1. Background
Benign prostatic hyperplasia (BPH) is a progressive proliferation of prostatic glandular and stromal tissues. BPH is an age related disease and is present in 20% of 40 yrs and 70% of 60 yr old men (Isaacs 1994). Currently, there is no complete effective treatment for BPH. In some severe cases, mostly transurethral resection of the prostate is the only effective intervention available today (Roehrborn 2005). Thus, new therapies are obviously needed. Despite the enormous burden of BPH on public health, its pathogenesis is incompletely understood. To date, we still have no precise knowledge of the cellular and molecular processes underlying the pathogenesis of BPH (Lee and Peehl 2004).

Aging and androgens are the two established risk factors for the development of BPH tissue remodeling in the prostate. Hyperplastic growth in BPH has been ascribed to an imbalance between androgen/estrogen signaling (Coffey and Walsh 1990). Although the influence of androgens and estrogens has been demonstrated, hormonal factors alone may not fully explain BPH development. Many researchers have reported the role of other factors which may play a role in the onset and development of BPH (Lin et al., 2007), overexpression of stromal and epithelial growth factors (Lucia and Lambert et al., 2008), hypoxia (Berger et al., 2003), epithelial to mesenchymal transition (Alonso-Magdalena et al., 2009) factors.

BPH frequently occurs concurrently with prostatitis, as shown by the finding of histological lesions in 40–90% of all cases (Soler Soler 1999). There are some evidences that prostatic chronic inflammation could be a key component in BPH progression and in the epithelial/stromal prostatic cells interactions (Kramer et al., 2007). Two of the major clinical studies on BPH Medical Therapy of Prostatic Symptoms (MTOPS and Reduce study) recently demonstrated a link between histological prostatic inflammation and enlargement (Roehrborn et al., 2005 and Nickel et al., 2008). These mediators are released in the prostatic gland by inflammatory cells that can be found on most of the surgery derived BPH specimens (Di Silverio et al., 2003). Many studies have shown that inflammatory infiltration T lymphocytes and macrophages in BPH patients, but the role of inflammation in BPH is not clearly understood (Gestinbluth et al., 2002).

A model of BPH in male rats can be produced by repeated injections of testosterone (Maggi et al., 1989). This model has been adapted for several studies (Scolnik et al., 1994, Liu et al., 2009, Rick et al., 2011). Because the mechanism of prostate growth is complex and heterogeneous in
different species, and the testosterone induced models of BPH show an epithelial hyperplasia the androgen-induced models of BPH have limitations. Alonso-Magdalena (2009) description of human BPH as predominantly of epithelial origin supports the use of a testosterone induced model of BPH with predominant epithelial hyperplasia. He also proposed that BPH is not a proliferative disease of the stroma but rather is an accumulation of mesenchymal-like cells derived from the prostatic epithelium and the endothelium (Alonso-Magdalena et al., 2009).

Daidzein belongs to the group of isoflavones. It is present in a number of plants and herbs. Soy isoflavones are a group of compounds found abundantly in soybean. Daidzein was known for its antioxidant, anti-inflammatory and chemopreventive properties. Moreover it is used as anti hypertension and in inflammatory diseases (Jackman et al., 2007). Luteolin belongs to the group of flavones. It is commonly found in many fruits and vegetables, well known for its antioxidant (Leung et al., 2006; Manju et al., 2005), and anti-inflammatory (Perwez Hussain and Harris, 2007; Brody et al., 2006; Karin et al., 2006) properties. Our aim was to study the protective role of daidzein and luteolin on inflammation, oxidative stress, and proliferative markers in the rodent model of testosterone propionate induced BPH progression.

