

Supplementation with Plasma Rich in Growth Factors Improves Short-Term *In Vitro* Maintenance of Human Fetal Testicular Tissue

Seyyed Amir Moradian¹, PhD; Zahra Amirkhani^{2,3}, PhD; Mansoureh Movahedin⁴, PhD; Seyyede Nazanin Zahra Gharib⁵, BSc; Hamed Hajipour¹, PhD; Kobra Hamdi⁶, MD; Behrooz Niknafs^{1,7}, PhD; Amir Fattahi¹, PhD

¹Department of Reproductive Biology, Faculty of Advanced Medical Sciences, Tabriz University of Medical Sciences, Tabriz, Iran;

²Department of Nursing, School of Nursing, Larestan University of Medical Sciences, Larestan, Iran;

³Student Research Committee, Larestan University of Medical Sciences, Larestan, Iran;

⁴Department of Anatomical Sciences, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran;

⁵Department of Biology Education, Shahid Bahonar Campus, Farhangian University, Shiraz, Iran;

⁶Women's Reproductive Health Research Center, Tabriz University of Medical Sciences, Tabriz, Iran;

⁷Department of Anatomy, School of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran

Correspondence:

Amir Fattahi, PhD;
Faculty of Advanced Medical Sciences, Tabriz University of Medical Sciences, University Campus, Golgasht St., Azadi St., Postal Code: 51666-53431, Tabriz, Iran

Tel: +98 41 33355789

Email: Amirfattahi@iran.ir

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What's Known

- Childhood cancer survivors and individuals with non-obstructive azoospermia (NOA) face a high risk of infertility.
- Current *in vitro* culture systems rely on xenogeneic supplements such as knockout serum replacement (KSR), which raise safety concerns. Alternatives, such as plasma rich in growth factors (PRGF), remain underexplored in human testicular tissue culture.

What's New

- Compared with KSR and higher plasma rich in growth factors (PRGF) concentrations, cultures supplemented with 5% PRGF showed superior preservation of seminiferous tubule integrity; higher numbers of promyelocytic leukemia zinc finger (PLZF)⁺, octamer-binding transcription factor 4 (OCT4)⁺, and DEAD-box helicase 4 (DDX4)⁺ germ cells; higher SRY-box transcription factor 9 (SOX9)⁺ Sertoli cell counts; greater steroidogenic acute regulatory protein (STAR) immunoreactivity in Leydig cells; increased proliferative labeling; and lower total Caspase-3 immunoreactivity.

Abstract

Background: Given the need for xeno-free fertility preservation strategies, this study aimed to evaluate plasma rich in growth factors (PRGF) as an alternative to knockout serum replacement (KSR) for short-term *in vitro* maintenance of human fetal testicular tissue.

Methods: This experimental study was conducted in May 2025 at Al-Zahra Hospital (Tabriz, Iran). Immature testicular tissue from three 26-week-old human fetal donors was allocated to four culture conditions: 10% KSR (control), 5% PRGF, 10% PRGF, and 20% PRGF. On day 15, 38 tissue fragments were analyzed per culture condition. Fragment-level data were analyzed using a mixed-effects model with culture condition as the fixed effect and fetal donor as the random effect, followed by Tukey's multiple-comparisons test.

Results: After 15 days of culture, 5% PRGF resulted in better preservation of seminiferous tubules (23.0±2.1%) than 10% KSR and higher PRGF concentrations (P<0.001). It also yielded the highest number of promyelocytic leukemia zinc finger (PLZF)⁺ (pro)spermatogonia (13.22±1.56 cells/tubule) compared with 10% KSR (11.00±1.65; P=0.0329) and higher PRGF concentrations (P<0.001). Total octamer-binding transcription factor 4/DEAD-box helicase 4 germ cells (OCT4⁺/DDX4⁺) were also higher with 5% PRGF than with 10% KSR (13.56±2.60 vs. 8.78±1.39 cells/tubule; P<0.001). In addition, 5% PRGF increased the number of SRY-box transcription factor 9 (SOX9)⁺ Sertoli cells (P=0.0352), steroidogenic acute regulatory protein (STAR) immunoreactivity (P=0.0121), and Ki-67⁺ cells (P=0.0168), while demonstrating lower total Caspase-3 immunoreactivity (P=0.0109).

Conclusion: PRGF is a promising xeno-free supplement for short-term culture of human fetal testicular tissue and merits evaluation in long-term functional studies.

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Keywords • Spermatogenesis • Fertility preservation • Organ culture techniques • *In vitro* techniques • Platelet-rich plasma

Introduction

Although childhood cancer cure rates now exceed 80%, many survivors experience long-term complications, including impaired fertility. Currently, approximately 1 in 530 young adults

is a childhood cancer survivor.¹ Gonadotoxic chemotherapy and radiotherapy can deplete the undifferentiated spermatogonial pool, with infertility rates reaching up to ~50% in high-risk groups.^{2,3} In addition, non-obstructive azoospermia (NOA), cryptorchidism, and Klinefelter syndrome can impair spermatogenesis, highlighting the need for fertility preservation strategies, particularly for prepubescent boys for whom sperm cryopreservation is not feasible.⁴ *In vitro* spermatogenesis has thus emerged as a promising approach.

Achieving functional spermatogenesis *in vitro* requires mimicking the testicular microenvironment.⁵ A key milestone came in 2011 when Sato and colleagues achieved complete spermatogenesis in mice using knockout serum replacement (KSR), a bovine-derived supplement.⁶ Although KSR can support complete *in vitro* spermatogenesis in some mouse strains,^{6,7} its efficacy appears to be strain-dependent,⁸⁻¹⁰ and it has so far failed to support full spermatogenic progression in rats,¹¹ primates,¹² and humans.¹³⁻¹⁵ In these models, including human testicular tissue, KSR was shown to disrupt germ–somatic cell interactions and promote apoptosis.^{16,17} Additionally, as a xenogeneic product, KSR poses risks of zoonotic pathogen transmission, which compromises its suitability for clinical application.¹⁸ Furthermore, standard culture media such as minimum essential medium (MEM) lack critical components present in human plasma.¹⁹ This non-physiological composition may further compromise cell function during long-term culture.

Given the limitations of xenogeneic supplements, such as KSR and conventional culture media, safer alternatives that better mimic the native testicular environment are required. Platelet-rich plasma (PRP) and plasma rich in growth factors (PRGF) have therefore emerged as promising xeno-free alternatives.²⁰ PRGF can be prepared from platelets and activated via calcium-induced degranulation, releasing a broad spectrum of bioactive factors, including platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factors (FGFs), vascular endothelial growth factor (VEGF), and insulin-like growth factor-1 (IGF-1), that are critical for regulating cell proliferation, survival, and differentiation.²¹ In contrast to KSR, PRGF is entirely xeno-free and has received regulatory approval in both Europe and the United States for clinical use,²² thereby offering a safer and more physiologically relevant option for translational research. In our previous study, we found that PRGF-supplemented media outperform KSR in supporting long-term culture

of immature mouse testicular tissue, preserving seminiferous tubule integrity, and enabling spermatogenesis to progress to the formation of flagellated spermatozoa.⁹

Based on these findings, this study aimed to evaluate whether PRGF can support short-term *in vitro* culture of human fetal testicular tissue and determine the optimal concentration for preserving tissue structure and the expression of germ and somatic cell markers, providing a basis for future preclinical and translational research.

Materials and Methods

Origin of Human Immature Testicular Tissue

In May 2025, immature human testicular tissues (n=3 donors) were obtained from three 26-week-old male fetuses who had died in the neonatal intensive care unit (NICU) at Al-Zahra Hospital, Tabriz, Iran. Donors with congenital anomalies or positive serology for infectious diseases were excluded. Written informed consent was obtained from the parents or legal guardians of the fetuses before tissue collection. This study was approved by the Ethics Committee of Tabriz University of Medical Sciences (IR.TBZMED.REC.1402.410) and conducted in accordance with the Declaration of Helsinki. A schematic overview of the experimental workflow is presented in figure 1.

Tissue Culture

Testicular tissues were collected within 5 hours postmortem via an inguinal incision, as the testes had not yet descended at 26 weeks of gestation (figure 2A). After washing in α -minimum essential medium (α -MEM, Gibco™, REF 11900-073; Thermo Fisher Scientific, Paisley, UK), the tunica albuginea was removed, and tissues were dissected under a stereomicroscope into ~2–3 mm³ fragments. The experimental design included testicular tissue from three fetal donors (table 1). For each donor, tissue fragments were allocated to four culture conditions: α -MEM supplemented with 10% KnockOut™ Serum Replacement (KSR; Gibco™, Catalog No. 10828-010; Life Technologies Corporation®, Grand Island, NY, USA; control), α -MEM supplemented with 5% PRGF, α -MEM supplemented with 10% PRGF, or α -MEM supplemented with 20% PRGF. In addition, three fragments per donor were fixed at day 0 for baseline analyses. Fragments were placed on pre-equilibrated 1.5% agarose blocks (w/v) (Catalog No. MR7740C; CinnaGen Co.®, Tehran, Iran) in 6-well plates and cultured using a gas–liquid interface system (figure 2B).

