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HISTOLOGICAL AND IMMUNOHISTOCHEMICAL STUDY ON THE EFFECTS OF WHEY PROTEIN ON TESTIS OF RATS

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ABSTRACT

Since many athletes consume large amounts of whey protein without consulting a nutritionist, this study was designed to determine the effect of whey protein on testicular tissue from a histological and immunohistochemical perspective. Thirty-six adult male rats were randomly assigned to three groups. The first group received 0.8 mg/kg of whey protein, the second group received 1.6 mg/kg, and the third group served as the control group. Testes were removed from the animals following slaughter for histological and immunohistochemical analysis. The findings revealed alterations in the testes, with fibrosis, increased interstitial space, a thickened layer of spermatogonia and primary spermatocytes, immature cells in the tubular lumen, increased Leydig cell proliferation, and atrophy and degeneration of the seminiferous tubules. Immunohistochemical staining showed positive CD3-T cell infiltration in the interstitial tissue of groups 1 and 2, contrary to the control group, which exhibited negative staining. In contrast to the control group, group 1 and group 2 showed negative staining for CD20-B lymphocyte infiltration in the interstitial tissue. In conclusion, testicular histopathological effects were induced by high whey protein concentrations. Additionally, T cells infiltrate the testis' interstitial tissue without B lymphocytes, stimulating the immunological response.

Key words: CD3, CD20, histopathological, Immunohistochemistry, Whey protein.

INTRODUCTION

The great nutritional, biological, and functional qualities of whey protein products have led to their widespread use as food additives (Kilara and Vaghela, 2018). Whey proteins are utilized in their natural state and are crucial structural elements in a variety of foods (Tunick, 2008; Famelart *et al*., 2018). Additionally, they function dependably as denatured or mixed proteins that are altered by heating methods. Both young adults and older persons take protein supplements daily (Keri, 2004). The majority of these individuals are dedicated gymnasts who regularly work out and strain their muscles. Protein is essential for the human body's strength, since it aids in the reconstruction of its muscles. The biggest percentage of branched-chain and protein source amino acids is seen in whey protein (Mann *et al*., 2019). Whey is a crucial

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component of the building blocks that the body utilizes to create the amino acids needed to develop lean muscle mass. Whey is taken into the bloodstream rapidly, since it is a fast-acting protein. Because whey protein shakes make nutrients and branched chain amino acids BCAAs easily accessible to the muscles (Saubenova *et al*., 2024), it is commonly thought that consuming one scoop after a workout will hasten muscle repair (Jovanovic *et al*., 2005; Saberi *et al*., 2016). In the scrotum, an outpouching of the abdominal wall, males have two testicles of comparable size. One testicle hangs lower into the scrotum than the other because of variations in vascular anatomy, which is known as scrotal asymmetry (Ross and Pawlina, 2006). The tunica albuginea, a strong membrane layer, covers the testes. Seminiferous tubules are highly coiled, extremely thin tubes located within the testes. From adolescence to old age, a layer of cells called germ cells, also referred to as male gametes, develop into sperm cells, which line the tubules (Garner and Hafez, 2000; Nieschlag *et al*., 2023). Spermatogenesis is the process of the formation and development of sperm cells within the testes, the male reproductive organs (Wistuba, 2007 and Batra *et al*., 2024). A complicated process of cell differentiation, spermatogenesis includes chromosomal recombination, reductive cell division to create haploid cells, expanding cell proliferation, dynamic changes in cellular shape and nuclear chromatin contents, and the shedding of residual bodies (Guraya, 2012 and Waqas, 2021). In the spermiogenesis process, round spermatids undergo significant morphological changes to develop into mature spermatozoa (Miyata *et al*., 2024). As the sperm develop, they pass through the seminiferous tubules, the efferent ducts, the rete testis in the mediastinum testis, and finally the epididymis, where the freshly formed sperm cells mature (Marty *et al*., 2003; Creasy and Chapin, 2013). Muscle contractions cause the sperm to exit the urethra after passing through the vas deferens and subsequently exiting the urethral aperture. Through the process of spermatogenesis, germ cells within seminiferous tubules grow into spermatogonia, spermatocytes, spermatids, and spermatozoon (O'Donnell *et al*., 2015). The seminiferous tubules real epithelium, Sertoli cells, are essential for promoting the development of germ cells into spermatozoa (Happ, 1992). The sperms and seminal fluid components were enhanced by whey protein supplementation. By raising the sperm mitochondrial membrane potential, they decreased sperm abnormalities. While simultaneously improving sperm motility and viability (Kandil *et al*., 2022). Whey protein's histopathological and immunohistochemical effects on male albino rats' testicles were the focus of this investigation.

