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Original Article

Whey supplementation alleviated ventral prostate damage and circulating hormones in aged rats

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Abstract

Many studies have focused on the aging related hypertrophy and prostate injuries. The use of natural product to prevent the prostate tissues degradation are of particular concern. The study's aim was to determine the biological significance of whey supplementation in optimizing aging-related changes in the structure and function of prostate of aged rats. Male Wistar albino rats aged 8 and 30-months were categorized into two main groups with or without whey supplementation (n=8). After two months of oral whey supplementation (2 ml, twice doses/day), the rats were euthanized, blood was collected and serum was separated for assessments of serum hormonal levels of testosterone and tri-and tetra-iodothyronin. The prostate was dissected and processed for histological, immunohistochemical and transmission electron microscopy. The studies showed that whey supplementation alleviated the shrieked and atrophy of prostate acini as well as hyperplasia of their lining epithelium. Whey supplementation also decreased expression of the caspase 3 and prostate specific antigen (PSA) immunohistochemistry. This was influenced by increased the immune reaction of proliferating cell nuclear antigen (PCNA).This were also associated with improved the serum hormonal levels of testosterone and triodo-and tetra-iodothyronine. Finally the author finally concluded that whey supplementation improved properties of aged properties and reduced hyperplasia, inflammatory lesions and collagenous infiltration. Increased testosterone and thyroid hormonal levels were also found.

1. Introduction

Aging resulted in benign prostatic hyperplasia (BPH) and benign prostatic enlargement (BPE in elderly men. Within the transition region, this was marked by of a nodular overgrowth of the epithelium and fibromuscular tissue within transition zone (1). Approximately 8% of men developed pathological BPH in their fourth decade of life. Furthermore , between the ages of 51 and 60 , 50% of the men experience pathological BPH. Men aged 70–79 years had a 4.6 fold increase in occurrence. BPH worsens as the prostate volume becomes larger and the maximum urinary flow rate decreases (2). The prostate epithelium overexpressed estrogen receptor β which regulate cell development, and apoptosis. Hyperplasia, intraluminal concretions, nuclear atypia and prostate intraepithelial neoplasias were found in the prostate of old rats (3). BMH afflicted More than 1 billion men throughout the world . The progression of BMH is closely linked to sexual hormones, such as progesterone, testosterone, dihydrotestosterone, and estrogen (4).

Whey protein (WP) manages immune functions by activation of lymphocytes, , antibody production and phagocytic activity (5) and increases the secretion of immunoglobulin A (IgA) from Peyer's patches (6). Aged male non-obese diabetic (NOD) mice ingested glycated whey protein were found to possess increased survival rate and decreased inflammation in of the prostate (7). There are no available work to discuss the assessment of bovine whey in the aged prostate. The available literature concentrated mainly in the in influence of lactoferrin a component of whey on prostate cancer. Lactoferrin was found to reduce the expression of steroid hormone receptors and Ki-67 in prostate cancer cell lines (8). Administration of lactoferrin conjugated with 3-diaminobutyric polypropylenimine (DAB) led to suppression of 70% of prostate cancer and 50% of human cell line tumors within one month-treatment (9). The aim of the study is to determine the therapeutic potential of whey in the old prostate by demonstrating histopathological and cytological changes as well as anti-apoptotic

activity and increases in testosterone and thyroid hormones.

