RESEARCH PAPER

Impact of bicalutamide, an anti-androgen on rat testis

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A B S T R A C T:

Bicalutamide/Casodex is a non-steroidal anti-androgen drug which used in treatment of prostate cancer. Castration by using anti-androgens such as bicalutamide became a successful strategy for prostate cancer metastasis suppression. The goals of the present work were to study the body weight, biochemical, histological and immunohistochemical changes induced by daily administration of bicalutamide 0.8mg/Kg body weight of rats. Treatment with bicalutamide caused non-significant decrease in relative testis weight, disturbing the histological architecture of the testis, decrease of sperm count, germinal layer depletion, increasing the apoptotic index of germinal layer cells and a decrease in testosterone level compared to control. In conclusion, bicalutamide treatment induced various biochemical, histological and immunological changes in rat testis.

KEY WORDS: Bicalutamide; Testes; Testosterone; Anti-androgen
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1. INTRODUCTION

Anti-androgen drugs have prospective to interfere abnormality in both human and animal male reproductive system development as well as functions (Sharpe, 2006, Metzdorff et al., 2007). Bicalutamide is an orally active and non-steroidal anti-androgen drug which used with luteinizing hormone-releasing hormone agonist to decrease symptoms in patients with a metastatic prostate neoplasm and used mostly during the initiation of androgen deprivation therapy (Hussain et al., 2014). The chemical formula of bicalutamide is (2RS)-4`-cyano-3-(4-fluorophenylsulphonyl)-2-hydroxy-2-methyl-3`-(trifluoromethyl)-propionanilide (Fig. 1) (Fradet, 2004).

Figure 1: The chemical formula of bicalutamide (Fradet, 2004).

The combination of anti-androgen drugs and castration inhibit androgen synthesis by testes and block residual adrenal androgens at the level of receptor (Hussain et al., 2014), thereby blocking the negative feedback mechanism that regulates testosterone hormone concentration (McLeod and Iversen, 2000), bicalutamide is a competitive inhibitor of androgens at the level of receptor (Furr et al., 1987) and it is pure anti-androgen that exhibits only antagonist activity (Térouanne et al., 2000).

Bicalutamide certainly reduced the mean testicular weight in the adult Sprague–Dawley rats (Khurshid et al., 2014). Researches on male rats have revealed that bicalutamide drug had few

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effects on serum luteinizing hormone and testosterone hormone concentrations (Freeman et al., 1989, Furr and Tucker, 1996). Bicalutamid had adverse effects like prostate, testis and seminal vesicle atrophy, as well as Leydig cell hyperplasia which resulting from suppressing of pituitary feedback by testosterone hormone (Iswaran et al., 1997). Reduction of testicular weight and associated histological changes were noted after administration of anti-androgen in an androgen-stimulated testis of rats (Kennel et al., 2003).

Although more information about use of bicalutamide in clinical research and castration mechanism available (Lee et al., 2018, Stanisławska et al., 2018, Sekino et al., 2019), there is more information we should know about castration by this anti-androgen drug and it’s histological and biochemical effects in the testes of experimental animals, therefore, the present investigation aimed to evaluate the histological and biochemical effects induced by bicalutamid which may be behind its castration and anti-androgenic role.

2. MATERIALS AND METHODS

2.1. Experimental Animals

Current investigation was carried out by using 12 adult healthy Wistar rats. They were weighing 200-270gm and 8-10 weeks old. The animals were bred in controlled temperature and light of 24 ± 3°C and 12/12 hrs light/dark respectively. Biology department animal house in college of science-Salahaddin University was used to conduct the study and all experiments were performed according to the protocols approved by the animal care ethic committee of Salahaddin University-College of Science (Erbil-Iraq).

2.2. Bicalutamide (Casodex)

Bicalutamide 50mg film-coated tablets (manufactured by AstraZeneca UK). Each tab contains 50mg of bicalutamide and during the current study, one dose of bicalutamide has been chosen which was 0.8mg/Kg of rat which orally administrated daily by gavage.

2.3. Experimental design

Rats were classified randomly into two groups, Group 1 (control group) which received 1 ml distilled water and Group 2 (treated group) which received bicalutamide at dose of 0.8mg/kg of rat dissolved in distil water (0.2 mg per rat which dissolved in 1 ml distilled water), and given orally via gavage for 45 days.

2.4. Relative testicular weight determination

From anesthetized and sacrificed rats, both testes were removed and then testicles were excised free of surrounding tissues and weighed by balance. Relative testes weight was calculated by following equation: Relative testis weight = (testis weight/body weight) x 100 (Mossa et al., 2015).

