

Testis Cell and Organ Culture

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Abstract

The testis, the male gonad in animals, produce male germ cells and sex hormones. It contains several primary cell types. Germ cells, Sertoli cells and peritubular cells are located within the seminiferous tubules. Germ cells are developed in the tubules supported by the two types of somatic cells, Sertoli and peritubular cells. In between tubules (interstitium) Leydig cells are present. Leydig cells are the location of steroidogenesis and synthesize hormones essential for germ cell differentiation. Different approaches to isolating and culturing testis cell types, and to performing organ culture on testis fragments, are reviewed. Cell culture and co-culture of individual cell populations from the testis as well as organ culture involving testis tissue has advanced the understanding of the processes of spermatogenesis, the interactions of specific cell types (signal transduction), and mechanisms of steroidal hormone actions.

Key Points

- Testis cell isolation and culture.
- Testis germ cell isolation and culture.
- Testis organ culture.
- Testis organoids.

Introduction

This chapter on testis cell and organ culture is to describe the general aspects of testis cell isolation and culture for use in experimental models to investigate testis cell biology. In addition, the new science in testis organ culture and organoid development is described to further advance the investigation of testicular cell biology and function. The utilization of these technologies for germ cell analysis and developmental biology of the testis and germ cells is invaluable as described.

Testis Cell Isolation and Culture

The testis, the male gonad in animals, produces male germ cells and sex hormones. It contains several primary cell types. Germ cells, Sertoli cells and peritubular cells are located within the seminiferous tubules. Germ cells are developed in the tubules supported by the two types of somatic cells, Sertoli and peritubular cells. In between tubules (interstitium) Leydig cells are present. Leydig cells are the location of steroidogenesis and synthesize hormones essential for germ cell differentiation (Christensen and Mason, 1965) (Fig. 1).

Testis Cell Biology

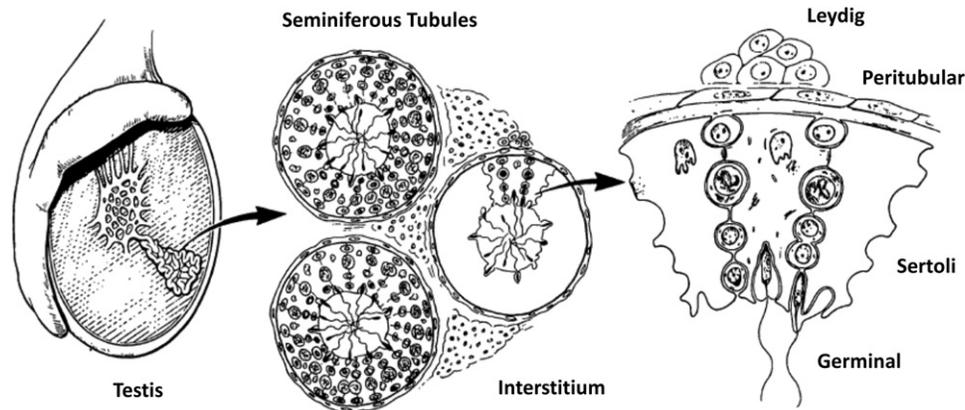


Fig. 1 Cell types in the testis. Modified from Skinner, M. K., 1991. Cell-cell interactions in the testis. *Endocrine Reviews* 12, pp. 45–77.

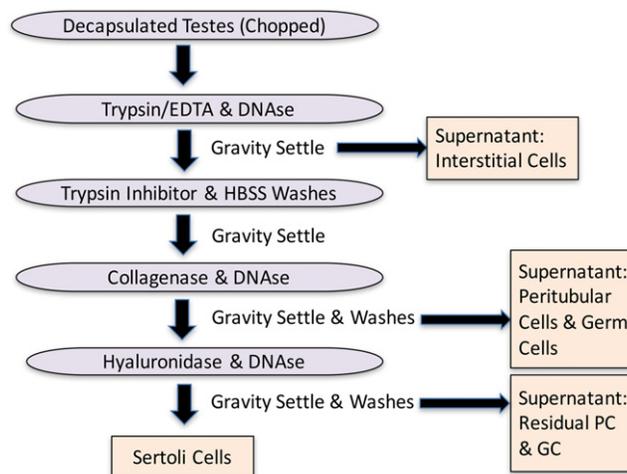


Fig. 2 Flowchart for isolation of different cell types from the testis.