2. Materials and Methods

2.1 Whey syrup supplementation

Fresh bovine whey syrup was freshly obtained from the Faculty of Agriculture, Cheese Fermentation Lab, and Mansoura University, Egypt. Rats orally supplemented freshly whey of 2 ml twice daily every other day for two months.The nutrient contents of whey were was investigated according to the study carried out by Kandil et al (10)

2.2 *Experimental animal*

This research and all procedures were accepted by the Egyptian Committee for Animal treatment and bioethics of the Faculty of Science, Mansoura University, Egypt. Twenty-four adult and old male Wistar albino rats (Rattus novergicus) at 8 and 30-M (months) old obtained from Hellwan Breeding Farm, Ministry of Health, Cairo, Egypt and used for experimental work. They were kept in an aerated room with approximately 12 hour of light and dark cycle and light intensity exposure at 180–200 lx. Free excess to of standard diet and water were allowed ad libitum. The animals were divided according into two main groups; 8 months (G1) and 30 months old (G2). Each group was further subdivided into two subgroups, whey supplemented (G1Y&G2Y) and non-supplemented group (G1&G2). For the whey supplemented group, each individual was supplemented 2 ml twice daily every other day for two months by interagastric tube. At the end of treatment, the rats were fasted overnight and euthanized by intraperitoneal injection of 60 mg ketamine/kg, sacrificed and dissected. Blood was collected in non-heparinized tubes and centrifuged at 2000 rpm. Serum was separated and kept in refrigerator. The ventral lobe of prostate of the studied groups were dissected and processed for the followings:

Light microscopy

The specimens were fixed in 10% phosphate buffered formalin (pH 7.4), dehydrated in ascending concentrations of ethyl alcohol, cleared in toluene and mounted in molten paraplast at 58-62 ºC. Five µm histological sections were cut, stained with Hematoxylin and eosin and viewed under a bright field light microscope

Transmission electron microscopy

Extra-specimens were fixed in 2% phosphate buffered glutaraldhyde (pH 7.4), then post-fixed in 1% osmium tetraoxide at 4 °C, dehydrated in ascending degrees of ethyl alcohol, and embedded in epoxy resin. Ultrathin sections were cut with a LKB Ultratome IV (LKB Instruments, Bromma, Sweden) and mounted on grids, stained with uranyl acetate and lead citrate, and examined on a Joel 100CXl transmission electron microscope at Mansoura University Lab.

Immunohistochemistry for Caspase-3, anti-proliferating cell nuclear antigen (PCNA) and anti-prostate specific antigen (PSA)

Five µm histological sections of formalin-fixed, paraffin-embedded tissue sections were dewaxed and rehydrated in descending concentration of ethyl alcohol. After clearing of endogenous peroxidase activity, the tissue sections were digested with drops of 0.05 % trypsin (pH 7.8), washed and incubated with the caspase 3 monoclonal mouse antibody (Cat #MA1-16843, ThermoFisher Scientific,USA 1:50,), primary antibody against proliferating cell nuclear antigen (PCNA) (Cat #MA5-11358, ThermoFisher Scientific,USA, at 1:50) and rabbit anti-prostate specific antigen (PSA, Cat. #RM-2104-S, Thermo Fisher Scientific Anatomical Pathology 46360 Fremont Blvd. Fremont, CA 94538, USA; dilution of 1:200 for 20 minutes). The slides were then treated with a secondary biotin linked anti-mouse antibody and with the streptavidin-peroxidase complex. The sections were then cleaned and incubated with developing solution (diaminobenzidine-hydrogen peroxide), and counterstained with hematoxylin. In the case of PCNA, the immunological reaction was visualized as brown reaction in nucleus , while in the case of caspase 3 and PSA, the immunological reaction was visible as brown reaction in the cytoplasm. Finally, the slides were examined using a digital canon camera and an Olympus microscope with a strong field illumination. Image processing was performed with at 40 X objective and an Olympus digital camera fixed on Olympus microscope with 1/2 X frame adaptor. The resulting images were analyzed using Video Test morphology software (Russia) on an Intel Core 5 based computer and the percentage area was calculated and reported.

