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REVIEW ARTICLE

Usage of the Cell Dissociation Technique in Reproductive Studies; Study on Mechanical Cell Dissociation in Male Mice in Maturity Course

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Abstract

Cell dissociation techniques are one of the main methods for separating different cells from various tissues. These techniques consist of two main approaches: chemical and mechanical separation. Each technique has its own advantages and disadvantages, and the most suitable method should be selected. In the chemical method, specific enzymes must be used. In the mechanical method, cells are separated by mechanical force. Normally, in most research studies, the chemical method using specific enzymes is more commonly applied. In this study, 15 mice were used during the maturity phase and were grown under standard conditions. These mice were operated on after 32 days, and the testes were extracted. The cells were dissociated using the mechanical method. Finally, the samples were stained with Giemsa and examined. The advantages of these techniques include the separation of undamaged cells, which were observed in both methods. However, the main focus of this paper was mechanical cell dissociation, which did not damage the primary cells. Moreover, this method can be used for chromatin studies by counting each cell type and separating them to investigate testicular functions. Therefore, using mechanical cell dissociation for the seminiferous tubule wall could be a useful and economical option as a low-cost method for comparative studies involving cells in testicular tissue. The findings suggest that mechanical dissociation provides an efficient and low-cost method for isolating intact testicular cells, making it suitable for histological and functional studies of seminiferous tubules.

1. Introduction

At this time, to understand the complex mechanisms of spermatogenesis, we study all the changes in surface antigens on the cells. Studying the expression patterns of various genes will molecularly evaluate the process of spermatogenesis, and these studies identify key factors in the control of spermatogenesis as well as other cytological aspects. Molecular studies require certain conditions, such as the isolation and identification of spermatogenic cells from testicular tissue, which is the main tissue examined here (Schneider *et al.*, 2015).

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According to all data, the first report on cell dissociation and identification of spermatogenic cells was published in 1977 (Bellvé *et al.*, 1977). They succeeded in obtaining a pure population of these cells from the testicular tissue of immature mice.

In this process of separating tissue cells from each other, they used mechanical methods and enzymatic digestion, and then applied SVUG (sedimentation velocity at unit gravity) as an appropriate technique to successfully extract pure populations of all six types of spermatogenic cells from the tissue during the growth and development of the testis in Balb/c mice (Schneider *et al.*, 2015; Zhang *et al.*, 2016). During the isolation phase of various populations of spermatogenic cells obtained from testicular tissue, they began using mechanical methods.

There are two methods for cell dissociation related to this function (Lima *et al.*, 2017).

1) Chemical method

2) Mechanical method

In the chemical method, enzymes like trypsin and collagenase are used for cell dissociation. In the mechanical method, special equipment with a specific technique called "sedimentation velocity at unit gravity" is used (Lima *et al.*, 2016; Tepperman *et al.*, 1975).

In addition to this, as a simpler and easier method, a tool such as scissors can be used to cut soft tissue such as the testes to access the inner cells such as spermatozoa (Montanari *et al.*, 2022; Goldberg, 2015).

2. Spermatogenic Cells and All Methods for Cell Dissociation Process

Spermatogenic cells that are present in cell cultures will be characterized according to their shape. Different elements include size, diameter, location of the nucleus, flagella cells, presence of acrosomal structures (e.g., haploid testicular cells), and their own staining quality (Schneider *et al.*, 2015; Zhang *et al.*, 2016). In the mechanical method, special equipment with a specific technique called "sedimentation velocity at unit gravity" is used. Alternatively, for soft tissues such as testes, which we are working with here, simple methods such as cutting with a tool like scissors can be used to isolate interior cells such as sperms (Montanari *et al.*, 2022).

Chemical dissociation methods using a variety of enzymes, such as different types of collagenases and trypsin, can be expensive. Studies have shown that cells obtained by enzymatic dissociation using enzymes like trypsin or pronase during trypsinization show limited proliferation in serum-free medium after washing with saline.

It is also possible that trypsin and pronase bind to the surface of cells and alter the surface structure, making it different. This function affects cell adhesion. Once this surface is altered, the cells may take some time to regain their ability to adhere to the dish and target, which can be observed when transferred to another container.