2. Treatment regimen

Rats were divided into seven groups with 8 animals in each group. Group I served as control. Testosterone propionate 7 mg/kg b.wt was given to group II from 14-28 days. Flutamide 30 mg/kg b.wt was given to group III animals from 14-28 days. Rats were pretreated with daidzein [group IV (20 mg/kg b.wt) & group V (60 mg/kg b.wt)] and luteolin [group VI (0.2 mg/kg b.wt) & group VII (0.4 mg/kg b.wt)] for first 14 days, followed by testosterone propionate 7 mg/kg b.wt in corn oil for 14 days (14-28) along with daidzein and luteolin treatments. All the groups were sacrificed after 28 days.
2.1 Material and methods

The levels of inflammatory (IL 1 β, IL 6, TNF α, IFN γ) cytokines and anti-inflammatory (IL 10) cytokines were measured by ELISA. The serum hormone levels of Testosterone, DHT, and PSA were measured by ELISA. The protein expression of inflammatory markers like NF-κB, iNOS, COX-2 and expression of proliferation by Ki-67 was observed by IHC staining. The level of inflammation was further studied by Mast cells staining by use of toluidine blue stain. We also checked the level of antioxidant enzymes like Catalase, GSH, LPO, XO, G6PD and SOD. For detailed methodology, please refer to chapter II (materials and methods).

3. Result

3.1 Effect of daidzein and luteolin on serum testosterone, DHT and PSA levels.

There was a significant increase in levels of Testosterone (p<0.05), DHT (p<0.001) and PSA (p<0.05) in group II when compared with control (group I). Group III showed higher levels of testosterone (p<0.05) then group II when compared with control. Whereas the serum DHT levels (p<0.05) in group III were lower than the group II when compared with control. In group III there was a significant decrease in serum PSA levels (p<0.05) when compared with control. Administration of diadzein at both the doses showed a significant decrease in serum testosterone and PSA levels group IV (p<0.05) and group V (p<0.05) respectively. Similarly we found a significant decrease in DHT levels in group IV and V (p<0.001). Luteolin administered at both the doses showed a significant decrease in the levels of serum testosterone and DHT levels in
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Group VI and VII (p<0.05) respectively. Similarly the levels of PSA were decreased in luteolin treated group's significantly and dose dependently group VI and group VII (p<0.05). (Figure 1)

![Graph A]

![Graph B]

![Graph C]

**Figure 1: Effect of daidzein and luteolin on serum hormone levels in BPH induced Wistar rats:** A. Serum Testosterone levels; B. Seum DHT levels; BPH treated group (II) and flutamide treatment group (III) resulted in increased in testosterone levels and DHT levels. Treatment of daidzein group (IV & V); luteolin group (VI and VII) showed dose dependent decrease in testosterone and DHT levles; C. Serum PSA levels: Serum PSA levels was elivated in BPH treated group (II) and flutamide treatment group (III) decreased serum PSA levels. Daidzein group (IV & V) and luteolin group (VI and VII) resulted in significant and dose dependendent decrease in serum PSA levels. Values are expressed in means ± S.E.M. (n =8). Significant differences are indicated by ###p < 0.001, #p < 0.05 when compared with control animals (Group I), and ***p < 0.001, *p < 0.05 when compared with BPH induced animals (Group II). +p < 0.05 when compared with control animals (Group I).

3.2 Effect of daidzein and luteolin on inflammatory (IL 1β, IL 6, TNF α, IFN γ) cytokines and anti-inflammatory (IL 10) cytokines.

There was an increase in the levels of IL 1β (p<0.01), IL 6 (p<0.001), TNF α (p<0.01), IFN γ (p<0.01) and decreased levels of IL 10 (p<0.001) when compared with control (group I). Similarly flutamide treated animals (group III) showed an increase in inflammatory cytokines IL 1β (p<0.05), IL 6 (p<0.001), TNF α (p<0.01), IFN γ (p<0.001) and decreased levels of anti-inflammator
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inflammatory cytokines IL 10 (p<0.001). Daidzein administration resulted in restoration of the cytokines levels to normal significantly and dose dependently, group IV and group V IL 1 β (p<0.001), IL 6 (p<0.001), TNF α (p<0.001), IFN γ (p<0.001), and IL 10 (p<0.001) respectively. Luteolin administration also resulted in restoration of the cytokines levels to normal significantly and dose dependently group VI, IL 1 β (p<0.01), IL 6 (p<0.001), TNF α (p<0.01), and IL 10 (p<0.01) respectively. Higher dose of luteolin (group VII) showed better restoration of cytokine levels to normal when compared to control, IL 1 β (p<0.001), IL 6 (p<0.001), TNF α (p<0.001), and IL 10 (p<0.001) respectively. Only higher dose of luteolin (group VII) showed a significant decrease IFN γ (p<0.01) respectively. (Figure 2)

