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Effect of Pumpkin Seed (*Cucurbita pepo* L.) Diets on Benign Prostatic Hyperplasia (BPH): Chemical and Morphometric Evaluation in Rats

Manal K Abdel-Rahman

Department of Nutrition and Food Science, Faculty of Home Economics,
65 Elmatbaea El-Ahlia St., Boulak, P.O. Box 11611, Cairo, Egypt

Abstract: Benign prostatic hyperplasia (BPH) is a common disease in elderly men. Although it is a non-malignant disease, it can have a significant impact on the quality of life of elderly men. The pumpkin seed is claimed to be useful in the management of BPH. This investigation analysed the chemical composition of pumpkin seeds and examined its effect on citral-induced hyperplasia of the prostate in Wistar rats. Citral was administered orally into stomachs of male rats to induce BPH to all rats except negative control group. A rat from each group was sacrificed after 15 days from study, protein binding prostate was determined in ventral prostate gland in order to ensure that BPH has been induced. Fifty adult Wistar male rats were divided into five groups as follows: negative control group that have no BPH and fed on basal diet (C-), positive group rats have BPH and fed on basal diet only (C+), the remaining groups had BPH and were fed on different level of pumpkin seeds, 2.5, 5 and 10%. Four weeks later all rats were sacrificed and several investigations have been conducted such as ventral prostatic growth, protein binding prostate (PBP) and the histology of testis. Citral significantly increased prostate weight (P<0.05). However, pumpkin seeds significantly inhibited enlarged prostate especially at high concentrations seed dose (10%) ($P < 0.02$). Results indicate that pumpkin seeds can alleviate the signs of BPH such as decrease of PBP levels, weight of ventral prostate size, improve histology of testis that may be beneficial in the management of mild stage of benign prostatic hyperplasia. For the first time we found a link between BPH and testis histopathology that needs more investigation.

Key words: Pumpkin seeds · Benign prostatic hyperplasia · Protein binding prostate · Rats · Histology

INTRODUCTION

Pumpkin seeds, known as pepitas, are flat, dark green seeds. Some are encased in a yellow-white husk, although some varieties of pumpkins produce seeds without shells. Pumpkin has been cultivated in Mexico and North America since at least 14,000 B.C. based on archaeological evidence [1]. Michael and co-worker [2] reported that roasted pumpkin seeds would improve the nutritional value of seeds, for example, the increases of the sterols and vitamin E during the roasting process that could be attributed to the changes of the seed meal, since at the end of the roasting the oil emerges from the seeds resulting in altered chemical behaviour of the extraction process.

The nutritional value of pumpkin seeds is based on high protein content (25–51%) [1] and high percentage of oil that ranging from 40% to 60%, up to 60.8%, of the oil is from the fatty acids oleic (up to 46.9%), linolenic (up to 40.5%), palmitic and stearic up to 17.4%, the ratio of monounsaturated to polyunsaturated acids from 0.60 to 0.75g [3].

There are approximately 1% phytosterols present in free and bound forms; squalene; (that can used as indicators when pumpkin seed oils suspected in adulteration with other oils) chlorophyll pigments, 4–5% minerals including selenium, zinc, calcium, copper, iron, manganese, phosphorous and potassium; approximately 30% pectins [1] tocopherols (β- and γ- tocopherol); carotenoids (lutein and β- carotene).

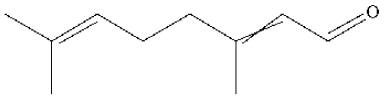
Pumpkin seeds have long been used as a "folk remedy" to treat irritable bladder and prostatic complaints. Recently, pumpkin seeds have become more popular as a medical treatment for urinary complaints associated with benign prostatic hyperplasia (BPH). Despite the acceptance of pumpkin seeds in medical

Corresponding Author: Dr. Manal K Abdel-Rahman, Department of Nutrition and Food Science, Faculty of Home Economics, 65 Elmatbaea El-Ahlia St., Boulak, P.O. Box 11611, Cairo, Egypt

practice, current knowledge of its efficacy as a treatment for complaints of BPH is limited to observational studies. In Egypt, pumpkin seeds are the alternative to the western popcorn, eaten for leisure as part of the Egyptian lifestyle.