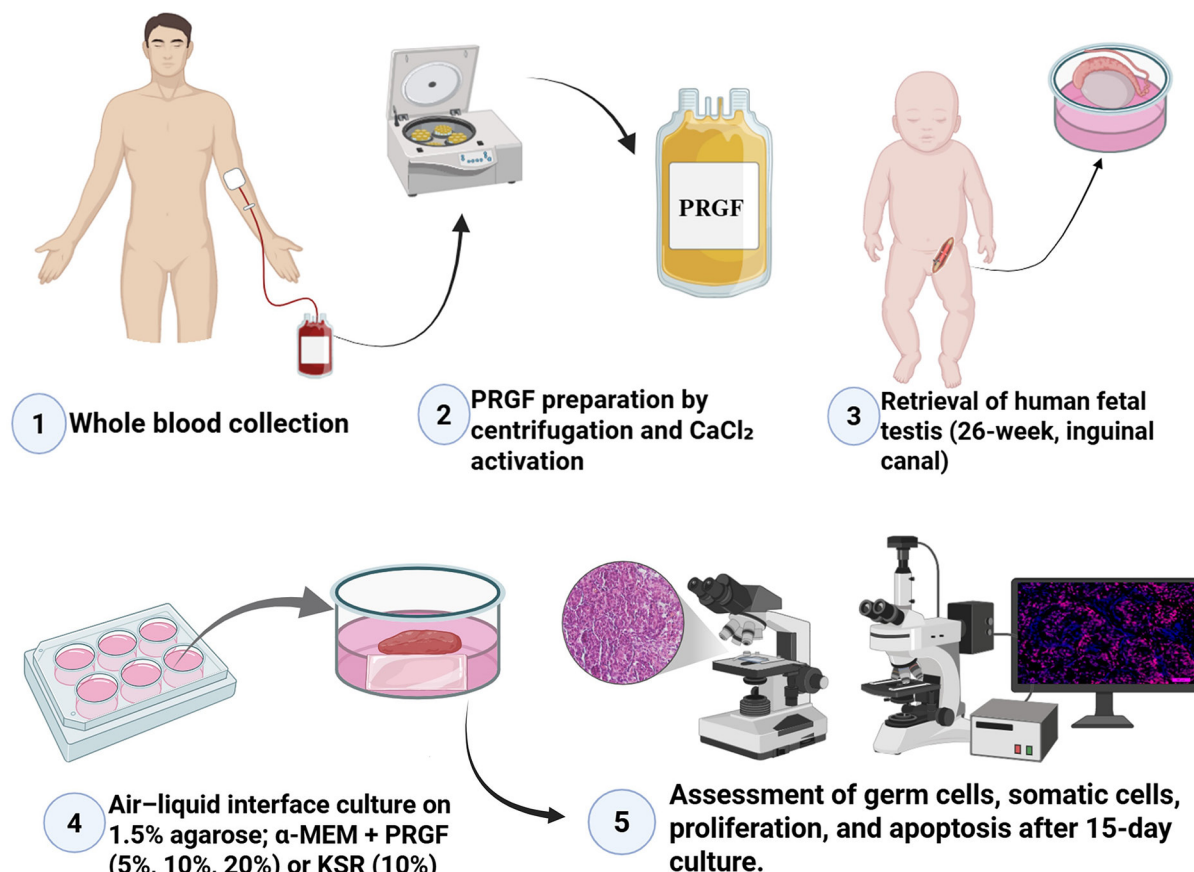


Figure 1: This schematic illustrates the experimental workflow, including PRGF preparation from whole blood and the culture of human fetal testicular tissue obtained at 26 weeks of gestation. Tissue fragments were cultured on 1.5% agarose at the air-liquid interface in α -MEM supplemented with 5%, 10%, or 20% PRGF, or with 10% KSR, followed by histological and immunofluorescence analyses after 15 days. PRGF: Plasma rich in growth factors; α -MEM: Alpha minimum essential medium; KSR: KnockOut serum replacement.

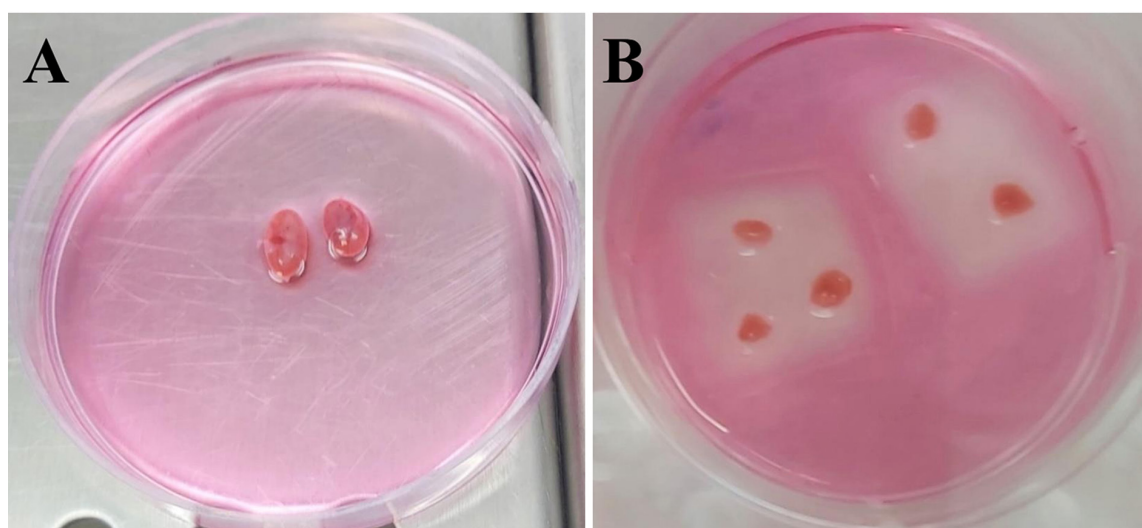


Figure 2: This figure shows the isolation and culture setup of human fetal testicular tissue. (A) Testicular tissue was obtained from fetuses at 26 weeks of gestation. (B) Tissue fragments were cultured on 1.5% agarose blocks using a gas-liquid interface system, with the medium level maintained slightly below the agarose surface.

At day 15, 12–14 fragments per donor per culture condition were available for analysis, resulting in 38 fragments per culture condition. Detailed fragment allocation is provided in tables 2 and 3. The control group received α -MEM containing

10% KSR, as used in previous testicular tissue culture studies.^{6, 15} All media were supplemented with 100 IU/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin (Bio Idea®, Tehran, Iran), and 50 $\mu\text{g/mL}$ gentamicin (Alborz Darou, Iran).

Table 1: Donor characteristics for 26-week human fetal testicular tissue samples used in this study

Donor ID	Gestational Age (weeks)	Postmortem Interval (hours)	Fetal Weight (g)	Exclusion Criteria
D1	26	4.5	750	None
D2	26	3.8	710	None
D3	26	4.2	735	None

Postmortem interval refers to the time elapsed between fetal demise and tissue collection/processing, before the initiation of culture. ID: Identification; g: Gram

Table 2: Number of donors and testicular tissue fragments per condition at day 0 and after 15 days of culture

Condition	Donor 1 (day 0/day 15)	Donor 2 (day 0/day 15)	Donor 3 (day 0/day 15)	Total fragments day 0	Total fragments day 15
10% KSR (control)	3/12	3/12	3/14	9	38
5% PRGF	3/12	3/12	3/14	9	38
10% PRGF	3/12	3/12	3/14	9	38
20% PRGF	3/12	3/12	3/14	9	38

Fragments collected at day 0 were used for baseline histological and immunofluorescence analysis. The remaining fragments were assigned to four experimental conditions and cultured for 15 days. KSR: KnockOut serum replacement; PRGF: Plasma rich in growth factors

Table 3: Number of tissue fragments used for H&E and immunofluorescence analysis at day 15 per culture medium and donor

Culture media	Donor 1 (H&E/ Immunofluorescence)	Donor 2 (H&E/ Immunofluorescence)	Donor 3 (H&E/ Immunofluorescence)	Total H&E/ Immunofluorescence fragments on day 15
10% KSR (control)	5/7	5/7	5/9	15/23
5% PRGF	5/7	5/7	5/9	15/23
10% PRGF	5/7	5/7	5/9	15/23
20% PRGF	5/7	5/7	5/9	15/23

H&E: Hematoxylin and eosin; KSR: KnockOut serum replacement; PRGF: Plasma rich in growth factors

Table 4: Blood parameters and platelet concentration for each donor before and after PRGF processing

Donor	Age (years)	Platelet ($\times 10^3/\mu\text{L}$)	WBC ($\times 10^3/\mu\text{L}$)	RBC ($\times 10^6/\mu\text{L}$)	Platelet concentration in PRGF ($\times 10^6/\mu\text{L}$)	PRGF to Whole Blood Platelet Ratio	Residual WBC after PRGF ($\times 10^6/\text{unit}$)
1	29	256	6.4	5.4	1.1	4.30	$<1 \times 10^6$
2	32	272	5.9	5.8	1.22	4.49	$<1 \times 10^6$
3	27	241	6.1	5.0	1.2	4.98	$<1 \times 10^6$
4	31	265	6.8	5.3	1.3	4.91	$<1 \times 10^6$

PRGF: Plasma rich in growth factors; WBC: White blood cell; RBC: Red blood cell; μL : Microliter

Cultures were maintained at 34 °C in a humidified incubator ($\approx 85\%$ humidity, 5% CO_2), and the medium was changed every 3 days.

PRGF Preparation

PRGF was prepared under sterile conditions in accordance with the Declaration of Helsinki. Whole blood was obtained from four healthy male donors (29.75 ± 2.21 years) following the provision of written informed consent (table 4). All donors were screened for infectious diseases, including HIV and hepatitis, and tested negative before donation. Whole blood units (450 mL) were collected into citrate–phosphate–dextrose (CPD) anticoagulant primary bags (≈ 63 mL CPD; approximately 7:1 blood-to-anticoagulant ratio) and processed within 8 hours using a quadruple blood bag system with an integrated

leukoreduction filter (LCR5, Ref. LQE615Q; Maco Pharma®, Mouvaux, France). After a first “soft” centrifugation ($2,000 \times g$ for 8 min at room temperature), the upper PRP layer (approximately 200–220 mL) was expressed from the primary CPD bag into the connected platelet bag.

The platelet bag then underwent a second “hard” centrifugation ($4,500 \times g$ for 5 min) to pellet the platelets. Following this spin, the supernatant platelet-poor plasma (approximately 150–170 mL) was transferred into the plasma bag, leaving a platelet pellet in the lower part of the platelet bag suspended in the remaining approximately 50 mL of plasma. This residual plasma was used to gently resuspend the platelet pellet, thereby generating a leukoreduced platelet concentrate (approximately 50 mL).

Platelet concentrates were stored at 22 ± 2 °C under gentle agitation, and routine blood-bank quality control met local standards (platelet yield $\geq 5.5 \times 10^9$ per unit, and residual leukocytes $< 1 \times 10^6$ per unit at the end of storage). Platelet concentrates from four donors were then pooled under aseptic conditions and processed to obtain PRGF as described below.

Pooled platelet concentrates were activated by adding 10% calcium chloride (50 μ L/mL; Catalog No. 1.02382.1000; Merck®, Darmstadt, Germany), incubated at 40 °C for 3 hours to allow clot formation, and the fluid phase was collected. This fluid phase (supernatant) was centrifuged at $8,000 \times g$ for 10 min to remove residual fibrin, and the resulting PRGF was aliquoted and stored at -80 °C. Before use, PRGF aliquots were thawed and centrifuged again at $10,000 \times g$ for 10 min at 4 °C to prevent gelling upon addition to the culture medium.

Quantification of Growth Factors in PRGF Using ELISA Kits

Six growth factors in PRGF were quantified using analyte-specific enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturers' instructions: PDGF-BB (Catalog No. DY220; R&D Systems®, Minneapolis, MN, USA), VEGF (Catalog No. DY293B; R&D Systems®, Minneapolis, MN, USA), EGF (Catalog No. E-EL-H0059; Elabscience®, Houston, TX, USA), acidic FGF (FGF1; Catalog No. DFA00B; R&D Systems®, Minneapolis, MN, USA), IGF-1 (Catalog No. DY391; R&D Systems®, Minneapolis, MN, USA), and hepatocyte growth factor (HGF; Catalog No. E-EL-H0084; Elabscience®, Houston, TX, USA). Absorbance was measured using a microplate reader (StatFax 4200, Awareness Technology Inc., Palm City, FL, USA) at 450 nm with a 630 nm reference wavelength. All ELISA measurements were performed in technical triplicate (three wells per analyte) using the same pooled PRGF sample.

Histological Examination

Fresh and cultured tissues were fixed in Bouin's fixative for 4 hours at room temperature, embedded in paraffin, sectioned at 5 μ m, and stained with hematoxylin and eosin (H&E) using standard procedures. The slides were then dehydrated, cleared, mounted, and dried overnight before microscopic evaluation.

Seminiferous Tubule Integrity Analysis

Tubular integrity was assessed in a double-blind manner using a semi-quantitative scoring system described by De Michele and

colleagues.¹⁴ At least 200 round or nearly round seminiferous tubule cross-sections were evaluated, using two sections from each of three tissue fragments per donor. The scoring included four criteria: adhesion of cells to the basement membrane, cellular cohesion, proportion of pyknotic nuclei ($< 5\%$), and distinct separation of germ and Sertoli cells. The seminiferous tubules were graded on a scale from 1 to 4, with "1" indicating poor preservation, "2" fair, "3" good, and "4" representing the best preservation (figures 3A–D).