Biochemical assessments of serum hormonal levels

The serum level of triiodothyronine (catalogue No. CSB-E05079r) and tetraiodothyronine (catalogue No. CSB-E05100r) and testosterone (catalogue No. CSB-E05100r) were determined by using ELISA kit (Cusabio Biotech Company, Houston, TX 77054, USA). The method based on placing 25 µl of serum samples to wells precoated with the biotin-conjugated polyclonal antibody of the assayed hormone. Avidin conjugated horse reddish peroxidase is added and incubated at room temperature (18-25 °C) for 90 minutes. The intensity of the colour development is proportional to the amount of hormones in the samples. The absorbance is measured spectrophotometrically at 450 nm.

Statistical analysis

Data were presented as a mean ± standard error (SE). The statistical analysis was performed with one-way post-hoc analysis of variance (ANOVA) using SPSS (version 16) software package for Windows, comparing the variations between studied groups and $P<0.05$ was considered statistically significant.

3. Results

Light and transmission electron microscopic observations

Histologically, the adult prostate possessed varying size and folded mucosa of the acini of the ventral lobe. There are three main types of epithelial cells. The luminal cells have apical brush borders with basal rounded nuclei, basal flattened cells and cells with pale cytoplasm suspected neuroendocrine (Fig. 1A and A1). Whey supplementation of the adult group preserved the histological picture (Fig. 1B and B1).

In comparison to adults, aged rats showed atrophied acini with a lack of luminal secretion .Inflammation is evident by intense clustering of leukocytes between the acini . Dense collagenous fibrils were also detected in between the prostate acini. There was significant severe hyperplastic pathological alterations in the epithelial lining cells. (Fig. 1A2 and A3).

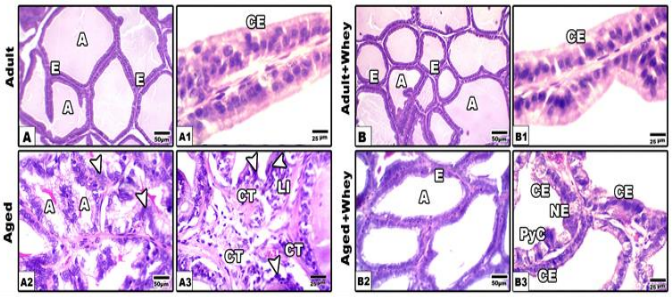

Figure 1. Photomicrographs of histological sections of prostate. A and A1. 8 month-old rat. B and B1. 8 month old rat supplemented whey. Note increased size of acini with colloid secretion and outlined by columnar eithelium. A2 and A3. 30 month old showing congested acini with hyperplasia of their lining epithelium. The acini infiltrated by collagenous fibrils and dense leukocyte infiltration manifested inflammation. B2 and B3. 30 month old rats supplemented whey showing restoration of the acini structure and the lining epithelium acquired cubical structure. H&E. Abbreviations; A, alveolar lumina; CE, cuboidal cell; CT, collagenous fibrils; E, Epithelium; HC, hypertrophied cells; LI, leukocytic infiltration; NE, necrotic epithelium; Pyc, pyknotic cell. Arrow head indicate hyperplastic epithelium

Whey supplementation to aged rats improved the histological picture associated with increased folding of the acini and restored the structural pattern of the lining epithelium. However, the epithelium took on a cubical form rather than the typical columnar structure (Fig. 1B2 and B3). In the aged rat, the prostate epithelial height was significantly reduced in comparison with more improvement after whey supplementation (Fig. 2).

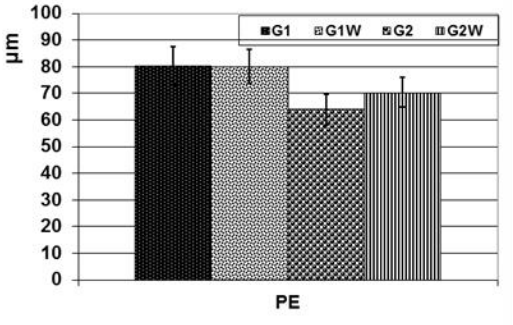

Figure 2. Chart illustrating apparent reduction of prostate epithelium (PE) height in 30 month aged rat and improved after supplementation of whey.