Benign prostatic hyperplasia (BPH) is an increase in size of the prostate gland without malignancy present and it is common to be normal with advancing age in up to 90% of men by the age of 80. BPH may defined as a life altering condition requiring urgent medical intervention, is a serious medical disorder associated with major complications, surgical intervention and severe lifestyle interference. It seems likely that the nature of BPH is a failure of apoptosis and that some of the drugs used to treat it may induce that process [6].

There is no clear statistics about the percentage of BPH in Egypt but in the Middle East, the incidence of prostate cancer is relatively low, however the disease is being increasingly encountered by urologists in the region [6-8].



Citral $C_{10}H_{16}O$

Citral induces benign or atypical hyperplasia in ventral prostate of rats by dermal, oral or encapsulated in feed. For example the recommended dose when applied dermally is ranging from one or more months [7,8].

MATERIALS AND METHODS

Sources and chemical analysis of pumpkin seeds: In the present experiment the pumpkin seeds were purchased from local Egyptian markets, the seeds were plain without shells. No heating treatment was applied to the seeds before mixing to the diets. Protein, carbohydrates and oil were determined in seeds. Fatty acids specifically were determined in pumpkin seeds according to [9].

Proximate Analyses: The moisture content was determined by heating two grams of ground air-dried sample in a vacuum oven at 700°C under pressure of 95mmHg to a constant weight [10]. The crude fat content was determined as follows: the oven-dried samples obtained from the moisture content determination was then extracted with petroleum ether (60–800°C) for 16 hours in Soxhlet-type extractor. The ether was evaporated and the residue dried to a constant weight at 95–1000°C and then cooled in a desiccator. The weight loss expressed as percentage gave the crude fat content [10].

The nitrogen percentage was determined by the improved Kjeldahl method described in AOAC [10] and the nitrogen content was converted to crude protein by multiplying with 6.25 [11]. The ceramic fibre filter method as described in AOAC [10] was used to analyse the crude fibre content. Briefly, two grams ground sample was defatted with petroleum ether then digested with 1.25% (v/v) H_2SO_4 and 1.25% (v/v) NaOH. The residues were ignited at 1300°C for 2 hours cooled in a desiccator and weighed. The ash content was determined using AOAC recommended method [10]. The carbohydrate content of the sample was determined by subtracting the sum of the percentages of moisture, crude fat, fibre, protein and ash from 100.

Derivatisation of fatty acids and GC analysis: Fatty acids were determined in pumpkin seeds by gas liquid chromatography (GLC). Within the sample collected in the study, variations were found in the chemical composition of the seeds specifically, fatty acids. The presented method of fatty acids were used according the analysis of which has been published by Mandl and co-workers [12]. It includes saponification of an accurate weight of seeds, the triglycerides followed by separation of the potassium salts of the fatty acids from the unsaponifiable fraction by adsorption chromatography. In order to enhance gas chromatographic properties of the analytes, the hydroxyl function of the sterols is derivatised with *N*-Methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) to the trimethylsilylether. Finally, the analytes were separated on a capillary column of medium polarity (HP 35 MS) in a temperature programmed run within 20 min. Detection of the analytes was done by flame ionization. Special attention was set onto the precision and repeatability of the method.

Animals: 7 week old rats, were purchased for use in experiments and were given the commercial diet before starting the experiments. This study was approved by the Animal Ethics of Ophthalmology institute research, Egypt, animals were weighed at the beginning of the experiment with masses ranging from 110-115 g. Fifty adult male Wistar rats were divided into five groups, including: negative control group (C-) which fed on pumpkin seeds free diet, Basal diet, Ain-93M diet

