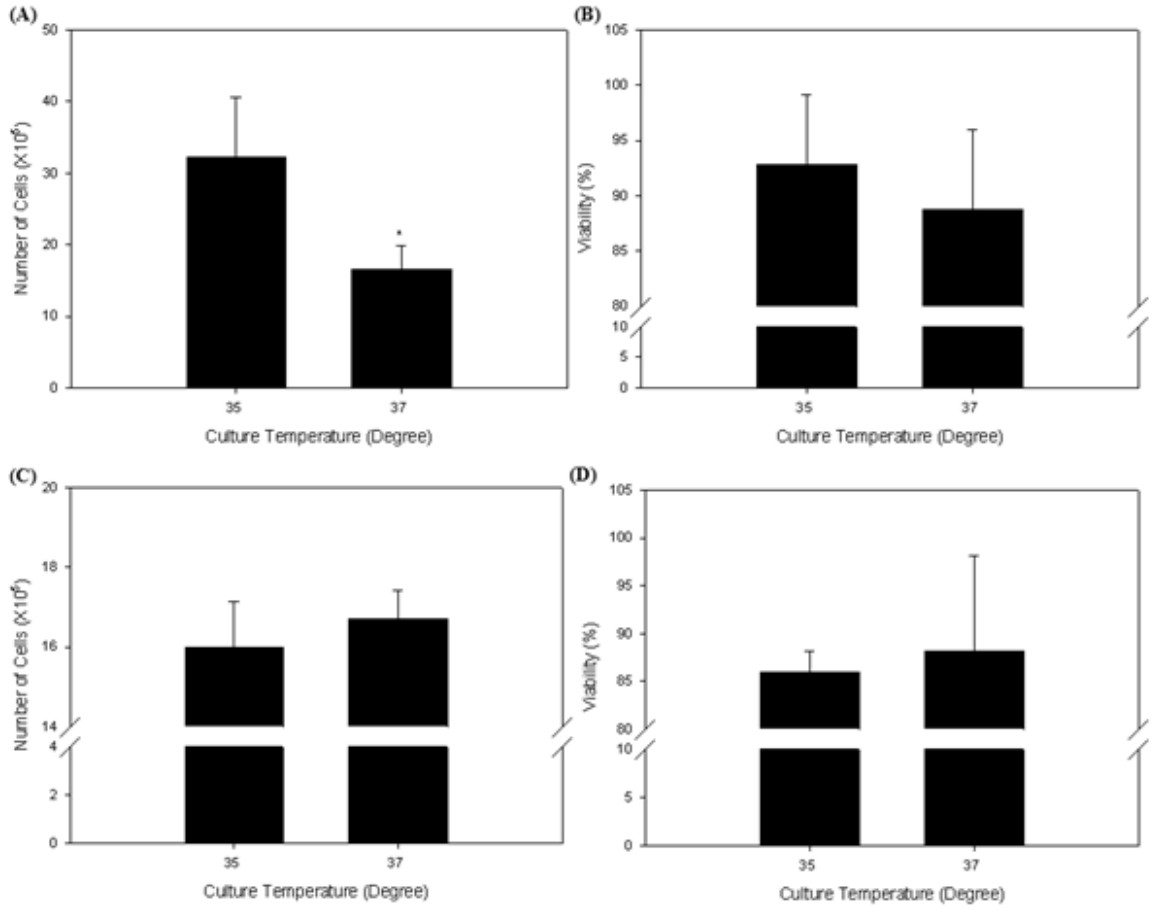


Fig. 3. Effects of different culture temperature on the proliferation and viability of testicular stromal cells (TSCs) derived from inbred DBA/2 male mouse. In order to conduct primary culture of TSCs retrieved enzymatically from testis, they were cultured for 7 days in TSC culture medium at 35 and 37℃, respectively. Subsequently, sub-passage was conducted by trypsinizing confluent TSCs cultured at each temperature and keeping the dissociated TSCs on being cultured at the same temperature as primary culture. Numbers and viability of TSCs per sub-passage were investigated by hematocytometry and trypan blue assay, respectively. As the results, primary TSCs cultured at 37℃ showed significant decrease of proliferation (A) and no significant difference in the viability (B), compared to those at 35℃. Moreover, significant differences in proliferation (C) and viability (D) of TSCs experiencing sub-passage were not induced by the difference of culture temperature. Error bars represent standard deviation (SD). n=3. *$p$<0.05.

ted at 37℃. From these results, primary TSC culture system could be systematically established according to before and after sub-culture and the difference of genetic background.

This study showed that culture temperature optimized to the proliferation and viability of mouse TSCs was different according to the difference of genetic background. Moreover, in the previous reports, temperature variation induced alteration of viability (Bollati-Fogolin *et al*., 2005) and the duration of the G1 and S stage, which are largely responsible for changing the over-all generation time (Sisken 1963; Rao and Engelber, 1965; Sisken 1965; Watanabe *et al*., 1967). Accordingly, the difference of TSC culture temperature condition optimized in between an inbred and outbred strain of mouse may result from the difference of cell cycle-related molecules activated per genetic background in the specific temperature.

