

# Potential Effect of Botulinum Neurotoxin A in the Treatment of Induced Benign Prostatic Hyperplasia in Male Rats

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## Abstract

**Background:** Researches carried out in humans, dogs, and rats showed that the injection of botulinum neurotoxin type A into the prostate gland reduce the size and other biomarker that indicate botulinum neurotoxin type A as a new promising treatment for benign prostatic hyperplasia.

**Aim of the research:** The purpose of the study is to evaluate the effects of botulinum neurotoxin type A in the treatment of induced BPH rat model, comparing it with the most powerful standard treatment available composed of alfuzosin and dutasteride (combination of  $\alpha 1$  adrenergic antagonist and  $5\alpha$  reductase inhibitor) for BPH rat model and also to describe the mechanism by which botulinum neurotoxin type A effect prostate growth by focusing on acetylcholine-prostate nicotinic receptor signal role in BPH.

**Method:** Five groups each of them composed of 10 rats. Normal group administered normal saline IP for 42 days. Induction group induced with BPH by testosterone 4 mg/kg/day SC injection for 14 days then administered normal saline IP for 28 days. Conventional treatment group induced BPH by testosterone 4mg/kg/day SC injection for 14 days then administered combination of alfuzosin and dutasteride daily through oral gavage for 28 days. Botulinum group induced BPH by testosterone 4 mg/kg/day SC injection for 14 days, then the injected inside the prostate at day 15 with botulinum neurotoxin type A and left free for 27 days. Nicotine group induced BPH by testosterone for 14 days, then the injected inside the prostate at day 15 with botulinum neurotoxin type A and administered nicotine orally for 27 days. At the end of the experiment rats weighed and sacrificed and blood and prostate samples were collected for PI, CPV, APV determination and sTNF $\alpha$ , sPSA, sCASP3, pTNF $\alpha$ , pPSA and pCASP3 ELISA testing.

**Results:** Botulinum, conventional treatment and normal groups had a PI (g/100 g) equal to  $0.239 \pm 0.056$ ,  $0.244 \pm 0.025$  and  $0.208 \pm 0.045$  respectively and showed a significant ( $P \leq 0.05$ ) reduced PI when compared with induction and nicotine groups which had PI equal to  $0.317 \pm 0.053$  and  $0.338 \pm 0.088$  respectively. Botulinum, conventional treatment and normal groups had CPV (mm<sup>3</sup>) equal to  $472.6 \pm 62$ ,  $468 \pm 123.4$  and  $370.1 \pm 100.3$  respectively and expressed a significant ( $P \leq 0.05$ ) reduced CPV in comparison with induction and nicotine groups which possessed CPV equivalent to  $997.9 \pm 148.6$  and  $845 \pm 177$  respectively. Botulinum, conventional treatment and normal groups had a APV (ml) equal to  $380 \pm 94.8$ ,  $385 \pm 127$  and  $330 \pm 67.4$  in that order and showed a significant ( $P \leq 0.05$ ) reduced APV when compared with induction and nicotine groups which had a APV equal to  $825 \pm 139.9$  and  $845 \pm 177$  respectively. Botulinum, conventional treatment and normal groups had a sTNF $\alpha$  (ng/ml)  $84.327 \pm 28.063$ ,  $99.545 \pm 27.78$  and  $78.2 \pm 34.195$  respectively and pTNF $\alpha$  (ng/ml) equal to  $41.272 \pm 6.78$ ,  $48.763 \pm 27.662$  and  $37.218 \pm 6.415$  respectively. Induction group and nicotine group possessed a sTNF $\alpha$  equivalent to  $139.236 \pm 34.691$  and  $157.836 \pm 24.517$  respectively and pTNF $\alpha$  equal to  $84.054 \pm 67.871$  and  $95.712 \pm 47.056$  respectively. Botulinum, conventional treatment and normal groups showed a significant ( $P \leq 0.05$ ) reduction in both sTNF $\alpha$  and pTNF $\alpha$  in comparison with induction and nicotine groups. Botulinum, normal groups had a sPSA equal to  $0.685 \pm 0.264$  and  $0.629 \pm 0.126$  respectively showed a significant ( $P \leq 0.05$ ) reduction in sPSA when compared with induction, nicotine groups which had a sPSA equal to  $1.023 \pm 0.423$  and  $1.328 \pm 0.356$  respectively. Botulinum, normal groups had a pPSA equivalent to  $0.339 \pm 0.213$  and  $0.315 \pm 0.164$  respectively expressed a significant ( $P \leq 0.05$ ) reduction on pPSA in comparison with induction and nicotine groups which possessed a pPSA equal to  $0.606 \pm 0.394$  and  $0.65 \pm 0.24$  respectively. Conventional treatment group had a sPSA equal to  $0.886 \pm 0.188$  and pPSA equal to  $0.411 \pm 0.197$  and showed a no significant difference ( $P > 0.05$ ) in both sPSA and pPSA when compared with normal, induction and botulinum groups, on the other hand showed a significant difference with nicotine group. Results showed no significant difference in sCASP3 ( $P > 0.05$ ) between all groups. Botulinum group express a pCASP3 equal to  $10.843 \pm 7.731$  and showed a significant increase ( $P \leq 0.05$ ) in PCASP3 when compared with normal, induction, conventional treatment and nicotine groups which had a PCASP3 equal to  $2.38 \pm 1.627$ ,  $2.151 \pm 1.582$ ,  $3.319 \pm 3.504$  and  $2.15 \pm 1.444$  respectively. The PPI of BPH of botulinum, conventional treatment and nicotine groups equal to 71.5%, 66.7% and -19.5% respectively.