The procedures for isolating individual testis cell populations for culture are very similar across the animal spectrum. [Hall *et al.* \(1969\)](#) were able to look at interstitial versus tubular cell types by manually dissecting the testis. [Welsh and Wiebe \(1975\)](#) introduced in 1975 an isolation method using the enzyme collagenase, which worked well for digesting the tissue without hurting viability of the isolated cells, and [Dorrington *et al.* \(1975\)](#) published a similar method in the same year that used the enzymes trypsin, desoxyribonuclease (DNase) and collagenase. [Tung *et al.* \(1984\)](#) improved the enzymatic digestion method by adding hyaluronidase which helped to reduce cross contamination of cell types. This method of using progressive steps of enzymatic digests has been used with slight modifications for isolation of individual testicular cell fractions from a wide range of species. The three enzymes trypsin, collagenase and hyaluronidase together with DNase are used for most protocols, but the order and combination in which they are used may change depending on the animal species and cell type to be isolated.

The following is a technique used for the isolation of testis cells from immature rats (around 20 days of age). In 20 d rats the Sertoli cells cease to divide and comprise a large fraction of the testicular cell types because spermatogenesis has not started yet and so the proportion of germ cell contamination in the Sertoli cell fraction is low ([Fig. 2](#)).

Sertoli Cell and Peritubular Cell Isolation and Purification ([Tung *et al.*, 1984](#) With Modifications)

The rats are sacrificed and testes removed from each rat. After rinsing the testes several times in sterile HBSS (Hank's Buffered Salt Solution) they are detunicated (the tunica albuginea encloses the testis) and minced finely. The initial enzymatic digest uses trypsin (protein hydrolysis) and DNase. The addition of DNase is necessary at every purification step for removal of the

DNA released from damaged cells, otherwise the cells would clump together into a gelatinous mass and be impossible to separate. The trypsin digest will separate the tubules, and interstitial cells (Leydig cells etc.) will be freed and can be separated from the tubules by gravity sedimentation. The tubules will settle to the bottom of the tube and the supernatant can be discarded. Several washes of the tubules using gravity sedimentation are needed to guarantee that most of the interstitial cells are removed. The next step in the procedure involves collagenase (serine protease) digestion, again with DNase added. This step will break the tubules into smaller pieces and release a large percentage of the peritubular cells. The tubule pieces (with Sertoli cells attached) can be separated from the peritubular cells by gravity sedimentation. The tubules will settle to the bottom and peritubular cells can be removed with the supernatant. The peritubular cells can be washed to remove cellular debris and enzymatic solution by pelleting the supernatant gently in a centrifuge, and then resuspending the peritubular cells in a physiological buffer. After several washes, they can be plated in appropriate medium for further study in cell culture. The remaining tubules need to be washed again several times to remove as many contaminating peritubular cells as possible and will then be further digested with hyaluronidase and DNase. This step will further break down the tubules, releasing the germ cells and remaining peritubular cells. In a microscopic check the Sertoli cells can be seen as small aggregates and after several washes using gravity sedimentation of the Sertoli cell aggregates, they can be plated in the appropriate medium. If further dissociation is needed before plating they can be homogenized by shearing using a hypodermic needle with small needle size and passed through a cell strainer which will result in a single cell suspension. Also, if needed, a hypotonic shock (using a 1:10 dilution of Hank's Balanced Salt Solution—HBSS with water) can be added after preparation of Sertoli cells which will not damage the Sertoli cells but will burst and get rid of remaining germ cells (Galdieri *et al.*, 1981). This is especially important in the preparation of Sertoli cells from adult testes since spermatogenesis is present at that time and contamination with germ cells could pose a problem in the cell culture. In addition, germ cells will not attach to the culture plate like Sertoli cells and will die after a few days in culture. So, the purity of Sertoli cells will increase after a few days in culture.

