Original Article

Generation of tail bearing sperm-like cells from in vitro spermatogenesis of farming goat testis

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Abstract

In vitro spermatogenesis offers a therapeutic modality for the treatment of male infertility associated with pre-or post-meiotic barriers to the spermatogenesis process. The present study reports the production of tail-bearing sperm-like cells from *in vitro* spermatogenesis of farming goat testis in an air-liquid inter-phase culture. The testicular fragments (1-2 mm^3) were placed on agarose scaffolds at air-liquid interphase and cultured *in vitro* for 60 days. Proliferating germ cell colonies were visible after 45-55 days of culture. Differentiation of cells to spermatogonia and spermatocytes was observed from the 40th day onwards. Tail bearing sperm-like elongated cells, with apical acrosome, were detected from the 45th day onwards. The differentiating germ-line cells were confirmed by stage-specific expression of positive markers (VASA, DAZL, SYCP1, SYCP3, C-Kit, POU5F1 and SOX-2). Histological sections of the cultured testicular tissue further confirmed the presence of tail-bearing sperm-like cells in the lumen of seminiferous tubules which was absent in non-cultured tissue. In conclusion, our study suggests that the air-liquid interphase organ culture might be useful *in vitro* generation of sperm in farming goats. The study could have a significant impact on the livestock management of the farmers and could improve their dependency on orthodox and natural breeding management.

Keywords: Air-liquid inter-phase, *In vitro* spermatogenesis, Organ culture, Germ-line stem cells, Goat, Differentiation

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Introduction

Infertility has been marked as a global problem with an approximation of influencing one out of every eight couples (Thoma *et al*., 2021). Broadly, 40-50% of the infertility cases are concomitant to male factors (Mehta *et al*., 2016; Szkodziak *et al*., 2016). Spermatogenic failure is pronounced as a foremost cause of infertility (~23%) in adult males (Kumar and Singh, 2015). To treat this medical condition diverse approaches have been exercised, however, the recovery rate from infertility remains a challenge. Regular treatments of this pathological ailment include supplementation or substitution of androgen and testicular prosthetic graft yet both the practices have been associated with complications (Bodiwala *et al*., 2007; Chen *et al*. 2016; Chen *et al*., 2019; Caires *et al*., 2012). Although researchers have been utilizing the process of xenograft using testicular tissue which has resulted in fertile sperms (Oatley and McLean 2005; Khadivi, and Shams-Esfandabadi 2014), the process is again associated with certain limitations, which include immunogenic rejection, inflammation and allergic reaction (Bodiwala *et al*., 2007; Chen *et al*. 2019; Schlatt *et al*., 2010).

Over the past decade, transplantation of germ-line stem cells (GS) has surfaced as a potential alternative for male fertility. GS cells have the capability to retain their spermatogenic capacity and, upon testicular transplantation, can repopulate the bare seminiferous tubules of infertile males to impart fertility (Duff Putu *et al*., 2016). Researchers have exhibited successful isolation of GS cells from both neonatal and adult testes (Jung *et al*., 2010; Kanatsu-Shinohara *et al*. 2004; Ning *et al*., 2012). The GS cells thus have the prospective for treating male infertility issues as well as impaired spermatogenesis. However, testicular transplantation of GS cells fails to cure male infertility in cases where the infertility is correlated with pre-or post-meiotic barriers associated with the process of spermatogenesis which accounts for 20-25% of spermatogenic disorders in humans (Sofikitis *et al*., 2005; Poongothai *et al*., 2009). In this regard, *in vitro* spermatogenesis could be a possibility in the generation of spermatids from GS cells. Moreover, despite all the efforts undertaken in optimizing numerous culture media and stimulating factors to accomplish an effectual *in vitro* environment, it remains a challenge (Yokonishi *et al*., 2012).

Recently, a new method was proposed by Sato *et al*., (Sato *et al*., 2012) for *in vitro* spermatogenesis from isolated spermatogonial stem cells (SSCs). This method implicated the transplantation of SSCs into the seminiferous tubules of germ cell-depleted testes, followed by organ culture (Sato *et al*., 2011; Song and Wilkinson 2012). The study demonstrated the migration of the transplanted SSCs into the basement membrane of the seminiferous tubule, which underwent proliferation after some days followed by differentiation of a few cells into elongated spermatids after two months. Yet, under the influence of the designated culture system, spermatogenic cells did not advance beyond the round spermatid stage.

More recently, GS cells have been successfully differentiated into round spermatids via an air-liquid interface system (Vardiani *et al*., 2020; Vermeulen *et al*., 2017). Various factors including media, growth factors, temperature and anti-retinoic factors have been analyzed to attain optimal culture conditions (Tetsuhiro *et al*., 2012; Sato *et al*., 2011). However, the inability of spermatogenic cells to convert into healthy and motile sperm has remained unaccomplished. *In vitro* differentiation of farming goats, SSCs have been reported to produce motile sperms (Deng *et al*., 2017; Yokonishi *et al*., 2013) but the rate of sperm production is much less.

In the present study, the organ culture method from farming animals is further refined and encompasses the proliferation of SSCs and their further development leading to healthy spermatids with a high production rate. The study could have a significant impact on the livestock management of farmers and could improve their dependency on orthodox and natural breeding management.

