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Research article

Human spermatogonia stem cells (SSCS) in a culture system with platelet rich plasma and correlations with spermatogenesis level

Silvia W Lestari[1*], Debby Aditya[2], Firda Asma'ul Husna[2], Gita Pratama[3], Dwi Anita Suryandari[1], Kanadi Sumapradja[3], Ria Margiana[4], Puji Sari[1], Ria Kodariah [5]

[1]Department of Medical Biology, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia,
[2]Master's Programme in Biomedical Sciences, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia,
[3]Department of Obstetric and Gynecology, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia,
[4]Department of Anatomy, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia,
[5]Department of Pathology Anatomy, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia.

ABSTRACT

There are instances when the sperm fail to be obtained from the testes in assisted reproductive technology (ART) of azoospermia infertility due to spermatogenesis arrest. Therefore, spermatogonial stem cells (SSCs) may be used as an alternative management. Platelet rich plasma (PRP) is a concentrated source of growth factors that support the proliferation and differentiation of stem cells in vitro. The objective of this study was to determine the efficiency of PRP in supporting SSCs proliferation and differentiation and to assess the correlation between the level of spermatogenesis and the potency of proliferation and differentiation of SSC in vitro. SSCs were isolated from seven testicular tissue samples using sperm extraction (TESA/TESE) and were then further cultured into two groups, PRP and FBS. The resulting cells were quantitatively assayed by qRT-PCR towards the expression of PLZF, OCT4, and CKIT. The level of spermatogenesis was assessed from the histology measurement using Johnson scoring. Apoptosis was evaluated using the TUNEL and immunocytochemistry methods. This study showed that PLZF, OCT4, and CKIT were expressed in the resulting cell culture. The difference was statistically insignificant among PRP and FBS. There was a positive correlation between the potency of proliferation and differentiation of SSCs toward the level of spermatogenesis. PRP may support the maintenance of proliferation and differentiation of SSCs in vitro and could be developed as an alternative of FBS in the SSCs culture system.

Keywords: spermatogonial stem cells, platelet rich plasma, fetal bovine serum, spermatogenesis, cell culture

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Correspondence: Silvia W Lestari* ✉ finallysilvia@gmail.com

Department of Medical Biology, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia

INTRODUCTION

Assisted reproductive technology (ART) by Intra Cytoplasmic Sperm Injection (ICSI) is the main management for azoospermia infertile men [1]. Direct sperm retrieval from the testes should be performed in cases of non-obstructive azoospermia (NOA). However, this sperm retrieval method may fail to obtain sperm due to spermatogenesis arrest. In these cases, the utilization of spermatogonial stem cells (SSCs) may be considered but the culture system should be optimally established.

It is difficult to develop spermatogonial stem cells (SSCs) due to the isolation techniques, identification, and culture conditions for the expansion of SSCs in vitro [2]. The small sample size obtained from testicular biopsy is inadequate and cells must be propagated in vitro [3]. The development of an optimal culture medium for SSCs has been carried out in recent years. A culture medium supplemented with serum, such as fetal bovine serum (FBS), is typically used but its utility is reduced because it contains animal-derived serum [4]. The platelet rich plasma (PRP), as an alternate to FBS, contains growth factors that may support the proliferation and differentiation of stem cells in vitro [5]. A study by Nicoletti et al. proved that PRP may promote increased tissue regeneration and vitality [6].

The use of PRP in SSC culture has never been performed. This study may determine the effectiveness of PRP on SSC cultures when compared to FBS with the addition of specific growth factors. The histological examination of testicular tissue was performed to determine the level of spermatogenesis based on Johnson scoring. The objective of this study was to determine the efficiency of PRP in supporting SSC proliferation and differentiation and to assess the correlation between the level of spermatogenesis and the potency of SSCs in self-renewal and differentiate in vitro.

MATERIALS AND METHODS

Ethics Approval

This research protocol was approved by Health Research Ethics Committee of the Faculty of Medicine with the letter number No.831/UN2.F1/ETIK/2017

Place and Time of Research

This research was conducted at Department of Medical Biology and Indonesia Medical Education and Research Institute (IMERI), Faculty of Medicine, University of Indonesia, and it took place from January 2018 through June 2020.