Depth Analysis of Intact Seminiferous Tubules

H&E-stained sections were analyzed in a blinded manner in ImageJ (NIH, Bethesda, MD, USA). For each condition, three fragments from each fetus were examined, with two non-adjacent sections per fragment. Six perpendicular transects were drawn from the tissue surface per section, and the maximum radial depth to the deepest well-preserved seminiferous tubule was recorded using the embedded scale bar for calibration.

Immunostaining

Tissue samples were fixed in 4% paraformaldehyde (Sigma-Aldrich®, St. Louis, MO, USA), embedded in paraffin, and sectioned at 5 μ m. Sections were deparaffinized, rehydrated, subjected to antigen retrieval in sodium citrate buffer, permeabilized with Triton X-100, and blocked with bovine serum albumin (BSA).

For single immunofluorescence staining, sections were incubated overnight at 4 °C with primary antibodies against promyelocytic leukemia zinc finger (PLZF; ZBTB16) for (pro) spermatogonia (Santa Cruz Biotechnology®, sc-28319; 1:100), SRY-box transcription factor 9 (SOX9) for Sertoli cells (Santa Cruz Biotechnology®, sc-166505; 1:100), steroidogenic acute regulatory protein (STAR) for Leydig cells (Elabscience®, E-AB-15419; 1:100), Ki-67 for proliferating cells (Elabscience®, E-AB-22027; 1:100), and total cysteine-aspartic acid protease-3 (Caspase-3) as an apoptosis-related marker (Santa Cruz Biotechnology®, sc-7148; 1:100). After washing, sections were incubated for 1 hour at room temperature in the dark with species-matched secondary antibodies: goat anti-mouse immunoglobulin G (IgG) heavy and light chains (H+L)–cyanine 3 (Cy3; Elabscience®, E-AB-1011; 1:100) or goat anti-rabbit IgG (H+L)–fluorescein isothiocyanate (FITC; Elabscience®, E-AB-1014; 1:100). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich®, D9542; 1:200). Negative control sections were processed in parallel by omitting

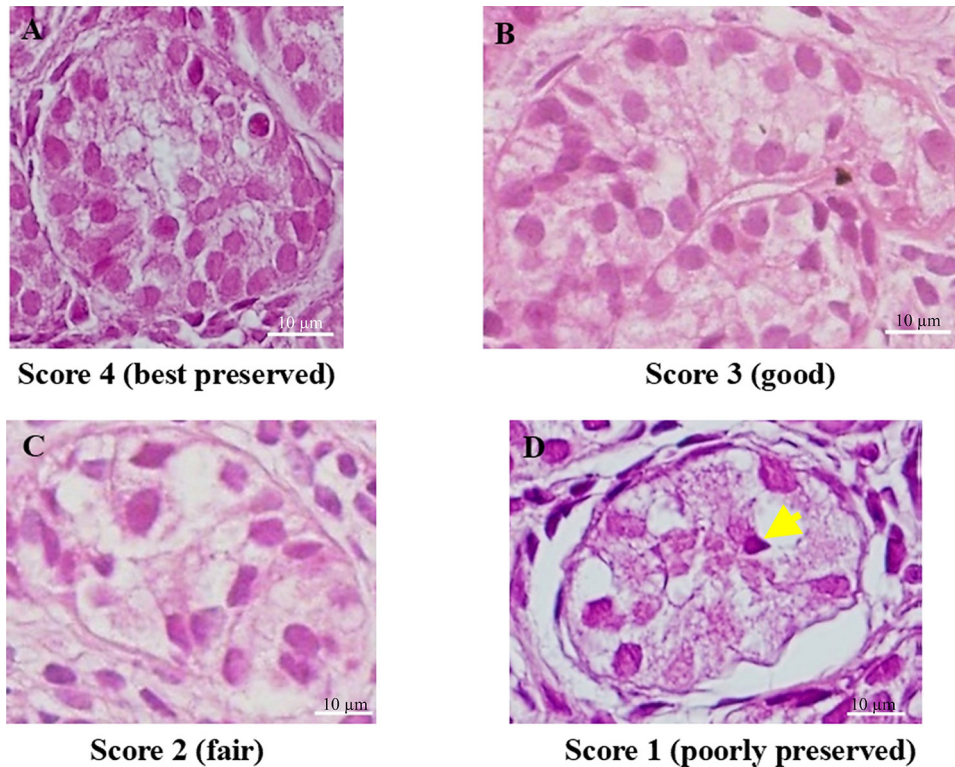


Figure 3: The figures show representative H&E-stained images used to define the seminiferous tubule integrity scoring system. (A) Score 4 indicates the best preservation, with firm cell attachment to the basement membrane, strong cellular cohesion, rare or absent pyknotic nuclei (<5%), and clearly distinguishable germ-Sertoli cell organization. (B) Score 3 indicates good preservation, with mostly intact basement membrane adhesion, good cellular cohesion, few pyknotic nuclei (<5%), and evident germ-Sertoli cell separation. (C) Score 2 indicates fair preservation, with partial cell detachment from the basement membrane, reduced cellular cohesion, and weak or partial germ-Sertoli cell separation. (D) Score 1 indicates poor preservation, with marked structural deterioration, loss of cell-basement membrane adhesion, poor cellular cohesion, increased pyknotic nuclei, and the absence of recognizable germ-Sertoli organization. The yellow arrow indicates a pyknotic nucleus, characterized by nuclear shrinkage and hyperchromasia. Scale bars: 10 μ m; H&E: Hematoxylin and eosin

the primary antibodies, confirming the absence of nonspecific fluorescence (figure 4).

For double immunofluorescence, sections were incubated with octamer-binding transcription factor 4 (OCT4; POU5F1; Abcam®, ab19857; 1:100) and DEAD-box helicase 4 (DDX4/VASA; Santa Cruz Biotechnology®, sc-48705; 1:100) to identify germ cells, followed by species-specific fluorescent secondary antibodies. Appropriate secondary antibodies conjugated to spectrally distinct fluorochromes were then applied as a cocktail for 1 hour at room temperature in the dark, including Cy3-conjugated Affinipure goat anti-rabbit IgG (H+L; Proteintech Group®, SA00009-2; 1:100) and mouse anti-goat IgG-FITC (Santa Cruz Biotechnology®, sc-2356; 1:100), according to the species of the primary antibodies. Nuclei were counterstained with DAPI, and stained sections were visualized using a fluorescence microscope. Details of antibodies are provided in table 5.

For each fetal donor and culture condition, three tissue fragments were analyzed. For each fragment, two non-adjacent sections (≥ 150 μ m apart) were examined, and five non-overlapping

random fields containing seminiferous tubules were captured per section at $\times 400$ magnification. Image acquisition and quantification were performed in a blinded manner with concealed group allocation. Measurements from fields were first averaged within each section and then across the two sections to obtain a single fragment-level value. Accordingly, individual data points that were shown in graphs represent fragment-level values (three fragments per fetal donor per condition).

Quantitative Analysis of Immunostained Sections

Positive cells for PLZF, OCT4, DDX4, SOX9, and Ki-67 were quantified per seminiferous tubule cross-section, as previously described for immature testicular tissue culture.^{9, 14} For STAR and total Caspase-3, whole-field mean fluorescence intensity (MFI) was measured in ImageJ on single-channel images using identical acquisition settings across groups. Tissue-containing regions of interest (ROIs) were selected to exclude blank areas, without gamma adjustment or thresholding.

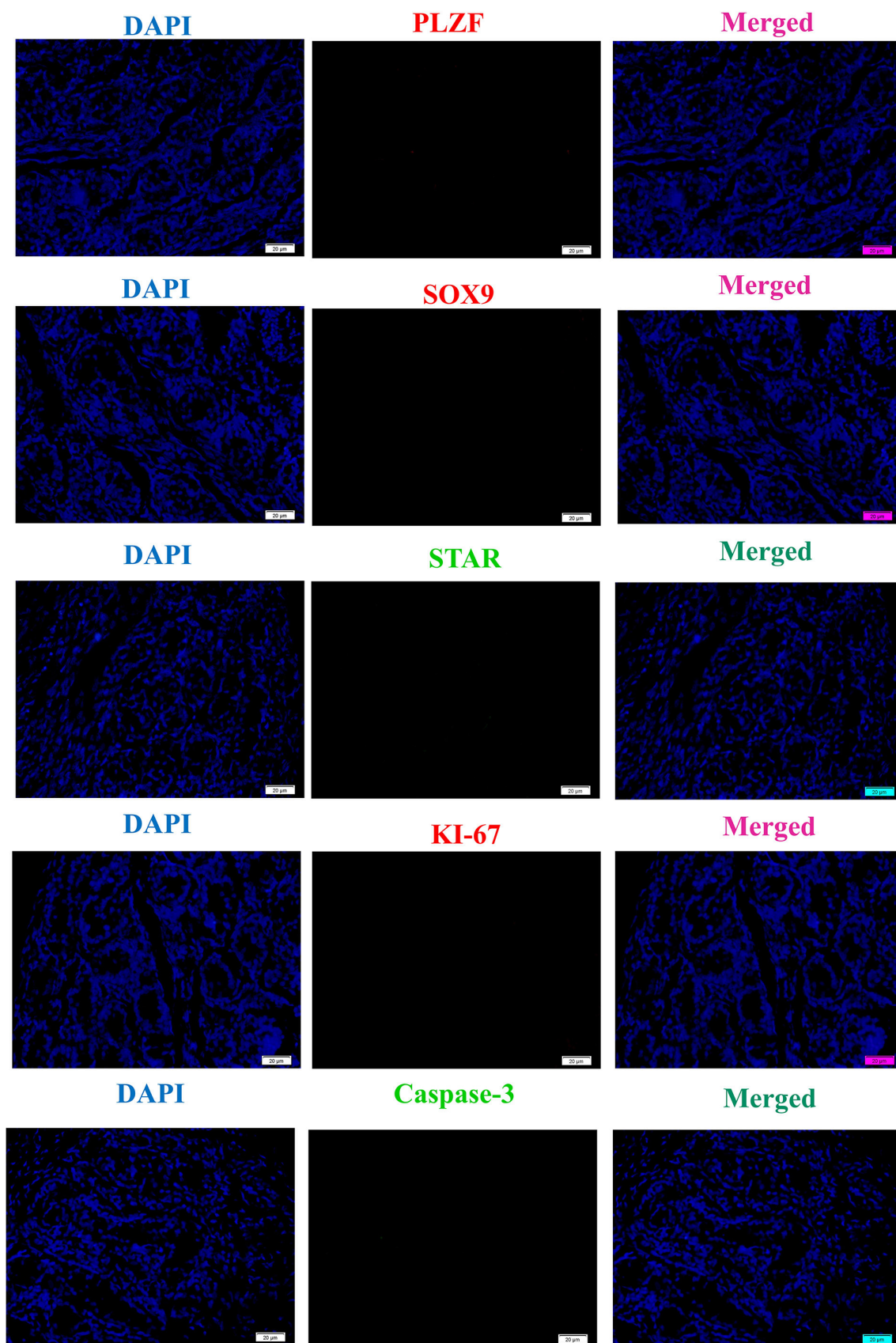


Figure 4: This figure shows representative negative control immunofluorescence staining in human immature testicular tissue for PLZF, SOX9, STAR, Ki-67, and Caspase-3. Sections were processed in parallel under identical conditions with the omission of primary antibodies, and nuclei were counterstained with DAPI (blue). Scale bar=20 µm; PLZF: Promyelocytic leukemia zinc finger; SOX9: SRY-box transcription factor 9; STAR: Steroidogenic acute regulatory protein; Ki-67: Ki-67 proliferation marker; Caspase-3: Cysteine-aspartic acid protease-3; DAPI: 4',6-diamidino-2-phenylindole

