Experimental Study of Apoptosis after Castration in Prostate Tissue

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Abstract: The prostate gland is one of the reproductive enclosure glands that its physiological function is necessary for successful reproduction in males. This gland depends on sexual hormones including androgens for its natural function and normal growth and development. So in the case of hyperplasia, hypertrophy or other prostate diseases, the most successful and efficient method of treatment is castration that in some cases is unavoidable. This thesis has been done to survey the effects of the androgen depletion states on the prostate gland and for determining the mechanism of cell death engaged in this state. In this thesis we used two groups of dogs that each group contained 5 dogs. These dogs were under care for 1 month. In this period of time they were surveyed for any possible disease. After this period the dogs in "treatment group were castrated for decreasing the level of the androgen hormones in the blood. The dogs in the "control group" were left intact. After a week of surgery, prostate glands of these dogs were extracted and used for preparing pathological cross sections. These sections have been colored in the TUNEL and H&E methods and then inspected with optical microscope for detecting apoptotic cells. We found that after castration, the size of the prostate gland decreases and microscopically changes of the gland include increased number of apoptotic cells. These results demonstrate that the type of the cell death engaged in prostate gland in androgen deprivation states are Apoptosis.

Key words: apoptosis, castration, prostate.

INTRODUCTION

The prostate gland is the origin of many diseases and disorders of reproductive system. Some of which such as benign prostate hyperplasia (BPH) have a very high prevalence among human population and some can even eventually result in death. In addition, as investigation and experimental researches on the causes and treatment of these diseases in human beings were difficult to do. Dog was used as a model for studying these diseases, the therapeutic effects of drugs and various therapeutic methods. Considering the above points, high prevalence of prostatic diseases and the use of castration as a treatment in many of these disorders, examination of procedures used in this therapeutic method and its success rate, are very important.

Apoptosis Caused by a Lack of Androgens in Prostate:
It is very important to understand the mechanism of apoptosis caused by a lack of androgens, because this factor is the only definitive treatment in the cause of prostate epithelial cells cancer. But this treatment because of increased resistance to androgen-dependent apoptosis in patients with metastatic form of prostate cancer has only temporary effects (Bagherzadeh, 2001). Prostate gland is the most important target organ for androgenic hormones. Existence of normal level of androgens in bloodstream and on the receptors in prostate is necessary for development and survival of this organ. Testosterone is the main androgen in systemic bloodstream that is excreted in a pheromone form by leydig cells and then in prostate is converted into dihydrotestosterone by 5-alpha reductase enzyme. As a result, the main ligand of androgenic receptors in prostate gland is dihydrotestosterone. Androgenic receptors are translational factors in genome and considered as steroid

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receptors. Production of abnormal steroids or the occurrence of mutations making the androgenic receptors inactive during embryonic period leads to the lack of development or defective development of prostate.

Lack of testosterone during growth, stops the growth of the gland, and this in turn, and causes extensive changes in prostate gland during adolescence. Therefore, proper function of androgen-androgenic receptor axis is necessary for the development and normal function of prostate gland. In addition, to regulating the proliferation and differentiation of prostate cells, androgens inhibit apoptosis occurrence in these cells. This characteristic is very noteworthy, because since 1940, apoptosis stimulation by decreasing androgens level, has been the main method in treating prostate cancer (Pousti, 1994).