Population and Research Subjects

Seven surplus testis samples were donated by azoospermic patients using a testicular sperm extraction (TESE) procedure from an in vitro fertilization (IVF) center (Smart-IVF ANNA Hospital). Written informed consent was obtained from all participants. Clinical data, including testicular volume and follicle stimulating hormone (FSH) serum concentration, were obtained from participants' medical records.

Testicular Sperm Extraction (TESE)

TESE was performed using a standard open surgical technique with local anesthetics, with or without intravenous sedation. A 2 cm long incision was made through the skin of the anterior scrotum, dartos, and tunica vaginalis. A 1 cm incision was made in the tunica albuginea. Once the testicular tissue was visible, 5 x 5 mm sections were removed with sharp scissors and were immediately placed in sperm culture media and Bouin's solution. Albuginea incisions were made at the top, middle, and bottom of the testicles to obtain several biopsy specimens of testicular tissue. The testicular specimens were sent to the laboratory for further analysis.

Histopathological Examination

A small amount of testicular tissue was fixed using a 10% formaldehyde solution. Histopathology preparation started with the dehydration of testicular tissue in a gradient of alcohol concentrations (70%, 80%, 90%, 95%, and absolute) for 1 hour each, followed by clearing in xylol I and II, respectively, for 1 hour. The samples were embedded in paraffin and the testicular tissue in paraffin was cut in 5 µm thicknesses using a microtome (Leitz, Wetzlar, Germany). The cut was stained with hematoxylin by dipping the cut in xylol I and II for 5 minutes, then rehydrated in a gradient of alcohol concentration (absolute for 5 minutes, 95% for 5 minutes, 80% for 3 minutes, 70% for 3 minutes) and rinsed with aquadest for 1 minute. The cuts were stained with hematoxylin for 2-3 minutes then rinsed in running water for 1-2 minutes. The samples underwent eosin staining for 1-2 minutes, followed by another dehydration process, were dipped in xylol I for 2 minutes and xylol II for 5 minutes, and were finally covered with entelan [7]. The stained testicular tissue was examined under a light microscope (Olympus, Tokyo, Japan). Johnson's method was used to determine the spermatogenesis process in the testicular tubules, with a score of 1 for the absence of spermatogonia and Sertoli cells up to a score of 10 for complete spermatogenesis [8]. Spermatogenesis was analyzed in 20 tubules with a 400x magnification.

Platelet Rich Plasma (PRP)

PRP was obtained from the Indonesian Red Cross, based on the procedure established by Jo et al. 2013. Whole blood was centrifuged at 900 g for five minutes to separate the plasma, then centrifuged again at 1500 g for 15 minutes to obtain a platelet concentration (PC) from the supernatant [9]. Two U/ml heparin (Inviclot, Tangerang, Indonesia) was added to prevent clotting. Then, PC was frozen at -20ºC for 30 minutes and thawed at room temperature (RT) for 15 minutes [10].

SSCs Culture Technique

Testicular tissue from biopsies was washed using a phosphate buffer saline (PBS) (Sigma, Merck, Germany) by centrifugation at 1200 rpm for 10 minutes. The tissue was minced using sterile scissors and digested by two-step enzymatic digestion including collagenase type IV (Gibco, Thermo Fisher Scientific, USA) 5 mg/Ml for 10 minutes in 37 ºC. Four ml of a culture medium composed of DMEM-F12 (Sigma, Merck, Germany), 1 % Glutamax (Gibco, Thermo Fisher Scientific, USA), 1 % Penicillin & Streptomycin (Gibco, Thermo Fisher Scientific, USA) and 1 % fungizone (Gibco, Thermo Fisher Scientific, USA) was added to inactivate trypsin. Dissociated cells were collected by 1200 rpm centrifugation for 5 minutes. The supernatant was discarded and the pellets were re-suspended with complete medium. Cell viability was determined and evaluated using Trypan blue stain (0.4%) (Gibco, Thermo Fisher Scientific, USA).