Table 1: Basal diet, Ain-93M

Ingredient	g/kg Diet
Cornstarch	465.692
Casein	140.000
Dextrinized corn starch	155.000
Sucrose	100.000
*Corn oil	40.000
Fibre	50.000
Mineral mix	35.000
Vitamin mix	10.000
L-cytesine	1.800
Cholin bitartarate	2.500
Tert-butylhydroquinone	0.008

*Soybean oil was replaced by corn oil

formulated for maintenance of adult rodents [15]. Table 1. Positive control group (C+) with BPH were fed on basal diet only. The rest of the groups were fed on different level of pumpkin seeds (PS), 2.5, 5% and 10 %. Rats were housed individually in stainless steel wire-bottom cages in a room maintained at 22–24°C and ~50% relative humidity. The room was lit from 0700 to 1900 h. Tap water was freely available. Blood was collected from the retroorbital sinus of rats under carbon dioxide anesthesia for chemical and clinical pathology and then euthanized with CO_2.

Inducing of BPH and its Markers

Chemicals: Inducing BPH in Wistar rats was implemented by Citral, (ß-substituted vinyl aldehydes) the compound has been shown to induce benign and atypical prostatic hyperplasia in rats.Citral (geometric isomer ratio of 2:1 geranial: neral) was obtained from Aldrich Chemical Company, Inc. (~ 97.6% pure) was used during the study. BPH was induced by oral gavage of citral at 200mg/kg/3days that reported to induce minimal to mild hyperplasia [13]. One rat from each group was killed after day 14 PBP levels were examined the prostate status. After the day 14 and ensure from symptoms of morphometric and protein binding prostate (PBP) levels; feeding experiment on different levels of pumpkin was started. Clinical findings were recorded weekly for rats. The animals were weighed initially, weekly thereafter and at the end of the study.

Protein Binding Prostate (PBP): Rats at risk of benign prostate hyperplasia can be quantifying type of protein called PBP in tissue rat samples. Quantification was based on a competitive inhibition assay [14] in which the anti-PBP antibody was incubated in solution with increasing concentrations (0-100 mg/ml) of pure PBP. With PBP coated in the wells, the reactivity of the inhibited antibody was then determined. With this used as a standard inhibition curve, it was possible to determine the inhibition capacity of the tissue samples when they replaced PBP as inhibitors. This standard curve allowed us to report the data as nanograms or micrograms of PBP per milliliter of solution. The percentage of inhibition was calculated as follows: % inhibition = [1 - (RFU with Inhibitor/RFU without inhibitor] x 100.

Histology: The right tissue testis were microscopically examined for microscopic examination, fixed and preserved in 10% neutral buffered formalin, processed and trimmed, embedded in paraffin, sectioned to a thickness of 4 to 6 μm and stained with hematoxylin and eosin. A complete histopathologic examination was performed on all core study untreated control vehicle control rats [16].

***In vitro* diagnostic testosterone kit:** Androgen is a term given to any steroid hormone that primarily influences the growth and development of the male reproductive system. Although there are other nature androgens, testosterone is the primary circulating androgen. Hormones, in particularly the androgens, are essential for the development, growth and maintenance of the prostate. The estimation of testosterone was carried out using the procedure enclosed with the kit purchased from GenWay Biotec, Inc. (catalog number 40-056-205042) (San Diego-USA) by using ELISA technique to determine free testosterone in serum based on the principle of competitive binding. The microtiter wells are coated with an antibody directed towards an antigenic site on the testosterone molecule. Endogenous Free testosterone of rat sample competes with a testosterone horseradish peroxidase conjugate for binding to the coated antibody. After incubation the unbound conjugate is washed off. The amount of bound peroxidase conjugate is inversely proportional to the concentration of free testosterone in the sample. After addition of the substrate solution, the intensity of colour developed is inversely proportional to the concentration of free testosterone in the rat sample as previously described [17]. The inter-and intra-assay coefficients of variation for the testosterone were <15%.