MATERIALS AND METHODS

Whey protein Collection:

The whey protein utilized in Iraq is always imported, as entire products that are made abroad. Whey protein was collected from the sport center in Baghdad. In this study, different concentrations of whey protein were used (0.8 and 1.6) mg\kg.

Ethical Approval:

The study procedure was admitted via the Department of Basic Science, College of Dentistry, Al-Iraqia University, ESAER-04-13-11-24.

Animal and Experimental Protocols:

The animals were obtained from the laboratory animal center of AL- Qadisiya University. The rats were housed in normal laboratory conditions and had free access to food and drinking water. 36 albino rats were randomly divided into 3 groups. Each group contained (12) rats in a separate cage. The concentration (0.8) mg\kg was given to group 1, and (1.6) mg\kg was given to group 2, and compared to the control group (given normal saline). The treatment continued orally for sixty days.

Histological and Immunohistochemical examination:

After sixty days, animals were sacrificed and testis were isolated for histological and immunohistochemical study. Parts from testes were fixed with a 10% formalin solution for 24 hours, prepared for paraffin embedding. The paraffin blocks were sectioned, and slides were stained with routine hematoxylin and eosin, according to Suvarna *et al*. (2018). Using an avidin-biotin complex with the monoclonal antibody CD3 and CD20 (Patho, Sito Leica company), immunohistochemical sections were prepared at 4 µm to detect T lymphocytes and B lymphocytes. The Olympus light microscope was used to examine the testis sections. After counting T and B- B-lymphocytes in ten high-power fields (400x), the mean percentage for each group was determined. The scores were recorded as: negative: - , mild: +, moderate: ++, severe: +++ and very severe: ++++ (Karamese *et al*., 2020).

Testicular Capsule Thickness:

Fiji software was used to measure the capsule thickness on sections. Three points were measured on each of the sex fields that were photomicrographed at X400 from the capsule's free surface (10 measurements for each animal, 10 per group) (Aire and Ozegbe, 2007).

Data Analysis

Excel software (2010) was used to analyze data, which was presented as mean ± standard deviation (SD), and when P-value <0.05 indicated a significant difference between the groups (Lanipekun *et al*., 2024).

RESULTS

Testicular histological sections of control rats revealed that the testes are composed of seminiferous tubules embedded in interstitial tissue that contain blood vessels and Leydig cells, which are characterized by their size and form. A complex stratified epithelium layer lines the seminiferous tubules, which are supported by a thin basal lamina. The sperm-forming cells of various phases (sperm progenitors, spermatogonia, and spermatids) that produce sperm, as well as the supporting cells (Sertoli cells), are found in the seminal epithelial tissue (Figure 1).