**Conclusion:** BTXA intraprostatic injection found to reduce hyperplasia by induction of apoptosis, reduce inflammation and decrease PSA. It also obvious from the results that BTXA as effective as the conventional treatment and have advantage of less side effects and be single injection instead of daily medication. Nicotine administration reverses the effects of Botulinum neurotoxin type A that's show the central role of nicotinic receptor stimulation in the pathogenesis of BPH.

**Keywords:** Benign prostatic hyperplasia • Alfuzosin • Dutasteride • Botulinum neurotoxin type A • Nicotine

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## Introduction

Benign Prostatic Hyperplasia (BPH) is a disease that mainly affects elderly men [1]. Lower Urinary Tract Symptom (LUTS) risk increased in BPH. LUTS can be divided into irritation symptom and obstructive symptoms [2]. BPH is characterized by increased both epithelial and stromal cells number in the area of the prostate gland. Although pathophysiology of BPH is not fully understood, it may be caused by androgenic stimulation that lead to overgrowth and adrenergic action that lead to contraction of smooth muscle [3,4]. Approximately 30% of human cancers occur as a result of tobacco and other pollutant. It has been found that tobacco smoking is specifically increase inflammation in prostate carcinoma. Nicotine forms up to 1.8% of the dry tobacco weight. IL8 expression can be stimulated in the human neutrophil by nicotine. It also found that tobacco smoker prostate cancer patient had increased expression of cytokine, B cell count and NF- $\kappa$ B in prostate gland [5-7]. The European Association of Urology and the American Urological Association recommended two classes of medication. The first group called  $\alpha$ 1 adrenergic receptor antagonists and the second group called  $5\alpha$  reductase inhibitors. Combination therapy of two drugs above result in reduction of progression of BPH significantly when compared with one class alone [8]. Over the past years, Botulinum neurotoxin (BTXA) found to have therapeutic effects in many diseases such as Strabismus and cosmetic etc., [9-11]. BTXA effects on the prostate not restricted to its action on the neuromuscular junction, it may be also associated with its effect on the neuro-glandular junction. Intraprostatic injection of BTXA can cause involution in the prostate without any complication like local inflammation and general systemic adverse effects [12]. BTXA is derived from type of bacteria called Clostridium [13]. Pharmacologically active BTXA comprising of a light chain and a heavy chain linked by a disulfide bond. Reduction of this disulfide S-S bond releases the light chain which has metalloprotease action [14]. BTXA then associate with catalytic activity towards SNAP25 (synaptosomal associated proteins 25) that lead to break of SNAR complex resulting in block of the release of the neurotransmitters from nerve ending [13-15]. The affected neurotransmitters by BTXA other than acetylcholine may include norepinephrine and others. BTXA showed effects on growth and dynamic contraction of the prostate [13]. The prostate gland is also regulated by parasympathetic system through the pelvic nerve that innervates the major pelvic ganglion and the post ganglionic neurons regulate the prostate gland. Acetylcholine expressed a relaxing effect on the induced prostatic contraction by phenylephrine in isolated prostate tissue from rabbit prostate gland. Studies showed Muscarinic 3 receptor subtype cause contraction in rat prostate tissue [16,17]. Nicotinic receptors found in cell membrane of all mammalian cells. These receptors are ionic channels receptor that found in the nervous system and muscle. Recent researchers found that these receptors also present in other tissue and play a role in many diseases such as prostate cancer. The  $\alpha$ 5 nicotinic acetylcholine receptors ( $\alpha$ 5nAChR) have a role in growth of prostate gland. When  $\alpha$ 5nAChR is activated lead to increase Akt. Akt showed to decrease apoptosis, increase expression of androgen receptor [18,19].