Classic function of androgenic steroids and their receptors, in molecular level, was recognized about one decade ago after production of androgenic receptors by genetic methods. After binding to the ligands, receptors separate from heat shock proteins (HSP) and undergo post-translational modifications including phosphorylation, dimerization and nuclear translocation. After entering the nucleus, androgen-ligand complex binds to the parts of promoter that relates to androgen-dependent genes and control their transcription. A non-genotropic process for androgenic receptors has also been discovered. These receptors by activating the intracellular processes like kinase-stimulating processes control the cellular functions such as proliferation and apoptosis inhibition. But the fact whether this non-genotropic process has a biological importance in prostate or not, has not been proved yet (www.dragon.zoo.utoronto.ca/). Fundamental method in treating prostate cancer is elimination of androgens. It seems that this method causes apoptosis in androgen-dependent cancerous cells. Unfortunately, this therapeutic method results in the survival of androgen-independent cancerous cells and these cells, in turn, in absence of androgen, continue their growth and proliferation leading to the aggravation of disease and finally death. This form of disease that happens when treatment with androgens fails is called androgen-independent form. In principle, androgen-independent prostate cancer is resistant to the induced apoptosis due to hormonal manipulation or other cytotoxic treatments. This form of disease is highly invasive. In all experimental cases, the occurrence of prostate adenocarcinoma has always been accompanied by resistance to induced apoptosis caused by androgen elimination. It is not completely clear that which of internal or external pathways after castration are responsible for apoptosis induction in prostate, though some findings clearly show the mitochondrial pathway intervention (Catz, 2003).

The Role of Rat Prostate Models in Understanding the Induced Apoptosis Caused by Androgen:

Epithelial cells with apoptotic form appear 24 hours after castration and their numbers reach to the maximum 48-72 hours after castration. Two weeks after castration, net weight of prostate clearly decreases and at most, 85% of cells are afflicted with apoptosis. Morphologically, events associated with the induced apoptosis caused by lack of androgens in secretory epithelial cells, have been carefully described (Kothakota, 1997) .They showed the general events including swelling of the upper membrane that was accompanied by cell deformation and chromatin assembly, formation of apoptotic bodies and separation from basal membrane and peripheral cells. Some genes that are expressed or suppressed by induced apoptosis conditions caused by castration have been biochemically recognized. After castration substances that are excessively excreted are testosterone repressed prostate message-2 (TRPM-2 or clusterin), transforming growth factor (TGF-beta), c-fos, c-myc, heat shock protein 70kDa (HSP70), fas, ventral prostate gene-1(RVP-1), matrix carboxy glutamic acid, glutathione S-transferase and Nurr77/TR (Doustar, 2004). Pre-apoptotic role for some of these molecules (such as fas, TGF-beta, c-myc, c-fos and Nur77) have been described. However, it seems that other molecules including clusterin acts as apoptosis-inhibiting factor in prostate cells that indicates the reaction of androgen complex to the process of cell life and death3. Among genes whose expressions are decreased by castration, epidermal growth factor (EGF) is of greatest importance. EGF is a cytogenic factor its absence in prostate along with an increase in TGF-beta gene expression is probably one of the mechanisms that induce apoptosis in prostate epithelial cells after castration. Further studies have also shown some changes in the expression of pre-apoptotic and anti-apoptotic genes of BCL-2 family. These studies demonstrated the importance of Bax/BCL-2 expression ratio in the occurrence of apoptosis. When Bax is predominant (that usually happens after castration) cells are affected with apoptosis. In comparison with this condition, after longer intervals and with predominance of BCL-2, apoptosis is not seen in these cells. Results obtained from the increase in the ratio of Bax/BCL-2, strengthen the hypothesis according to which, castration causes apoptosis through mitochondrial pathway, because Bax gene is one of the strongest inducers of this pathway. Prostate of castrated animals is restored after androgen administration. Biopsy from prostate before and after administration of androgen provides useful samples in recognizing the functions of genes have been induced or repressed after treatment with androgens, because it is supposed that the genes which are recognized in restored tissues are involved in anti-apoptotic function and cellular proliferation. Wang and his colleagues recognized 25 genes with

increased expression and 4 genes with decreased expression in the prostate of castrated rats.

For instance, the expression of calreticulin which is an important regulator of apoptosis and protects the cell against apoptosis due to increased calcium recruitment increases. When with a greater increase in the buffering activities than that of calcium inside the cell, calreticulin inhibits the apoptosis due to calcium recruitment into the cell caused by A23187 calcium ionophore. Therefore, it can be imagined that in normal conditions, androgen causes the inducement of calreticulin production and thereby maximizes the buffering effect of the cell as compared to the intracellular calcium and consequently inhibits apoptosis. After castration, intracellular calreticulin decreases and consequently the ability of the cell in buffering action against the calcium ion also diminishes and thus more cells are subjected to apoptosis8.