2.5. Determination of testosterone concentration

Serum testosterone concentration of all rats were estimated by using Cobas 6000 instrument in Bio Lab private, Erbil. The assay principle of estimate testosterone hormone was combines an enzyme immunoassay competition method with a final fluorescent detection. Finally, the serum testosterone concentration results were calculated in relation to the calibration curve and the results were expressed by ng/ml.

2.6. Histological preparations

2.6.1. Paraffin method

After anesthetising rats, testes were surgically removed by opening abdomen, cleaned, directly fixed in 10% buffered formalin for 48 hrs, then dehydrated through gradually increased ethanol concentrations (50%, 70%, 80%, 95%, 100% and 100%). Xylene used for clearing and after infiltration by using paraffin wax, paraffin blocks were prepared from pieces of testes by embedding in the same wax. About four micrometer thick slices were acquired by using rotary microtome machine (Bright, MIC) from paraffin block and then stained by haematoxylin and eosin stains (H&E) (Bancroft et al., 1977). Specimens were viewed and photographed by using digital light microscope (digital binocular compound microscope 40x 2000x, built-in 3MP USB camera).

2.6.2. Immunohistochemistry

The DakoCytomation En Vision®+Dual link system-HPDAB(+)+HRP staining procedure was used for immunostaining to detect p53 protein and was applied to paraffin embedded testes tissue which
fixed by formalin. After preparing of slides, deparaffinization, rehydration, monoclonal mouse anti-human p53 protein antibody, incubation with enzyme and substrate-chromogen solution diaminobenzidine have been done. Sections were rinsed with distilled water and counterstained with haematoxylin stain. After wash with running tap water, testes sections on slides were dehydrated via gradually increased ethanol concentration and examined under digital light microscope.

2.6.3. Plastic method
Small pieces of testes which about 1 mm$^3$ were fixed in primary fixative which it is 2.5% glutaraldehyde in 0.1 cacodylate buffer pH 7.2-7.4 for 1 hr, postfixed in secondary fixative which it is 1% osmium tetroxide for 1 hr, dehydrated through gradually increased of acetone concentrations (50%, 70%, 80%, 95%, 100% and 100%). Then infiltrated by using acetone with resin mixture (3:1) for 1 hr, acetone with resin mixture (1:1) for 1 hr, acetone with resin mixture (1:3) for overnight, and eventually embedded only in resin medium to make plastic block (Durcupan ACM mixture epoxy resin: 10gm; Hardener: 10gm; Accelerator (dimethoxypropane) DMP 30: 0.3gm; and Di-n-butyl phthalate Plasticizer: 0.2gm). All of these chemicals have been got from Fluka AG, Bucha SG. Oven at 60°C for 72 hrs used to polymerization. Plastic blocks were sectioned by ultra-microtome into 1μm thick sections and then stained by using 1% toluidine blue in 1% Borax for light microscopic examination (Glauret, 1965).

2.7. Apoptotic index
The counting of apoptotic cells was achieved by using a 40x objective lens in which 100 numbers of cells were counted randomly throughout the field of the testis and then the number and percentage of apoptotic cells have been calculated. To get an accurate result, counting of cells repeated ten times in different fields.

2.8. Sperm counting
After sacrifice of rats, cauda epididymis were removed and cut to release sperms into 20ml of normal saline in small petri dish and then carefully minced by using manual glass homogeniser. Sperm numbers were measured by using haemocytometer slide and expressed by a number of sperm per millilitre.

2.9. Measurement of seminiferous tubule diameter and germinal layer thickness
About 10 seminiferous tubules were chosen randomly from each rat testes paraffin section which they were round or nearly round. Seminiferous tubule diameter and germinal layer thickness were measured at 100x magnification by using a scale of a digital light microscope.

2.10. Statistical analysis
All data were expressed as means ± standard error of mean (M ± SE) and statistical analyses were done by using statistically available software of Graph Pad Prism 6. To find significant differences between groups, T-test was performed.

3. RESULTS AND DISCUSSION

3.1. Relative testes weight
In the current study, no change was observed in the color, consistency and appearance of testes in both control group and bicalutamide treated group. However, the difference was noted in the weight of the testes. The present investigation showed a non-significant decrease in relative testes weight of bicalutamide treated rats when compared with control group rat testes (Fig. 3.1). This result was compatible with other studies that showed bicalutamide reduced the mean testicular weight in the adult Sprague – Dawley rats (Khurshid et al., 2014). In another investigation conducted by (Leonelli et al., 2011), concerning the effect of flutamide (another anti-androgen) on rats, a significant reduction in testicular weight was noted. Similarly, reduction of testicular weight and associated histological changes were noted after administration of anti-androgen in an androgen-stimulated testes of rats (Wason et al., 2003).
3.2. Effect of bicalutamide on serum testosterone concentration