The purpose of the study is to evaluate the effects of botulinum neurotoxin type A in the treatment of induced BPH in rat model, comparing it with the most powerful standard treatment available composed of alfuzosin and dutasteride (combination of  $\alpha$ 1 adrenergic antagonist and  $5\alpha$  reductase inhibitor) for BPH rat model and also to describe the mechanism by which botulinum neurotoxin type A effect prostate growth by focusing on acetylcholine-prostate nicotinic receptor signal role in BPH

## Materials and Method

### Experimental animals

50 Adult male Wister rats 250-350 gram purchased from the Iraqi center for cancer and medical engineering research. Animals had kept in the animal house in normal room temperature approximately 21 and sustained

at 12 dark/light cycle with maintenance of free food and water access.

### Materials

Testosterone Propionate (MH pharma. Co. LTD. Pakistan), Pure Olive Oil, Alfuzosin (Xatral-Sanofi-France), Dutasteride (Avodart-GSK company-UK), Botulinum neurotoxin vial (HUTOX inj-Huons Global Co.Ltd-South Korea). Nicotine lozenges 2mg (Nicorette® Gum-Johnson & Johnson-USA), xylazine (kepro-Holland) and ketamine (Bremer Pharma GmbH-Germany).

### Dose calculation and preparation

Dutasteride and alfuzosin dose calculation and preparation: The dose of dutasteride used 0.044 mg/Kg/day. For conversion of the human dose to rat dose, we multiply human dose with 6.2 (conversion factor according to FDA) [20]. For 70 Kg human administer 0.5 mg/day, so the dose will be 0.00714 mg/Kg/day. Multiplying the dose by conversion factor between rats and human will give the dose for rats:  $0.00714 \text{ mg/Kg/day} \times 6.2 = 0.044 \text{ mg/Kg/day}$ . (Avodart capsules diluted with olive oil to prepare 0.11 mg/ml).

Alfuzosin dose For BPH in 70 Kg human who take 10 mg per day. The dose will be 0.142 mg/Kg/day. Rat dose calculated by Multiply the human dose by the conversion factor. Rat dose= $0.142 \text{ mg/kg/day} \times 6.2$ . Rat dose= $0.88 \text{ mg/kg/day}$ . Alfuzosin diluted with distilled water to prepare stock solution of 2.2 mg/ml.

Testosterone propionate dose and preparation: Testosterone propionate added olive oil to prepare stock solution of 20 mg/ml. The dose for BPH induction was 4 mg/kg/day for 14 days [21].