Materials and Methods

Air-liquid inter-phase organ culture: Testis pairs were collected from pre-pubertal farming goat (*Capra aegagrus hircus*), 1-2 months old, brought from a slaughterhouse. Testes with attached epididymis were collected in PBS having a 1% combination of penicillin, streptomycin and amphotericin (Himedia- A002A), transported to the laboratory and maintained within 1-2 h at 37 °C. The testes were then washed with PBS and the outer layer of tissues and epididymis were aseptically removed. The animal studies were performed as per the guidelines of the Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA), India. The experimental protocols had been approved by the Institutional Animal Ethical Committee of the National Institute of Technology Rourkela India.

Organ culture was performed at the air-liquid interface by a previously described method (Sato *et al*., 2011; Yokonishi *et al*., 2013) with modifications. Briefly, the testes were decapsulated, tunica albuginea and visible connective tissues removed and they were cut into small fragments (approximately 1–2 mm^2) and placed on a low melting agarose matrix (1.5%; w/v). The testis tissue fragments were submerged with culture media up to half to four-fifths of the height in a six-well culture plate (Fig. 1) and kept in a humidified atmosphere of 5% CO_2 at 37 °C. The culture medium composition was α minimum essential medium (α-MEM, Himedia: AL080) supplemented with nucleosides, 10% FBS (Himedia: RL811), 0.04% (w/v) AlbuXL (Himedia: TC 265) and antibiotics 1% combination of penicillin, streptomycin and amphotericin. The organ culture was maintained over 60 days with tissue fragments positioned at the air-liquid interface while changing media once a week. The cultured tissues were monitored once in five days for colony formation using an inverted phase-contrast microscope (Olympus, Japan) equipped with a high sensitivity camera (Olympus, Japan) operated on Q-capture pro-7 software.

Morphometry analysis of colony assessment: Images of cultured testis fragments were taken on the 35th, 40th,

45th, 50th, 55th, and 60th day for gross morphologic evaluation and quantified using the ImageJ software (National Institutes of Health, USA). Different types of cells and colonies were identified according to their morphological character (Santos Nassif Lacerda *et al*., 2013); (Osuru *et al*., 2014). Further, meiotic progression was observed which corresponds to the assumption that spermatocytes start meiosis at around the 35th day of the culture. The size and shape of the identified cells were compared as per the adopted method and confirmed for spermatocytes and spermatozoa (Santos Nassif Lacerda *et al*., 2013); (Osuru *et al*., 2014).

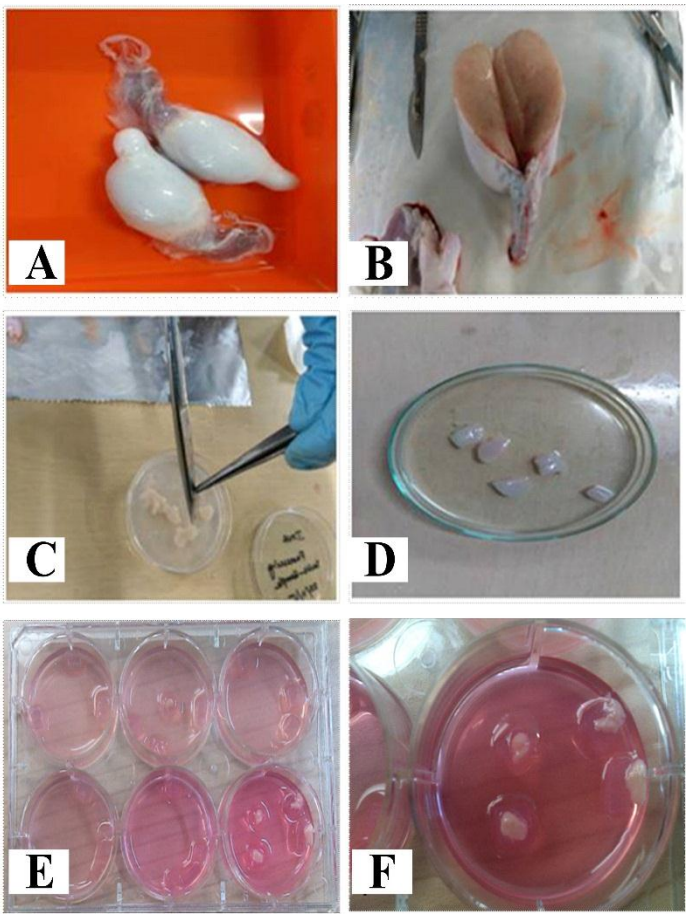

Figure 1 Various steps followed by air-liquid interphase organ culture from testis tissue.

Histology of organ cultured tissue: The organ cultured testicular tissues were harvested after the 40th, 45th, 50th, 55th, and 60th day of culture. The weight of the testes fragment was measured to an accuracy of ± 0.1 mg using a digital weighing machine (Wensar Instruments, India). The testis was then fixed in Bouin's fixative, kept for 24 h, and processed for paraffin embedding as per the standard protocol. Paraffin-embedded tissue was sectioned into small pieces of 5-7 µm thickness by rotary microtome (Scientific instrument, India) and mounted on glass slides. The sections were deparaffinized and then stained with Hematoxylin-Eosin and were analyzed under a bright-field microscope (Bhaskar *et al*., 2017). A hundred sections of testis were randomly selected from each group for histopathological and morphometric measurements using an image analysis system (Bhaskar and Mohanty, 2014) (Image J Software).