Figure 2: Effect of daidzein and luteolin on serum cytokine levels: There was an increase in inflammatory cytokines, decrease in anti-inflammatory cytokines in both BPH induced groups II and flutamide treated group III. Daidzein group (III & IV) and luteolin (group V & VI) administration restored the cytokine levels to normal. But lower dose of luteolin did not show any significant reduction in IFNγ levels. Values are expressed in means ± S.E.M. (n =8). Significant differences are indicated by #p < 0.05, ##p < 0.01, ###p < 0.001 when compared with control animals (Group I), and *p < 0.05, **p < 0.01, ***p < 0.001 when compared with BPH induced animals (Group II). +p < 0.05, ++p < 0.001, +++p < 0.001 when compared with control animals (Group I).
3.3 Effect of daidzein and luteolin on SOD, catalase and glutathione peroxidase levels in BPH induced Wistar rats.

BPH induced group II showed decreased activity of SOD, Catalase and GPX when compared to control \((p<0.001)\). Daidzein at both the doses (group IV and group V) and luteolin (group VI and group VII) administration restored the levels of SOD, Catalase and GPX induced by BPH to normal \((p<0.001)\) significantly and dose dependently. (Figure 3)

3.4 Effect of daidzein and luteolin on GSH, GR and LPO levels in BPH induced Wistar rats.

GSH, lipid peroxidation and GR were ased to study the effect of daidzein and luteolin on induction of BPH. BPH induced group II showed decreased GSH levels, GR activity and increase in lipid peroxidation when compared to control \((p<0.001)\). Daidzein at both the doses (group IV and group V) and luteolin (group VI and group VII) administration restored the levels of GSH, GR and LPO levels to normal \((p<0.001)\) significantly and dose dependently. (Figure 4)

3.5 Effect of daidzein and luteolin on XO activity and G6PD levels

BPH induced rats showed an increase in activity of XO and depleted the levels of G6PD when compared to control group (II) \((p<0.001)\). Flutamide (Group III) administration also alleviated XO \((p<0.05)\) activity and decreased the levels of G6PD \((p<0.001)\). Daidzein administration at both the dosed [group IV \((p<0.05)\) and group V \((p<0.01)\)] and luteolin [group VI \((p<0.05)\) and group VII \((p<0.01)\)] dose dependently restored XO activity to normal. There was a significant increase in the levels of G6PD by administration of Daidzein [group IV \((p<0.05)\) and group V \((p<0.01)\)] and luteolin [group VI \((p<0.05)\) and group VII \((p<0.001)\)] (Figure 5).
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Figure 3: Effect of daidzein and luteolin on antioxidant levels in BPH induced Wistar rats:
A. SOD levels ; B. Catalase levels ; C. Glutathione Peroxidase levels : BPH induced group II and flutamide treated group III depleted the SOD, Catalase, GPx activity and daidzein (group IV & V); luteolin (group VI & VI) restored the activity significantly to normal. Values are expressed in means ± S.E.M. (n=8). Significant differences are indicated by #p < 0.001 when compared with control animals (Group I), and *p < 0.001 when compared with BPH induced animals (Group II), +p < 0.001 when compared with control animals (Group I).
Figure 4: Effect of daidzein and luteolin on antioxidant levels in BPH induced Wistar rats:
A. GR;  B. GSH levels; BPH induced group II and flutamide treated group III depleted the GR, GSH levels and daidzein (group IV & V); luteolin (group VI & VII) restored the significantly the levels to normal. C. LPO: Daidzein group (IV & V), luteolin group (VI & VII) significantly restored the lipid peroxidation induced by BPH induction to normal. Values are expressed in means ± S.E.M. (n =8). Significant differences are indicated by #p < 0.001 when compared with control animals (Group I), and *p < 0.001 when compared with BPH induced animals (Group II). +p < 0.001 when compared with control animals (Group I).
3.6 Effect of daidzein and luteolin on NF-kB, iNOS, COX-2, and Ki-67 protein expression