At this time, it is necessary for the cell surface to be remodeled or repaired to resolve the problems (Montanari *et al.*, 2022). Two enzymes that are useful and can be quite powerful for chemical dissociation are collagenases and trypsin.

3. Collagenase

This enzyme may be divided into two kinds that are primarily and totally based on its presence in distinct pathways:

1) Endogenous human collagenase

2) Pharmaceutical collagenase

The human body naturally produces some collagenase inside tissues such as gums, epithelial tissue, and synovial surfaces. These various types of collagenases play an essential role in breaking down collagen within the body during intra-distal processes, and this kind is called endogenous collagenase (Schol *et al.*, 2024).

4. Different Types of Collagenases and their Applications and Functions of Collagenase

These types and functions exist in numerous forms, which are summarized in Table 1 (Worthington, 1988). Some of the more general types include Collagenase I-V (Wu *et al.*, 2023).

4.1. Collagenase I

This enzyme is used for many tissues such as epithelium, lung, fat, and the isolation of adrenal tissue cells. It is appropriate for digesting tissues and converting them to a unicellular form. so, it is useful for separating mammalian cells (Worthington, 1988; Wu *et al.*, 2024).

4.2. Collagenase II

This enzyme is a suitable and effective agent for tissues such as liver, bone, thyroid, heart, and salivary glands (Worthington, 1988; Shajib *et al.*, 2022).

4.3. Collagenase III

Collagenase type III is a bacterial enzyme capable of degrading collagen types I, II, and III, particularly in dense connective tissues. It enables efficient cell isolation while preserving membrane integrity, making it

suitable for tissue engineering and cell culture applications (Wu *et al.*, 2023).

4.4. Collagenase IV

This kind of enzyme consists of at least seven protease additive factors with molecular weights ranging from 68 to 103 kDa.

In addition to all these properties, this enzyme can also digest various forms of tissue (Wu *et al.*, 2023).

4.5. Collagenase V

This enzyme contains at least seven protease components with molecular weights ranging from 68 to 103 kDa.

It may be used to separate pancreatic islets from all parts of the main tissue. It is also possible to isolate unique cells with this enzyme.

When an organization wants to digest and separate hard tissue consisting of connective tissue or collagen, cell dissociation with trypsin is much less effective. Therefore, collagenase may be used for more complete and specific separation.

Interstitial digestion of cells through collagenase is possible and has minimal impact on epithelial cells. Consequently, it is a highly appropriate enzyme for isolating and digesting fibrous tissue, epithelial cells, cancerous tissue, and collagen fibers. It allows separation without harming or damaging the cells (Worthington, 1988; Alipour *et al.*, 2016).

5. The Mechanical Approach

This method is a way to carry out cell dissociation without using any enzymes or chemical materials. For example, in the article by C. Broadley and colleagues, cells were separated by cutting the sample into small parts and placing them in a centrifuge at high speeds (Schneider *et al.*, 2015; Shetab-Boushehri and Shetab-Boushehri, 2024; Broadley *et al.*, 1994). In other articles, such as "A Plasma-Deposited Surface for Cell Sheet Engineering: Advantages over Mechanical Cell Dissociation", Heather E. and her colleagues compared these techniques and concluded that there is a relationship with the mechanism of cell death.

These results show that mechanical cell dissociation contrasts with apoptotic mechanisms, because it causes lipid membrane rupture, leading to the discharge of intracellular contents and contributing to irritation and swelling.

Therefore, cells obtained by the mechanical method are used in studies where destructive mediators are present. In sequence analysis studies, it is common to use enzymatic methods and cells obtained through enzymatic dissociation (Canavan *et al.*, 2006).

6. Material and Methods

The main goal of this study is to become familiar with different cell dissociation techniques that are used in various studies, such as reproductive research. In addition, it aims to examine mechanical cell dissociation as an economical technique for studying different testicular cells at minimal cost.

The main study was performed on 15 male mice, all of which were kept under standard temperature and nutritional conditions. In these experiments, mice were euthanized after 32 days, and according to the image in Fig. 1, their testes were successfully extracted (Fig. 1; Abbasi-Malati *et al.*, 2018). In this process, we weighed the mice before surgery and weighed the right testis before and after fixation. In addition, the measurements of length, width, and diameter were recorded and presented in charts.