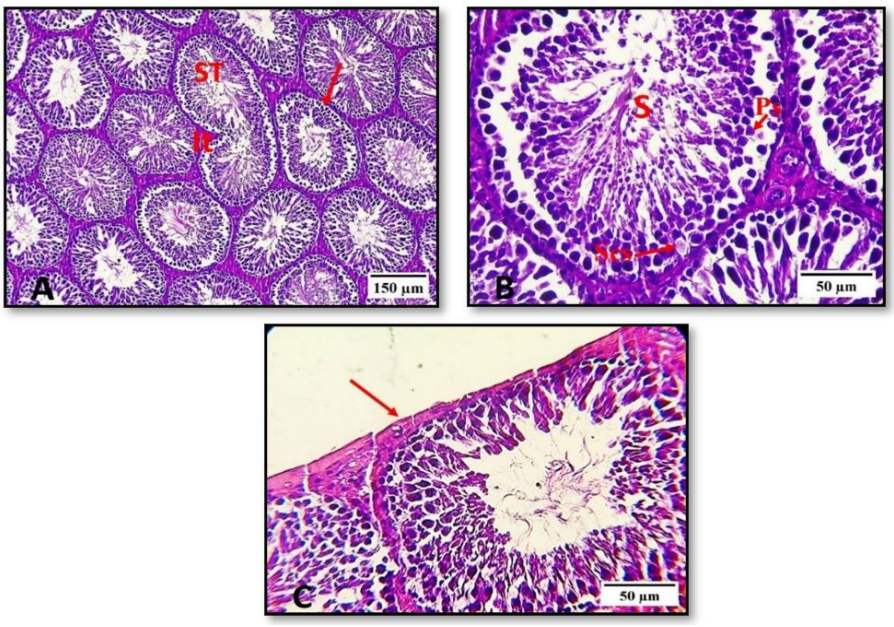

Figure 1: A section of the testicular tissue of an animal from the control group showing. A: the normal seminiferous tubules (ST) with arrangement of spermatogonia (arrowhead) and normal Interstitial Tissue (It.). B: Sertoli cells (Scs). Normal structure of primary spermatocytes (Ps) and mature sperm (S). C: Normal capsule (arrow) (H&E stain).

Whey protein-treated group showed degeneration and atrophy of seminiferous tubules, with fibrosis and increased interstitial space, more distribution of Leydig cells, thick layer of spermatogonia and primary spermatocyte, immature cells in the lumen of seminiferous tubules and thickness of capsule of 7.41±0.65 µm (Figure 2) compared to control group (3.89±1.30 µm). While the testis in group 2 treated with whey protein showed a capsule of 9.42±1.28 µm thickness, atrophy of seminiferous tubules, sloughing, increased interstitial space, and the interstitial cells showed atrophy, thickened the lining epithelium of seminiferous tubules, and thick layers of spermatogonia and primary spermatocytes. There were congestions of blood vessels. In addition, the presence of some cells in the lumen of some seminiferous tubules has been observed (Figure 3).

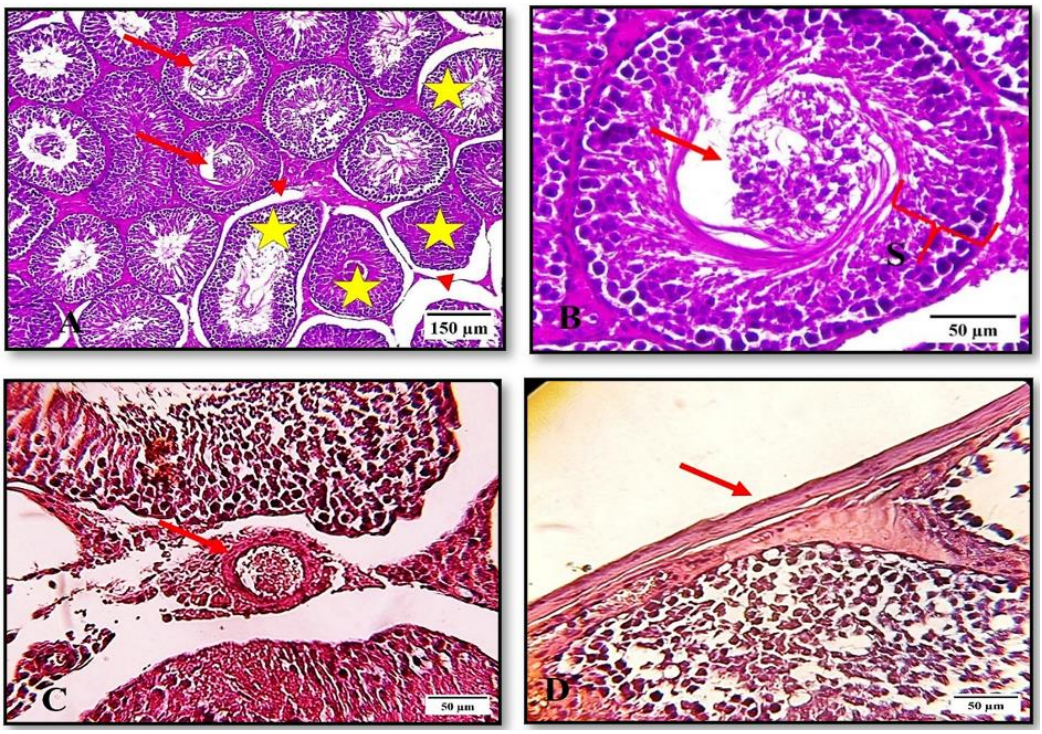

Figure 2: A section of the testicular tissue of an animal from group 1 showing: A: mild atrophy of testicular tubules (stars), immature cells in the lumen (arrow), increased interstitial space, and more distribution of Leydig cells in the space (arrowhead). B: thick layer of spermatogonia and primary spermatocytes (S) and immature cells in the lumen of seminiferous tubules (arrow). C: congestion of blood vessels (arrow). D: Thickness of capsule (arrow) (H&E stain).