Botulinum neurotoxin type A (BTXA): The vial was dissolved with 0.9% normal saline to prepare stock solution of 25 unit/ml [22]. Rats anesthetized with 50 mg/Kg ketamine Intra-Peritoneal (IP) injection plus IP injection of 12 mg/Kg xylazine. The lower abdominal hair removed by shaver and the incision area dis-infected with povidone iodine 10% and 70% ethyl alcohol. A lower abdominal small incision about 2 cm was made at the midline and the prostate gland together exposed for injection syringe by remove of the surrounding tissue carefully. Ventral lobe of the prostate gland injected with 5U (0.2 ml) of botulinum toxin stock solution. Care took to inject 2.5 (0.1 ml) into each lobule of the ventral lobe of the prostate. The injection area then cleaned. After that, wound of the skin and wall of abdomen closed using 0/4 PGA suturing and disinfected with povidone iodine 10% [23].

Nicotine preparation and administration: Nicotine solution was prepared by dissolving one lozenge containing 2 mg of nicotine in distilled water to prepare stock solution of 0.25 mg/ml. the dose used was 0.1 mg/Kg/day orally [24].

### Experimental design

50 Rats were divided into five groups, each group composed of 10 rats. The first group which is the normal group (Group 1) administered IP 0.5 ml/kg/day of 0.9% normal saline for 42 day. The second group is the induction group (Group 2) administered testosterone propionate subcutaneous injection 4 mg/Kg for 15 day to induce BPH and then administered IP 0.5 ml/Kg/day of 0.9% normal saline for 28 day. The third group is the conventional therapy group (Group 3) administered subcutaneous injection of testosterone propionate 4 mg/Kg daily for 15 day to induce BPH. Then rats on this group administered combination therapy of alfuzosin hydrochloride 0.88 mg/Kg/day and dutasteride 0.44 mg/Kg/day. This combination administered by oral gavage daily for 28 day. The fourth group which is the botulinum group (Group 4) composed administered subcutaneous injection of testosterone propionate 4 mg/Kg /day for 15 day to induce BPH. After that, rats in this group administered intraprostatic injection of 5U into the ventral lobe of the prostate [25,26]. Then rats in this group were monitored for any behavior or other abnormality for 27 day. The fifth group is the nicotine group (Group 5), BPH induced by subcutaneous injection of testosterone propionate 4 mg/Kg daily for 15 day. Then rats administered intraprostatic botulinum toxin 5U into the ventral lobe of the prostate at day 15. Rats in this group then administered 0.1 mg/Kg/day nicotine orally for 27 days. Rats on all groups

had weighed and then, sacrificed by IP injection of 200 mg/Kg phenobarbital [23]. Then the prostate had harvested, weighed and cut into two parts, one part fixed in formaldehyde 10% for histopathological examination and the other homogenized for ELISA testing.

### Parameter to be measured

**Prostate Index (PI) and Percentage of Prostate Inhibition (PPI) calculated from the formula:**  $PI = (\text{Prostate weight} \times 100) / \text{Rat body weight}$ . Unit of PI=gram/100 gram body weight [21].  $PPI \text{ of Group X (PPIX)} = \{100 - [(MPI.X - MPI.C) / (API.I - API.C)]\} \times 100\%$  [20]. [Where is: MPI.X=Mean PI of X, X can represent conventional treatment or botulinum or nicotine group, MPI.C=Mean PI of normal group, MPI.I=Mean PI of induction group] [20].

**Calculated Prostate Volume (CPV) and Actual Prostate Volume (APV):** After the prostate dissected a vernier caliper used to detect the length, width and height of the prostate gland and the CPV calculated by the formula  $CPV = (\text{Length} \times \text{width} \times \text{height}) / 2$  [20,21]. APV is calculated using fluid displacement according to Archimedes' principle. The Actual prostate volume is calculated from the formula:  $(APV) = \text{Final volume} - \text{Initial volume}$ .

**Plasma ELISA testing for the following marker:** ELISA a kit for serum purchased from Sunlong Biotech Co LTD-China. According to the instruction blood collected from heart by 5 cc syringe. Blood allowed clotting by leaving it undisturbed at 25 °C for 20 minutes. The clot had been removed by centrifuging at 3,000 rpm for 20 minutes. ELISA test done for serum concentration of the following biomarker: Tumor Necrosis Factor  $\alpha$  (TNF $\alpha$ ), Prostate Specific Antigen (PSA), Caspase 3.