Effect of inflammation was studied in terms of the expression of NF-kB, iNOS, COX-2, and Ki-67. Testostreone treatment resulted in increased cell proliferation which was measured in terms of Ki-67 expression and resulted in increased expression of NF-kB, iNOS, and COX-2 positive cells. Flutamide administration led to decreased in cell proliferation (Ki-67 expression) but there was an alleviated expression of NF-kB, iNOS, and COX-2 positive cells. Administration of daidzein and luteolin resulted in decreased prostatic hyperplasia (Ki-67 expression) and NF-kB, iNOS, and COX-2 expression dose dependently. (Figure 6)
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COX-2  iNOS  NFκB

I

II

III

IV

V

VI

VII
Figure 6: Effect of daidzein on COX-2, iNOS and NFκB expression in BPH induced Wistar rats. In order to study the role of inflammation in testosterone propionate induced BPH in wistar rats we checked the expression of COX-2, iNOS and NF-kB by IHC. There was a marked increase in the expression of COX-2, iNOS and NF-kB expression in BHT induced group II rats and flutamide treatment (group III) also resulted in the ellivation of COX-2, iNOS and NF-kB expression. Daidzein treatment (group IV & V) and luteolin (group VI & VII) decreased the expression of COX-2, iNOS and NFκB expression. (Positive stained cells and negative stained cells 40X)

3.7 Effect of daidzein and luteolin on Ki-67 and histopathological alterations.

There was an increase in cell proliferation seen by the alleviated expression in ki-67 positive stained cells and also evident in the prostate secretory cell hyperplasia in H&E stained cell when compared to control animals (group II). Flutamide treated group III also showed ki-67 expression but there was no hyperplasia in the secretory cells (H&E staining). Daidzein administration resulted in decreased in expression of Ki-67 stained cells dose dependently. Only higher dose of luteolin was better able to attenuate the expression of Ki-67 in prostate cells. The photomicrographs of H&E staining showed that, testosterone propionate (group II) treated rats resulted in prostate hyperplasia. Flutamide administration drastically reduced the prostate hyperplasia almost to normal. Higher doses of both the drugs daidzein (group IV) and luteolin (group VI) showed restoration of prostatic hyperplasia to normal. Daidzein higher dose proved to be more effective in retarding the prostate hyperplasia. (Figure 7)
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Ki-67 vs H&E

I

II

III

IV

V

VI

VII
Figure 7: Effect of daidzein on Ki-67 expression and histopathology changes in BPH induced Wistar rats: To study the prostatic hyperplasia we checked the expression of a proliferation marker Ki-67. The expression of Ki-67 was greatly increased in BPH induced group II. Antiandrogen (flutamide) treated group III showed less intensity of Ki-67 positive stained cells. There was a marked decrease in Ki-67 expression in both doses of daidzein treatments (group IV and V) and luteolin treatments (group VI and VII). Histopathology clearly revealed that the testosterone propionate treated group II resulted in a prostate hyperplasia (increase in number of secretory cells). Flutamide (group III) an antiandrogen drug restricted the cell proliferation of prostate secretory cells to a greater extent. There was a greater decrease in the prostate hyperplasia in higher dose of daidzein (group V) when compared with that of lower dose (group IV) and luteolin treatment resulted in restoration of hyperplasia to normal dosed dependently (group VI and VII). (Ki-67 Positive stained cells and negative stained cells (40X))
3.8 Effect of daidzein and luteolin on mast cell infiltration.