At ultrastructural level, the adult prostate epithelium had a typical pattern distribution of nuclei with characteristic chromatin distribution. The secretory vesicles were also eventually distributed throughout the apical cytoplasm. The microvilli covered the basal lamina of the prostate epithelium. Within the prostate stroma, fine collagenous fibers were found. Near the apical margin of the prostate lining epithelium ,secretory vesicles were detected . Mitochondria and rough endoplasmic reticulum were distributed throughout the cytoplasm (Fig. 3 A & B). Whey supplementation to adult rat modulated the structural pattern of prostate epithelium as in adult (Fig. 3 B, B1 & C1).

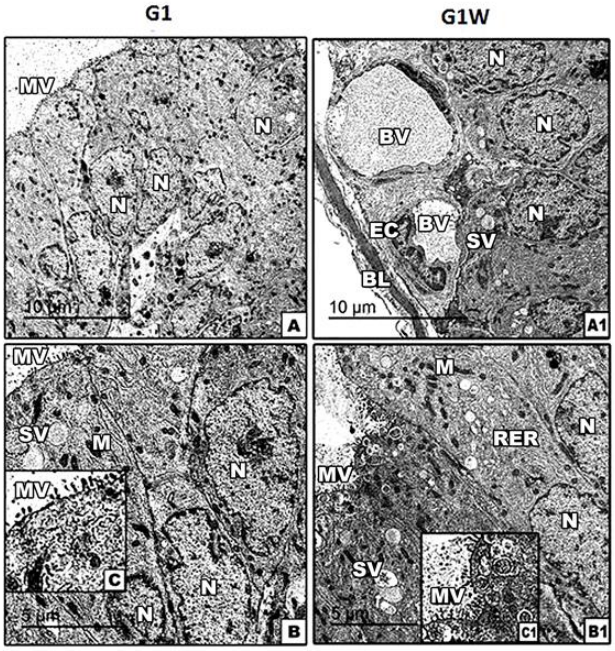

Figure 3. Transmission electron micrographs of normal prostate of 8 month old rats. A-C. Adult rat showing columnar epithelial cells with characteristic nuclei.Note abundant microvilli in the brush border. A1-C1. Adult rat supplemented whey showing blood capillaries in contact with thin basal lamina. Microvilli is abundant in peripheral border. Abbreviation; BL, basal lamina; BV, blood vessel; EC, endothelial cells; M, mitochondria; N, nuclei; MV, microvilli; RER, rough endoplasmic reticulum; SV, secretory vesicle.

In the aged prostate, there was a considerable thickening of the collagenous sheath covering the acini. Myoid cells were spreading in the collagenous tissue. A comparative reduction in epithelial height and the absence of microvilli was observed. Hyperplasia of the epithelial lining cells having pleomorphic nuclei and convoluted nuclear. Pyknosis was seen in some of these cells, which had densely compacted nuclear heterochromatin. Lysosomes were detected in the cytoplasm of the injured epithelium , surrounding the secretory vesicles. Mitochondrial damage and vesicuolated rough endoplasmic were also observed (Fig. 4 A-C). Whey supplementation restored thinning of the collagenous sheath of basal lamina in the aged rats . Lysosomes were more evenly distributed. The nuclei of the epithelium had a typical chromatin distribution pattern. The secretory vesicles were

oriented properly and relocated to the apical border of the cells (Fig. 4 A1, B2 & C1).