**Prostate tissue homogenate ELISA testing for the following parameter:** ELISA a kit for tissue purchased from Sunlong Biotech Co LTD-China. According to the instructions prostate tissue were cut and homogenized after adding Phosphate Buffer Saline (PBS) 9 times the prostate tissue weight. The supernatant collected after centrifuging at 3,000

rpm for 20 minutes. ELISA test done for prostate tissue concentration of the following biomarker: Tumor necrosis factor  $\alpha$ , Prostate Specific Antigen (PSA) and Caspase 3.

**Histological analysis and staining procedure:** The prostate tissue cut and fixed in 10% formaldehyde. Prostate tissue then treated with alcohol 70%, 80 %, 90 % and absolute concentration of alcohol. The slide immersed in paraffin. Paraffin cleared using xylene. The slide cut at 4  $\mu$ m using microtome. The slide then immersed in absolute, 90%, 80% and 70% alcohol in that order. The slide washed in water for 1 minute, stained with hematoxylin and eosin. The slide after that washed with water. The slide treated with 70%, 80%, 90% and absolute concentration of alcohol. At last Canada balsam added and the slide cleared using xylene. The slide read using light microscope.

### Statistical analysis

The results analysis by using SPSS and value of each result expressed as a mean  $\pm$  SD (Standard Deviation). The post hoc test was used, and  $p \leq 0.05$  was considered significant.  $P > 0.05$  was considered as no significant difference.

## Results

The mean of PI, CPV and APV in all groups showed in Table 1.

The mean of sTNF $\alpha$ , sPSA and sCASP3 in all groups showed in Table 2.

The mean of pTNF $\alpha$ , pPSA and pCASP3 showed in Table 3.

The degree of Prostate Percentage of Inhibition (PPI) for botulinum, conventional treatment and nicotine groups were 71.5%, 66.7% and -19.5% respectively.

**Table 1.** The means value of PI, CPV and APV of each group with standard deviation.

Rats group	Mean PI g/100 g $\pm$ S.D	Mean CPV mm <sup>3</sup> $\pm$ S.D	Mean APV ml $\pm$ S.D
Normal	0.208 $\pm$ 0.045	370.1 $\pm$ 100.3	330 $\pm$ 67.4
Induction	0.317 $\pm$ 0.053	997.9 $\pm$ 148.6	825 $\pm$ 139.9
Conventional treatment	0.244 $\pm$ 0.025	468 $\pm$ 123.4	385 $\pm$ 127
Botulinum	0.239 $\pm$ 0.056	472.6 $\pm$ 62	380 $\pm$ 94.8
Nicotine	0.338 $\pm$ 0.088	845 $\pm$ 177	845 $\pm$ 177

**Note:** SD: Standard Deviation; PI: Prostate Index; CPV: Calculated Prostate Volume; APV: Actual Prostate Volume; g: gram; mm<sup>3</sup>: cubic millimeter and ml: microliter.

Botulinum-Normal and conventional treatment groups showed a significant reduction in PI, CPV and APV when compared with induction and nicotine groups in post hoc ANOVA test.

Significant differences when ( $p \leq 0.05$ ).

**Table 2.** The mean and SD of sTNF $\alpha$ , sPSA and sCASP3 of each group.

Rats group	Mean sTNF ng/ml $\pm$ S.D	Mean sPSA ng/ml $\pm$ S.D	Mean sCASP3 $\pm$ S.D
Normal	78.2 $\pm$ 34.195	0.629 $\pm$ 0.126	3.004 $\pm$ 3.172
Induction	139.236 $\pm$ 34.691	1.023 $\pm$ 0.423	2.024 $\pm$ 0.505
Conventional treatment	99.545 $\pm$ 27.78	0.886 $\pm$ 0.188	3.639 $\pm$ 3.176
Botulinum	84.327 $\pm$ 28.063	0.685 $\pm$ 0.264	3.702 $\pm$ 2.547
Nicotine	157.836 $\pm$ 24.517	1.328 $\pm$ 0.356	1.882 $\pm$ 0.338

**Note:** SD: Standard Deviation; sTNF $\alpha$ : Serum TNF $\alpha$  level; sPSA: Serum PSA level and sCASP3: Serum caspase 3 level.