Table 5: Primary and secondary antibodies and reagents used for immunostaining

Marker/Target	Antibody Details	Company	Catalog No.	City	Country
OCT4 (POU5F1)–gonocyte marker	Rabbit polyclonal	Abcam®	ab19857	Cambridge	UK
PLZF (ZBTB16)–spermatogonial stem cell marker	D-9, mouse monoclonal	Santa Cruz Biotechnology®	sc-28319	Dallas, TX	USA
VASA (DDX4)–germ cell marker	K-14, goat polyclonal	Santa Cruz Biotechnology®	sc-48705	Dallas, TX	USA
SOX9 (Sertoli marker)	E-9, mouse monoclonal	Santa Cruz Biotechnology®	sc-166505	Dallas, TX	USA
STAR (Leydig marker)	Rabbit polyclonal	Elabscience Biotechnology®	E-AB-15419	Wuhan	China
Ki-67 (proliferation marker)	Clone 8K5, mouse monoclonal	Elabscience Biotechnology®	E-AB-22027	Wuhan	China
Caspase-3 (apoptosis marker)	H-277, rabbit polyclonal	Santa Cruz Biotechnology®	sc-7148	Dallas, TX	USA
Secondary (Mouse)	Goat anti-Mouse IgG (H+L), CY3-conjugated	Elabscience Biotechnology®	E-AB-1011	Wuhan	China
Secondary (Goat IgG)	Mouse anti-Goat IgG, FITC-conjugated, affinity purified	Santa Cruz Biotechnology®	sc-2356	Dallas, TX	USA
Secondary (Rabbit)	Goat anti-Rabbit IgG (H+L), FITC-conjugated	Elabscience Biotechnology®	E-AB-1014	Wuhan	China
Secondary (Rabbit)	Cy3–conjugated Affinipure Goat Anti-Rabbit IgG (H+L), polyclonal	Proteintech Group®	SA00009-2	Rosemont, IL	USA
Nuclear counterstain	DAPI (4',6-Diamidino-2-phenylindole)	Sigma-Aldrich®	D9542	St. Louis, MO	USA

OCT4: Octamer-binding transcription factor 4; POU5F1: POU class 5 homeobox 1; PLZF: Promyelocytic leukemia zinc finger; ZBTB16: Zinc finger and BTB domain containing 16; VASA/DDX4: DEAD-box helicase 4; SOX9: SRY-box transcription factor 9; STAR: Steroidogenic acute regulatory protein; Ki-67: Ki-67 proliferation marker; IgG: Immunoglobulin G; FITC: Fluorescein isothiocyanate; Cy3: Cyanine 3; DAPI: 4',6-diamidino-2-phenylindole

Images were taken from the preserved peripheral rim to minimize artifacts from central degeneration. MFI values were reported as field-level immunoreactivity and may reflect both staining intensity and the proportion of marker-positive cells within the analyzed field.¹⁰

Statistical Analysis

Data were presented as mean±SD. For each fetal donor and condition, three tissue fragments were analyzed. Measurements from microscopic fields and two non-adjacent sections were averaged within each fragment to generate one fragment-level value. Fragment-level data were analyzed using a mixed-effects model with culture condition as a fixed effect and fetal donor as a random effect (fragments nested within donor) to account for within-donor clustering. *Post hoc* pairwise comparisons among culture conditions were performed using Tukey's multiple-comparisons test. For selected comparisons involving only two groups, a two-tailed Student's *t* test was used. Normality of model residuals and homogeneity of variance were assessed using Shapiro–Wilk and Levene's tests, respectively. A *P* value<0.05 was considered statistically significant. Analyses were performed using GraphPad Prism 10

(GraphPad Software Inc., USA).

Results

Measurement of Platelet and Growth Factor Concentrations in PRGF

Platelet counts in whole blood were $258.5 \pm 13.38 \times 10^3/\mu\text{L}$, whereas the platelet concentration in PRGF was $1.20 \pm 0.08 \times 10^6/\mu\text{L}$, indicating effective platelet enrichment. The mean PRGF-to-whole-blood platelet ratio was 4.67 ± 0.28 , corresponding to an approximately 4- to 5-fold increase in platelet concentration. Residual leukocytes after PRGF preparation remained below 1×10^6 cells/unit for all preparations, confirming effective leukoreduction.

The concentrations of VEGF, EGF, FGF1, HGF, PDGF-BB, and IGF-1 in PRGF were quantified by ELISA. Mean concentrations were as follows: VEGF, 87.67 ± 3.56 pg/mL; EGF, 262.9 ± 6.10 pg/mL; FGF1, 2567 ± 58.59 pg/mL; HGF, 303.3 ± 5.77 pg/mL; PDGF-BB, 16730 ± 423.0 pg/mL; and IGF-1, 190227 ± 1961 pg/mL (figure 5). Based on these values and the dilution factors, the estimated final concentrations in the 5%, 10%, and 20% PRGF-supplemented media are provided in table 6.

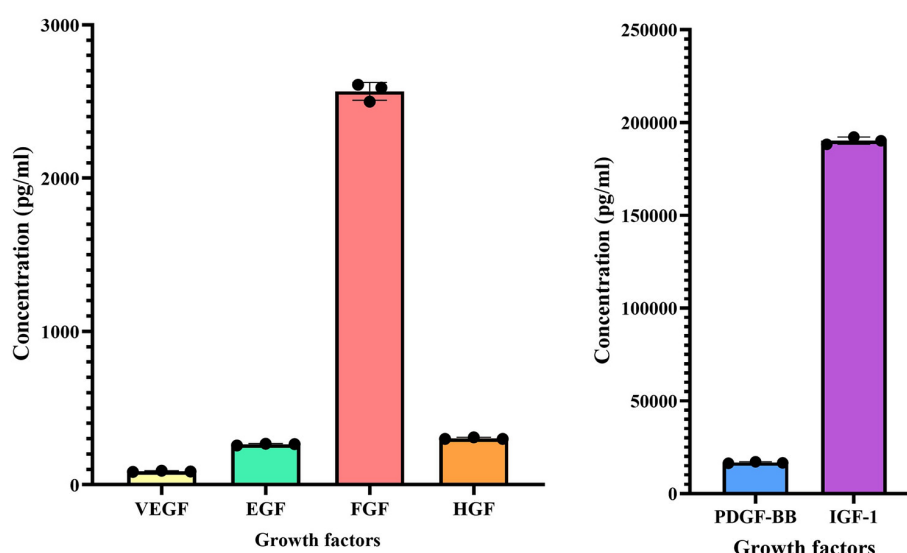


Figure 5: This bar graph shows the concentrations of six growth factors quantified in PRGF, expressed as pg/mL. Data points represent technical replicates (n=3), with each growth factor measured three times from the same pooled PRGF sample, and error bars indicate the SD. PRGF: Plasma rich in growth factors; VEGF: Vascular endothelial growth factor; EGF: Epidermal growth factor; FGF1: Fibroblast growth factor1; HGF: Hepatocyte growth factor; PDGF-BB: Platelet-derived growth factor BB; IGF-1: Insulin-like growth factor 1; SD: Standard deviation

Table 6: Final concentration of growth factors in each PRGF culture group

Growth Factor	Concentration in PRGF (pg/mL)	5% PRGF (pg/mL)	10% PRGF (pg/mL)	20% PRGF (pg/mL)
VEGF	87.67±3.56	4.38±0.18	8.77±0.36	17.53±0.72
EGF	262.9±6.10	13.15±0.31	26.29±0.61	52.58±1.22
FGF1	2567±58.59	128.35±2.93	256.7±5.86	513.4±11.72
HGF	303.3±5.77	15.17±0.29	30.33±0.58	60.66±1.15
PDGF-BB	16730±423.0	836.5±21.15	1673±42.3	3346±84.6
IGF-1	190227±1961	9511.35±98.05	19022.7±196.1	38045.4±392.2

PRGF: Plasma rich in growth factors; VEGF: Vascular endothelial growth factor; EGF: Epidermal growth factor; FGF1: Fibroblast growth factor 1; HGF: Hepatocyte growth factor; PDGF-BB: Platelet-derived growth factor BB; IGF-1: Insulin-like growth factor 1

Baseline Morphology and Cellular Composition of Fresh Fetal Testicular Tissue

Histological and immunofluorescence analyses were performed on baseline (day 0, pre-culture) testicular tissues from 26-week-old male fetuses. The H&E staining (figure 6A) showed dense, well-preserved seminiferous tubules with no obvious signs of necrosis or tissue degeneration. Germ cells exhibited large, round nuclei, and Sertoli cells appeared elongated.

Effect of Culture Medium Composition on Seminiferous Tubule Integrity

After 15 days of culture, all groups exhibited a central degenerative zone marked by disorganized seminiferous tubules, fragmented basement membranes, reduced cell–cell adhesion, numerous pyknotic nuclei, and collapse of adjacent supporting structures, resulting in marked germ cell depletion. In contrast, the peripheral rim remained relatively intact and was selected for further analysis. Within this preserved rim, tubules exhibited intact

basement membranes and a more organized germ–Sertoli cell arrangement.

In the 10% KSR group (figure 6B), tubules displayed partial integrity with a mosaic of preserved and disorganized areas. In the 5% PRGF group (figure 6C), tubules maintained a round-to-oval architecture with well-defined boundaries and radial cellular alignment. In contrast, tissues cultured with 10% and 20% PRGF (figures 6D–E) showed pronounced structural disorganization, widened interstitial spaces, and thinning or collapse of basement membranes.

Quantitative scoring of seminiferous tubule integrity (figure 6F) showed that 5% PRGF yielded a higher proportion of well-preserved tubules (scores 3–4) than 10% and 20% PRGF ($P<0.001$), whereas poorly preserved tubules (score 1) were most prevalent at higher PRGF concentrations ($P<0.001$). Moreover, the percentage of score-4 tubules was higher in 5% PRGF ($23.0\pm2.1\%$) than in 10% KSR ($17.0\pm2.1\%$; $P<0.001$). However, 10% KSR still outperformed 10% and 20% PRGF ($P<0.001$).