Following all procedures, after collecting the right testicular tissue, each testis was placed in 1 ml of physiological serum and cut into small pieces with sharp scissors, as small as possible, to release spermatozoa cells from the seminiferous tubules.

Table 1
Classification of collagenases and their substrates (Worthington, 1988; Alipour *et al.*, 2016).

Row	Enzyme	Matrix metalloproteinase	Group	Substrate
1	Collagenase 1	MMP-1	Collagenases	Collagens 1, 3, 7, 8, 10, gelatin, L- selectin, interleukin-1, entactin, ovostatin, MMP-2, MMP-9, proteoglycans, aggrecan
2	Collagenase 2/Neutrophil collagenase	MMP-8	Collagenases	Collagens 1, 3, 5, 7, 8, 10, fibronectin, gelatin, aggrecan
3	Collagenases 3	MMP-13	Collagenases	Collagens 1, 4, 9, 10, 14, fibronectin, MMP-9, gelatin, plasminogen, aggrecan, perlecan osteonectin
4	Collagenases 4	MMP-18	Collagenases	Type I collagen

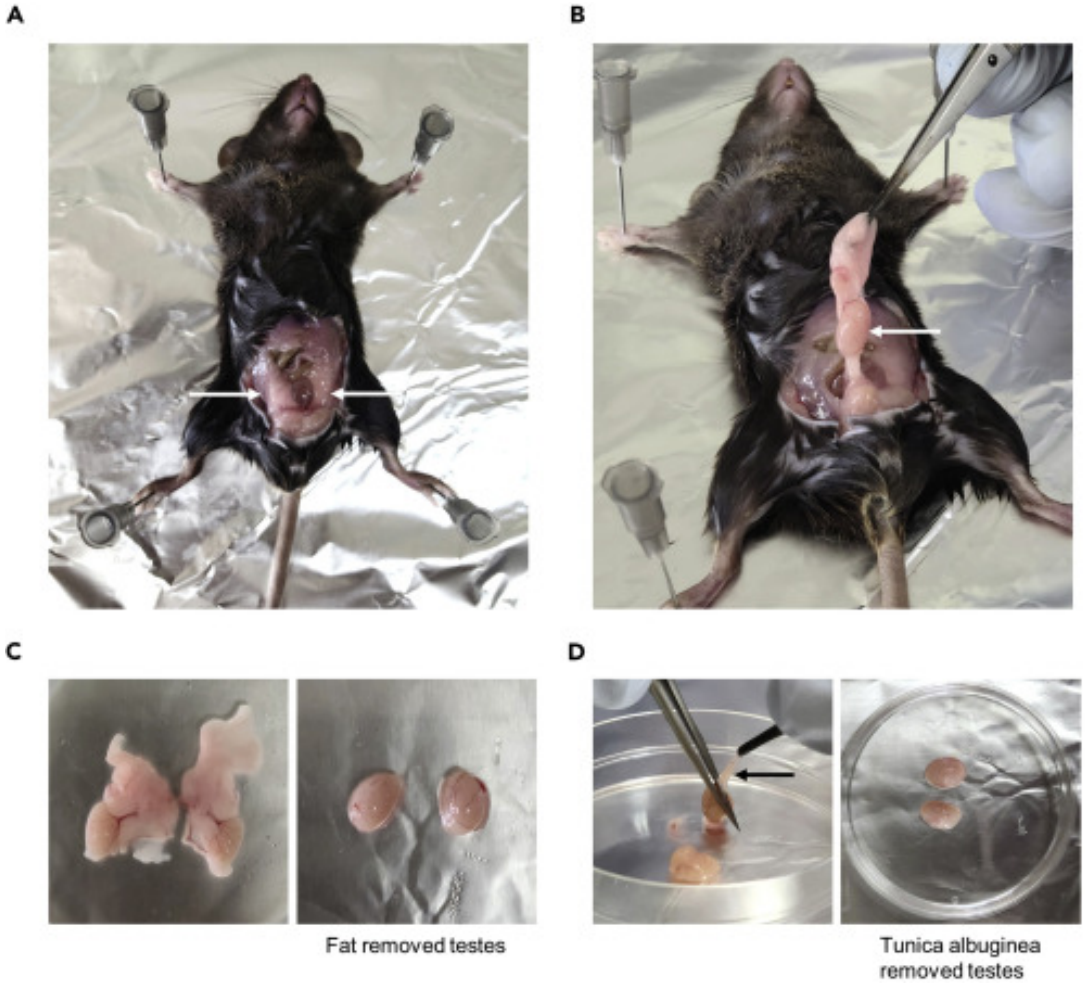

Fig. 1. Mice surgery for extracting testes tissue and making it ready for mechanical cell dissociation (Abbasi-Malati *et al.*, 2018).