Cells were suspended in a complete medium with 10% FBS (Gibco, Thermo Fisher Scientific, USA) and were incubated overnight to reduce the somatic cells (myoid and Sertoli cells). Approximately 10^6 floating cells were collected and cultured in a medium that contained the following supplements and growth factors: 10ng/mL human GDNF (Sigma, Merck, Germany), 10 ng /mL bFGF (Sigma, Merck, Germany), and 20 ng/mL EGF (Sigma, Merck, Germany) in 20 µl/ml laminin (Sigma, Merck, Germany) at density 30,000/cm^2. The samples were cultured in coated dishes at 37 ºC in 5% CO_2 with the addition of PRP or FBS (Gibco, Thermo Fisher Scientific, USA) according to the study group. The culture was observed daily and the medium was changed every two days. Confluent cultures were harvested using TrypLE Select (Gibco, Thermo Fisher Scientific, USA), then combined and counted. A portion of the result of the primary culture was expanded in T25 flasks (seeding around 5000/cm^2). After harvest, the cells were cultured and passaged three times during 14 days.

Gene Expression

The total RNA was isolated using the total RNA mini kit for cell culture (Geneaid, Taiwan). We synthesized RNA into cDNA using ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo, Japan). RNA isolation and cDNA synthesis were performed in according to the manufacturer's directions. qRT - PCR was assayed by using the Sensi FAST SYBR kit for master mix qPCR to a specific marker of SSCs including PLZF, OCT4, CKIT. A whole testis was utilized as a positive control and β-actin was utilized as a housekeeping gene for this assay. The primers were amplified for 40 cycles with denaturation on 95 ºC for 12.5 seconds, annealing on 55 ºC for 10 seconds, and elongation on 72 ºC for 20 seconds. (Table 1) The Ct value was analyzed using the Livak method to show the level of relative expression.

Table 1: Primers of PLZF, OCT4, and CKIT genes used for RT-PCR

Genes	Primer Sequence
PLZF	F: GAG CAG CAG CAA CT CTC TT
	R: GCT CTG AGG AGG GGT AAA GG
OCT4	F: AGT GAG AGG CAA CCT GGA GA
	R: GCC GGT TAC AGA ACC ACA CT
CKIT	F: GTG GGA AAA CAC TGC CAT CT
	R: GGA AAG GTG CGA GAG CAT AG
β actin	F: AGA AAA TCT GGC ACC ACA CC
	R: CCA TCT CTT GCT CGA AGT CC

TUNEL Assay

An apoptosis assay was performed using the terminal deoxynucleotide 1 transferase (TdT-mediated dUTP nick end labeling (TUNEL) method (Sigma, Merck, Germany). Cells that were harvested were washed using PBS, then fixed with 4% paraformaldehyde (PFA) in PBS for 30 minutes. Cells were washed using PBS, then fixed with 4 % paraformaldehyde (PFA) in PBS for 30 minutes. Cells were washed using PBS then permeabilized using 0.1% Triton X-100 at 0.1% sodium citrate at 4 ºC for 30 minutes. Cells were then washed with PBS and labeled with 50 µl TUNEL mixture mix (45µl label solution and 5 µl enzyme solution) and placed in an incubator at 37 ºC for one hour in the dark. Samples were washed using PBS, dripped on an object glass, covered by a cover glass, then observed using a confocal microscope (Zeis LSM 700, USA). The positive control was demonstrated by adding 5 µl DNAse at 4 ºC for 30 minutes before being mixed in TUNEL mixture mix. We performed counter staining to observe the nucleus using DAPI staining. The apoptotic index was defined as the number of apoptotic cells per total number of cells in 100 cells. The observation was done twice by two observers.