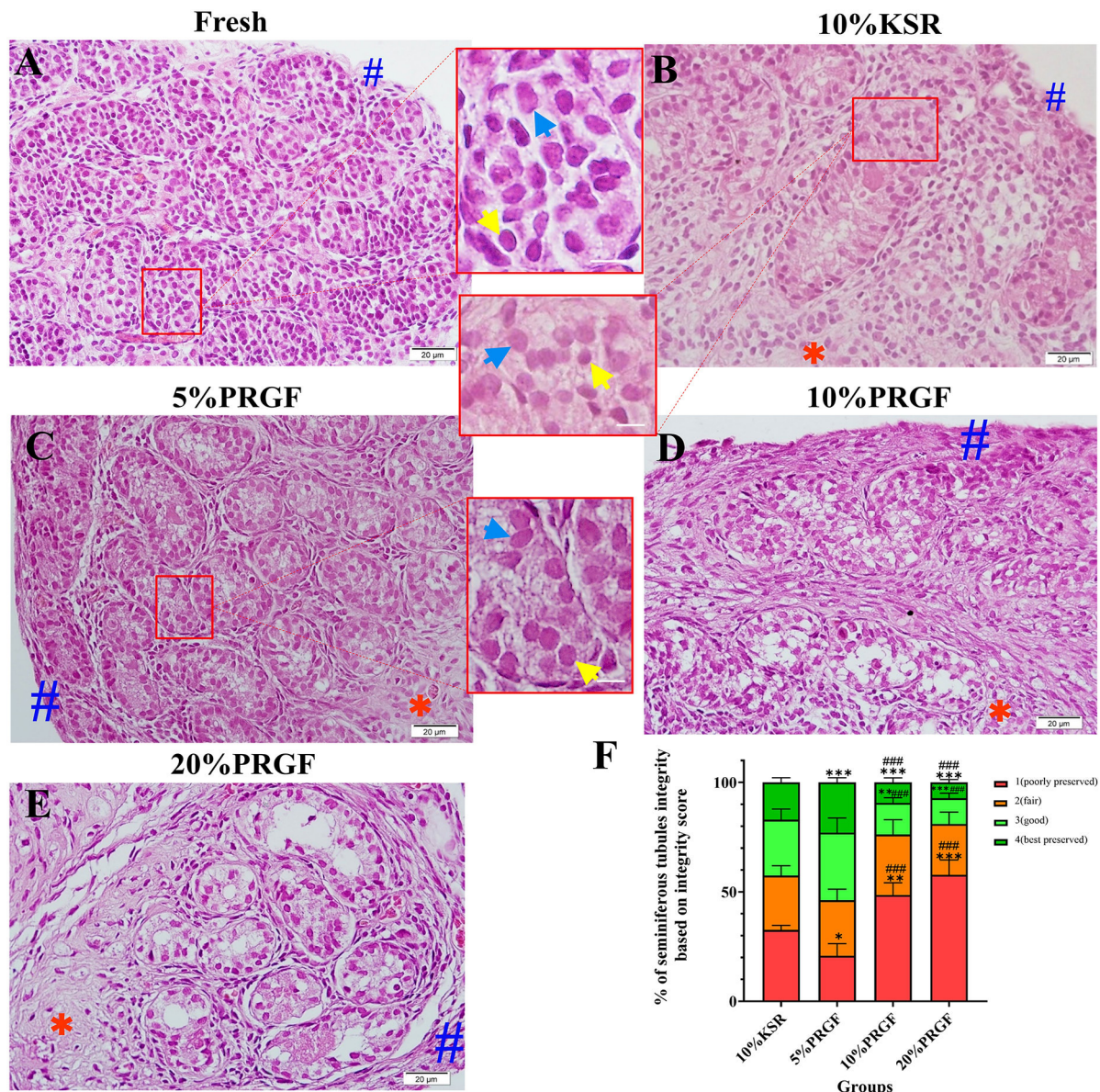


Figure 6: This figure shows H&E staining of fetal testicular tissue and quantitative assessment of seminiferous tubule integrity. (A) Baseline fresh tissue at day 0 shows well-organized seminiferous tubules. After 15 days of culture, tissue cultured with 10% KSR (B) shows areas of disorganization, although some tubules remain relatively intact, whereas tissue cultured with 5% PRGF (C) shows better preservation of tubule architecture. Tissues cultured with 10% PRGF (D) and 20% PRGF (E) show marked structural disorganization. In cultured groups (B–E), central degenerative zones are indicated by red asterisks, and peripheral better-preserved regions are marked by blue hash symbols. Insets show higher-magnification views of the boxed regions. Morphologically identified germ cells and Sertoli cells are indicated by yellow and blue arrows, respectively. Scale bars: 20 μ m (A–E) and 5 μ m (insets); (F) The stacked bar plot shows the percentage distribution of seminiferous tubules across integrity scores. Statistical analysis was performed using a mixed-effects model followed by Tukey's multiple-comparisons test. Statistical significance is indicated as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for comparisons between 10% KSR and PRGF concentrations; ### $P < 0.001$ for comparisons between 5% PRGF and 10% or 20% PRGF. H&E: Hematoxylin and eosin; KSR: KnockOut serum replacement; PRGF: Plasma rich in growth factors

Measurement of the depth of the preserved peripheral rim revealed values of 118.8 ± 41.8 μ m in the 10% KSR group and 227.0 ± 40.7 μ m in the 5% PRGF group, indicating significantly better preservation with 5% PRGF ($P < 0.001$; figure 7).

Immunostaining Analysis: 5% PRGF Supports Improved Preservation of Cellular Markers
Quantitative Assessment of PLZF⁺ and

OCT4⁺/DDX4⁺ Germ Cells: PLZF, a marker of (pro)spermatogonia, showed the highest abundance in the 5% PRGF group among the cultured conditions (13.22 ± 1.56 PLZF⁺ cells per tubule cross-section), predominantly localized along the basement membrane (figure 8A). This value was significantly higher than in the 10% (2.88 ± 1.26) and 20% (3.88 ± 1.26) PRGF groups ($P < 0.001$) and also exceeded the 10% KSR group (11.00 ± 1.65 ; $P = 0.0329$).

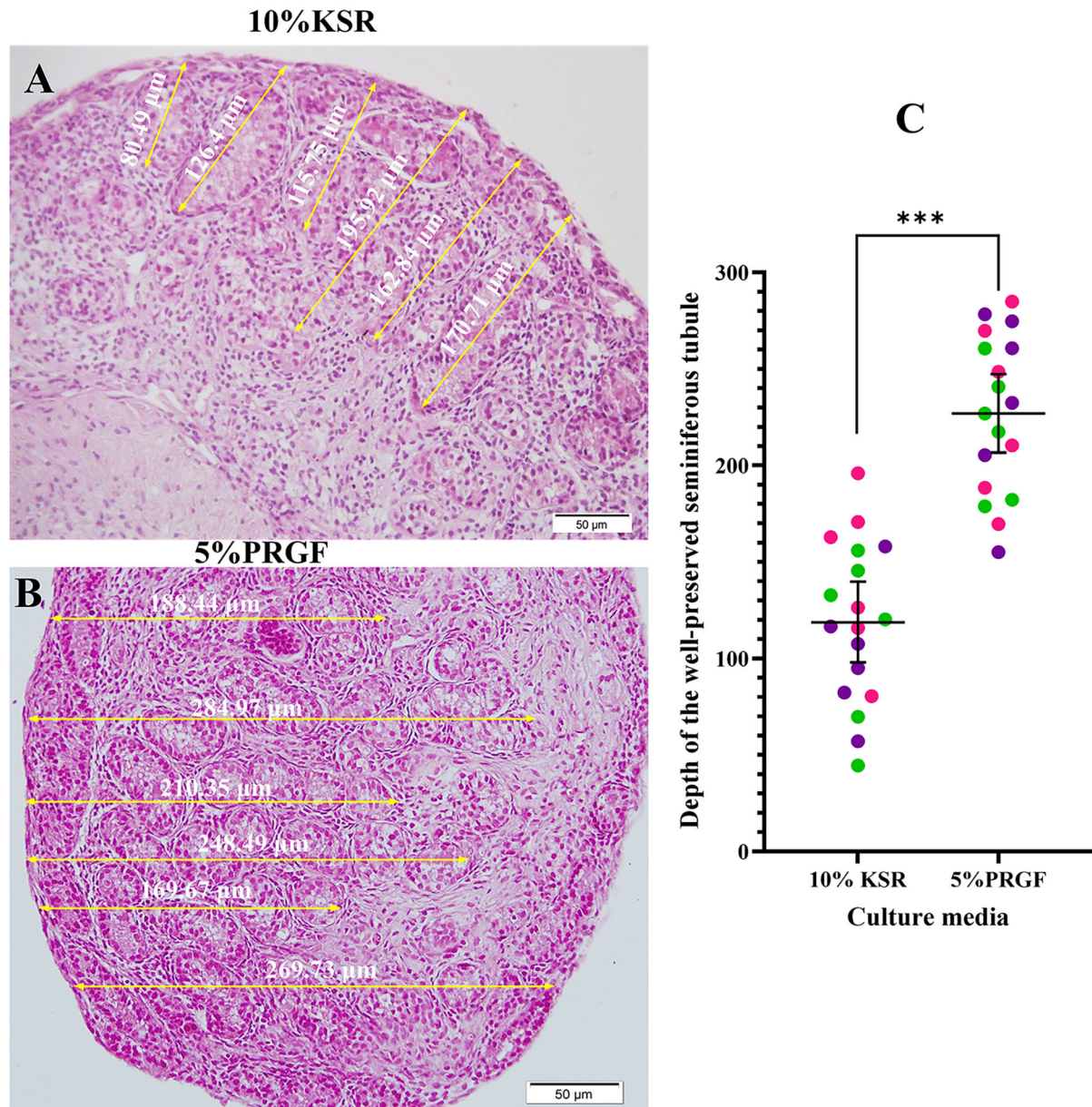


Figure 7: This figure shows the depth of the well-preserved seminiferous tubule rim in tissues cultured with 10% KSR or 5% PRGF. (A, B) Representative H&E-stained images from fetal donor 2 are shown. Scale bar=50 μ m. (C) The graph shows rim depth quantification in micrometers. Dots represent tissue fragments, with three fragments analyzed per donor and two non-adjacent sections examined per fragment; colors indicate individual donors. Data are presented as mean $\pm$ SD. Statistical analysis was performed using a two-tailed Student's *t* test. ****P*<0.001; H&E: Hematoxylin and eosin; KSR: KnockOut serum replacement; PRGF: Plasma rich in growth factors; SD: Standard deviation

The 10%KSRconditionyieldedhigherPLZF $\square$ cell counts than the 10% and 20% PRGF conditions (*P*<0.001). PLZF $\square$ counts in the 5% PRGF and 10% KSR groups were not significantly different from fresh tissue (11.22 $\pm$ 1.92; figure 8B).

OCT4, a marker of germ cells (figure 9A), showed a mean of 7.78 $\pm$ 2.11 OCT4 $\square$ cells per tubule cross-section in fresh tissue. This number was reduced in the 10% KSR group (5.44 $\pm$ 1.01; *P*=0.0372), whereas the 5% PRGF group maintained OCT4 $\square$ cell numbers (8.11 $\pm$ 2.26) comparable to fresh tissue and higher than those in the 10% KSR (*P*=0.0160; figure 9B).

DDX4 immunostaining revealed a mean of 3.89 $\pm$ 1.36 positive cells per tubule cross-section in fresh tissue, which was lower in the 10% KSR group (2.33 $\pm$ 0.87). In the 5% PRGF group, DDX4 $\square$ cell counts (6.44 $\pm$ 1.67) were higher than in both fresh tissue (*P*<0.001) and the 10% KSR group (*P*<0.001; figure 9B), indicating higher DDX4 $\square$ germ cell numbers under this condition.