Analysis of gene expression: The total RNA was extracted from the cultured testis fragment by the TRIzol (Invitrogen™: 15596026) method and DNase I treatment (Invitrogen™: 18047019) was carried out according to the manufacturer's protocol prior to cDNA synthesis. Genomic DNA was removed during RNA isolation prior to cDNA synthesis (Kumar *et al*., 2015). RNA quantification and purity were examined using a spectrophotometer (Multiskan-Go, Thermo-Fisher). First-strand cDNA was synthesized from the High-Capacity cDNA Reverse Transcription Kit (Invitrogen™: 4368814) as per the manufacturer's instruction. Gene amplification analysis was performed using the gene-specific primers listed in Table 1. The cDNA products were amplified using PCR master mix (AmpliTaq Gold™ 360 Master Mix, Applied Bio-systems) according to the manufacturer's protocol. The PCR amplification consisted of an initial denaturation for 7 mins at 95 °C, followed by 30 cycles of 15 secs denaturation at 95 °C, annealing for 60 s at the desired temperature, and extension for 30 s at 72 °C. The final extension step was carried out at 72 °C for 7 mins. The PCR reactions were performed on five different samples in duplicates.

The different types of cells were analyzed with a positive marker of pluripotent cells (Conrad *et al*., 2014) (VASA, SOX2, POU5F1, and C-kit), primary spermatogonia (DAZL SYCP1, SYCP3) and spermatocyte (SYCP1, SYCP3) at different time points. RNA from the testis was used as a positive control. The

GAPDH marker was used as an internal control. The PCR amplicons were analyzed in a 0.8% agarose gel electrophoresis with 0.5 µg/ml of ethidium bromide and image analysis software.

Statistical Analyses: Each experiment was statistically analyzed using Prism 5 statistical software and reported as a final result. All values were represented as Means ± SEM. Data was analyzed by one-way Analysis of Variance method (Repeated measure ANOVA) together with post-test (Dunnett test). Significant values were given depending on the values obtained (p <0.05, p <0.01, p <0.001).

Table 1 Details of primer pairs used for RT-PCR analysis

Gene Name	Strand	Sequence (5′→ 3′)	Amplicon size (bp)	Annealing Temperature
gDAZL	Forward	GCCAGCACCCAGTCTTCTT	539	54
	Reverse	AACTCCTTTGTTCCCCAGCA		
gc-Kit	Forward	GACTGAAGGAGGCACTTACACA	214	54
	Reverse	CCCAACAGGAACAGAACACC		
gSYCP1	Forward	GGAATGAACTGGAATCTGTGAG	189	51
	Reverse	GCTGCTTGCTTTCTGCTGT		
gSYCP3	Forward	AAGTCTGCGAAACCATCCGT	521	52
	Reverse	TGATTCTTCTCCAAGTCCTCCA		
g POU5F1	Forward	AGTGAGAGGCAACCTGGAG	332	53
	Reverse	TGACAGACACCGAGGGAAA		
gGAPDH	Forward	GCCGTAACTTCTGTGCTGTG	234	53
	Reverse	CTTCCCGTTCTCTGCCTTG		
gSOX2	Forward	ATGATGGAGACGGAACTGGA	784	52
	Reverse	TGGAGTGGGAAGAAGAGGT		
gVASA	Forward	TGGTAGTGGAAGTGGACGAG	152	54
	Reverse	CAGGTGGAGGAGGTGGTATG		

Results

Phenotypic Characterization: During the organ culture, the weight of the testicular tissue gradually decreased by 18-24% (p<0.05) on the 40th day of culture. The drop-in organ weight increased to nearly 55-60% of cultured tissue on the 60th day of culture. The reduction in organ weight was due to the dispersion of cells away from the tissue. The first evidence of cell dispersion was observed after the 35th day of culture. It was observed that the dispersed cells were a mixed population of cells including somatic cells, germ cells and spermatogonia (Fig. 2: A). The putative germ cells were aggregated in different forms of colonies such as single, clusters, circular to the oval and mulberry shaped after the 40th day and continued to grow during the culture period (Fig. 2; A-F). The putative germ cell colonies had unclear borders and were found on the surface of agar scaffolds (Fig. 3; A-F). The putative germ cell colonies were increased in mulberry-shaped or rosette-shaped colonies from a single cell (Table 2). The colonies were hanging on a weak physical connection to the feeder monolayer by a stalk; on the other hand, cultures with a mixed population of somatic cells were confluent (Fig. 2; B). The morphology of colonies was of three types, mainly cluster, radial, and mulberry colonies. The size of the cluster colonies significantly altered during the 50th to 60th day, whereas radial and mulberry colonies did not change significantly from the 40th day to the 50th day but significantly decreased on the 60th day of culture. The number of radial and mulberry-type colonies was fewer than cluster colonies on the 40th to 50th day of culture. Table 2 summarizes the mean areas occupied by the colonies along with their numbers.