Another important change that occurs in prostate of castrated rats is a considerable decrease (50% in the first 24 hours after castration) in blood distribution to this tissue. However it is noteworthy that castration doesn't cause any changes in blood distribution to other androgen-dependent tissue and testosterone administration in castrated animals prevents the occurrence of any change in blood distribution to prostate. As the blood flow changes is accompanied with apoptosis and these changes occur sooner than secretory cells apoptosis. Some of scientists believe that prostate epithelium injuries are due to hypoxic and ischemic conditions caused after castration (Pierre Allard *et al*, 1997).

Use of Apoptotic Molecules for Inducing Therapeutic Apoptosis in Prostate Cancer:

As androgen-independent prostate cancer has a high prevalence and is not curable, researchers are under great pressure to find new therapeutic methods. A new method based on using genes involved in apoptosis is under investigation. When these genes become active, they trigger a process of effective and irreversible suicide inside the cell, so they can be considered as potentially therapeutic substances.

Scientists continue their studies to find if they can benefit from manipulation of activities and expression of these molecules to induce the apoptosis of grown cancerous cells in vivo and in vitro (www.vetmed.wsu.edu/ClientED/anatomy/dog_ug.asp#prostate).

MATERIALS AND METHODS

Ten male dogs aged 8-10 with average 23-27 kg were selected, then they were divided in two control and treatment groups. The treatment group was openly castrated by operation, the control group was not but it had open testicle operation, the operation condition was the same for both mentioned groups. Seven days passed when we began controlling and sampling of the treatment and control group. The tissues were fixated, dehydrated and clarified and then they were saturated in paraffin to mold and prepare 5-6 micron tissue sections. When some sections were colored with usual hematoxylin eosin, the others were colored based on private apoptosis diagnosis technique. Mann-whitney U test was used for their statistical analysis.

Firstly 1mg/kg dose of pre-anesthesia, acepromazine was injected to the muscle of the dog and then the hairs of operation position were completely cut. The anesthesia instillation was done by 10mg/kg 2.5% sodium thiopental, the dog was laid at backward position during the operation and it was injected sugary-salty serum, the anesthesia was controlled by 2% halothane. The operation position was disinfected marked, and then the scrotum was cut with scalpel as required. Testicles were removed whit a little squeeze after cut of dartus and tunica vaginalis. Tunica vaginalis was cut since the operation was open and proper ligament testis were cut too to remove the testicles. The testicle joint and deferens ductus were stitched by the non-absorbable zero no ligatures silk yarn, they were cut and the testicle was put out. The next testicle was also removed by using this approach. Then the hypodermic tissues were stitched by absorbable zero no ligatures catgut and the dermis was simply stitched by no.1 silk yarn.

40mg/kg cefazolin was injected into the vein before the operation, after the operation 1mg/10kg ketoprofen was injected into the muscle of the dog for 4 days.

TUNEL Diagnosis Technique:

Firstly HCL/Tris produced proteinase k solution is being prepared by PH 7.5-8, 10-20 micro liter of the concerned girdle is incubated at 21-37ºC for 15-30 minutes. After the incubation process with girdle proteinase k , it is washed with PBS solution two times, it takes 5 minutes each time, whereas the girdle is dried and washed by PBS again, the respective girdle is incubated in ice for two minutes during permeability. This is the most important step in diagnosis technique: 50 micro liter of TUNEL reaction mixture is added to each sample when it is incubated at 37ºC in 60 minutes. To prevent the dryness of the solution over the girdle and to observe the performance of the technique conditions, the girdles are covered by paraffin.