For OCT4/DDX4 co-staining, total germ cell counts (cells positive for OCT4 and/or DDX4) were the highest in the 5% PRGF group (13.56 $\pm$ 2.60 cells per tubule cross-section), compared with fresh tissue (10.44 $\pm$ 2.79) and 10% KSR (8.78 $\pm$ 1.39).

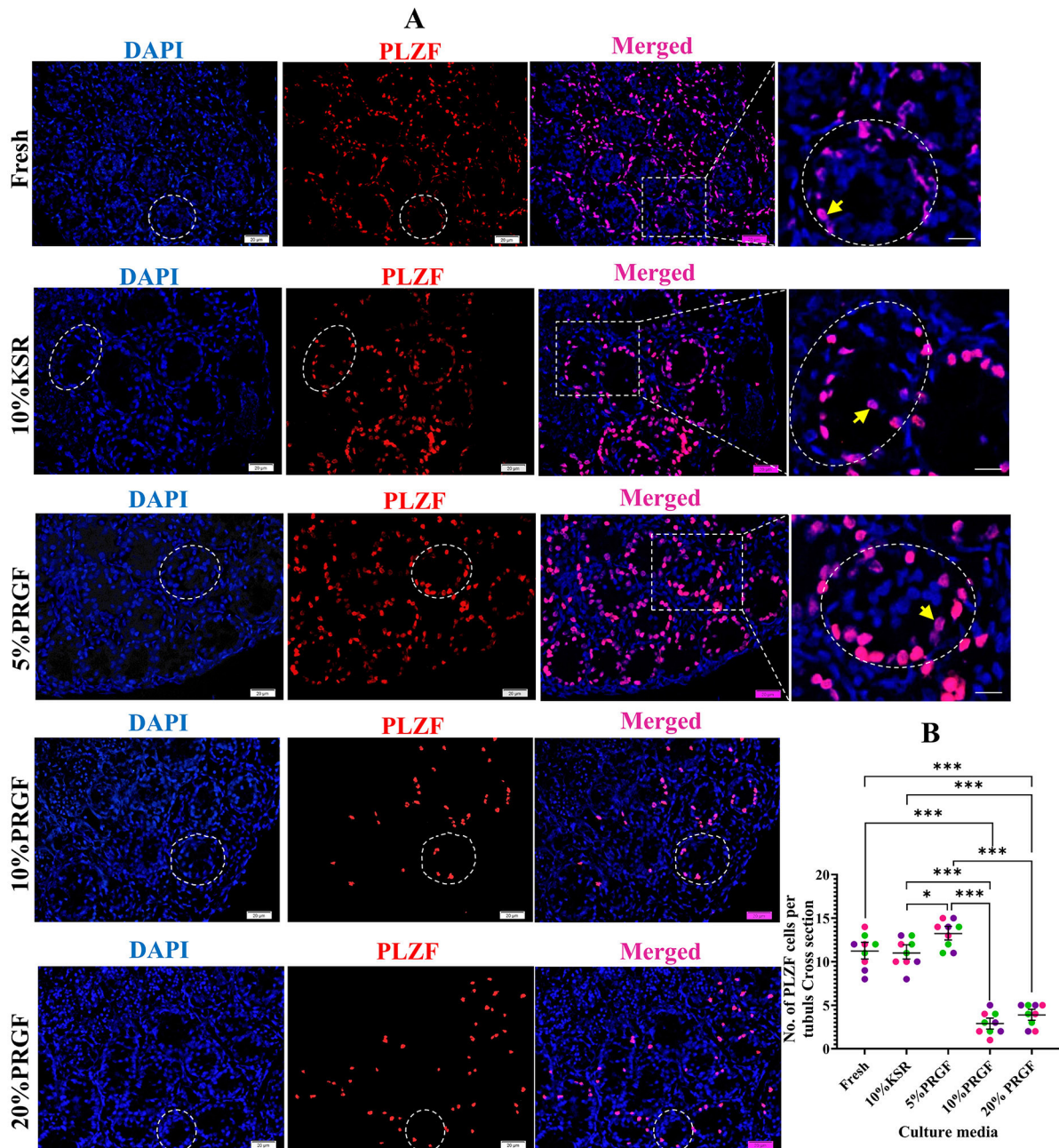


Figure 8: This figure shows PLZF immunofluorescence in fresh and cultured human fetal testicular tissue. (A) Representative images show PLZF-positive (pro) spermatogonia in fresh tissue and after 15 days of culture in media supplemented with 20%, 10%, or 5% PRGF or with 10% KSR. Nuclei were counterstained with DAPI (blue); dashed circles indicate seminiferous tubules, and the yellow arrow indicates a PLZF-positive (pro) spermatogonium. Images are from fetal donor 2. Scale bars: 20 μ m for main images and 10 μ m for insets. (B) The graph shows quantification of PLZF-positive cells per seminiferous tubule across conditions. Each dot represents one tissue fragment, and colors indicate individual biological donors (n=3). Data are presented as mean $\pm$ SD. Statistical analysis was performed using a mixed-effects model followed by Tukey's multiple-comparisons test. Statistical significance is indicated as *P<0.05 and ***P<0.001; PLZF: Promyelocytic leukemia zinc finger; PRGF: Plasma rich in growth factors; KSR: KnockOut serum replacement; DAPI: 4',6-diamidino-2-phenylindole

The 5% PRGF group showed significantly higher total germ cell counts than 10% KSR ($P<0.001$) and was also higher than fresh tissue ($P=0.0251$; figure 9B).

Quantification of SOX9⁺ Sertoli Cells: SOX9 immunostaining (figure 10A) showed that the 5% PRGF condition yielded significantly higher SOX9⁺ Sertoli cell counts per tubule cross-section

(11.11±1.26) than the 10% (3.22±2.33) and 20% (3.66±1.73) PRGF (P<0.001) and 10% KSR (7.22±1.30; P=0.0352). The 10% KSR condition also supported higher SOX9⁺ cell counts than the 10% PRGF group (P=0.0285). Fresh tissue indicated the highest Sertoli cell count (30.56±5.10), significantly exceeding all cultured conditions (P<0.001; figure 10B).

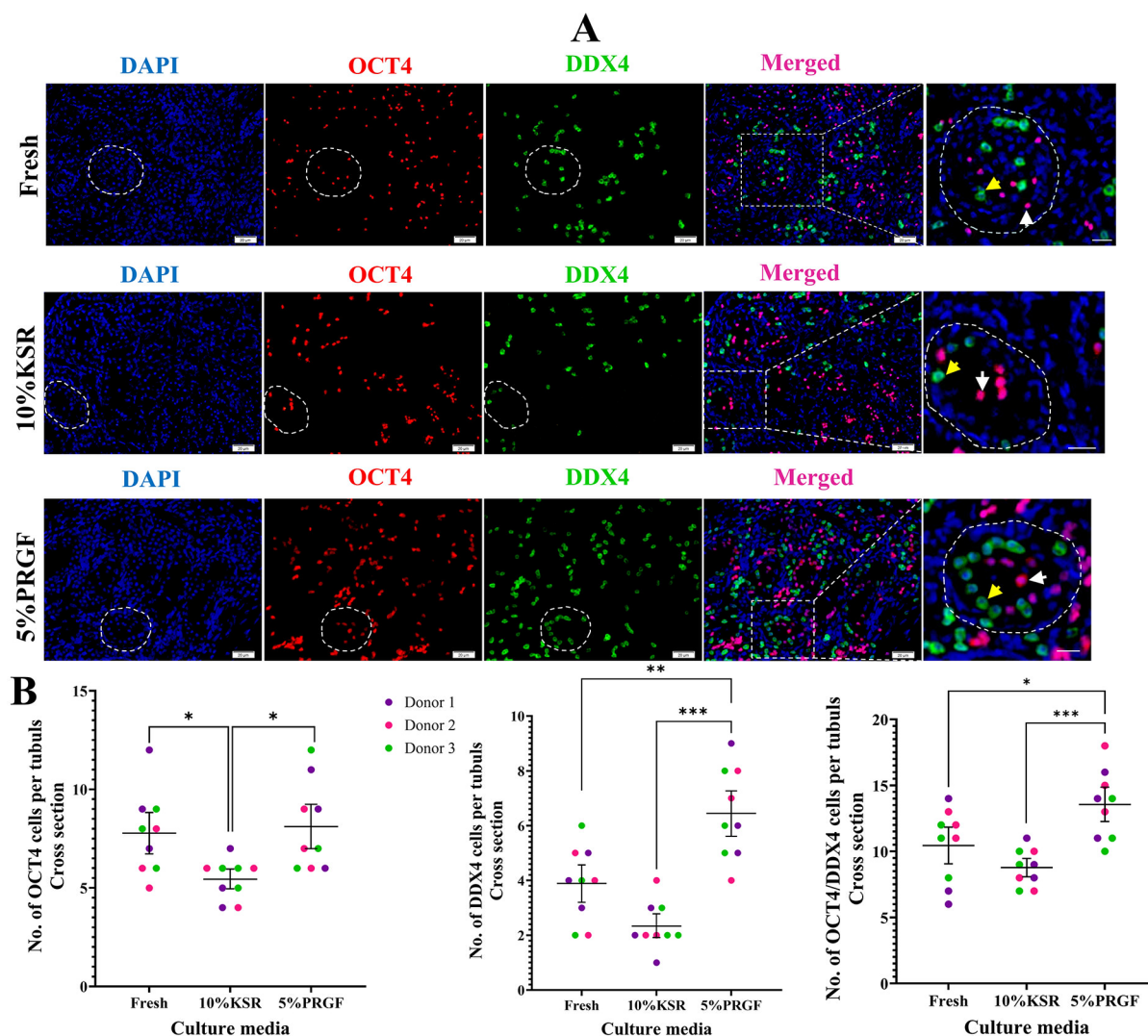


Figure 9: This figure shows co-immunostaining of OCT4 and DDX4 in human fetal testicular tissue. (A) Representative immunofluorescence images show OCT4-positive germ cells (red) and DDX4-positive germ cells (green) in fresh tissue and after 15 days of culture in 10% KSR or 5% PRGF. Nuclei were counterstained with DAPI (blue); dashed circles indicate seminiferous tubules, and insets show higher-magnification views. White arrows indicate OCT4-positive germ cells, and yellow arrows indicate DDX4-positive germ cells. Images are from fetal donor 2. Scale bars: 20 μ m for main images and 10 μ m for insets. (B) The graph shows quantification of OCT4-positive and DDX4-positive germ cells per seminiferous tubule across conditions. Each dot represents one tissue fragment, and colors indicate individual biological donors (n=3). Data are presented as mean $\pm$ SD. Statistical analysis was performed using a mixed-effects model followed by Tukey's multiple-comparisons test. Statistical significance is indicated as *P<0.05, **P<0.01, and ***P<0.001. OCT4: Octamer-binding transcription factor 4; DDX4: DEAD-box helicase 4; KSR: KnockOut serum replacement; PRGF: Plasma rich in growth factors; DAPI: 4',6-diamidino-2-phenylindole; SD: Standard deviation.