Measurements: Serum samples from Wistar males were assayed in triplicate by using radioimmunometric assay (Diagnostic Systems Laboratories, Webster, TX). Assay performance was monitored by standard curve parameters and quality control samples. Three Diagnostic

Systems Laboratories kit controls and one Bio-Rad Immunoassay plus Control (Hercules, CA) were assayed at the beginning and end of each batch of serum samples. Triplicate samples with coefficient of variation (CV) > 10% were reassayed. Intra-assay CVs for the quality control samples were 4.0% to 5% for all results. Inter-assay CVs were ranged from 2.7% to 3.6 % at all results reported.

Statistical Analysis: All results were expressed as the mean ± S.E.M. Statistical analyses were performed using analysis of variance, comparisons between negative and positive groups for the data in Tables 3 were made by using one-way analysis of variance (ANOVA). Statistical analyses for possible dose related effects on rats were used [18]. Ventral Prostate weight data were analyzed with the parametric multiple comparison procedures [19].

RESULTS

The chemical composition of pumpkin seeds: The chemical properties and fatty acid composition of the seed oil were examined. The pumpkin seeds are a good source of protein (~38%), carbohydrates (~37%) and oil (~33.7%). The four dominant fatty acids found are: palmitic C16:0 (11.50%), stearic C18:0 (7.0%), oleic C18:1 (31.20%) and linoleic C18:2 (47.0%) as shown in Table 2.

Morphomertic changes of prostate in normal, BPH and BPH with different levels of pumpkin seeds in rats

Table (3) and Fig. (1) shows that 10 % pumpkin seeds level has reduced weight of ventral prostate gland to a maximum level compare to negative control group. There was an increase in prostate gland weight in positive control (threefold increase) with BPH compare to negative control. The weight of prostate of positive Wistar rats

Table 2: The chemical composition summary of contents in pumpkin seeds per (100gm) (three different localities)

Fatty acid	Amount %
Palmitic	11.50
Stearic	7
Linoleic	47
Oleic	31.20
Total fat	33.7

Given values are means of triplicate analyses:

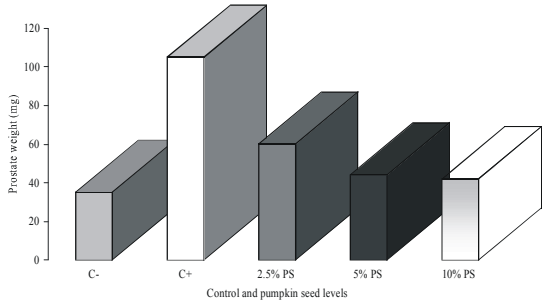


Fig. 1: Correlation between prostate weight (mg) and different levels of pumpkin seeds levels

progressively increased, while increased slowly in the presence of 2.5, 5% PS and approached from negative control with 10% PS addition.

Results in Table (4) shows that there is no significant correlation between benign prostatic hyperplasia (BPH) in rats and level of testosterone in all experimented groups.

Table (5) shows the effect of feeding pumpkin seed levels (2.5%, 5% and 10%) on the concentration of protein binding prostate in ventral lobes of prostate in Wistar rats. feeding 10 % level of pumpkin. Treatment-related decreases in BPP were observed (Table 5) and were associated with the marked suppression in mean of prostate weight, suggesting that pumpkin seeds have a curing effect.

DISCUSSION

This study was undertaken to analyse the chemical composition of pumpkin seeds and investigate the effects of different pumpkin seed levels on the mild status of benign prostatic hyperplasia (BPH) in Wistar male rats. Several investigations have been carried out such as tissue protein binding prostate (PBP), serum testosterone, prostate growth and histology of testis.

Chemical composition of pumpkin seeds: Chemical analysis of pumpkin seeds was carried out for protein carbohydrates and oil Table (2). Fatty acids were quantitated by GLC. The current study indicated that pumpkin seeds analysis contains an appreciable amount

Table 3: Ventral prostate lobe weight % (mg) increase over negative control after feeding different pumpkin seed levels

[n]Group of Rats	(C-)	%	(C+)	%↑	2.5% PS	%↑	5% PS	%↑	10% PS	%↑
Weight of Prostate (mg)	35±0.20	0	[a]105±0.08	300	[a]60±0.11	171	[b]44±0.14	125	[b]42±0.33	120

↑ means percentage of increase of prostate weight over negative control, (C-) negative control, (C+) positive control, (PS) pumpkin seeds, [a] Results are statistically significant from the control groups of rats (P<0.001). [b] Results are statistically significant from the control groups of rats (P<0.05). ± Standard Error of Mean, values are the mean of 9 results, n=9.