The morphology of various germ cells appearing during organ culture could be divided into XII stages, Stage I: spermatogonia were seen as round or oval-shaped cells with condensed chromatoid bodies and no evidence of pre-acrosomal granules. Spermatogonia formed one or more pre-acrosomal granules, which were present near to nucleus in Stage II. Chromatin rim was present in the inner nuclear membrane (Fig. 3; G). Intermediate spermatogonia (Stage III) were smaller with big pre-acrosomal granules and contained more heterochromatin compared to Stage II. Intermediate spermatogonia had a triangular-shaped acrosome (Stage IV) (Fig. 3; H). The cells were comparatively larger than in the previous stage (Stage III). The shape of the acrosome in these cells was spread over the surface of the nucleus (Stage V) and displayed dark stains near the nuclear body (Fig. 3; I). Stage VI showed acrosome size and formed a cap-like structure while covering at least half of the spermatocyte nucleus (Fig. 3; I) with acrosome occupying more than one-third of the nucleus. A dark heterochromatin appeared as the lining of the inner nuclear membrane of the nucleus in Stage VII (Fig. 3; I). The acrosome was observed with maximal spreading over the nucleus and exhibited a polarity towards the basement membrane (Fig. 3; I). The spermatocytes had a thread-like appearance of chromatid of leptotene nucleus. The sperm head was curved at the dorsal side and was flat from the ventral side (Fig. 3; J). The leptotene-like spermatocytes changed into an elongated and flattened sperm head, which was elongated from the dorsal surface of the head (Fig. 3; K). The shape of the sperm appeared to be

more elongated and slender compared to stage X. The sperm head appeared to be at full length due to acrosome expansion towards the dorsal surface of the head region (Fig. 3; L). The acrosome covered the entire head region of spermatids and the elongating tail appeared at stages XII (Fig. 3; M).

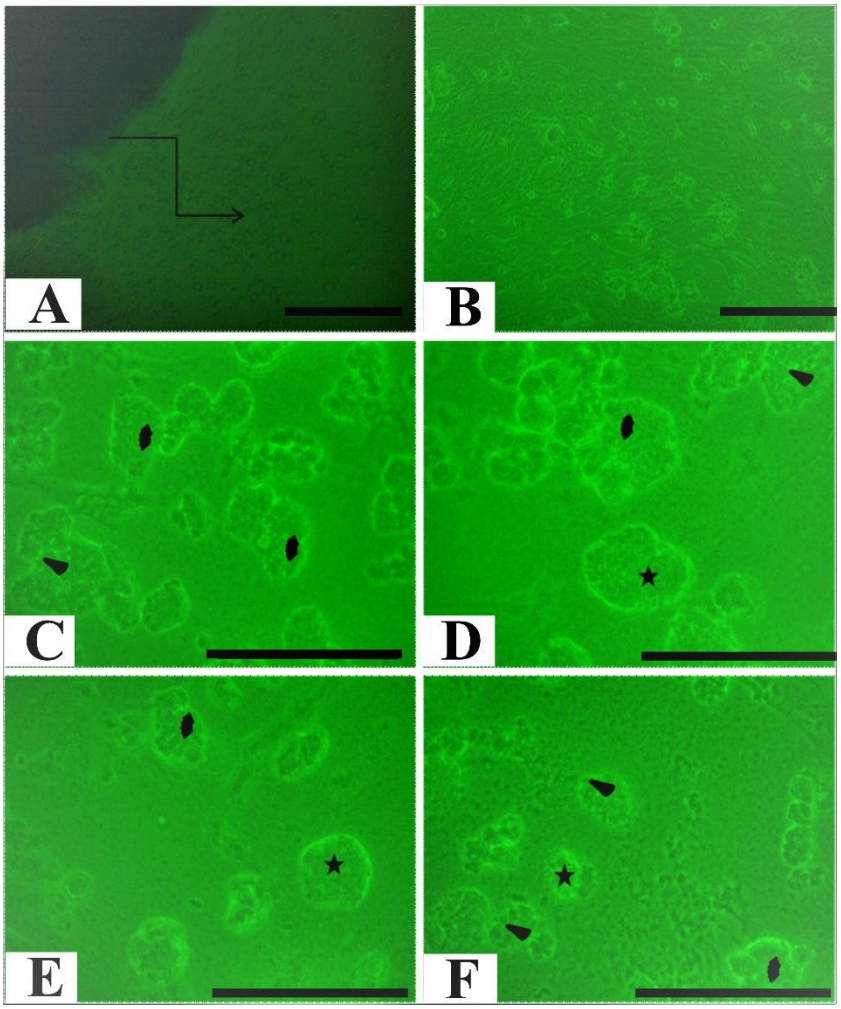

Figure 2 Stereomicroscopic view of cultured adult goat testicular cells. (A) Goat testis tissue fragments on culture day 35, (B) testis tissue fragments on culture days 40, and (C-F) testis tissue fragments on culture days 40 to Day 60 respectively. Note Cluster colony ( ), radial colony ( ) and mulberry colony ( ). Scale bars: 50 µm.

Table 2 Morphometry of cell colonies observed during in air-liquid interface culture of the testicular fragment.

Colony Morphology	Day 40	Day 45	Day 50	Day 55	Day 60
No. of Colony / LPF					
Cluster	19.5±2.1	13±2.9*	9.28±2.4**	15±0.18*	16.21±3.6
Radial	3.3±0.8	4±1.2	2.24±.04**	3.33±0.24	2±0.12*
Mulberry	4.8±1.2	6.8±1.87*	3.73±0.97*	3.33±0.81*	3.8±0.98
Area of Colony perimeter (µm)					
Cluster	5371.42±279	5705.33±482	6769.58±286*	5623±229*	2969.76±107**
Radial	3235.17±174	2777.66±97	3922.99±162**	3598.08±258	2202.6±197**
Mulberry	8152.11±597	8595.44±781	8341.45±721	10691.5±869**	8415.65±586*

*$P < 0.05$, **$P < 0.01$ significantly different compared to respective rows.