1. This stage is optional. PBS is used to wash three times; they are reviewed under fluorescent microscope at 515-565 nanometer wavelength, where the apoptotic cells are shown green.
2. No.3 solution, the same POD converter is used, 50 micro liters is added to each samples, and then it is incubated at 37ºC in 20 minutes.
3. In this stage, the respective girdle is washed three times with PBS.
4. DAB chromogen is used 50-100 micro liter is added on each sample and it is incubated at 15-25ºC in 5-20 minutes.
5. For the last time in this stage the respective girdle is washed by PBS, three times, it is allowed to be dry.
6. The TUNEL technique is finished in this stage but the researchers could study the tissue based on the secondary staining, where the apoptotic cells are seen in brown, the concerned optional and general staining in our experiment was H&E.
7. At last the girdles could be easily seen under optical microscope.

The Quantitative Results Obtained from Microscopic Studies of Prostate Gland in Treatment and Control Groups:

Mann-whitney U test was used for statistical analysis of figures obtained from counting the number of apoptotic cells in both treatment and control groups. Results of this analysis are shown in tables 1. According this table, the mean ranks of the number of apoptotic cells in treatment group and control group are 5 and 0.8 respectively. According to diagram 1, the med number of apoptotic cells in treatment group is 5 and in control group is 1 and according to standard, ($z=2.6$ and $p<0.008$) difference between the cells in treatment and control group is significant.

Qualitative Results of Apoptosis Changes in Prostate Tissue:

In the following pictures, photomicrographs of prostate tissue are shown. Apoptotic cells, as it can be clearly seen, are marked by small dark-colored apoptotic bodies.

Table 1: Mean ranks of the number of apoptotic cells in treatment group and control group.

| Parameter	Treatment	Control
mean	5.00	0.80
Standard deviation	1.58	0.84
Standard errors	0.71	0.37

DISCUSSION AND RESULTS

Classic function of androgenic steroids and their receptors in molecular level has been described about one decade before the production of androgenic receptors by genetic method. As prostate gland is the main target tissue for androgenic hormones, the presence of normal quantity of androgens in bloodstream and prostate is necessary for development and survival of in men lifetime (Johnston, 2000; Meuten, 2002).

In addition, to regulating the proliferation and differentiation of prostate cells, androgenic hormones inhibit the occurrence of apoptosis in these cells which is considered as the basis of all therapeutic methods in medical sciences. One of the therapeutic methods in prostate cancer is elimination of androgenic factors. It seems that this method causes apoptosis in androgen-dependent cancerous cells. Castration induces the regression of tumor growth in patients with benign hyperplasia and prostate cancer through elimination of androgenic factors by apoptosis induction (Gyton, 2003). Degree of apoptotic changes in prostate tissue of treatment group in comparison with control group shows that elimination of androgenic hormones, in prostate tissue can play an effective role in death of cells, sensitive to a decrease in the amount of androgenic hormones. For this reason, in castration, a decrease in expression of EGF gene and an increase in expression of TGF-beta gene is probably one of the castration-related mechanisms in prostate. Along with the above mentioned factors, expression of Bax and BCL-2 genes that are involved in the formation of mitochondrial membrane canals of prostate epithelial cells is another important factor in inducing apoptosis in castrated cases (Johnston, 2000).

Considering the above mentioned points and the data gathered from both treatment and control groups, the results of the analysis of statistical data with Mann-whitney U test method were significant and the number of apoptotic cells significantly correlates whit castration operation in above groups. This increase in the number of apoptotic cells will be gradually accompanied by a decrease in the general size of prostate gland which is the main goal in treating the diseases such as BPH.