STAR Immunoreactivity: The MFI of the STAR signal (figure 11A) was quantified per image field (whole-field MFI) as a measure of STAR immunoreactivity. The 5% PRGF group (14.41 $\pm$ 1.25) showed significantly higher STAR MFI per field than the 10% and 20% PRGF groups (P<0.001) and was also higher than the 10% KSR group (12.47 $\pm$ 1.48; P=0.0121). The 10% KSR group, in turn, exhibited higher STAR MFI than the 10% and 20% PRGF conditions (P<0.001). Fresh tissue showed the highest STAR signal (15.84 $\pm$ 1.58), exceeding that in the 10% KSR group and the 10% and 20% PRGF groups (P<0.001; figure 11B).

Ki-67-Based Evaluation of Proliferation: Ki-67 staining (figure 12A) showed significantly higher Ki-67 $^+$ cell counts per tubule cross-section in the 5% PRGF group (9.00 $\pm$ 1.00 Ki-67 $^+$ cells per tubule) than the 10% (2.55 $\pm$ 1.74) and 20% (2.11 $\pm$ 1.90) PRGF groups (P<0.001) and the 10% KSR group (6.88 $\pm$ 0.78; P=0.0168). It was also higher than fresh tissue (1.33 $\pm$ 1.00; P<0.001). The 10% KSR group exhibited higher Ki-67 $^+$ cell counts than the 10% and 20% PRGF groups (P<0.001) and also exceeded fresh tissue levels (P<0.001; figure 12B). Since Ki-67 was assessed by single staining, the specific identity of the proliferating cells (germ vs. somatic) could not be determined.

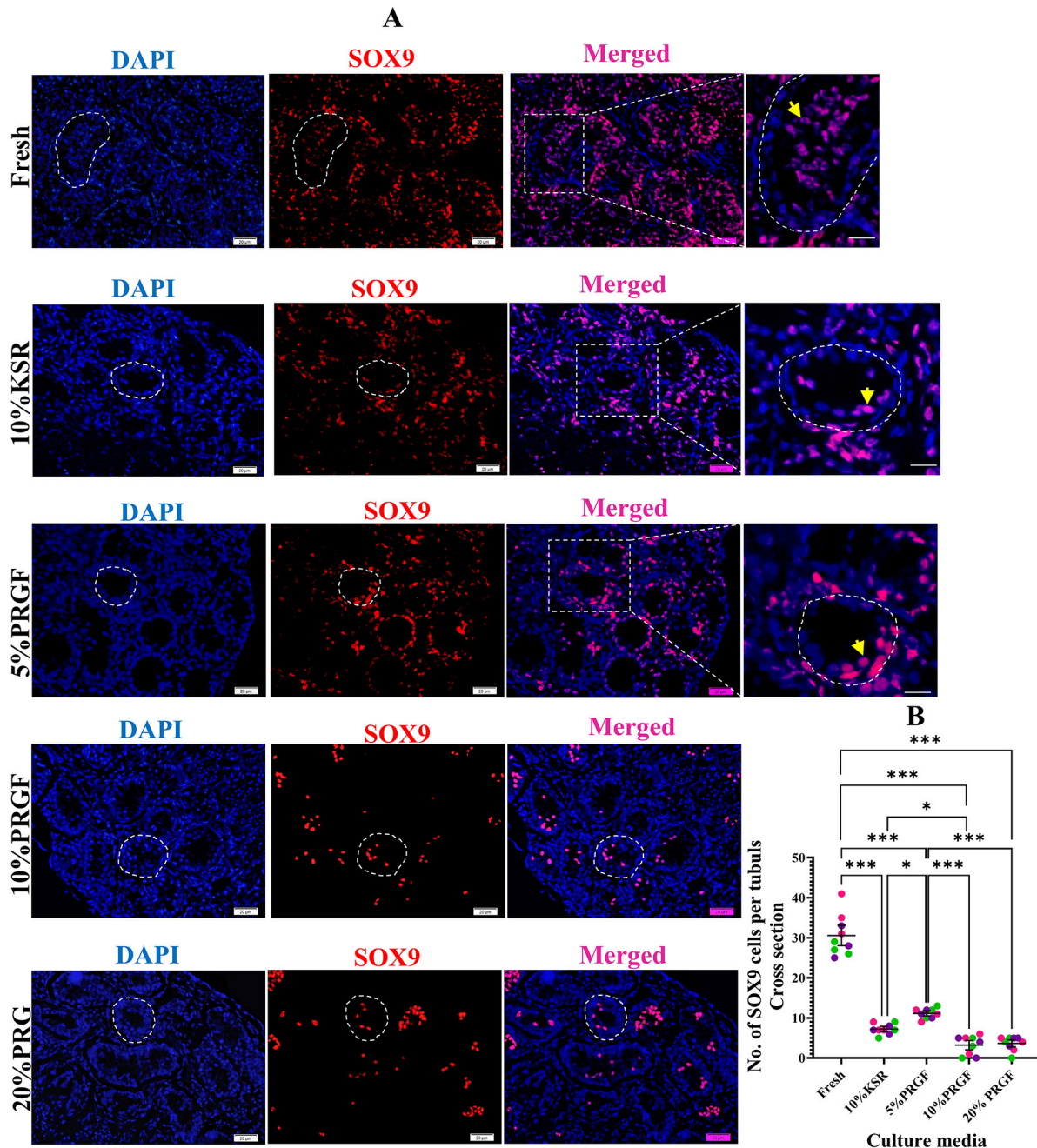


Figure 10: This figure shows SOX9 immunofluorescence and quantification of Sertoli cells in human fetal testicular tissue. (A) Representative images show SOX9-positive Sertoli cells in fresh tissue and after 15 days of culture in media supplemented with 20%, 10%, or 5% PRGF or with 10% KSR. Nuclei were counterstained with DAPI (blue); dashed circles indicate seminiferous tubules, and the yellow arrow indicates a SOX9-positive Sertoli cell. Images are from fetal donor 2. Scale bars: 20 μ m for main images and 10 μ m for insets; (B) The graph shows quantification of SOX9-positive Sertoli cells per seminiferous tubule cross-section across conditions. Each dot represents one tissue fragment, and colors indicate individual biological donors (n=3). Data are presented as mean $\pm$ SD. Statistical analysis was performed using a mixed-effects model followed by Tukey's multiple-comparisons test. Statistical significance is indicated as *P<0.05 and ***P<0.001. SOX9: SRY-box transcription factor 9; PRGF: Plasma rich in growth factors; KSR: KnockOut serum replacement; DAPI: 4',6-diamidino-2-phenylindole; SD: Standard deviation

Total Caspase-3 Immunoreactivity

Caspase-3 immunostaining (figure 13A) showed that the 5% PRGF group (4.66 ± 0.92) exhibited the lowest total Caspase-3 signal among the cultured conditions. The MFI in the 5% PRGF group was significantly lower than in the 10% (18.00 ± 1.57) and 20% (29.62 ± 2.55) PRGF groups (P<0.001) and lower than in the

10% KSR group (7.28 ± 1.63 ; P=0.0109). The 10% KSR group also showed lower total Caspase-3 MFI than the 10% and 20% PRGF groups (P<0.001). Fresh tissue exhibited significantly lower total Caspase-3 immunoreactivity than the 5% PRGF group (1.72 ± 0.58 ; P=0.0032) and the other cultured groups (P<0.001), consistent with baseline levels (figure 13B).

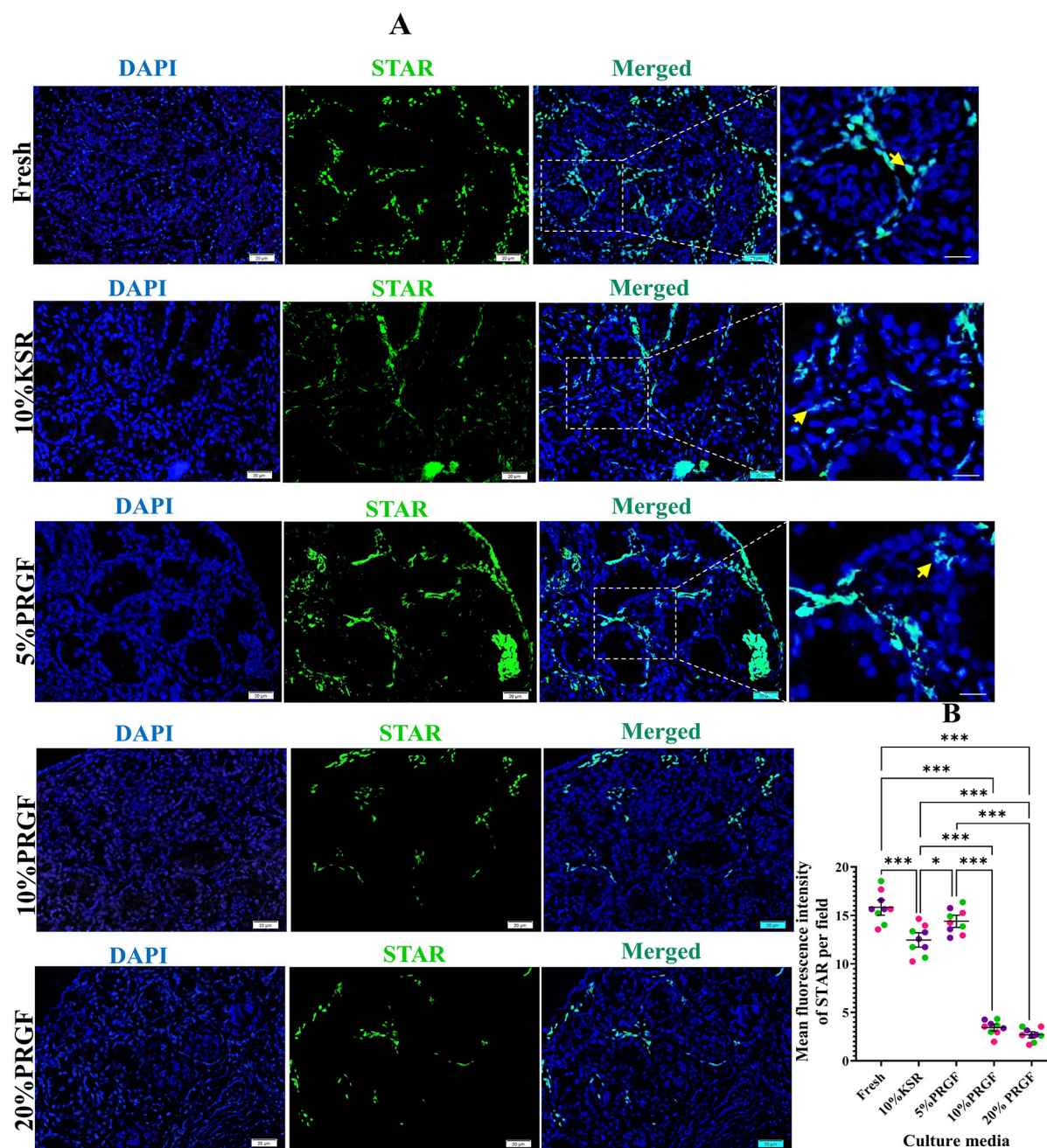


Figure 11: This figure shows STAR immunofluorescence in human fetal testicular tissue. (A) Representative images show STAR-positive Leydig cells in fresh tissue and after 15 days of culture in media supplemented with 20%, 10%, or 5% PRGF or with 10% KSR. Nuclei were counterstained with DAPI (blue), and the yellow arrow indicates a STAR-positive Leydig cell. Images are from fetal donor 2. Scale bars: 20 μ m for main images and 10 μ m for insets; (B) The graph shows quantification of STAR immunoreactivity as mean fluorescence intensity per image field across conditions. Each dot represents one tissue fragment, and colors indicate individual biological donors (n=3). Data are presented as mean $\pm$ SD. Statistical analysis was performed using a mixed-effects model followed by Tukey's multiple-comparisons test. Statistical significance is indicated as *P<0.05 and ***P<0.001. STAR: Steroidogenic acute regulatory protein; MFI: Mean fluorescence intensity; PRGF: Plasma rich in growth factors; KSR: KnockOut serum replacement; DAPI: 4',6-diamidino-2-phenylindole; SD: Standard deviation

Discussion

The findings of the present study indicated that 5% PRGF, a platelet-derived cocktail of multiple growth factors, was the most favorable short-term culture supplement for immature human testicular tissue, supporting seminiferous tubule

preservation, the maintenance of germ and somatic cell markers, higher Ki-67 labeling, and lower total Caspase-3 immunoreactivity. The improved tubule architecture and cellular cohesion observed with 5% PRGF suggested that a more physiologically relevant microenvironment supports tissue integrity.