After that, according to our method for investigating these cells, 2–3 drops of the resulting suspension were taken from the main sample and placed on a gelatin-coated slide to achieve the desired spread without drawing another slide over it.

The selected slides were placed in a laboratory environment to dry, and after drying, pure methanol was added to cover the entire surface of the slides. The methanol remained on the sample until only a thin layer of moisture was visible on each slide, indicating the staining time.

The samples were stained with Giemsa stain for 15 minutes (Othman *et al.*, 2023; Tsouloufi *et al.*, 2020). After staining, the samples were first rinsed with distilled water, then placed in xylene, and finally dried in the laboratory environment (Schneider *et al.*, 2015).

After drying, a larger cover slip was attached to the slide using Entellan glue to preserve the sample under standard conditions. The results of cell dissociation from mouse testicular tissue were studied under a microscope.

The mice used in these experiments belonged to the control group and did not receive any treatment.

7. Results

According to the experiment and all procedures performed, mechanical cell dissociation of spermatogenic cells from mouse testes was successful, showing different cell types in the prepared slide samples. In this way, different cell types were clearly observed and easily distinguished from each other.

Finally, images of the prepared samples (Fig. 2) are provided here. Different shapes of spermatogenic cells were captured and can be seen in the images.

The analysis of testis size before and after the fixation process was conducted to determine whether the mechanical procedure caused any structural damage to the tissue. Based on the recorded measurements and statistical analysis, the p-value was greater than 0.05, indicating no significant difference in testis dimensions ($P>0.05$).

According to all date of weights and measurements, we have some charts, and after studying all charts, we can see that the mice are in the same condition in terms of testis and size information. You can see the charts below (Figs. 3, 4, 5, 6).