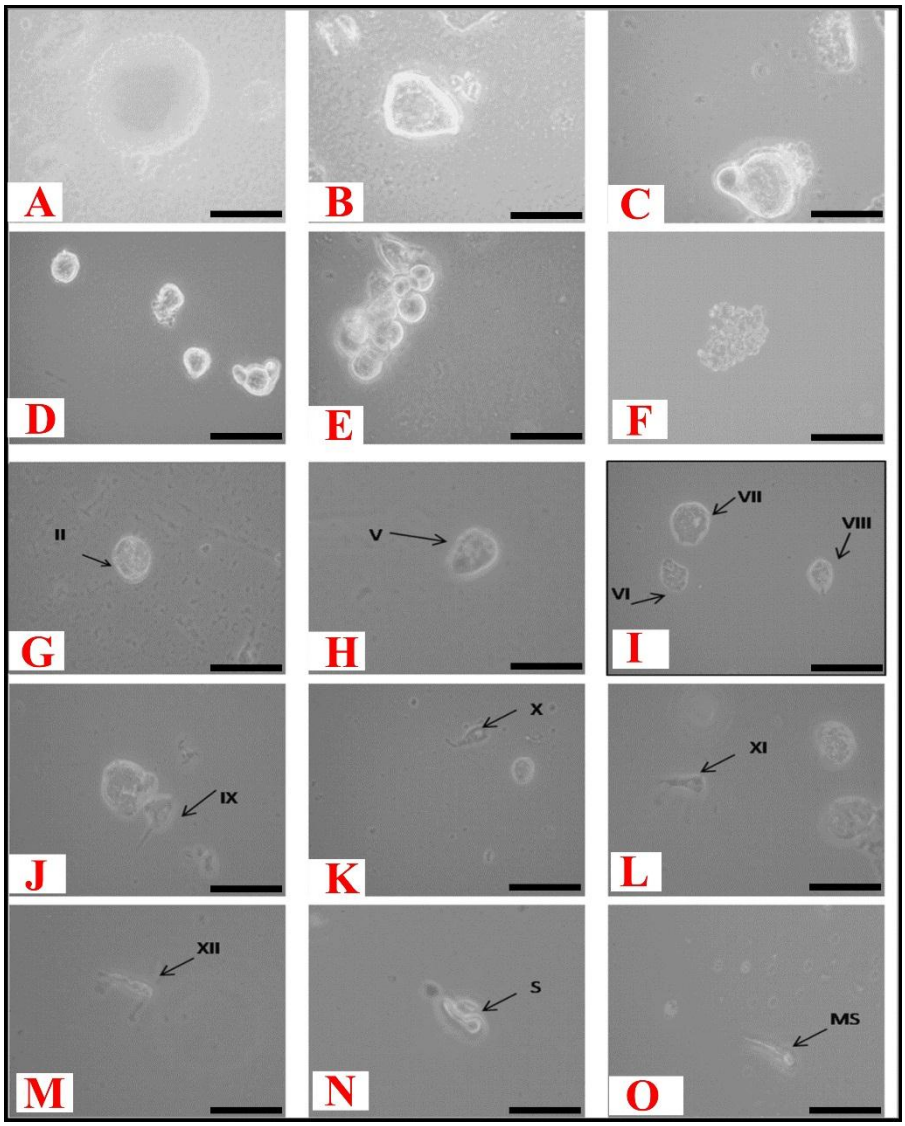

Figure 3 Stereomicroscopic view of cultured adult goat testicular cells under air-liquid interface culture. After 35 days of culture, the putative GS cells were seen as single (A- B), paired (C), aligned (D), and cluster (E-F). At the onset of culture, spermatogonia were round cells with a spherical nucleus, a high nucleus: cytoplasm ratio, and many cytoplasmic inclusions, mostly concentrated at one side of the cell. Scale bars- 50 µm.

Histopathology of organ cultured testicular tissue: A significant reduction in the diameter of seminiferous tubules ranging from 18 to 36% was observed. The seminiferous tubules showed a small lumen with mixed populations of spermatogonia, mostly localized in the center of the lumen. There were a few germ cells with disrupted alignment and debris accumulated in the lumen. Sperm-like cells with a tail-like formation were present in the lumen of the seminiferous tubule during the 40th to 60th day but were absent in the control testes (Fig. 4).

Analysis of Gene Expression: To investigate the properties of the colony-forming cells or putative germ cells, we examined the presence of the VASA, POU5F1 and SOX2 gene transcripts in cultured cells (Fig. 5). POU5F1 gene was expressed on the 50th day and 55th day, respectively but SOX2 was expressed on the 45th-55th day. RT-PCR results confirmed that transcription of the germ-line specific gene such as VASA, POU5F1 and SOX2 occurred in the experimental group of cells. A similar gene expression profile was also observed in the case of control testes. VASA gene transcript was detected at all stages except for the 60th day, whereas the SOX2 gene was expressed in all the stages of the culture. C-kit was highly expressed on the 45th day and 50th day of culture but was not detected on the 40th day and 60th day, respectively (Fig. 5). VASA was also expressed at all the stages of culture with maximum expression recorded on the 55th day except the 60th day where negligible expression was observed. DAZL gene was expressed at all stages except on the 60th day. It was also evaluated that the SYCP1 gene was expressed on the 40th-60th day but the expression of SYCP3 was witnessed on the 40th, 50th and 60th day respectively. The control testis expressed the transcription factors at the study point (Fig. 5).