Table 4: The effect of different levels of pumpkin seeds in normal, BPH, on testosterone level in rats

Group of rats	Testosterone levels pg/ml
(C-)	45±0.12
[c](P+)	43±0.15
[c]2.5% PS	42±0.80
[c]5% PS	45±0.76
[c]10% PS	46±40.3

Values are the mean of 3 results, ± Standard Error of Mean, values are the mean of 9 results, n=9. [c] not statistically significant from the control groups of rats

Table 5: The effect of different levels of pumpkin seeds in normal and BPH rats, on the protein binding prostate (PBP) level in prostate rat's tissue

[n]Group of rats	PBP levels mg/ml
(C-)	3.6±0.12
(P+)	9[a]±0.15
2.5% (PS)	7[a] ±0.80
5% (PS)	5[a] ±0.76
10% (PS)	3.8±0.40

Values are the mean of 3 results, ± Standard Error of Mean, n=9. [a]Statistically significant (P<0.001) from the control groups of rats

of unsaturated fatty acids mostly linoleic acid. Our results were in agreement with [9] and [3] our results indicated to a variation in the chemical composition of seeds this may be due to different cultivars.

Citral to induce BPH: Citral has been extensively studied for its effect on the induction of benign and atypical hyperplasia in the ventral prostate of male rats Figure (4) [7,8]. Some studies, found after careful examination that citral did not reveal any effect on male accessory glands, including all lobes of the prostate [21].

However, a comparative study of citral-induced benign and atypical hyperplasia in Wistar, Sprague-Dawley, Fischer 344 and ACI/Ztm rats demonstrated that strain genotype and endocrine background play a role in the development of this disease [22]. The animal model chosen for the current study, the Wistar rat, was shown to be responding to citral-induced prostatic hyperplasia our results were in agreement [22].

Protein binding prostate (PBP) markers: PBP is a major androgen-sensitive secretory product of the rat ventral prostate and its measurement provides a good assay for the study of prostate secretion [23]. The level of this glycoprotein was ascertained to determine the effects of BPH on rats. The BPH results of rats showed an increase in the levels of PBP. This result is consistent with the hyperplasia assessment reported by [14]. Our results indicate an inverse relationship between levels of

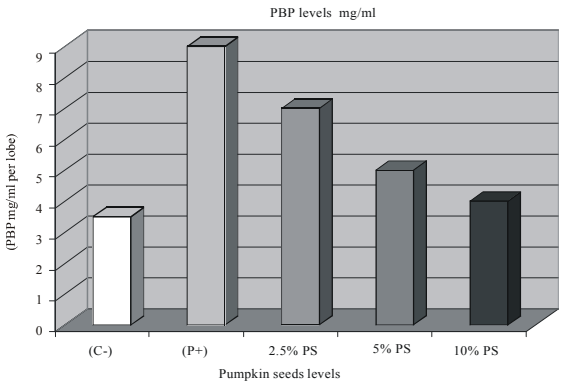


Fig. 2: The effect of different levels of pumpkin seeds in normal and BPH rats, on the protein binding prostate (PBP) level in prostate rat's tissue

pumpkin seeds and level of tissue protein binding prostate. Presumably, the 10% pumpkin seed in rat group shows no change in PBP levels because markers of BPH were observed in the increase levels of protein binding prostate (PBP) compared to negative control. The PBP was significantly increased about three fold compared to normal rats (C-) Table (5) and Fig. (2), our results are in agreement with [14].