Leydig Cell Isolation and Purification (Risbridger and De Kretser, 1986; Klinefelter *et al.*, 1987 With Modifications)

The testis interstitium contains a mix of Leydig cells, macrophages, epithelial cells, and fibroblasts. As mentioned before, Leydig cells synthesize hormones important for sexual development and germ cell differentiation and are needed for study in that context. Testes are prepared as described above and digested with collagenase until the testes start to dissociate into tubules, thus releasing the interstitial cells. Over-digestion needs to be avoided in order to keep the tubules intact and avoid contamination of the Leydig fraction with tubular cells. The interstitial cells will be in the supernatant after letting the tubules settle out and are removed for further processing. Density gradients such as Percoll gradients are used to separate Leydig cells from other interstitial cells. The interstitial mix will be loaded on a Percoll gradient and spun at low g. The Leydig cells will concentrate at a certain Percoll density and can be isolated as a pure fraction. The Percoll needs to be washed off before plating the cells for cell culture.

Testicular Macrophage Isolation and Purification (Yee and Hutson, 1983)

Testicular macrophages are the main immune cells of the mammalian testis. There are two different populations which differ in morphology and localization (in the interstitium or on the surface of seminiferous tubules). They are involved in classical immune functions but also in organogenesis, spermatogenesis and male hormone production.

The protocol in the original paper describing isolation of testicular macrophages from mammalian testis (Yee and Hutson, 1983) is still being used with minor modifications. After decapsulating the testes and washing them in PBS, the testes are minced and then incubated with a digestive enzyme like trypsin or collagenase with DNase added. The seminiferous tubules are allowed to sediment and the supernatant is collected and centrifuged. The pellet containing a mixture of interstitial cells is resuspended in serum free medium or PBS and plated on plastic cell culture dishes. Under these conditions the macrophages will attach, but not the other cells. After only a short incubation period of about 20–30 min the non-adherent cells are washed off and the remaining—adherent—cells constitute the macrophage fraction which has a purity of over 95% (Winnall *et al.*, 2011).

Germ Cell Isolation and Purification (Bellvé, 1979 With Modifications)

Germ cells at different stages of spermatogenesis can be isolated depending on the age of the animal used for preparation. But especially for mammals it is difficult to grow germ cells in culture by themselves, with the exception of spermatogonial stem cells. Sertoli cells are essential for the structural support and paracrine control (through for example testosterone and FSH) of germ cells during spermatogenesis. Therefore, Sertoli cells are often used in a co-culture with germ cells to study the stages and regulation of spermatogenesis. Still, it is difficult to achieve complete spermatogenesis in cell culture, but it can be done in organ cultures using testis fragments as described below in section Testis Organ Culture.

Isolation of germ cell fractions follows essentially the same enzymatic digestion protocol as used for somatic testis cells. An initial enzymatic digest with trypsin or collagenase will release interstitial cells and the majority of peritubular cells, which will stay

in the supernatant during gravity sedimentation and can be discarded while tubule fragments and Sertoli cell/germ cell clusters will be in the pellet. Further enzymatic digestion with trypsin/EDTA and DNase will fully dissociate the seminiferous tubules and release Sertoli and germ cells.

A germ cell fraction can be isolated different ways. Panning is an efficient method for crude separation of germ cells from Sertoli cells and relies on the fact that Sertoli cells will stick to a culture plate while germ cells will not. So, after plating and a 2–3-day incubation the germ cells can be removed from the top of the plate leaving the Sertoli cells behind. Germ cells can then be further purified based on their density using a gradient made with BSA (bovine serum albumin) or Percoll. Another approach is to remove Sertoli cell clusters with a cell strainer which will result in a single cell germ cell fraction in the filtrate. Again, this fraction can be further purified using a gradient.

Sometimes it is necessary to isolate germ cells at a specific developmental stage and this can be done using a Sta-put (Romrell *et al.*, 1976; Mccarrey *et al.*, 1992) apparatus, which is a velocity sedimentation cell separator allowing the isolation of cell fractions based on their differences in gravity sedimentation rate. Germ cells at different stages of development have different sedimentation rates.