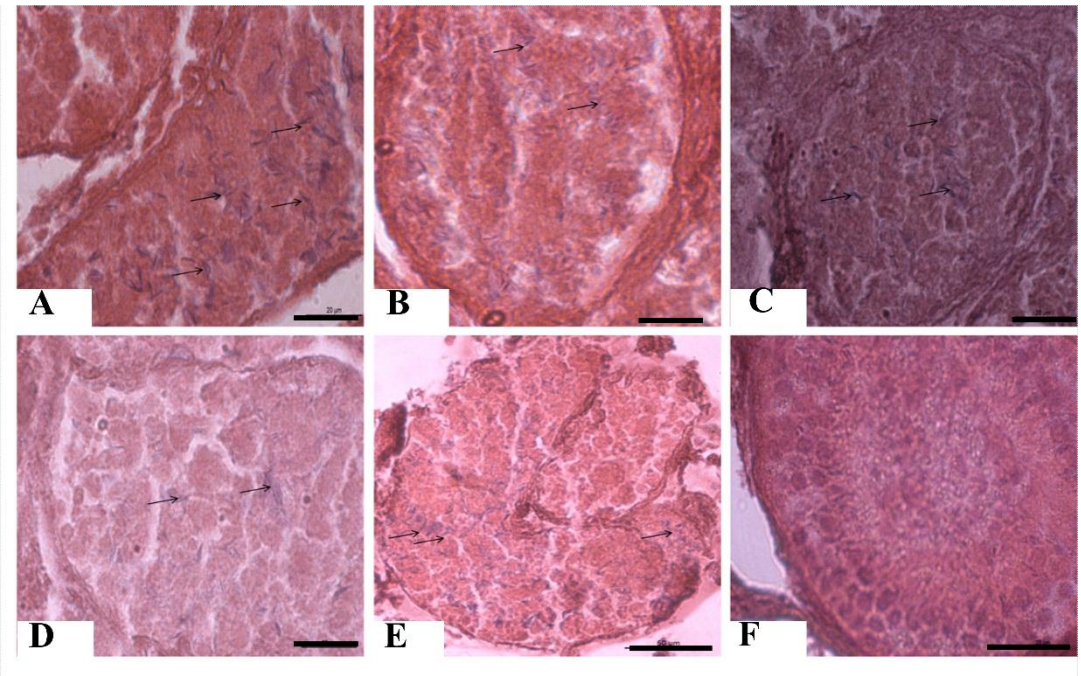

Figure 4 Histological sections of goat testes stained with Hematoxylin and Eosin. The different sizes of spermatogonia and the absence of central lumen are noticeable. (A) Goat testis tissue fragments on culture day 40, (B) testis tissue fragments on culture day 45, and (C-F) testis tissue fragments on culture Day 50 to Day 60, respectively. Note tail-bearing sperms-like cells (↗). Scale bars: 20 µm.