Immunocytochemistry (ICC) Assay

We performed immunocytochemical assay after manufacturing blocks of harvested cells. The procedure for immunocytochemical staining in this study used the existing protocols in the Paramount Universal Detection Kit (Biogear Scientific, Iowa, USA). Cell blocks cut to a 3µm thickness were placed on a slide, then dried at 37 ºC and heated on a warmer plate at 60 ºC for 30 minutes. Next, the slides were paraffinized in xylol and rehydrated in graded alcohol. After that, cell preparations were marked with a pap pen and incubated with a paramount block reagent for 10 minutes. The slides were then pre-treated with Tris-EDTA (TE) buffer pH 9 (Sigma, Merck, USA) in a decloaking chamber at 96 ºC for 20 minutes, then cooled. Subsequently, the slides were washed with phosphate buffer saline (PBS) (Sigma, Merck, USA), blocked with paramount ostium blocker, and incubated for 20 minutes. After that, the slides were incubated with caspase 3 (primary antibody) (Santa Cruz, USA) for 1 hour at room temperature, then washed with PBS. The slides were incubated with a secondary link for 30 minutes at room temperature, then incubated with paramount HRP for 30 minutes, and washed with PBS. The slides were then incubated with DAB mixture for 10 minutes and washed with water. We performed counter-staining with hematoxylin on the slide and washed it with water. Next, the slides were rinsed with saturated lithium carbonate and water and dehydrated with graded alcohol. The clearing process was conducted by dipping the slide in xylol. We then mounted the sample on a slide and covered the sample with a glass cover. The staining results were observed under a Leica DM 750 microscope with magnifications of 100x and 400x. The percentage of caspase 3 expression was defined by the percentage of positive caspase cells compared to the total number.

Statistical Analysis

Data were measured using one-way analysis of variance (ANOVA) to analyze the difference between proliferation and differentiation among PRP and FBS. The correlation between the level of spermatogenesis and the potency of proliferation and differentiation of SSCs was analyzed using the Spearman's test.

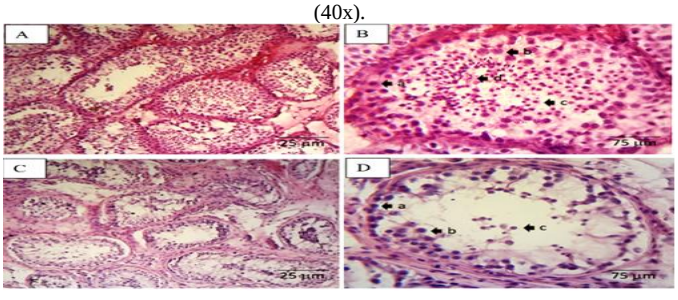
RESULTS AND DISCUSSION

Sampling

The analysis was performed on 3 non-obstructive azoospermia (NOA) infertile men. The clinical data obtained from medical record disclosed for the testicular volume 12.33 ± 2.51 mL (normal values 12-25 mL), FSH concentrations 10.63 ± 2.73 mIU/mL (normal values 2-12 mIU /mL).

Histopathological examination

Figure 1: The histopathology of testis. (A). Control with Johnson score 8 (10x), (B). Control with Johnson score 8 (40x), (C). Hypo spermatogenesis with Johnson score 6 (10x), (D). Hypo spermatogenesis with Johnson score 6 (40x).



The pattern of biopsy testis was hypo spermatogenesis, with scoring Johnson was ranging from 6 to 7.

The pattern performed a decrease in all of the elements of spermatogenic cells including the absence of spermatozoa in the seminiferous tubules, many spermatocytes and a few of spermatid (Fig. 1 C&D). As a control, the histological measurement with scoring Johnson 8 was presented with a few spermatozoa, many spermatocytes and spermatid (Figure 1 A&B).

SSCs culture

An average of 1.108.000 cells were isolated from each sample with high viability (91.97%) (Table 2). The floating cells obtained after two steps of enzymatic (Fig. 2A), attached cells appeared 4 hour to overnight after initial seeding (Fig. 2B). The resulting cells were capable of being propagated in vitro, and the fibroblastic-like cells appeared to attach and formed monolayer. After 14 days of culture, there were no colonies or clumps of visible cells growth among group PRP (Figure 3).

Table 2: Cell viability after two-step enzymatic digestion

Series number	Total count ($x10^3$)	Viable count ($x10^3$)	Dead cell count ($x10^3$)	Viability rate (%)	Dead cell rate (%)
1	677	630	47	93.05	6.95
2	867	809	58	93.31	6.69
3	1.781	1.595	186	89.55	10.45
Mean	1.108	1.011	97	91.97	8.03

Figure 2: The morphology of the testicular culture cell after isolation. (A). Cell culture after two steps digestive enzymatic (10x), (B). Cell culture after incubation for overnight (10x). Black arrow presented the attachment of fibroblast-like somatic cells.