Orally administration bicalutamide decreased testosterone level in treated group rats when compared with control group rats (Fig. 3.2). The result of the current investigation was similar to the results of Hashimoto et al. (2010) on bicalutamide. Another study which achieved on human, used another type of pure non-steroid anti-androgens has been shown a decrease in testosterone levels (Morse et al., 1973), while another study found no change in testosterone level, after treatment with bicalutamide (Chandolia et al., 1991, Morgante et al., 2001). Recently, bicalutamide treatment caused decrease level of testosterone hormone slightly (Han et al., 2018). Bicalutamide has effect through the competitive inhibition of androgen receptors, and it was shown to have slight impact on serum testosterone hormone concentration (Furr and Tucker, 1996). Bicalutamide might affect Leydig cells and this may be the reason behind testosterone decrease as other drugs did (Chiao et al., 2002).

3.3. Histological effect of bicalutamide on testis.

The histological structure of seminiferous tubules in control group rats was normal with well-formed spermatozoa in their lumen (Fig. 3.3). Administration of bicalutamide caused alteration of the histological structure of testes such as the appearance of vacuoles and sloughing of germ cells from the germinal layer of seminiferous tubules into their lumens which caused decrease of germinal epithelium thickness. Spermatids detachment and accumulation of the desquamated spermatocytes together with spermatids and cellular debris were seen in the seminiferous tubule lumens (Fig. 3.4). In plastic sections, dying germ cells, the formation of the apoptotic body, peripheral chromatin condensation in nuclei which are the characteristic of cell death were noted and apoptotic cells were shed into the seminiferous tubule lumen (Fig. 3.5). Bicalutamide caused a decrease in testosterone concentration which led to sperm death. Deprivations of gonadotropin or testosterone induce apoptosis in germ cells (Sinha Hikim et al., 1997). In some seminiferous tubules of the experimental rat group, detachment of the seminiferous tubule epithelial cells, presence of immature germinal cell and loss of germ cells was observed following administration of bicalutamide (Chandolia et al., 1991). Long-term bicalutamide monotherapy showed to have a little effect on the structure of testes, maturation of sperms and morphology of Sertoli and Leydig cells (Morgante et al., 2001). Administration of bicalutamide led to significant decrease of germ cells number and impacts of bicalutamide on the development of germ cells were due to contribute with testicular androgen actions (Russell and Clermont, 1977). Viguier-Martinez et al. (1983) noticed a slight decrease of spermatocyte count after administration of flutamide.

3.4. Immunohistochemical analysis

Immunohistochemistry technique for detection of p53 was done to demonstrate cells died by apoptosis mode of cell death in testes. As illustrated in Figure (3.6), sections of the control group was shown negative reaction, while bicalutamide treated rat testes was shown a positive p53 reaction (brown to black color cells) in the cytoplasm and nuclei of germinal layer.
cells. The multinucleated giant cells of the germinal layer were seen having a strong reaction. In adult rats, germ cell apoptosis due to bicalutamide could be associated with an increase in the expression and activation of p53. Expression and activation of enzymes such as caspases have a major role in apoptosis. Most of the characteristics of apoptosis such as cell shrinkage and nuclear condensation were observed in the current study. Apoptosis is essential to control cell number in adults, development in early life of multicellular organisms such as human and protects the organism by removing or eliminating cells which damaged by aging, disease, genetic mutation, infection, and exposure to toxic substances (Saikumar and Venkatachalam, 2009). P53 was first reported to be expressed and to promote apoptosis in primordial germ cells (Matsui et al., 2000), type A1 spermatogonia (Beumer et al., 1998) and primary spermatocytes (Yin et al., 1998). Exposure to flutamide which caused apoptosis in germ cells related in increase in the expression and activation of caspases-3 and caspases-6 enzymes in rats, which they are two major elements in the process of apoptosis (Omezzine et al., 2003).

As shown in Figure (3.7), bicalutamide caused a significant increase (P<0.0002) of apoptotic cells in testes of rats in comparison to the control group rats. The percentage of apoptosis in testes of bicalutamide treated rats was six folds when compared to the testes in control group. Bicalutamide caused decrease in testosterone level which is essential to growth of germ cells in testes. Omezzine et al. (2003) found that the number of apoptotic germ cells in adult rat testes were increased significantly after flutamide exposure. Decline of testosterone hormone level induced apoptotic cell death mode of germ cells (Sinha Hikim et al., 1997). Giant multinucleated cells (most often round spermatids, occasionally spermatocytes) result from failed integrity of the cellular bridges serving as partitions, which appeared in the bicalutamide treated rat seminiferous tubule which are a kind of apoptotic germ cells (Anton, 2003, Rasul and Aziz, 2012, Luo et al., 2013, Vidal and Whitney, 2014,).