Immunohistochemical sections of testis showed mild positive staining for CD3-T lymphocyte infiltration in the interstitial tissue of group 1 (treated with 0.8 g of whey protein), while group 2 (treated with 1.6 g of whey protein) were moderate positive staining compared to control group which were negative to the staining (Table1) (Figure4).

Table 1: Semiquantitative assessment of CD3 and CD20 lymphocytes in the testis of treated and control groups:

Groups	N.	CD3 T - lymphocytes	CD20 B- lymphocytes
Control	12	-	-
Group 1	12	+	-
Group 2	12	++	-

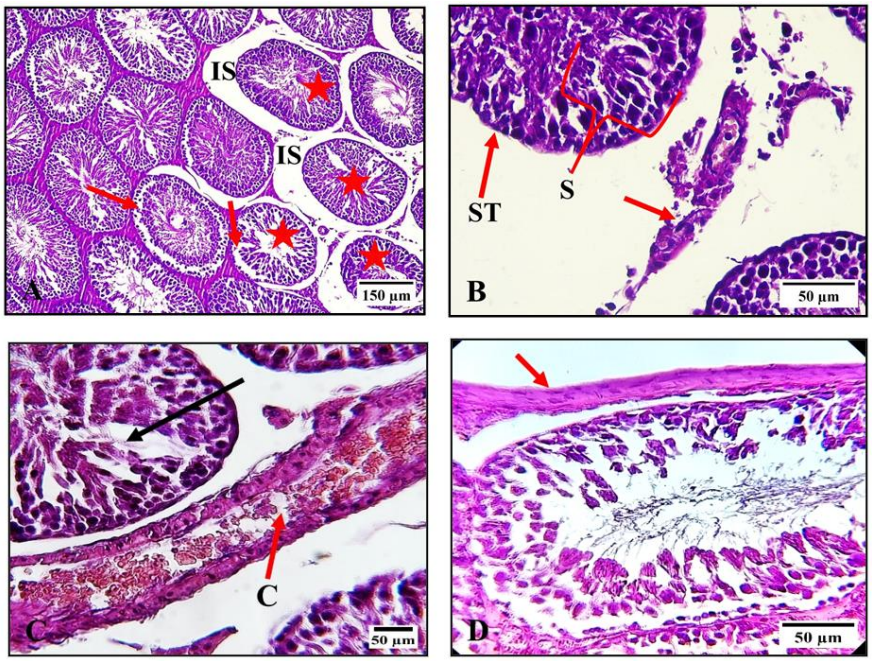

Figure 3: sections of the testicular tissue of an animal from group 2 showing: A: atrophy of seminiferous tubules (stars), increased interstitial space (IS) and sloughing (arrows). B: The interstitial cells showed atrophy (arrow), thickened the lining epithelium of seminiferous tubules (ST), thick layers of spermatogonia and primary spermatocytes (S). C: congestions of blood vessels (C) and immature cells in the lumen of seminiferous tubules (black arrow). D: Thickening of capsule (arrow) (H&E stain).