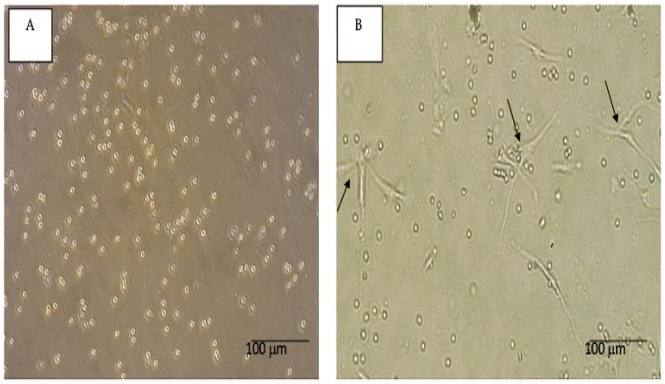
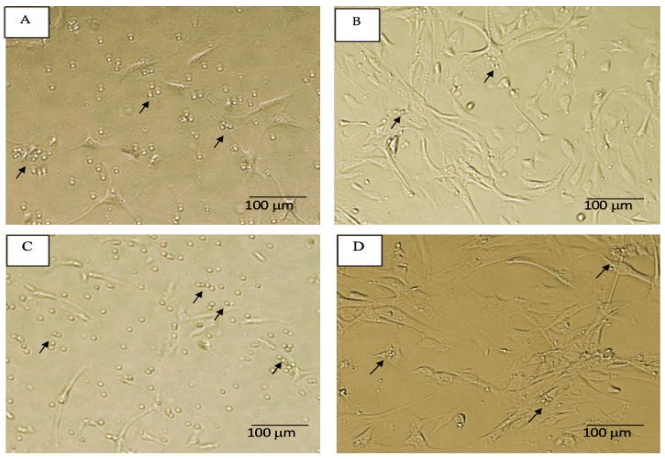


Figure 3: The morphology of the testicular culture cell (A) on day-7, (B) on day-14 in FBS, (C) on day-7 and (D) on day-14 in PRP. Black arrow suggested as the spermatogonial-like stem cells.



Apoptosis

In addition, this study also shows that the apoptosis of spermatogenic cells in a culture medium with FBS is significantly higher than that with the addition of PRP (Fig. 4) Furthermore, apoptosis results obtained by using the TUNEL method were confirmed by the expression of caspase-3 in immunocytochemistry results, which also showed that the expression of caspase-3 in spermatogenic cells in culture medium with the addition of FBS is higher than that with the addition of PRP (Figure 5).

Figure 4: The apoptosis of spermatogenic cells resulted in TUNEL (A-C) in FBS, (D-F) in PRP and (G) as apoptotic index.