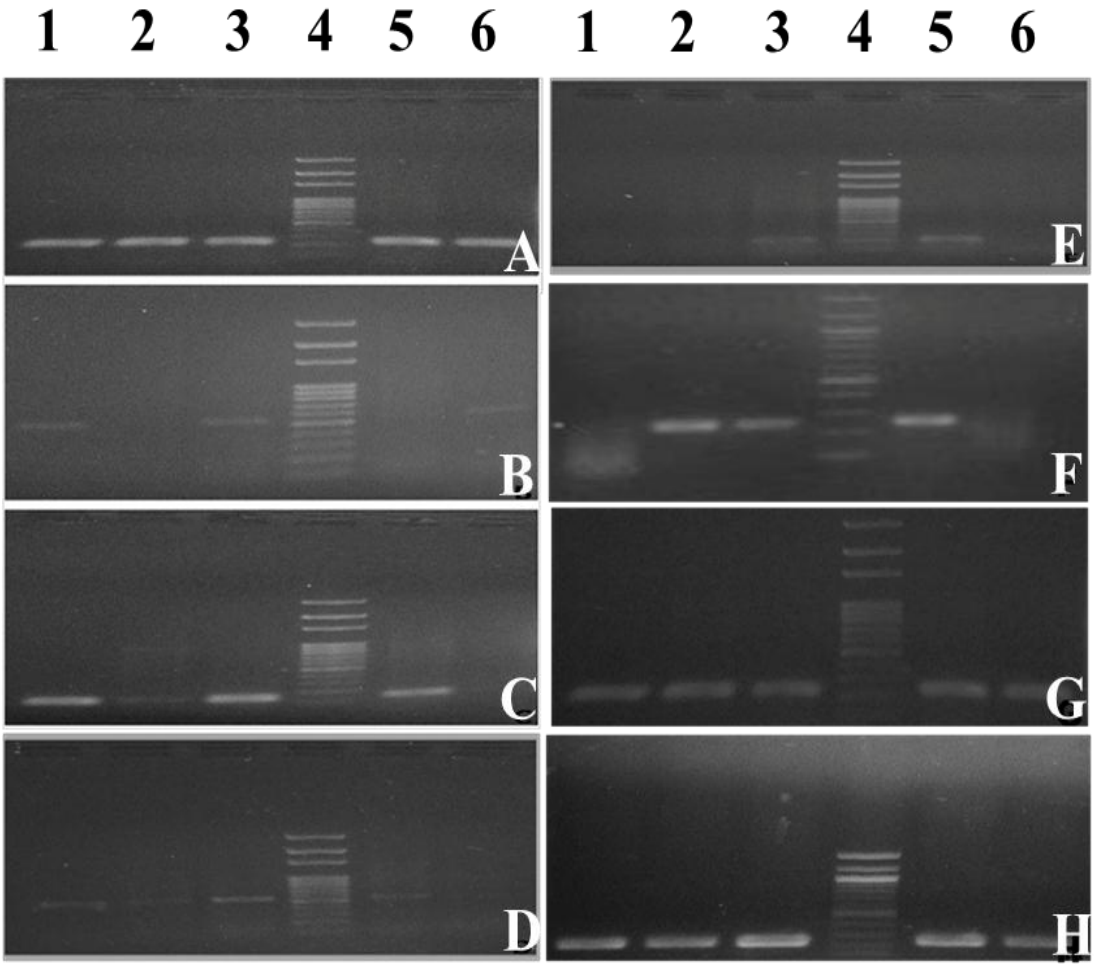

Figure 5 Amplification of SYCP1 (A), SYCP3 (B), VASA (C), DAZL (D), c-Kit (E), Sox2 (F), Pou5f1 (G), and GAPDH (H) mRNA transcripts in testicular tissue cultured in air-liquid interphase organ culture for the various duration. Lane 1: Day 40; Lane 2: Day 45; Lane 3: Day 50; Lane 4: 1kb DNA ladder; Lane 5: Day 55 and Lane 6: Day 60.

Discussion

Mammalian spermatogenesis is a complex biological process comprising sequential cell division and differentiation of germ cells producing millions of spermatozoa throughout life (Ko *et al*., 2010). The completion of the whole process has not been successfully accomplished in *in vitro* conditions (Gassei and Schlatt, 2007). In the present study, we reexamined the air-liquid interface culture method that is reported to produce tail-bearing sperm-like cells. Steinbergers *et al*., 1960 developed the basic principle of the present method. However, Sato *et al*., 2010 were successful in producing round spermatids through the air-liquid interface method. The volume of the culture medium was balanced to a position underneath the groove of the agarose gel bed. Air-liquid phase cultures are considered important to ensure an adequate supply of oxygen (Gholami *et al*., 2018; Hasegawa *et al*., 2020). In the filtration process, the micropores provide sufficient oxygen and nutrients (Bhaskar, 2021; Bhaskar and Gupta, 2020). These results indicate that our organ culture was differentiated SSCs into tail-bearing like sperm cells. Gene expression was also induced from testis fragments, indicating that this organ culture system was useful in elucidating the production of in vitro spermatid. In the present study, further improvements in this method were made by adding nucleosides for the production of motile sperm-like cells in *in vitro* conditions. The first evidence of cell dispersion was observed on the 35th day from cultured tissue and colony formation was started on the 40th day of culture. The putative GS cells were discovered in different forms such as single, paired and clusters in the culture plate. The somatic cells including myoid cells, were fully confluent and acted as a feeder layer for putative GS & SSCs cells. Testicular somatic cells are the most vital part of the proliferation and differentiation of GS cells in evolving a successfully developed spermatozoon (de Rooij, 2017).

Our results represented the maintenance and derivation of GS colonies, the proliferation of spermatogonia and the development of spermatids in farming goats. Previously *in vitro* spermatid production has also been reported from neonatal mouse testes via the organ culture method (Sato *et al*., 2011) and differentiated round spermatids from rat spermatogonia (Reda *et al*., 2016). In the current study, different sizes of spermatogonia were perceived as having small round cells with 1-3 irregular nucleoli, the large spermatogonia (A1–A16) consists of differentiating spermatogonia subpopulations. A progressive development of colony size was observed in putative GS cells. Putative GS colonies started dividing and lineage differentiation of cells was also observed which is also well documented (Koruji *et al*., 2009). The spermatogonia were further differentiated into intermediate spermatogonia. The morphology of spermatozoa was compared with the chromatin threads of the nucleus of spermatogonia which reflected maximal spreading of acrosome over the nucleus and also exhibited a polarity towards the basement membrane (Santos Nassif Lacerda *et al*., 2013). Furthermore, round spermatids were recognized owing to their smaller size when compared to the other cells (SSC and somatic cells) present in the air-liquid culture system. It was observed that the pre-leptotene spermatocytes had been converted into leptotene spermatocytes which could be identified by the presence of thread-like appearance of chromatin, which is also well documented (Osuru *et al*., 2014). The appearance of the sperm head was recorded due to acrosomal expansion toward the dorsal surface of the head region as shown in (Fig. 3-L). The acrosome covered the entire head region of spermatids and the elongating tail started to appear at stage XII (Fig. 3-M) and converted into mature spermatids (Fig. 3-N) and tail bearing sperm (Fig. 3-O). This trend was in coherence with previous reports (Santos Nassif Lacerda *et al*., 2013; Osuru *et al*., 2014).