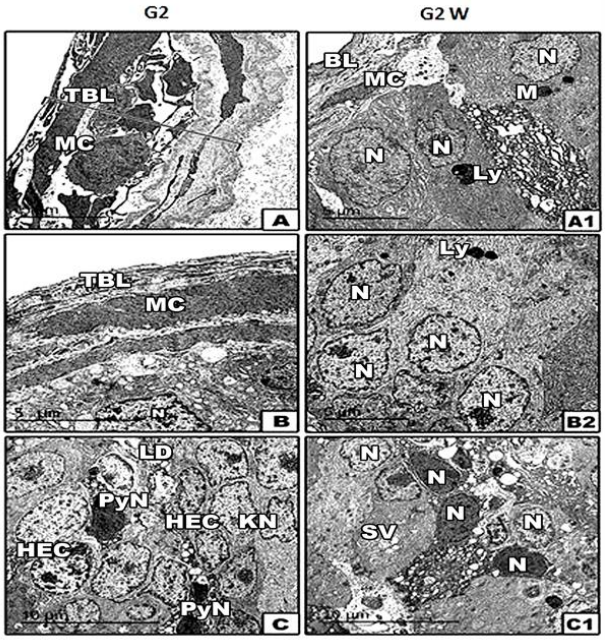

Figure 4. Transmission electron micrographs of prostate aged rat 30 month-old. A-C. Aged rat prostate showing thickened basal lamina and deposition of collagenous fibrils infiltrated by myoid cell (A and B). C. Showing hyperplasia of prostate epithelium characterized by presence of grouping of epithelial cells with comparatively varying sizes. Some cells appeared binucleated with indication of mitotic cell division. A1-C1. Aged rat supplemented whey. A1.Showing less thickened basal lamina with atrophied myoid cells B1. Showing improved epithelial lining cells with normal characteristic nuclei. C1. Showing hypertrophied epithelium with pyknotic nuclei. Abbreviation; HEC, hypeplastic epithelial cells; LD, lipid droblet; M, mitochondria; Ly, lysosome; MC, myoid cells; MV, microvilli; N, nucleus;PyN, pyknotic nuclei; SV, secretory vesicle; TBL, thickened basal lamina.

Immunohistochemistery of PCNA, PSA and caspase 3

The prostate epithelium in both the adult and whey supplemented groups, had upregulation of the proliferating cell nuclear antigen (PCNA) immunohostochemical reaction (Fig. 5 A and B). However in aged rats, there was a significant reduction of the immunological reaction (Fig. 5C). Whey supplementation to the aged group restored the immunohistochemical reaction of the epithelial lining cells but the intensity of the immune reaction was still less in comparison with the adult group (Fig. 5D). Image analysis revealed increased intensity of the PCNA in adult groups in comparison with aged group (Fig. 6).

The immunohistochemical reaction to cysteine-aspartic acid protease 3 (caspase-3), was abundantly identified in the epithelial lining cells of aged rats (30 M-old) compared to a negligible immunological reaction in the adult. Aged rat's supplemented whey possessed a reduced immune reaction but it was still detected more than that of adult supplemented whey (Fig. 5). When comparing the aged group to that supplemented whey, image analysis demonstrated increased caspase-3 intensity. The immunohistochemical reaction was least expressed in the adult group (8 M-old) and its whey supplementations group (Fig. 6).

Concerning PSA, the immunohistochemical reactions in the prostate epithelium in aged rats (30 M-old) were much higher than in adult group. Compared to adult group, elderly rats treated with whey had a reduction in the immune reaction (Fig. 5). Image analysis showed that the immunological reaction was increased in the aged group than in the whey supplementations group (Fig. 6).