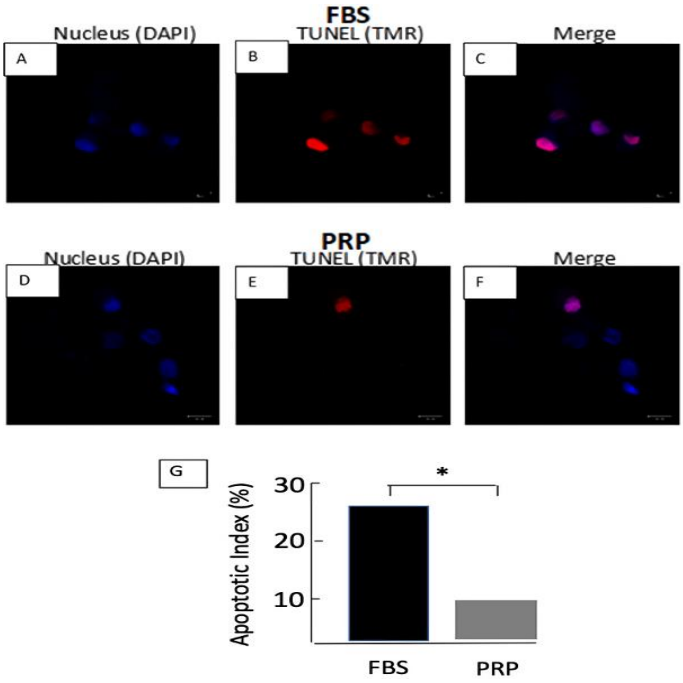
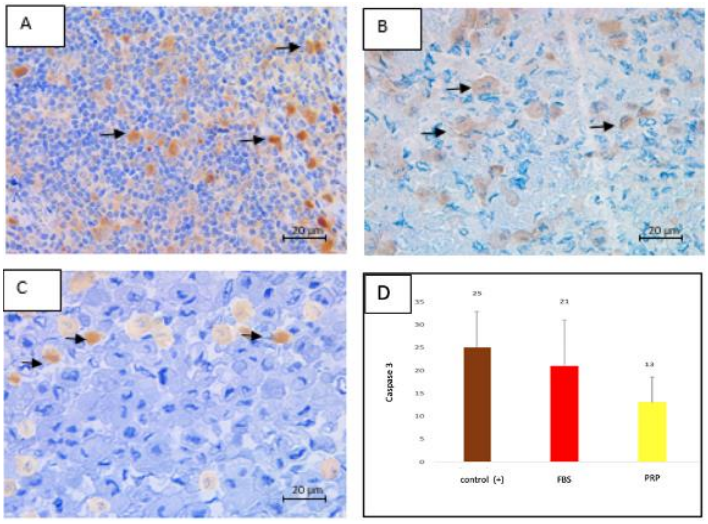


Figure. 5: The caspase 3 expression in spermatogenic cells resulted in ICC (A) in control, (B) in FBS and (C) in PRP.



Gene expression

After 2 weeks culture, the resulting cell were characterized using qRT-PCR. Total RNA was extracted and RT-PCR was performed to analyze the expression of specific SSCs markers, including PLZF, OCT4 and CKIT. The relative expression was

carried out using Livak method yielding the increasing expression of PLZF, OCT4, CKIT among PRP and FBS groups compare to whole testis (Fig. 6). There were no significant differences in PLZF ($p = 0.13$), OCT4 ($p = 0.28$) and CKIT ($p = 0.08$) among the groups.

The correlation between the potency self-renewal and differentiation of SSCs in vitro and the level of spermatogenesis

There was a very weak positive correlation on PLZF, and a strong positive on OCT4 and CKIT to the level of spermatogenesis through Johnson's assessment in PRP group. In the FBS group there was a strong positive correlation on PLZF and CKIT, and a negative correlation on OCT4 to the level of spermatogenesis through scoring Johnson.

Spermatogenesis appearance in non-obstructive azoospermia

Primary testicular failure as a major etiology of non-obstructive azoospermia (NOA) is classified as hypergonadotropic in hypogonadism. It is indicated by a high concentration of FSH and small testicular volumes [11]. In this study, FSH was approaching the upper values of the normal threshold (10.63 ± 2.73 mIU/mL), while the testicular volume was approaching the lower end of the threshold (12.33 ± 2.51 mL). Testicular failure affects the functionality of spermatogenesis resulting in hypospermatogenesis, the arrest of maturation, or Sertoly-cell-only syndrome (SCOS) [11,12]. Histological analyses presented no spermatozoa, many spermatocytes, and few spermatids. The results of testicular biopsy showed that the subjects in this study were categorized as NOA with hypo spermatogenesis, which was further categorized by a Johnson score of six. The results of the study are in line with Lestari et al, which found that the majority of azoospermia infertile men in an IVF laboratory in Jakarta, Indonesia experienced hypo spermatogenesis [13]. In addition, research by Steger et al. demonstrated that testicular biopsies with a score of seven to one, obtained from azoospermia men, showed a decrease in the number of spermatogonia compared to normal testis [14].