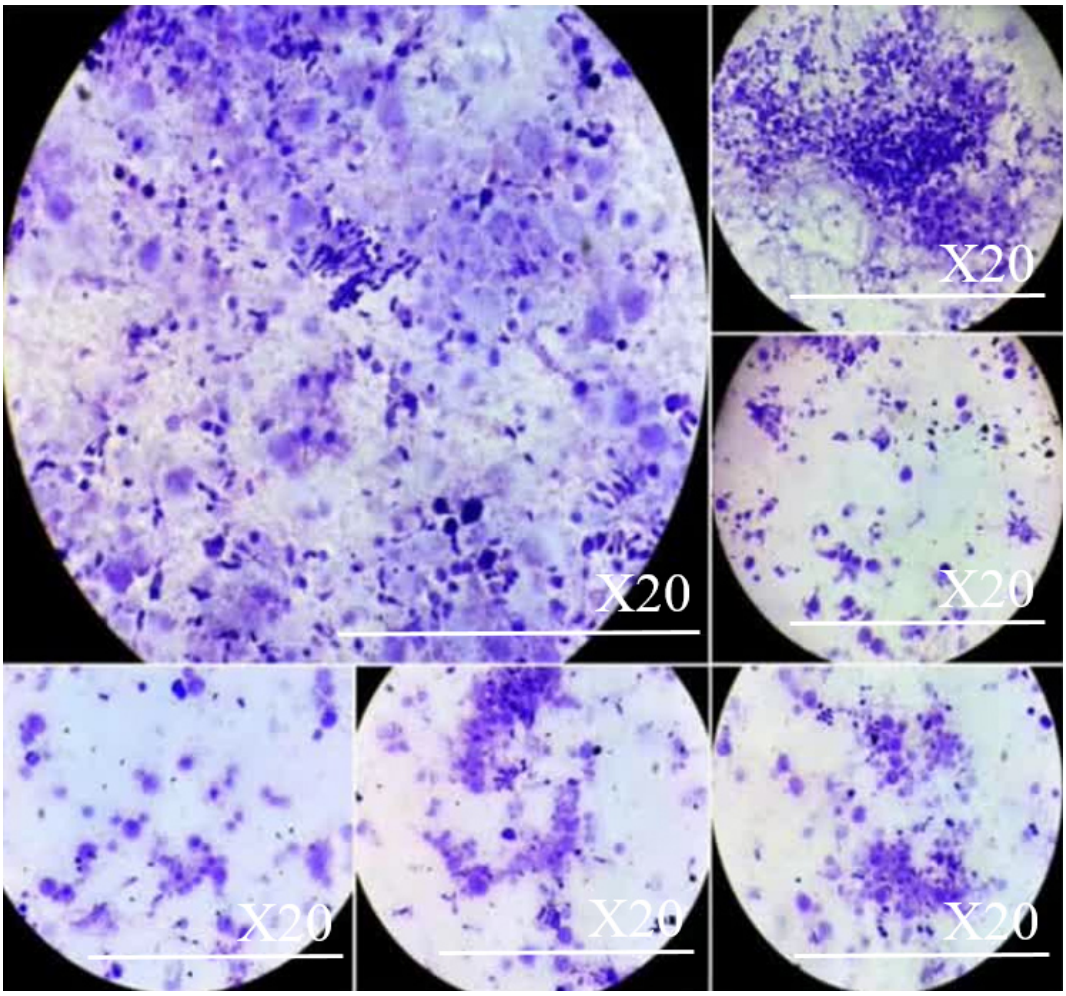

Fig. 2. Different types of sperm in different steps, with different shapes. These cells were seen in mechanical cell dissociation samples, they were derived from 15 mice without any treatment.

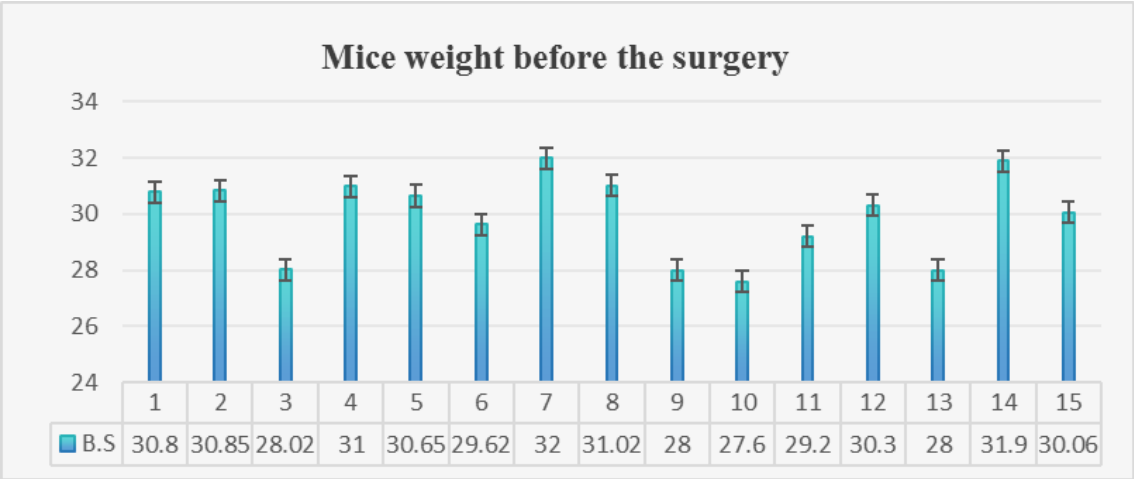

Fig. 3. Mice weight before surgery at 32 days old. All the mice were in the same condition, and their weights were nearly the same, and similarity was observed. "B.S." in the chart is the main group before the surgery process.