Healing effect of pumpkin seeds: Long an ancient Egyptian and Eastern European folk remedy for the prostate problems of men, the seeds and oils have in fact been shown to improve symptoms associated with an enlarged prostate due to benign prostatic hyperplasia. Components in pumpkin seed oil appear to interrupt the triggering of prostate cell multiplication by testosterone and DHT. It is questionable whether eating the seeds whole in snack quantities, rather than taking therapeutic doses of the pumpkin seeds, would provide any prostate benefit.

In a multi centre controlled study involving more than two thousand subjects, a product containing pumpkin seeds was evaluated for the treatment of benign prostate hyperplasia (BPH). The results indicated that, not only were pumpkin seeds effective in reducing symptoms associated with BPH, especially in its early stages, but also no side effects were reported by the patients involved in the trial [6].

In a Swedish study involving 53 patients, pumpkin seed reduced symptoms related to BPH, without any side effects. Other clinical trials also show that pumpkin seeds, along with other herbs, have a positive effect against mild to moderate BPH. Pumpkin seed oil has shown to possess strong antioxidant properties in animal experiments [24].

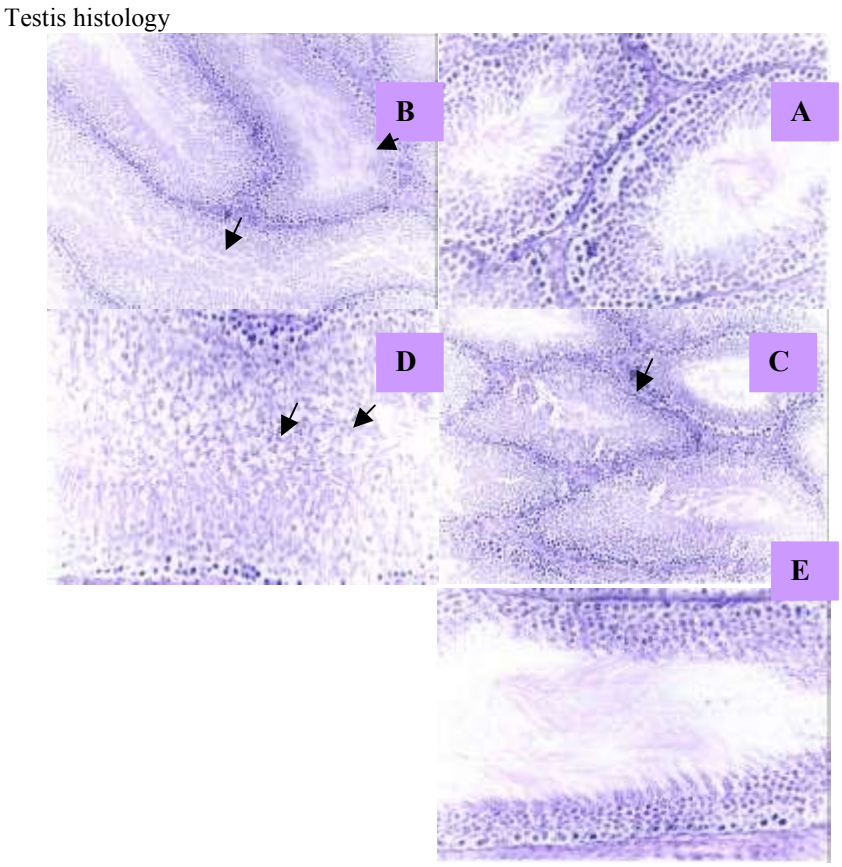


Fig. 3: Testis of rats of control group showing no histopathological signs (H and E X 200) (A) (Grade 0). Testis of positive control (C+) with BPH group showing hyperplasia and activation of spermatogoneal cells lining semineferous tubules (B) Grade 4). Testis of rats 2.5 % PS showing degeneration of spermatogoneal cells lining semineferous tubule (C) (Grade 2) Testis of rats from 5 % PS groups showing slight testicular degeneration and desquamation of spermatogoneal cells lining semineferous tubules (D) (Grade 1.5). Testis of pumpkin 10% group showing no histopathological changes (E) (Grade 0).