Toluidine blue staining was used to visualize the inflammation cells in prostate interims of mast cell infiltration. Testosterone propionate treatment (group II) resulted in increased mast cell (violet cells), flutamide (group III) also showed mast cells infiltration but they were less when compared to group II. Administration of daidzein and luteolin showed decreased in mast cell dose dependently. (Mast cells) (Figure 8)

Figure 8: Effect of daidzein and luteolin on mast cell infiltration in BPH induced Wistar rats:
There was an increase in mast cell infiltration in BPH (group II) and flutamide (group III) treated group. Daidzein and luteolin both the compounds showed a decrease in mast cell infiltration. This led to the decreased expression of inflammatory cytokines, retarding the hyperplasia of the prostate. (40X)
4. Discussion

BPH is the most frequently occurring disease in majority of the population of old individuals. With increase in age there is a gradual decline in the level of androgen. Researchers have reported that in BPH patients there is an increase in serum testosterone and DHT and its levels are directly proportional to the size of the prostate (Partin et al., 1991). BPH frequently occur concurrently with prostatitis, as shown by the finding of histological lesions indicating prostatitis associated with BPH in 40–90% of all cases (Soler Soler 1999, Bedalov 1994).

We have found an increase in level of inflammatory cytokines (IL 1β, IL 6, TNF α and IFN γ) and decreased expression of IL 10 in BPH rats. Luteolin inhibited TNF α, IL-6 and IL-1β secretion induced in BPH rats (Comalada et al., 2006). Luteolin inhibited the mast cells and reduced the activation of iNOS and COX-2. Our results are in accordance with Monika et al. (2010) who reported that luteolin inhibits COX 2, TNF α, IL-6 and IL-1β secretions. Daidzein also inhibited the secretion of inflammatory cytokines IL 1β, IL 6, TNF α, IFN γ and prevented the activation of NFκB. On the other hands, studies in our laboratory also revealed that attenuation in the COX-2, TNF α, IL-6, IL-1β, iNOS expression and inhibition of NF-κB activation by soy isoflavones (khan et al., 2012), similar reports were published by Mari (2007) (Mari et al., 2007).

BPH induction resulted in increase in mast cell infiltration. This massive infiltration of inflammatory mast cells resulted in increased expressions of iNOS and COX-2 via poly (ADP ribose) polymerase 2 (PARP2) activation (Vannacci et al., 2004). Daidzein retarded the induction of iNOS and activation of COX-2 (Hämäläinen et al., 2007 and khan et al., 2012). Immigration of T cells into the area is attracted by increased production of pro-inflammatory cytokines such as IL-6, IL-8 and IL-15 (Lee and Peehl 2004, Handisurya et al., 2001). In addition to infiltrates, this may also upregulate a set of pro-inflammatory cytokines in BPH tissue, particularly IL-15 in stromal cells (Handisurya et al., 2001), IL-17 in infiltrating T-cells (Steiner et al., 2003), IFN γ in basal and stromal cell (Royuela et al., 2000) and IL-8 in epithelial cells (Giri and Ittmann, 2001).

In another report by Penna (2009), human prostate stromal cells act as antigen presenting cells, activating allo-antigen specific CD4+ T cells to produce IFN γ and IL-17 (Penna et al., 2009). Secondly the pro-inflammatory cytokines released from adjacent inflammatory cells induced the expression of COX-2 in epithelial cells, which then elevated the proliferation rate of cells in the prostate. Kramer reported that, 79% of patients with BPH has increased IL-17 expression,
activated T-cells leading to the induction of COX 2 expression (Wang et al., 2008, Kramer et al., 2006).