According to the researches carried out12 on castrated rats, it was found out that about 24 hours after castration, apoptotic cells appear in prostate tissue and after about two weeks, approximately 85% of prostate secretory epithelium cells will be affected by apoptosis and thus the size of prostate gland greatly diminishes (Zhang, 2002). This corresponds with the results obtained in our research. Researchers on this area are still continuing. It is hopeful that in a near future, scientists will introduce effective therapeutic methods by their new research findings on cell death.
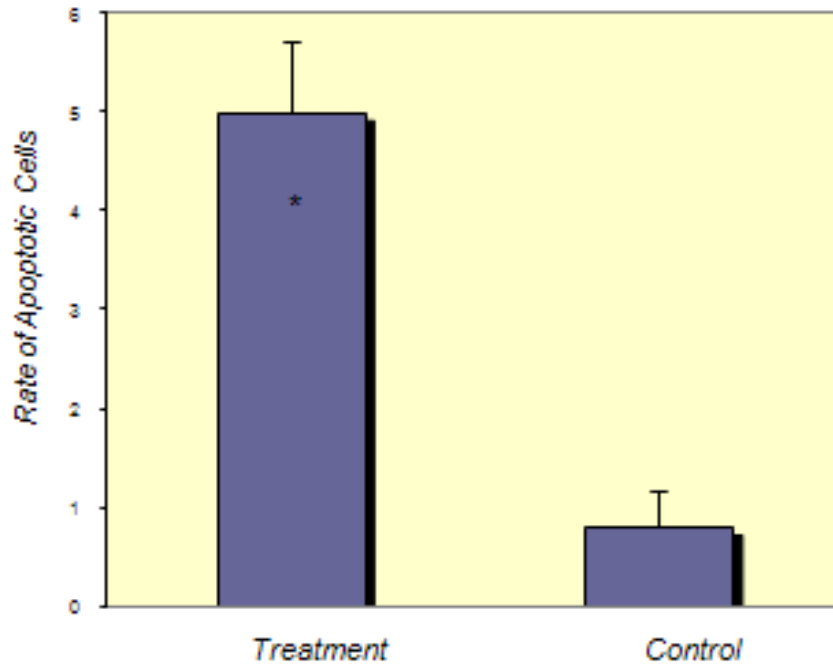

Diagram 1: The average of apoptotic cells obtained from 10 microscopic field of glandular prostate epithelium by TUNEL in castrated dogs. Data were rendered as mean ± standard deviation. P < 0.005 considered as significant difference.
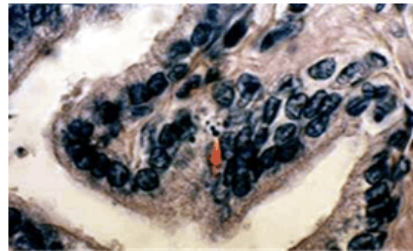

Fig 1: Microscopic view from prostate tissue of treatment group, dark blavk nuclear fragmens (arrow) is seen. Adjacent epithelial cells are normal. (H&E ×100).
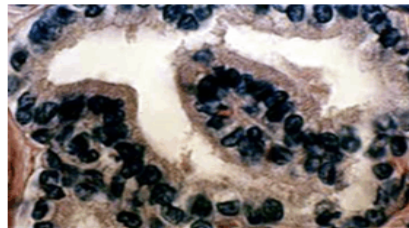
Fig. 2: Microscopic appearance from prostate tissue of control group in which glandular epithelial cells are normal. (H&E ×100).
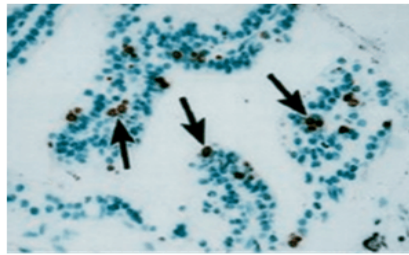
Fig. 3: Microscopic view from prostate tissue of treatment group in which numerous positive TUNEL cells are prominent (arrows). (H&E ×100).
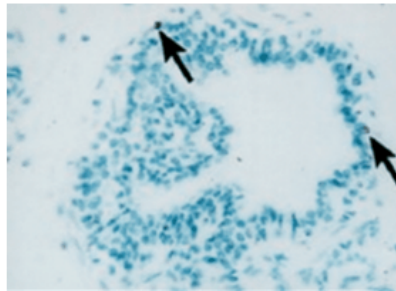
Fig. 4: Representative section from prostate tissue of control group in which there are few positive TUNEL cells (arrow). (H&E ×100).

Conclusions:

The pathology girdles were obtained from prostate tissue of treatment and control groups, the tissues were studied to diagnosis the presence of apoptotic cells. The number of apoptotic cells was enumerated in 5 random wise selected microscopic fields, the average enumerated apoptotic cells are shown at table1.

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