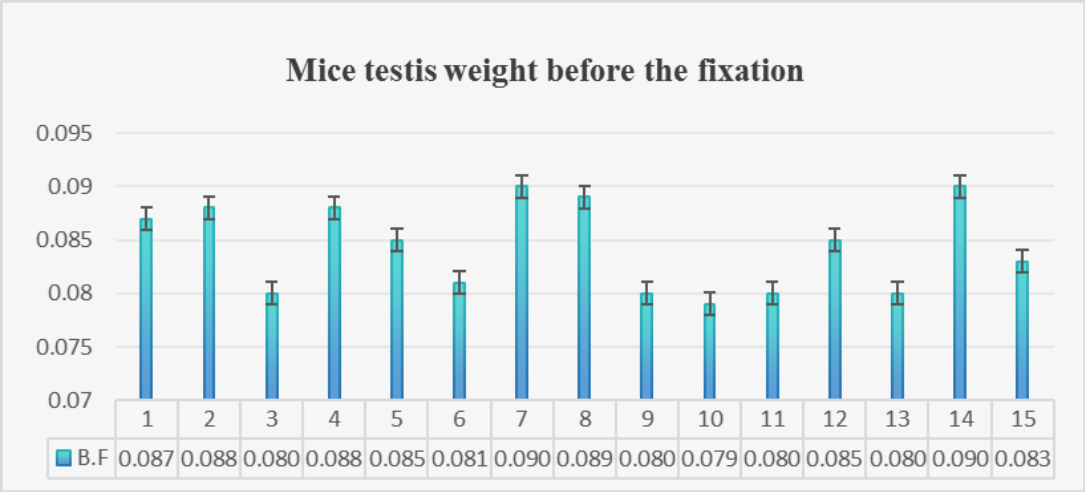

Fig. 4. Mice testis weight before fixation was more than after fixation. "B.F." in the chart is the main group before the fixation process.

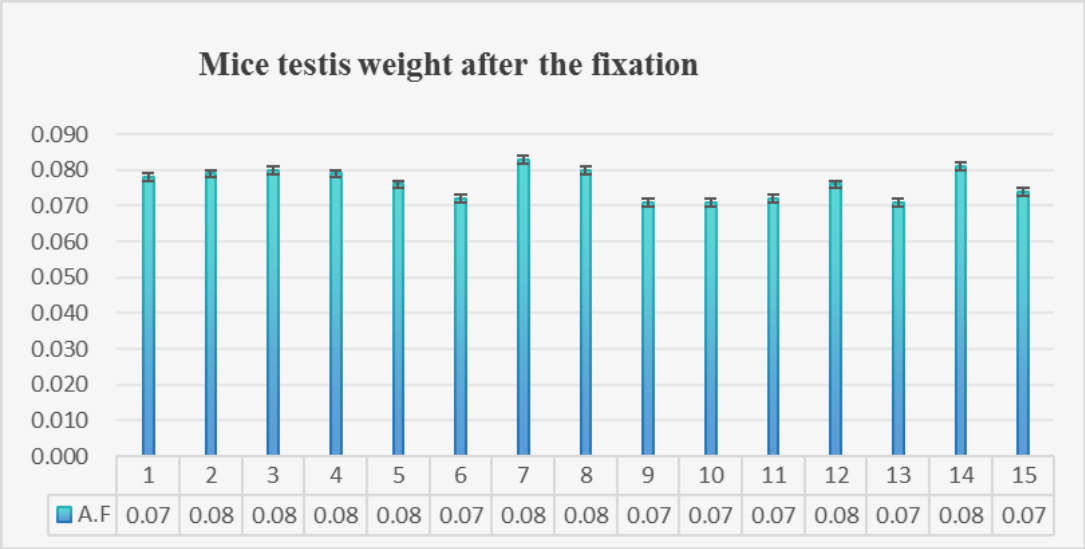

Fig. 5. Mice testis weight after fixation was less than before fixation. "A.F." in the chart is the main group after the fixation process.