The seeds are used against benign prostatic hyperplasia (BPH), sometimes in conjunction with other herbs; especially Saw Palmetto (*Serenoa repens*) seed extract [25].

Plasma testosterone in the current study concentrations in adult Wistar male rats showed no correlation between BPH and level of testosterone within groups Table (4). Plasma Analysis of variance showed no significant difference between all of these groups compared to the negative control groups.

The results of this study refocused on the importance of pumpkin seeds in reducing protein binding prostate tissues, due to the seeds being rich in unsaturated fatty acids such as omega 3, 6 and 9 as well as its high protein content and uncertain functional and electrophoretic properties [26], recent studies indicated the preventative effect of pumpkin seed to inhibit the conversion of testosterone into dihydrotestosterone (DHT) in cultures of human fibroblasts. The mechanism for this inhibition, however, was characterized as different from other herbal extracts used for the treatment of benign prostatic hyperplasia (BPH).

Components in pumpkin seeds, specifically fatty acids appear able to interrupt the triggering of prostate cell multiplication by testosterone and DHT, although the exact mechanism for this effect is still a matter of discussion (Wikipedia).

Pumpkin seeds are rich in unsaturated fatty acids therefore studies show that rats fed diets rich in monounsaturated fats had greater 17 β-dehydrogenase activity (a key enzyme in the testosterone synthesis

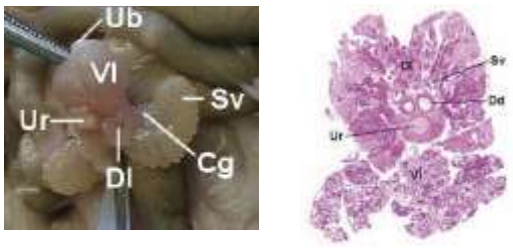


Fig. 4: Removal of prostate, urinary bladder and seminal vesicles as a unit. (Cg: coagulation gland, Dl: dorsolateral lobe of prostate, Sv: seminal vesicle, Ub: urinary bladder, Ur: urethra, Vl: ventral lobe of prostate [20].

pathway in the male rat) [27] and plasma androgen concentrations compared to rats fed diets rich in saturated and polyunsaturated fats [28].

Prostate glands proliferating activity: In this study, results of histology of testis revealed to a degree of histopathological alteration in rats with BPH Fig. (4). The microscopic examination of the ventral prostate of the animals (data not shown) showed definite lesions: hyperplasia in few acini was seen after 15 days of treatment, thereafter, expanding with continued treatment. After 37 days of citral treatments for all rat groups (Except C-) which means for example, positive control showed that the enlarged acini were more crowded and reached a back-to-back arrangement while separated by dense connective tissue, which is characteristic of glandular hyperplasia. For the first time we have found that the histopathological results of rat testis have a direct link with BPH conditions.

Testosterone: Testosterone is the principal hormone necessary for insuring the completion of normal spermatogenesis. The fall in testosterone production with increasing age is largely caused by a reduction in Leydig cell mass and function. Testosterone production is influenced by a variety of factors including age, [29] training status, [30] amounts of body fat stores [29] and diet. We have shown that there is no relation between BPH and level of testosterone in rat serum, the results were in agreement with [31] who studied the relationship between serum testosterone and measures of benign prostatic hyperplasia in aging men.

The serum free testosterone levels were evaluated and did not correlate significantly with ventral prostate weight.

CONCLUSION

The pumpkin seed is claimed to be useful in the management of BPH. This investigation seeks to examine the effect of pumpkin seed on citral-induced hyperplasia of the prostate in Wistar rats. Citral was administered orally into male rats to induce BPH. Chemical analyses of pumpkin seeds were undertaken to investigate the effects of pumpkin seeds on ventral prostatic weight, protein binding prostate (PBP) and the histology of testis in rats. We conclude that pumpkin seed at 10% can inhibit citral-induced hyperplasia of the ventral prostate lobe as observed in reducing protein binding prostate levels, weight of ventral prostate lobe and improve histology of testis therefore may be beneficial in the management of benign prostatic hyperplasia.

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