Testicular histopathology revealed the disrupted spermatogenic compartments as indicated by reduced diameter of the seminiferous tubule and disrupted alignment of germ cells at all stages. Sperm-like structures were located in lumens of seminiferous tubules from 45th-60th days. Our findings are in line with the results of a previous study in which spermatozoa were observed in the culture of rodents, goats and humans (Pence *et al*., 2019; Deng *et al*. 2017; Sato *et al*., 2011). Further, the differentiation of GS cells in our study was confirmed by transcription factors such as VASA, DAZL, SYCP1, SYCP3, C-kit, OCT4, SOX2 and GAPDH (Conrad *et al*., 2014). To evaluate the properties of the GS cells, we observed the presence of the POU5F1, VASA, and SOX2 gene transcripts in cultured testis tissue. These factors play an important role in reprogramming the GS cells. Similar expression patterns of mRNAs were also observed in the control testis, supporting the expansion of the spermatogonial population *in vitro* condition (Jung *et al*. 2010). Early studies evidenced that the expression of POU5F1 and SOX2 along with some important transcriptional factors for regulating pluripotency and self-renewal was observed in germ-line stem cells (Kashyap *et al*., 2009; Gillis *et al*., 2011). POU5F1 was also reported to be expressed in undifferentiated spermatogonia (Liao *et al*. 2019). VASA was detected at all stages except the 60th day but SOX2 and OCT4 were reported to be expressed from the 40th to 55th day which indicates continuous progression of the germ cells through meiosis of spermatogenesis. The C-kit expression was comparatively higher on the 45th day and 50th day in the cultured tissues. C-kit transcripts have been reported in bovine SSC (Bedford-Guaus *et al*., 2017; Gillis *et al*., 2011) and have also been identified in differentiating spermatogonial germ cells (Morimoto *et al*., 2009; Rossi, 2013). The DAZL gene family encodes for potential RNA binding proteins expressed in prenatal and postnatal male germ cells and is used to identify pre-meiotic germ cells (Kee *et al*., 2009). This gene is transcribed as a member of the depleted azoospermia-like (DAZL) protein family. The encoded proteins are expressed in the pluripotent stem cells, which is an indication of spermatogenesis. It is well known that SYCP1 is required for normal meiotic chromosome synapsis during spermatocyte development and fertility (El Zowalaty *et al*., 2015; Ollinger *et al*., 2005). SYCP3 is expressed in early unpaired cores, in the chromosome cores at the diplotene stage and also in the lateral domains of the

synaptonemal complex (El Zowalaty *et al*., 2015). The results of SYCP1 and SYCP 3 expression obtained in the present study indicates continuous and progressive lineage division of spermatogonia. The analysis of these markers points towards the presence of a synaptonemal complex in the cultured tissue that could further support the formation of spermatid. Our results of gene expression suggested that GS cells, spermatogonia, and spermatocytes were present in the culture. Many studies have reported a similar pattern of organ culture (Sato *et al*., 2012; Kanatsu-Shinohara *et al*., 2004; Gohbara *et al*., 2010; Stukenborg *et al*., 2008). Nucleosides are mandatory for spermatogenic cell proliferation and differentiation. Kato *et al*. 2005, reported the nucleoside uptake mechanism through Sertoli cells. Sertoli cells are to supply supplements and metabolic antecedents to spermatogenic cells in the seminiferous tubule, nucleosides are probably utilized for DNA and RNA synthesis in Sertoli cells and spermatogenic cells (Kato *et al*., 2005). The mechanism by which nucleosides flow from Sertoli cells into the seminiferous tubular lumen to spermatogenic cells has not yet been established (Hasegawa *et al*., 2020). The present work demonstrates the importance of nucleoside in enhancing the formation of tail-bearing sperm-like cells.

In conclusion, the study revealed the production of the tail-bearing sperm-like cells from *in vitro* spermatogenesis of farming goat testis in an air-liquid inter-phase culture system. This system presents the potential for being a platform for understanding the mechanism of spermatogenesis and future clinical applications. The obtained spermatids and sperm might also be useful for producing reproductively competent offspring through ART technologies. The study could have a significant impact on the livestock management of farmers and could improve their dependency on orthodox and natural breeding management. Future studies will determine the fertilizing ability of these sperms produced by *in vitro* spermatogenesis.

Compliance with ethical standards: The animals used in the study (*Capra aegagrus hircus*) were humanely treated as per the guidelines of the Committee for the Purpose of Control and Supervision of Experimental Animals, Ministry of Environment and Forests, Government of India (CPCSEA) and the experimental protocols were approved by the Institutional animal ethical committee of National institute of technology Rourkela India.

Author Contributions: Conceptualization and Methodology, R.B., and M.K.G.; Formal Analysis, R.B., B.J., and M.K.G.; Investigation, R.B., and M.K.G. Writing-Original Draft, R.B., and M.K.G. and S.S.H. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest: The authors declare that they have no conflict of interest.

List of abbreviations:

ART	:	Assisted reproductive technology
Dazl	:	Deleted in azoospermia like
ES	:	Embryonic stem cells
FBS	:	Fetal bovine serum
GAPDH	:	Glyceraldehyde 3-phosphate dehydrogenase
GDNF	:	Glial cell-derived neurotrophic factor
GFP	:	*Green fluorescent protein*
GnRH	:	gonadotropin-releasing hormone
GS	:	Germ-line stem cells
MEM	:	Minimum Essential Medium
Mvh	:	Mouse Vasa Homologue
Oct4	:	Octamer-binding transcription factor 4
Sox2	:	Sex determining region Y-box 2
SSCs	:	Spermatogonial stem cells
Stra8	:	Stimulated by retinoic acid gene 8 protein homolog
SYCP1	:	Synaptonemal complex protein 1
SYCP3	:	Synaptonemal complex protein 3
Zfp-42	:	Zinc Finger Protein 42 Homolog
α-MEM	:	α is a modification of Minimum Essential Medium

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