Human SSCs in PRP and FBS culture system

The primary culture of testicular tissue was characterized by two main populations: fibroblastic-like somatic cells that tend to attach to the surface of the culture dish, and cells that are round and small, such as germinal cells. Germinal cells tend to attach to fibroblastic-like somatic cells that divide and form colonies that are believed to be lost in the initial passage because adherent cells develop rapidly [15]. It is believed that SSCs will disappear in large numbers in the early stages of culturing, accompanied by increased expression of testicular somatic cells. Testicular cells play an important role in supporting the growth of SSCs but also have a deleterious effect due to their excessive growth. The results of cell isolation showed that the morphology and distribution of spermatogenic cells, which specified that spermatogenesis had occurred. Spermatogonia-like stem cells are morphologically detected among other cells by round cells with large nuclei that attach to adherent cells, when compared to the cytoplasmic ratio.

Human SSCs increased in number by self-renewal in vitro. Growth factors used in our culture medium including glial cell-derived neurotrophic factor (GDNF), basic fibroblast growth factor (bFGF), and epidermal growth factor (EGF) that were able to support SSCs survival and proliferation [16]. The crucial factor towards SSCs pluripotency is GDNF and Sertoli cell-derived factor, which controls self-renewal or differentiation. GDNF family receptor alpha 1 (GFRA1) is expressed by SSCs, implying that spermatogonia responded to GDNF and Sertoli cells capable of regulating the quantity of SSCs. The addition of bFGF and EGF encouraged the influence of GDNF although the underlying mechanism is undefined [17-20].

The utilization of serum supports a culture system by providing nutrients and acting as a matrix [14]. FBS, known as a universal serum, is commonly used in stem cell culture. FBS is rich in growth factors and hormones that stimulates cell proliferation and facilitates cell differentiation. FBS has several limitations, including that its composition has not been completely identified, the risk of contamination by fungi, bacteria, viruses, and mycoplasmas, and the high risk of rejection to xenogeneic antigens [21]. PRP, also known as platelet rich growth factors (GFs) and platelet rich fibrin (PRF) matrix, could be used as an alternative to FBS. The activation of the MAP kinase signal cascade is the cellular mechanism in serum responsible for stimulating cell proliferation. Mitogen including growth factors and serum (FBS, PRP) triggers the activation of Raf which phosphorylates MEK (MAPK/ERK kinase), and triggers the specific phosphorylation of ERK 1/2. As a result, the terminal kinase activates specific transcription factors that initiate the expression of genes, which play a role in cell division, proliferation, and differentiation [22].

The resulting cell culture appeared as floating cell and did not form a cluster or colony in both groups in the first week of culture. In the second week, small round cells were discovered on the cell surface attached to both groups. We did not measure the proliferation rate in our study but there were no differences in the morphological appearance of both groups. This result reflected the results of Mohammad Zadeh et al, which found no SSC colony in the group with FBS [23]. To the best of our knowledge, this study was the first study using PRP in cell culture systems from testicular tissue compared with FBS, to analyze the potency of SSC proliferation and differentiation in vitro. The use of PRP could result in differences, triggered by the number of platelets, concentration of growth factors, platelet survival rate of preparations, and donor variations [24]. The efficacy of PRP was based on the production and release of growth

factors when platelets were activated. The differences in the concentration of growth factors and the presence of small amounts of debris may have an impact on the proliferation and differentiation of culture cells.

Apoptosis of human SSCs in PRP and FBS culture system

Furthermore, this study also showed the apoptosis of spermatogenic cells in culture system. This study shows that the apoptosis of spermatogenic cells in a culture medium with FBS is significantly higher than that with the addition of PRP. Apoptosis results obtained by using the TUNEL method were confirmed by the expression of caspase-3 in immunocytochemistry results, which also showed that the expression of caspase-3 in spermatogenic cells in culture medium with the addition of FBS is higher than that with the addition of PRP. Our results are supported by the results of other studies such as Fukaya et al. (2012), which found that PRP is able to inhibit apoptosis better than FBS in vitro [25]. Asjid et al. (2019) found that PRP is able to induce apoptotic inhibition in chondrocytes [26]. Caspase-3 expression was also in accordance with the findings of Moussa et al. (2017), which determined that increasing the PRP dose (5%, 10% and 20%) can induce a significant reduction in caspase-3 expression and significantly increase Bcl2 expression [27]. The mechanism underlying the results of this study was revealed in Yang et al. (2016), which showed that the administration of PRP can reduce the regulation of pro-apoptotic factors and increase the expression of anti-apoptotic factors [28].