3.5. Sperm counting

Daily administration of bicalutamide showed a statistically significant decrease (p<0.0001) of sperm count in bicalutamide treated group when compared with control group (Fig. 3.8). Spermatozoa produced from germ cells in a germinal layer of seminiferous tubules were consistently decreased after administration of bicalutamide (Chandolia et al., 1991). Morse et al. (1973) showed decrease sperm count in human after administration of cyproterone acetate (which is another type of anti-androgen drug). The reduction in sperm count might be via the partial arrest of spermatogenesis and also due to oxidative stress induced by the drug (Ghosh et al., 2002, Srinivasulu and Changamma, 2017).

3.6: Effect of bicalutamide on seminiferous tubule diameter and germinal layer thickness

Seminiferous tubule diameter and germinal layer thickness were shown statistically significant differences between two groups. Bicalutamide drug led to statistically significant increase (p<0.0001) in the diameter of seminiferous tubules, while it caused statistically significant decrease in the germinal layer thickness (p<0.05) in treated group as compared with control group (Table1). Other studies showed marked reduction in the germinal layer thickness in the experimental group of animals that received bicalutamide as compared to the control group (Khursheed et al., 2011). Flutamide, which it is another anti-androgen, didn’t cause change in diameter of seminiferous tubules in experimental group while it led to a significant reduction in the germinal layer thickness (Bustos-Obregón et al., 2006). Degeneration of the germinal cells in response to bicalutamide administration may the reason behind the depletion in germinal layer thickness.
Figure 3.3: Sections in the testes of control adult rats. A and B) Paraffin sections revealed normal histological structures of seminiferous tubules and spermatozoa in their lumen, H&E. 100x and 400x respectively. C and D) Plastic sections through the seminiferous tubules showing healthy features of the germinal epithelial cells, toluidine blue. 400x and 1000x respectively. ST Seminiferous tubule, SZ Spermatozoa, L Lumen, GL Germinal layer, SG spermatogonia and PS Primary spermatocyte.
Figure 3.4: Sections of seminiferous tubules in the testes of rats treated with bicalutamide A) Paraffin section shows certain histological alterations such as decreased thickness of the germinal epithelium layer (GL), decrease of spermatids number and accumulation of cellular debris (arrow) in the center of the seminiferous tubules. H&E. 100x B) Vacuoles (V) and depleted germinal layer with reduction of sperm number have been shown in the seminiferous tubule. H&E. 400x, C) Damaged spermatozoan tails forming clusters of filaments (arrow). H&E. 400x, D) Damaged germinal layer and cellular debris (arrow) are noted in the seminiferous tubule lumen. H&E. 400x.
Figure 3.5: Plastic sections of seminiferous tubules in the testes of rats treated with bicalutamide A) Approximately no spermatids were seen in seminiferous tubule (ST). Toluidine blue 400x. B) The appearance of apoptotic nuclei with margined chromatin (arrows). Toluidine blue 1000x. C) Apoptotic bodies shed (thin arrow) into seminiferous tubule lumen. In the germinal layer, a number of apoptotic-like nuclei with margined chromatin (thick arrow) are seen. Toluidine blue 1000x. D) Destructed sperms (arrows) in the lumen of the seminiferous tubule were noted. Toluidine blue 1000x.
**Figure 3.6:** Sections of seminiferous tubules demonstrating the immunohistochemical reaction of p53. A) Control group revealed no p53 immunohistochemical reaction. 400x. B, C and D) Treated group showed positive immunohistochemical reactions in the cells of germinal layer epithelium of seminiferous tubules. Reactions are seen in the germ dead cells (thick arrow) and multinucleated giant cells (thin arrow) 400x.
Figure 3.7: Apoptotic index in both control and bicalutamide treated rats. *** indicates that the value is significant compared to control at $P<0.0002$

Figure 3.8: Effect of bicalutamide on sperm count. **** indicates that the value is significant compared to control at $P<0.0001$

Table 1. Effect of bicalutamide on seminiferous tubules diameter and germinal layer thickness.

| Measured regions(µm)           | Control                | Bicalutamide          |
|-------------------------------|------------------------|-----------------------|
| 80.62 ± 1.708                 | 80.62 ± 1.708          | 106.0 ± 1.322 ****    |
| Seminiferous tubules diameter |                        |                       |
| Germinal layer thickness      | 16.19 ± 0.5500         | 14.47 ± 0.4631 *      |
|                               |                        |                       |

Data represented as mean ± S.E. * mean significant differences at level $P<0.05$, **** mean significant differences at level $P<0.0001$ compared with control.
4. CONCLUSIONS

According to the present results, bicalutamide treatment caused castration in rats represented by the appearance of various biochemical, histological and immunohistochemical changes.

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Conflict of Interest
I do not have any conflict of interest.

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