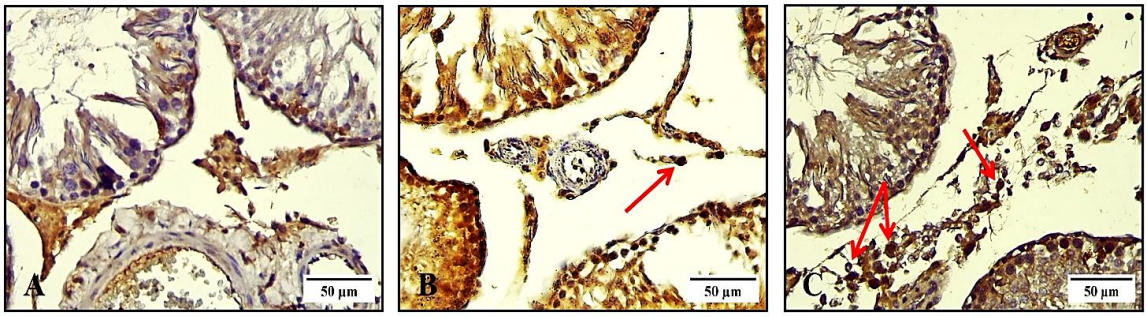

Figure 4: Immunohistochemical staining exhibiting CD3-T lymphocyte in the interstitial tissue of the testis in: A: the control group was negative staining, B: group 1 was mildly positive staining (arrow). C: Group 2 was moderately positive staining (arrow).

The animals treated with different doses of whey protein, the testicular immunohisto-chemical sections showed negative staining due to no infiltration of CD20-B lymphocyte in the interstitial tissue of groups 1 and 2 compared to the control group (Table 3) (Figure 5).

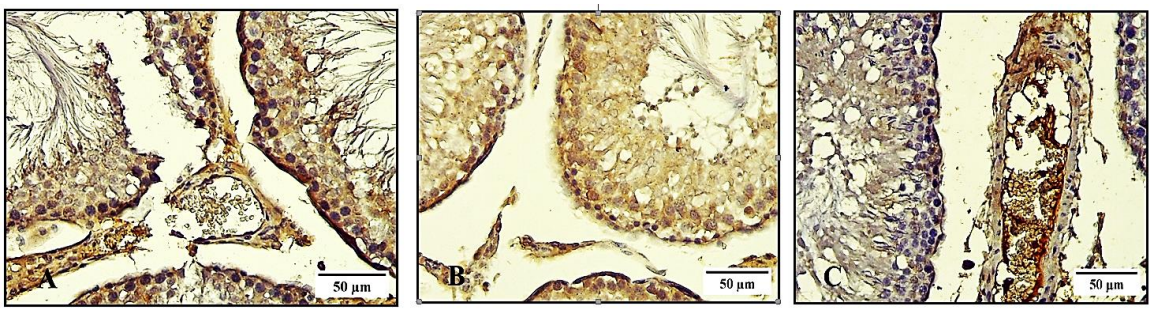

Figure 5: Immunohistochemical staining for CD20-B lymphocytes in the interstitial tissue of the testes showed negative staining in all groups **(A:** Control, **B:** Group 1**, C:** Group 2).

DISCUSSION

Whey protein-treated group 1 displayed seminiferous tubule atrophy, fibrosis, and increased interstitial space. There were also more Leydig cells distributed throughout the interstitial tissue, alterations to the spermatogonium and primary spermatocyte layers, and immature cells in the seminiferous tubule lumen. Degeneration may result from the effects of whey protein components on cell membranes, or it may be the result of a hormonal imbalance (Creasy *et al*., 2012). Atrophy of the seminiferous tubules in supplement-treated rats was confirmed by Yamada et al. (2007). However, the testis of animals in group 2 that received whey protein treatment displayed thicker capsules, thicker lining epithelium of seminiferous tubules, and thick layers of spermatogonium and primary spermatocytes, sloughing of the germinal layer, increased interstitial space, and atrophy of the interstitial cells. Blood vascular congestion was present. Furthermore, the presence of cells in the lumen of certain seminiferous tubules may be attributed to several factors. These cells are believed to be either immature germ cells or cells that have detached from the testicular epithelium, it is believed that they are cells separated from the testicular epithelium or immature germ cells (Johenson, 2014). Another proposed explanation is that they originated from weak cell divisions due to the high concentration of whey protein use for a long time, which led to these damages. Another proposed explanation that has been effects resulted from weak cell divisions brought on by prolonged high doses of whey protein. Whey protein and reactive oxygen species (ROS) have a connection; the supplement created histological risks in the testicular epithelium because reactive oxygen species damaged the cell membranes (Mitra *et al*., 2013). Other studies suggest that protein supplements contain silicone dioxide and soy protein, which cause DNA damages affecting on spermatogenesis and destroy a testicular tissue (Safdar *et al*., 2021). Congestion and a few other histological abnormalities discovered in the testes could be related to reactive oxygen species and oxidative stress brought on by whey protein supplement consumption. Dietary soybean protein consumption caused decreased testosterone level in men that led to spermatogenesis disorders (Habito *et al*., 2000). The damage of the seminiferous tubules observed in the testes of rats given dietary supplements has been confirmed by previous studies (Momeni *et al*., 2012; Abdelatty *et al*., 2020). Casein induced higher serum levels of tumor necrosis factor alpha (TNF-α) and interleukin-10 (IL-10), which can cause degenerative changes in the seminiferous tubules, where the epithelium is composed of a layer of Sertoli cells affecting spermatogenesis, spermiogenesis, and few sperm, as well as chronic inflammatory conditions and pathological changes in testicular tissue (Zhao *et al*., 2017). Immunohistochemical sections scored mild positive staining in treated animals' group1 and moderate positive staining in group 2 due to T-lymphocytes infiltration in the interstitial tissues of the testis, it's possible that consuming varying amounts of whey protein damages tissue, which in turn triggers the release of cytokines. This immunological reaction encourages lymphocytes to gather at the site of inflammation. An alternative reason is that L-arginine amino acids slightly raised the production of interleukin IL-2, which may boost T cell proliferation in mice by boosting the expression of certain receptors and the use of IL-2 to improve cellular immune response (Ochoa *et al*., 2001). Some studies suggest that dietary supplements was associated with an increased CD3 T-lymphocytes count (Ivkovic *et al*., 2004; Shakoor *et al*., 2021). Additionally, there was weak infiltration of B-lymphocytes. The study provides compelling evidence that proteins can