Gene expression of human SSCs in PRP and FBS culture system and its correlation to spermatogenesis level

In addition, another result of this study was the gene expression in SSCs culture. The relative expression was carried out using the Livak method and showed an increased expression of PLZF, OCT4, CKIT among the PRP and FBS groups, compared to whole testis. Meanwhile, there were no significant differences in PLZF ($p = 0.13$), OCT4 ($p = 0.28$) and CKIT ($p = 0.08$) among the groups. PLZF as a SSC-specific transcription factor in the testis is required to regulate pluripotency [29-34]. This transcription factor exerts growth-suppressive activities complemented by the accumulation of cells in the G0/G1 compartment of the cell cycle. PLZF control the epigenetic repression of chromatin domains that are necessary for cell differentiation. Our results are congruent with those of Ardekani et al [35]. which stated that PLZF was expressed in primary testicular cultures. The expression of the pluripotency OCT4 gene indicated that there were SSCs populations. OCT4 and PLZF were expressed in the first week of culture [29-34]. Akhondi et al. found that testicular tissue cultures showed high expressions of OCT4 [36]. The expression of CKIT, a differentiation marker of SSCs, were strengthened in the second week, suggested that the culture system could support the differentiation in vitro. Azoospermia patients with hypo spermatogenesis have a decreased spermatogonia population. There was a 23.6% decrease in spermatogonia in hypo spermatogenesis due to the depletion of the stem cell population or impaired proliferation of SSCs in vivo. In the case of maturation arrest there was a significant decrease of Sertoli cells (26.6%) and all spermatogonia (45.3%) from azoospermia male testicular biopsy compared with normal spermatogenesis [37, 38]. The loss of germ cells, spermatocytes, and spermatids is also suspected due to initial meiosis, a mitotic disorder, and excessive apoptosis. Deficiencies in the stem cell pool and meiotic abnormalities indicate that both aspects are very important in functional spermatogenesis [37, 39].

Moreover, this study showed the correlation between the potency of self-renewal and differentiation of SSCs and the level of spermatogenesis. There was a strong positive correlation on OCT4 and CKIT, and no correlation on PLZF, for the level of spermatogenesis through Johnson's assessment in the PRP group. The impairment of functional spermatogenesis in vivo may influence the ability of SSCs in proliferation and differentiation in vitro. However, there was a strong positive correlation on PLZF and CKIT, and a negative correlation on OCT4 for the level of spermatogenesis through Johnson’s assessment in the FBS group. Negative correlations implied that low mRNA expression would be inversely proportional to a high Johnson score. Conversely, a high expression of mRNA would be inversely proportional to a low Johnson score. A Johnson score of six on the right or the left testicular biopsy also indicated a discordant pattern. There is a difference in the results depending on the part of the tubules obtained for testicular or culture biopsy. A discordant pattern greatly affects the culture results and the correlation between the level of spermatogenesis as shown by Johnson scoring. Others possible bias factors could be due to the limited sample size.

Our study was limited by the lack of facilities for sorting cells and the limited antibodies used. We tried to use simple techniques to purify the SSCs but were hindered by the excessive growth of somatic cells. The culture system we developed did not use a feeder cell or matrix system and we used limited supplementation to nurture the cells. It is challenging to maintain the appropriate conditions for the sustainability of human SSCs during culturing, which was suggested by the condition of the culture. Despite the study limitations, this study was the cornerstone to develop and establish further research, particularly in the utilization of human SSCs culture in Indonesia. In conclusion, PRP may support the proliferation and differentiation of SSCs in vitro and could be developed into an alternative for FBS in the human SSC culture system.

CONCLUSIONS

In conclusion, PRP may support the proliferation and differentiation of SSCs in vitro and could be developed into an alternative for FBS in the human SSC culture system.

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CONFLICT OF INTERESTS

The authors report no declarations of interest

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