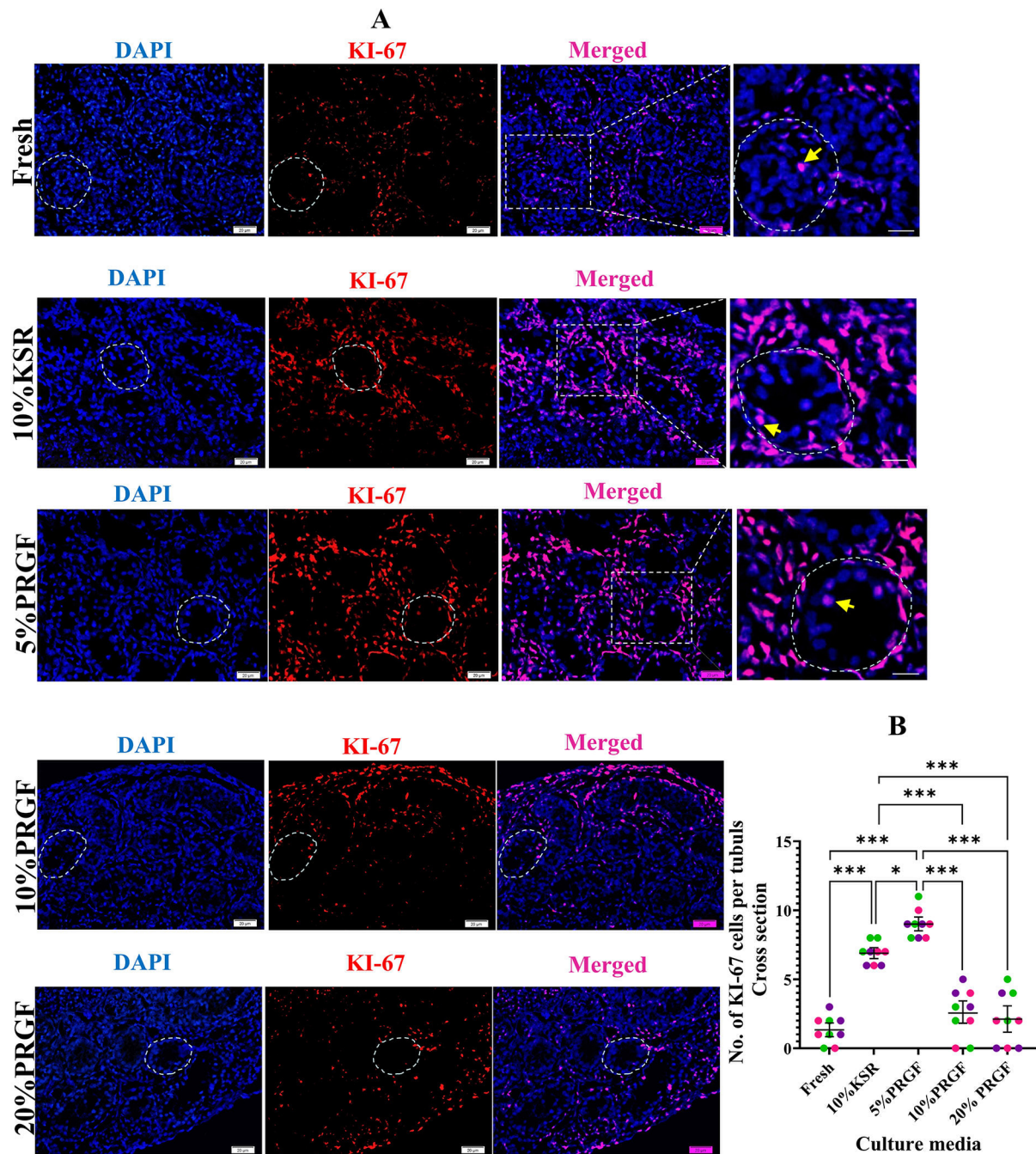
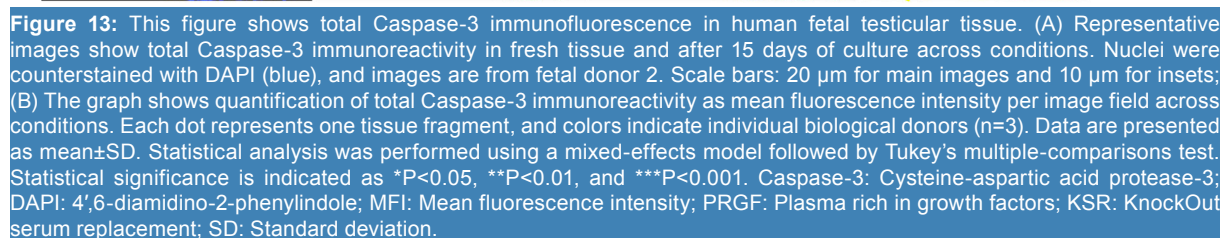


Figure 12: This figure shows Ki-67 immunofluorescence and quantification of proliferating cells in human fetal testicular tissue. (A) Representative images show Ki-67-positive cells in fresh tissue and after 15 days of culture in media supplemented with 20%, 10%, or 5% PRGF or with 10% KSR. Nuclei were counterstained with DAPI (blue), and dashed circles indicate seminiferous tubules. Images are from fetal donor 2. Scale bars: 20 μ m for main images and 10 μ m for insets; (B) The graph shows quantification of Ki-67-positive cells per seminiferous tubule cross-section across conditions. Each dot represents one tissue fragment, and colors indicate individual biological donors ($n=3$). Data are presented as mean $\pm$ SD. Statistical analysis was performed using a mixed-effects model followed by Tukey's multiple-comparisons test. Statistical significance is indicated as * $P<0.05$ and *** $P<0.001$. Ki-67: Ki-67 proliferation marker; PRGF: Plasma rich in growth factors; KSR: KnockOut serum replacement; DAPI: 4',6-diamidino-2-phenylindole; SD: Standard deviation

This finding was consistent with tissue engineering principles and previous evidence that growth factors such as VEGF could promote seminiferous tubule preservation and cell survival *in vitro*.^{23, 24}

The decline in tissue integrity observed at 10% and 20% PRGF suggested that higher concentrations of growth factors might disrupt

cellular homeostasis. Excessive levels of growth factors, including TGF- β or HGF, were reported in other systems to inhibit cellular proliferation or induce apoptosis, potentially through activation of stress-response pathways such as p38 MAPK and the upregulation of cell cycle inhibitors such as p21Waf1 and p27Kip.^{25, 26}



The increase in PLZF⁺ (pro)spermatogonia in the 5% PRGF group might indicate improved maintenance of undifferentiated germ cells, potentially mediated by growth factors, such as VEGF and HGF, which supported germ cell survival and proliferation *in vitro*.^{29, 30}

Consistent with the second-trimester transition from OCT4⁺ gonocytes to VASA/DDX4⁺ prespermatogonia,³¹ 5% PRGF preserved OCT4⁺ cells while increasing DDX4⁺ cells. However, changes in marker expression were insufficient to confirm stage progression. PRP-related growth factor signaling might also enhance spermatogonial proliferation and stemness markers,³² while increased SOX9⁺ Sertoli cells might reflect better Sertoli cell preservation and support for germ cell survival.³³

Similarly, the higher STAR immunoreactivity observed in the 5% PRGF group might suggest improved preservation of STAR-positive interstitial cells and/or steroidogenic marker expression. STAR is essential for mitochondrial cholesterol transport, which constitutes the rate-limiting step in steroidogenesis.³⁴

Lower total Caspase-3 immunoreactivity in the 5% PRGF group reflected a diminished overall signal. However, this finding alone did not signify reduced apoptosis, as the antibody detects both pro- and cleaved forms. Growth factors present in PRGF, including VEGF, PDGF, and HGF, bind their cognate receptors and activate the PI3K–Akt signaling pathway.^{26, 35} This pathway leads to the suppression of pro-apoptotic proteins and the upregulation of anti-apoptotic factors such as Bcl-2 and XIAP,^{9, 36} suggesting that the observed reduction in total Caspase-3 signal may reflect a potential anti-apoptotic effect of 5% PRGF.

Conversely, increased Ki-67 expression in the 5% PRGF group indicated higher proliferative labeling within seminiferous tubules. Growth factors, including EGF, IGF-1, and BMP4, are known to stimulate the proliferation of both germ and somatic cells in testicular cultures.^{37, 38}

Although 10% KSR supported partial preservation of marker-positive germ and Sertoli cell populations, it was consistently inferior to 5% PRGF across the morphological and immunostaining-based endpoints assessed in this study. These findings were consistent with our previous study in immature mouse tissue,⁹ and underscored the limitations of conventional supplements, which might contain xenogeneic components that compromise clinical translatability.

A key strength of this study was the use of rare human fetal testicular tissue as proof-of-concept for PRGF-supported organ culture. However, the small number of donors, short culture duration, limited marker panel, and unassessed batch-to-batch variability of PRGF constrained the interpretability of our results. Larger, longer-term studies incorporating expanded molecular

and functional analyses are required to validate these findings.

Conclusion

Overall, 5% PRGF appeared to be a promising xeno-free supplement for short-term culture of immature human testicular tissue, demonstrating better preservation of tissue architecture and marker-positive cell populations than KSR and higher PRGF concentrations. However, further studies with larger cohorts, extended culture periods, and functional analyses are required to confirm these preliminary findings.

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Authors' Contribution

S.A.M. performed the *in vitro* experiments and contributed to manuscript writing. B.N., A.F., Z.A., and M.M. contributed to study design, obtained funding, supervised the project, and edited the manuscript. S.N.G. carried out *in vitro* experiments, analyzed the data, and contributed to manuscript editing. H.H. and K.H. were involved in data interpretation, validation, statistical analysis, and manuscript review. All authors have read and approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Declaration of AI

The authors declare that they have not used AI-generated work in this manuscript.

Conflict of Interest: None declared.

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