The upregulation of COX-2, an inducible isoform of COX that converts arachidonic acid to pro-inflammatory prostaglandins, has been noted in macrophages and in epithelial cells in BPH tissue (Wang et al., 2004). The inflammatory cells that arrive in the prostate, the inducible iNOS is the principal factor activating reactive nitrogens that can damage cells (Baltaci et al., 2001). Gradini (1999) characterized NOS expression in human prostate tissue and, particularly for iNOS, they found an increased immunostaining in the epithelial cells of BPH patience (Gradini et al., 1999). COX-2 activity has been detected in all inflammatory cells in the epithelium and interstitial spaces of human prostate tissue and increased in proliferative inflammatory lesions, generating pro-inflammatory prostaglandins (Palapattu et al., 2004, Sugar 2006). In human BPH tissue, Di Silverio (2005) showed that COX-2 inhibition can produce a significant increase in prostate cell apoptotic activity.

We found a decrease in serum hormone levels (testosterone and DHT) by Daidzein and luteolin administration. It has already been reported that increased levels of Testosterone and DHT are responsible for increased cell proliferation (Arnold et al., 2005). The anti-proliferative effect of daidzein and luteolin may be attributed to decreased levels of serum testosterone and DHT. PSA is also a marker of cell proliferation and in our study we found that supplementation of daidzein and luteolin resulted in decreased levels of serum PSA which further supports the antiproliferative effects of daidzein and luteolin (Roehrborn et al., 1999).

Chronic inflammation has been demonstrated to be a source of oxidative stress that leads to tissue injury in infiltrated areas (Victor et al., 2004). Daidzein and luteolin restored depleted levels of SOD, Catalase, glutathione, glutathione dependent enzymes and increased lipid peroxidation in BPH induced rats. Macrophages produce reactive oxygen species (ROS) such as superoxide anion (O2-) and hydrogen peroxide (H2O2) by activation of nicotinamide adenine dinucleotide phosphate-reduced (NADPH) oxidase (Forman and Torres, 2002). This increased oxidative stress has also been monitored in prostatic patients with chronic prostatitis (Shahed and Shoskes, 2000). Local hypoxia can play a role as one of the inflammatory mediators by inducing lower levels of reactive oxygen species, which can promote neovascularization (Wang et al., 2008).
Data show that chronic inflammation can induce proliferative events and posttranslational DNA modifications in prostate tissue through oxidative stress (Naber and Weidner 2000). In fact, repeated tissue damage and oxidative stress related to this event may provoke a compensatory cellular proliferation with the risk of hyperplastic growth or also of neoplastic modifications (Palapattu et al., 2004, Sugar 2006). It is well accepted that regions of prostatic inflammation can generate free radicals, such as nitric oxide (NO) and various species of oxygen. In particular, macrophages and neutrophil infiltrations provide a source of free radicals that can induce hyperplastic or precancerous transformations through the oxidative stress to the tissue and DNA (Palapattu et al., 2004). A feature of these oxidative stress reactions is the production of arachidonic acid from membranes, a process associated with the generation of new reactive oxygen radicals (Palapattu et al., 2004). It can also be converted by the COX enzymes to various eicosanoids, in particular, prostaglandins that have long been recognized as important factors in the regulation of prostate cell proliferation (Palapattu et al., 2004). Many researchers have reported that luteolin as a potent antioxidant, alleviated the antioxidant enzymes. Luteolin and daidzein restored the BPH induced antioxidant depletion (Manju et al., 2005, Samy et al., 2006). Jin (2010) and our laboratory reported the free radical scavenging and restoring the antioxidant status of the cell (Jin et al., 2010, khan et al., 2012). We propose that BPH is an age and inflammation associated disease, therefore, addition of some anti-inflammatory agents in diet may prolong the onset of the disease.

Daidzein and luteolin proved to be good anti-inflammatory and anti-proliferative agents. Use of these agents as dietary supplements may help in better management of patients suffering from BPH and may lead to delayed onset of BPH.