In conclusion, *in vitro* culture of TSCs playing an important role in maintaining undifferentiation and stimulating self-renewal of SSCs (Seandel *et al*., 2007; Kim J *et al*., 2008) is a very fundamental technique making it possible to persistently conduct basic researches and applications related with SSCs in the future. Accordingly, genetic background-specific TSC culture system developed in this study will greatly contribute to the production of qualified SSCs in diverse strain and be able to lead to a great interest in SSCs with potentials like propaga-

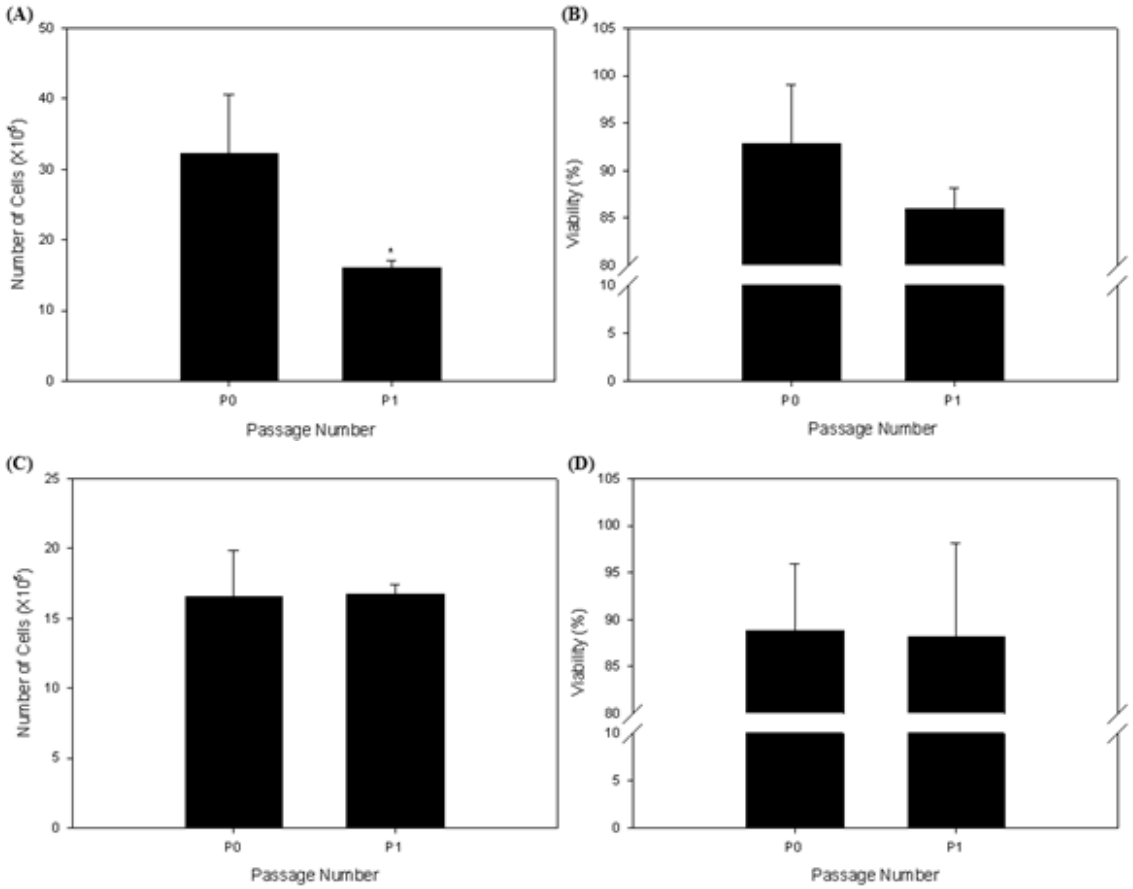


Fig. 4. Comparison of the proliferation and viability of inbred DBA/2 mouse-derived primary testicular stromal cells (TSCs) before or after sub-passage at the specific culture temperature. Primary culture of TSCs retrieved from testis in TSC culture medium for 7 days was conducted at 35 and 37℃, respectively. Subsequently, sub-passage was conducted by culturing confluent TSCs dissociated by trypsinization at the same temperature as primary culture. Analysis of numbers and viability of TSCs per sub-passage were performed by hematocytometry and trypan blue assay. Sub-passage of TSCs maintained at 35°C induced significant decrease in proliferation (A) and no significant difference in viability (B). Moreover, no significant differences in proliferation (C) and viability (D) of TSCs by sub-passage were observed at 37℃. Error bars represent standard deviation (SD). n=3. *$p$<0.05.

tion and cell therapy.

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