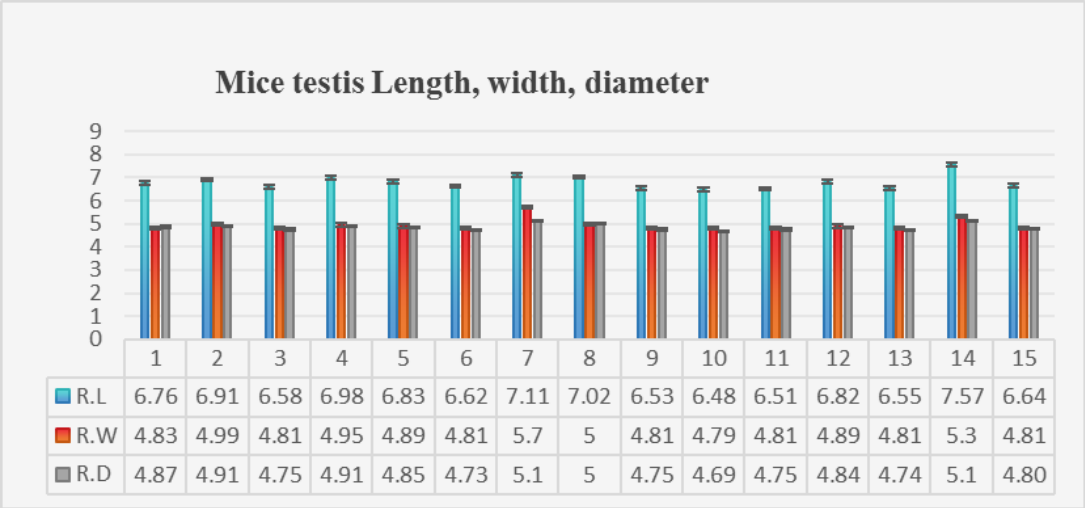

Fig. 6. In this chart, the first column represents the right testis length in mice and is labeled as "R.L.". The second column shows the right testis width, labeled as "R.W.", and the third column indicates the right testis diameter in mice, labeled as "R.D.".

8. Discussion and Conclusion

In this paper, according to the introduction of the cell dissociation methods for studying different cell types of the seminiferous tubule wall of testis tissue, it can be chosen as an ideal and economical method for histological techniques. Therefore, this method, as demonstrated in this article, can be used to isolate and separate different cells of main tissues for different purposes and targets, such as reproductive studies. This allows for the achievement of appropriate and efficient results at a low cost.

According to the results of this experiment and study, different cell types were observed within the field of view, and they were countable.

so, it is possible to study the causes of infertility by counting the gametes in mice that were treated with different dietary or non-dietary substances. This may help to study and determine whether the cause of infertility is due to a reduction in the number of gametes or because of changes in their normal morphology. Also, it is important to know the ages of mice at which these substances have significant effects on the gamete cells.

In this study, mechanical cell dissociation was evaluated as a practical and economical method for isolating spermatogenic cells from testicular tissue. The analysis of testis size before and after the fixation process was conducted to determine whether the mechanical procedure caused any structural damage to the tissue. Based on the recorded measurements and statistical analysis, the P-value was greater than 0.05, indicating no significant difference in testis dimensions. This suggests that the mechanical dissociation method preserves tissue integrity and does not induce measurable damage to the seminiferous tubules (Abbasi-Malati *et al.*, 2018).

The absence of significant morphological changes following fixation supports the reliability of this approach, particularly in studies where enzymatic reagents are either unavailable or undesirable due to cost, accessibility, or potential chemical interference with cellular structures. Unlike enzymatic methods, which may alter cell surface proteins or affect downstream molecular analyses, mechanical dissociation offers a non-invasive alternative that maintains the native architecture of the cells.

Furthermore, the ability to isolate intact and countable spermatogenic cells using this method opens new avenues for reproductive research. For example, in studies investigating infertility, quantifying gametes and assessing their morphology in response to dietary or pharmacological interventions is essential. The mechanical method enables researchers to perform such analyses without introducing chemical artifacts, making it especially valuable in toxicological, developmental, and histopathological contexts (Othman *et al.*, 2023).

Additionally, this technique may be particularly beneficial in resource-limited settings, where access to specialized enzymes such as trypsin or collagenase is restricted. The simplicity of the protocol using basic laboratory tools such as scissors and physiological serum makes it adaptable for routine use in both academic and clinical laboratories. Its cost-effectiveness and reproducibility further enhance its appeal for large-scale studies and comparative analyses (Tsouloufi *et al.*, 2020).

In conclusion, mechanical cell dissociation represents a viable and efficient method for testicular tissue processing. It ensures minimal tissue disruption, preserves cellular morphology, and provides a reliable platform for downstream applications such as cell counting, functional assays. Given its advantages, this method can be considered a suitable alternative to enzymatic dissociation, especially when the preservation of tissue integrity and reduction of experimental costs are prioritized.

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