Botulinum-conventional treatment and normal groups showed a significant reduction in sTNF $\alpha$  induction and nicotine groups. Botulinum and normal group expressed a significant reduction in sPSA in comparison with induction and nicotine groups. Conventional treatment showed no significant reduction difference in sPSA in relation with induction and normal groups.

There is no significant difference in sCASP3 between all groups. Significant differences when ( $p$  value  $\leq 0.05$ ). Post hoc ANOVA test used to analyze the results.



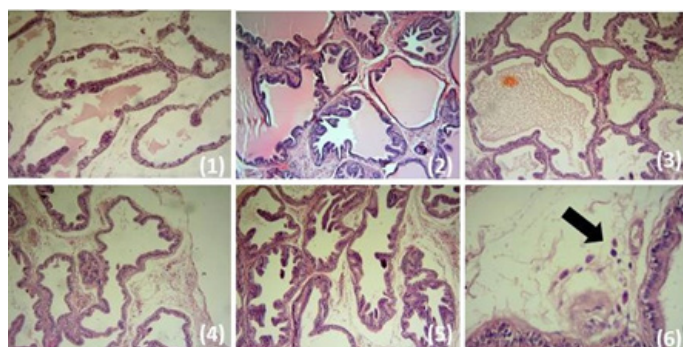
**Table 3.** The mean and SD of pTNF $\alpha$ , pPSA and pCASP3 of each group.

Rats group	Mean pTNF $\alpha$ ng/ml $\pm$ S.D	Mean pPSA ng/ml $\pm$ S.D	Mean pCASP3 $\pm$ S.D
Normal	37.218 $\pm$ 6.415	0.315 $\pm$ 0.164	2.38 $\pm$ 1.627
Induction	84.054 $\pm$ 67.871	0.606 $\pm$ 0.394	2.151 $\pm$ 1.582
Conventional treatment	48.763 $\pm$ 27.662	0.411 $\pm$ 0.197	3.319 $\pm$ 3.504
Botulinum	41.272 $\pm$ 6.78	0.339 $\pm$ 0.213	10.843 $\pm$ 7.731
Nicotine	95.712 $\pm$ 47.056	0.65 $\pm$ 0.24	2.15 $\pm$ 1.444

Note: S.D: Standard Deviation; pTNF $\alpha$ : Prostate TNF $\alpha$  concentration; pPSA: Prostate PSA concentration and pCASP3: Prostate caspase 3 concentration. Normal; conventional treatment and botulinum groups showed significant reduction in pTNF $\alpha$  when compared with induction group. Normal; botulinum groups showed significant reduction in pPSA when compared with induction group. Conventional treatment group showed no significant difference in pPSA with all other groups. Botulinum group increase pCASP3 significantly when compared with all other groups. Significant when  $p \leq 0;05$ .

### Histopathological examination

The section of the prostate tissue of the normal group showed unremarkable histopathological changes without sign of inflammation or hyperplasia. The induction group showed increase in proliferation of the glandular and stromal tissue with hyperplastic changes and increased number of the glands and cells. There are strong inflammatory reaction with numerous vascular dilation and congestion. The conventional treatment group showed mild hyperplastic changes with mild inflammatory reaction of the stroma. The investigated therapy group showed mild inflammatory reactions with mild hyperplasia and decrease prostate glands secretion with appearance of scattered apoptotic body. The nicotine group showed hyperplasia with strong stromal inflammatory reaction and focal hemorrhage with vascular congestion. Figure 1 showed those entire histological features.