Cell culture of germ cells can be accomplished by co-culturing them with feeder cells like Sertoli cells. This co-culture can be improved by growing the cells on specific substrates like Matrigel, collagen or in a soft-agar culture. Also, growth in 3-D culture systems providing “scaffolding” for the germ cells has shown promise in improving germ cell viability and growth (Hunter *et al.*, 2012; Stukenborg *et al.*, 2009).

Testis Organ Culture (Dores *et al.*, 2012)

Cell culture systems are very helpful because they reduce a complex structure into smaller parts and therefore help one to understand complicated processes by examining and manipulating the single parts of the system. An in vitro cell culture system is indispensable for studying cell–cell interactions, roles of hormones and growth factors, gene expression and morphological changes. Some processes however, for example spermatogenesis, need a more intact system to function properly in order to fully finish their developmental events. So far it has not been possible to produce functioning spermatozoa using an in vitro cell culture system alone.

Organ culture on the other hand preserves the proper niche of testicular structure and paracrine exposures which are needed for proper spermatogenesis. Organ culture of testis fragments can be maintained for several weeks. Testis organ culture’s main use has been to study spermatogenesis with clinical implications for help with reproductive issues.

Organ culture protocols often use the air–liquid interface method (Trowell, 1954) in which a filter is carefully set on top of the appropriate cell culture medium into a well of a multi-well cell culture plate (Levine *et al.*, 2000). It is important that the filter float on top of the medium and is not submerged. A small piece of testicular tissue or a whole embryonic testis are placed on the filter floating on cell culture medium. The testis piece or embryonic whole testis will be covered with a drop of medium to prevent it from drying out. This places the organ culture close to the atmosphere in the incubator, and allows for higher oxygen concentrations in the cultured organs. Instead of just using liquid medium, other supporting substrates like agarose can also be used (Stukenborg *et al.*, 2008). These more solid substrates can be saturated with medium with the testis piece placed on top. Incubation of the organ culture in a CO₂ cell culture incubator can continue from a few days to several weeks with frequent medium changes.

Human testicular pieces have been cultured this way for about 2 weeks with decreasing germ cell numbers throughout this time (Roulet *et al.*, 2006). Nakamura *et al.* (2017) could maintain a mouse testis organ culture with spermatogenesis for over 2 months. One of the most important features to maintain a fully functioning organ culture seems to be the proper medium and additives. For example, a change in the medium from serum to serum replacements like GlutaMax® and AlbuMax® made a big difference in successfully maintaining organ cultures for longer periods of time and achieving full spermatogenesis. These experiments have been mostly done with mammalian tissue, but with appropriate culture conditions could probably be extended to other species.

Testis Organoids

Although individual cells can be isolated and cultured, construction of mixed cell populations of in vitro organ cultures is limited. In vitro maturation of somatic cells and spermatogonia in organ culture systems have been reported. However, this has been difficult for organoids derived from dissociated testis cells. The generation of testicular organoids in microwell culture for months have been shown with lower oxygen and higher temperatures to allow somatic cell maturation and spermatogonial cell differentiation in culture. These organoid cultures allow in vitro cell differentiation and spermatogenesis processes to be investigated. The impacts of environmental exposures to toxicants have also been described (Sakib *et al.*, 2019). Therefore, the microwell derived testicular organoids with mixed cell populations provide a novel culture platform to study testis cellular maturation and potentially allow in vitro spermatogenesis (Sakib *et al.*, 2022).

Summary

Cell and organ culture of testis cells or testis tissue from a variety of species is advancing the understanding of processes in the testis on both a molecular and whole system level. Cell and organ culture together provide means in basic and applied research to investigate this complex organ in detail providing information on organogenesis, male germ cell production, the specific cell types and their ways of interacting, and the signaling pathways controlling testis function.

This knowledge is invaluable for finding out more about the mechanisms of spermatogenesis, which provide the means to develop new diagnostic tools and therapies for restoring male fertility and for assisted reproduction. It will also be helpful, for example, for preservation of gametes, maintenance of livestock and species conservation. Organ cultures in particular are needed for research where the intact testis structure is required, as in toxicological studies and for in vitro maturation of sperm cells.

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