affect immune function via a variety of mechanisms, including impacts on B cells and local immune cells. Lymphocytes use glutamine at a rapid rate. In athletes, it is oxidized as fuel and for DNA and RNA synthesis (Venkatraman and Pendergast, 2002).

CONCLUSION

The research aimed to investigate the effects of whey protein on testis of male rats. The high concentration from whey protein causes changes in the male reproductive system include testicular histopathological effect. In addition, stimulates immune response through T lymphocytes infiltration in the interstitial tissue of testis.

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هديل كامل خليل ، مختار خميس حبه

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نظراً لأن العديد من الرياضيين يستهلكون كميات كبيرة من بروتين مصل اللبن دون استشارة أخصائي تغذية، فقد صُممت هذه الدراسة لتحديد تأثير بروتين مصل اللبن على أنسجة الخصية من منظور نسيجي ومناعي كيميائي. قُسِّم ستة وثلاثون جرذاً ذكراً بالغاً عشوائياً إلى ثلاث مجموعات. تلقت المجموعة الأولى ٠,٨ ملغم/كغم من بروتين مصل اللبن، وتلقت المجموعة الثانية ١,٦ ملغم/كغم، وشكلت المجموعة الثالثة مجموعة الضبط لمدة ٦٠ يوما. أُزيلت الخصيتان من الحيوانات بعد التضحية بها لإجراء التحليل النسيجي والمناعي الكيميائي. أظهرت النتائج تغيراً في الخصية، مع تليفها، وزيادة المساحة الخلالية، وطبقة سميكة من الخلايا المنوية والخلايا المنوية الأولية، وخلايا غير ناضجة في تجويف الأنبوب، وزيادة تكاثر خلايا لايديغ، وضمور وتنكس الأنابيب المنوية. على عكس المجموعة الضابطة، التي أظهرت صبغة سلبية، أظهرت المقاطع المناعية النسيجية للخصية صبغة إيجابية لخلايا CD3-T التي ترشحت إلى النسيج الخلالي للمجموعتين ١ و٢ من بروتين مصل اللبن. وعلى عكس المجموعة الضابطة، أظهرت المجموعتان ١ و٢ صبغة سلبية لترشيح الخلايا الليمفاوية CD20-B في النسيج الخلالي. نستنتج من ذلك وجود تأثيرات نسجية مرضية على الخصية بسبب التراكيز العالية من بروتين مصل اللبن. بالإضافة إلى ذلك، ترشح الخلايا التائية إلى النسيج الخلالي للخصية دون الخلايا الليمفاوية البائية، مما يحفز الاستجابة المناعية.

الكلمات المفتاحية: CD3 , CD20 , النسجي المرضي , المناعية الكيميائية , بروتين مصل اللبن.