**Figure 1.** Hematoxylin-Eosin (H-E) stain of rat prostate gland. \*Sections of the prostate gland at 10X.

a) Normal group b) Induction group c) Conventional treatment group d) Botulinum group e) Nicotine group \*f) Section of the prostate gland at 40X of the investigated therapy group showed apoptotic body marked by black arrow.

### Discussion

The results of this research suggest that use of BTXA for the treatment of BPH in rats showed a significant improvement in the measured parameters. It also clarify that there is a central role for nicotine in BPH progression and that effect of BTXA may be in large part due to inhibition of acetylcholine from stimulating prostate nicotinic receptor as nicotine administration reverse BTXA effects. It is also showed that BTXA decrease inflammatory cytokine TNF $\alpha$ , reduce prostate weight and volume and induce apoptosis. The study showed that continuous daily administration of conventional treatment with alfuzosin and dutasteride showed similar effect to single botulinum neurotoxin injection except that botulinum neurotoxin more powerful in reduce PSA and induce apoptosis.

When compared with induction group. Botulinum group showed a significant reduction in PI, CPV and APV which mean BTXA decreases weight and size of the prostate. This may be due to reduce number of cells in the prostate gland as a result of growth inhibition and apoptosis induction action. BXTA induce apoptosis in the prostate (26) by increasing pCASP3 as shown in Table 3 and the appearance of apoptotic body as shown in Figure 1a-1f. There is no significant difference in the sCASP3 between all groups. In apoptosis, the apoptotic body formed which consist of intact plasma membrane surround the cytoplasm and organelles, this apoptotic body mainly removed through phagocyte engulfment. The cell component not released into interstitial area surrounding the cells so, that's explained why inflammation not occur in apoptosis [27]. These findings suggest sCASP3 is not reliable biomarker for testing actual prostate apoptotic activity.

BTXA growth inhibition action in the prostate may be due to interruption of  $\alpha 5$  nAChR and Muscarinic receptor signals through inhibition of acetylcholine release from the parasympathetic nerve ending in the prostate gland. Inhibition of  $\alpha 5$  nAChR in the prostate gland may cause decrease in Akt and ERK1/2 expression resulting in decrease prostate cells proliferation [28]. Akt shown to increase expression of AR receptor in androgen dependent prostate cancer [29]. It is also obvious that Akt inhibition cause increase the process of apoptosis as Akt cause inhibition of caspase 9 activation and at the premitochondrial level before release of cytochrome c [30]. Inhibition of ERK signaling in human prostate cancer cell result in down regulation of prostate cancer [31]. Activation of M3 receptor in prostate gland activate Ca<sup>2+</sup>/calmodulin signal lead to proliferation of prostate epithelial cells, increase prostate secretion and cause prostate smooth muscle contraction [32]. BXTA inhibit norepinephrine release leading to increase TGF- $\beta$  and decrease expression of NF- $\kappa$ B, AR and 5 $\alpha$  reductase [33,34].

In comparison with induction group, Botulinum group showed a significant reduction in sTNF $\alpha$  and pTNF $\alpha$  as shown in Tables 2 and 3 that may be an indicator that BTXA decrease systemic as well as local inflammation. TNF $\alpha$  is a cytokine have a central role in inflammation [35]. BTXA have anti-inflammatory effect in reducing mast cell degranulation in induced arthritis in rats [36]. Antagonizing the muscarinic receptor in rat's leukocyte decreases T-cells and reduces chemoattraction to lymphocyte, monocyte and other neutrophil [37].

When compared with induction group, Botulinum group showed significant decrease in sPSA and pPSA in Tables 2 and 3. BTXA showed to decrease PSA in humans [38]. PSA is secreted from prostate gland [39]. This result is supported by evidence of decreased secretion in the glandular part of the prostate tissue histopathological examination in Figure 1a-1d.