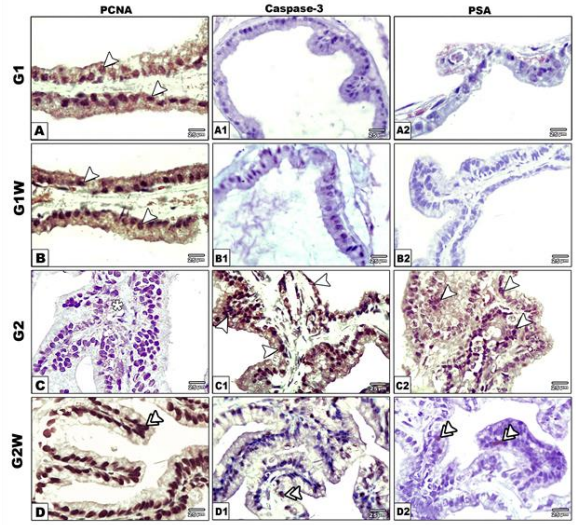

Figure 5. *Photomicrographs of formalin fixed histological sections of prostate immunohistochemical stained with PCNA (A-D), caspase 3 (A1-D1) and PSA (A2-D2).G1 (A-A2), G1Y (B-B2), G2 (C-C2) and G2Y (D-D2). Note increased PCNA immunohistochemical reaction in G1 & G1W compared with decreased the immune reaction in G2. G2W exhibited improving of the immune reaction. Also, caspase 3 and PSA overexpressed in G2 compared with G1 and G1W. The immune reactions is decreased in G2W. Arrow head pointed to the dense immunohistochemical reaction. Star means decreased PCNA immunological reaction. Double arrow means improved.*

Serum levels of testosterone, T3 and T4

Whey supplementations modulated the blood hormonal levels of triiodothyronine, tetra-iodothyronine, and testosterone to the 8 months old rats. On one hand, aged rats exhibited abnormal low level of the measured hormones. On the other hand, whey supplementations restored the measured serum hormonal levels, although they were still remained lower than the control group (Fig. 7).

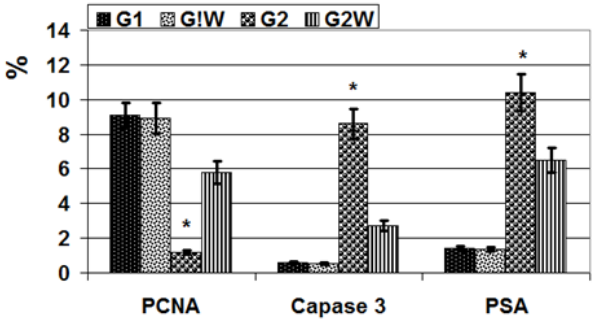

Figure 6. *Chart illustrating image analysis of PCNA , caspase 3 and PSA in in prostate of adult & old rats with or without whey supplementations. Note over expression of caspase3 and PSA in aged rats and improved in experimental group supplemented whey. Star means significant compared to control at P<0.05.G1; 8month old; G1W,8 month old supplemented whey; G2, 30 month old; G2W, 30 month old supplemented whey.*

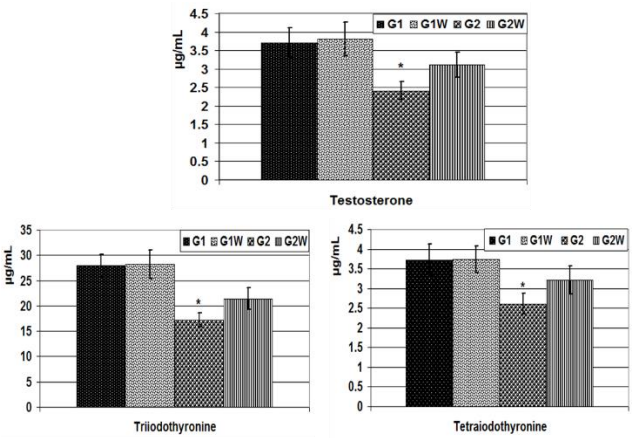

Figure 7. *Chart illustrating serum testosterone and triodo-and tetradiodothyronine of aged rat with or without whey supplementation. Note decreased serum hormonal levels in aged group (G2) in comparison with G2 supplemented whey group (G2W). Adult rat supplemented whey (G1W) showed similar serum hormonal levels as in adult group (G). Star means significant decrease in comparison with the control.*

4. Discussion and conclusion

FThe observed findings revealed that the ventral lobe of the prostate gland of aged rats possessed acini with folded mucosa in contrast with adult rats. The lumina of the acini become atrophied due considerable atrophy and hyperplasia of their epithelial lining cells. The present prostatitis is in agreement with the investigations carried out by Lau *et al*. (11) in the ventral lobe of senile patients.