Conventional treatment group significantly reduce PI, CPV and APV when compared with induction group. Results also showed that despite this effect conventional treatment group has not shown a significant increase in caspase 3 level in tissue or blood as shown in Tables 2 and 3. These

finding suggest that conventional treatment group reduce size and weight of the prostate gland by a process other than apoptosis.  $\alpha_1$  adrenergic antagonists shown to decrease proliferation by activity that include its action on blocking  $\alpha_1A$  receptors and by other mechanism that is not related to  $\alpha_1A$  receptor like DNA damage as shown in [40]. Dutasteride is an  $5\alpha$  reductase inhibitor and researches showed that these type of drugs cause autophagy through down regulation of Insulin like Growth Factor 1 (IGF-1) in the stromal cells of the prostate glands [41]. Conventional treatment group showed a significant reduction in sTNF $\alpha$  and pTNF $\alpha$  when compared with induction group as shown Tables 2 and 3. Dutasteride treatment restored the expression of Estrogen Receptor  $\beta$  (ER $\beta$ ) that was downregulated in the inflamed prostate. ER $\beta$  showed anti-inflammatory effect in human prostate [42]. Alfuzosin showed to reduce chronic prostatitis [43]. The conventional treatment group showed moderate reduction in both sPSA and pPSA as it showed no significant difference with both normal and induction group. The histopathological finding suggests that conventional treatment group not reduce prostate secretion as shown in Figure 1. Alfuzosin show no significant difference in PSA level between untreated patient group and patient group administered alfuzosin for at least one month [44]. It is well known that dutasteride reduce PSA level significantly after six months of treatment [45].

Nicotine group inverse the action BTXA on the prostate gland and showed significant increase in PI, CPV and APV when compared with botulinum, normal groups, on the other hand expressed no significant difference with induction group. Dr. Silva study suggests that the effect of this BTXA in the prostate related to its activity on the adrenergic not cholinergic system. The limitation of this study that it is just focused on study the muscarinic receptor implication through bethanicol which is a direct muscarinic receptor agonist but without give attention to prostate nicotinic receptor. It was thought that nicotine proliferative effect on prostate gland attributed to stimulation of nicotinic receptor located on the ganglia of neurons terminate on the prostate that lead to secretion of norepinephrine from nerve terminal [46]. For the BTXA used to block the release of norepinephrine from nerve terminal to just focus on effect of nicotine on the prostate nicotinic receptor as botulinum toxin block acetylcholine, norepinephrine and ATP release from nerve terminal [13].  $\alpha_5nAChR$  activation increases proliferation and decreased phosphorylation of level of AKT and ERK1/2 [28]. Evidences in both *in vivo* and *in vitro* animal model studies suggest that treatment with nicotinic acetylcholine receptor blocker such as  $\alpha$ -Bungarotoxin could reverse proliferation induced by nicotine [47]. Nicotine smoking is also known to inhibit aromatase enzyme responsible for conversion of androgen to estrogen so that is explain the reduction of estradiol 17 $\beta$  on female rats after chronic exposure to nicotine [48]. Nicotine increased testosterone level because rats that given aromatase inhibitor showed high testosterone level [49]. Nicotine reverse BTXA reducing inflammation effect as shown in Tables 2 and 3 that nicotine administration increase sTNF $\alpha$  and TNF $\alpha$ . Nicotine exposure from smoking increase inflammation [6]. Nicotine group showed significant decrease in pCASP3 as shown in Table 3. Nicotine suppresses apoptosis induced by cisplatin in lung cancer by causing bcl2 stability [50].

## Conclusion

BTXA intraprostatic injection found to reduce hyperplasia by induction of apoptosis, reduce inflammation and decrease PSA that mean it eliminate the main key players in the pathogenesis of BPH. It also obvious from the results that BTXA as effective as the conventional treatment and have advantage of be more powerful in causing prostate growth inhibition, less side effects and a pharmaco-economic advantage of be single injection instead of daily medication. Nicotine administration reverses the effects of Botulinum neurotoxin type A that's prove the central role of nicotinic receptor stimulation in the pathogenesis of BPH. Further studies need for studying the effects of repetitive doses of BTXA as well as to study the duration of action of BTAX in BPH treatment. The nicotinic receptor may be a new target for the future researches to develop new effective drugs to treat BPH.

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