Also, the prostatitis of aged rats were associated by dense leukocyte infiltration forming semi-like granulomatous lesions and infiltrated by collagenous materials. The present findings supported the studies carried out by Bianchi-Frias *et al.* (12) in aged mice reported that inflammation led to damage of prostate epithelium associated with collagenous infiltration which may be predicted to the development of prostatic cancer.

There was a significant thickening of the collagenous sheath enveloping the acini and invaded by myoid cells during the ultrastructural examination of the aged prostate. There was a noticeable drop in epithelial height as well as the missing of microvilli. Hyperplasia of epithelial lining cells with pleomorphic nuclei and convoluted nuclear envelope. Pyknotic nuclei were seen in several of these cells. The cytoplasm surrounded by many pleomorphic storage vesicles. Lysosomes were seen in the injured epithelial cytoplasm and near the secretory vesicles.

Similar findings of atrophied prostate epithelium and missing of microvilli were reported in diabetic SD rats (13).

Experimental studies carried out on ventral prostate of diabetic mice (14) exhibited similar alterations of the prostate epithelium.

The observed Inflammatory lesions of the prostatitis were explained by increase apoptotic cell assessed by overexpression of caspase 3 and PSA immunohistochemistry and reduced expression of PCNA in the epithelium of ventral lobe of prostate. The present findings agree with the studies carried out by O'Neill et al. (15) who reported increase caspase 3 in both basal and secretory epithelial cells of benign prostatic hyperplasia (BPH). Both inflammation and apoptosis were carried out parallel with each other in BPH of aged men (16).

The pathogenicity of the prostate epithelium may be represented by increased PSA immunohistochemistry. PSA is produced is produced by the transition zone of the hyperplastic benign prostatic epithelium (17). There is a great association with increased PSA and increased prostate volume and BPH (18).

The present findings confirmed the studies carried out by in patients with prostate benign prostatic hyperplasia in prostate of diabetic rats (13). At the same time, aged rats exhibited reduction of the serum levels of tri-and tetra-iodothyronin and testosterone in comparison with the adult individuals.

The present finding confirmed the study carried out by Mani Maran et al. (19) following prostate ectomy. The present findings are closely similar to the studies of testosterone dysfunction carried out in elderly patients (20, 21) and old rats (22).

Aging related prostatitis may result from impairment of triiodothyronine, and total testosterone level (11). Whey supplementation restored the integrity of the lining epithelium in aged rats however the epithelium took on a cubical shape rather than the regular columnar shape. The epithelium possessed distinctive nuclei with vesicular chromatin material. The secretory vesicles were normally oriented and relocated to the apical cell boundary.

The increased whey contents of a-lactalbumin, b-lactoglobulin, lactoferrin, lactoperoxidase, and serum albumin. (Madureira *et al*., (23) and amino acids such as phenylalanine, isoleucine, cystine and threonine and high amounts of sulphur containing amino acids (24) may facilitated improvement and reduced inflammation due to amphoteric characteristic of amino and carboxyl groups for scavenging free radicals. These findings agree with the studies carried out by Corrochano *et al.* (25) who reported that whey products have increased antioxidant and radical scavenging activity.

Diabetic mice received camel whey protein exhibited decreased the levels of pro-inflammatory cytokines, reactive oxygen species and activating transcription factor-3 (26).

Finally the author finally concluded that whey supplementation enhanced the antioxidant quantities of aged individuals and alleviated the inflammation as evidenced by increased testosterone and thyroid hormones which improved the prostate structure and function.

CONFLICT OF INTEREST

There is no conflict of interest in this study.

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