CHAPTER -3

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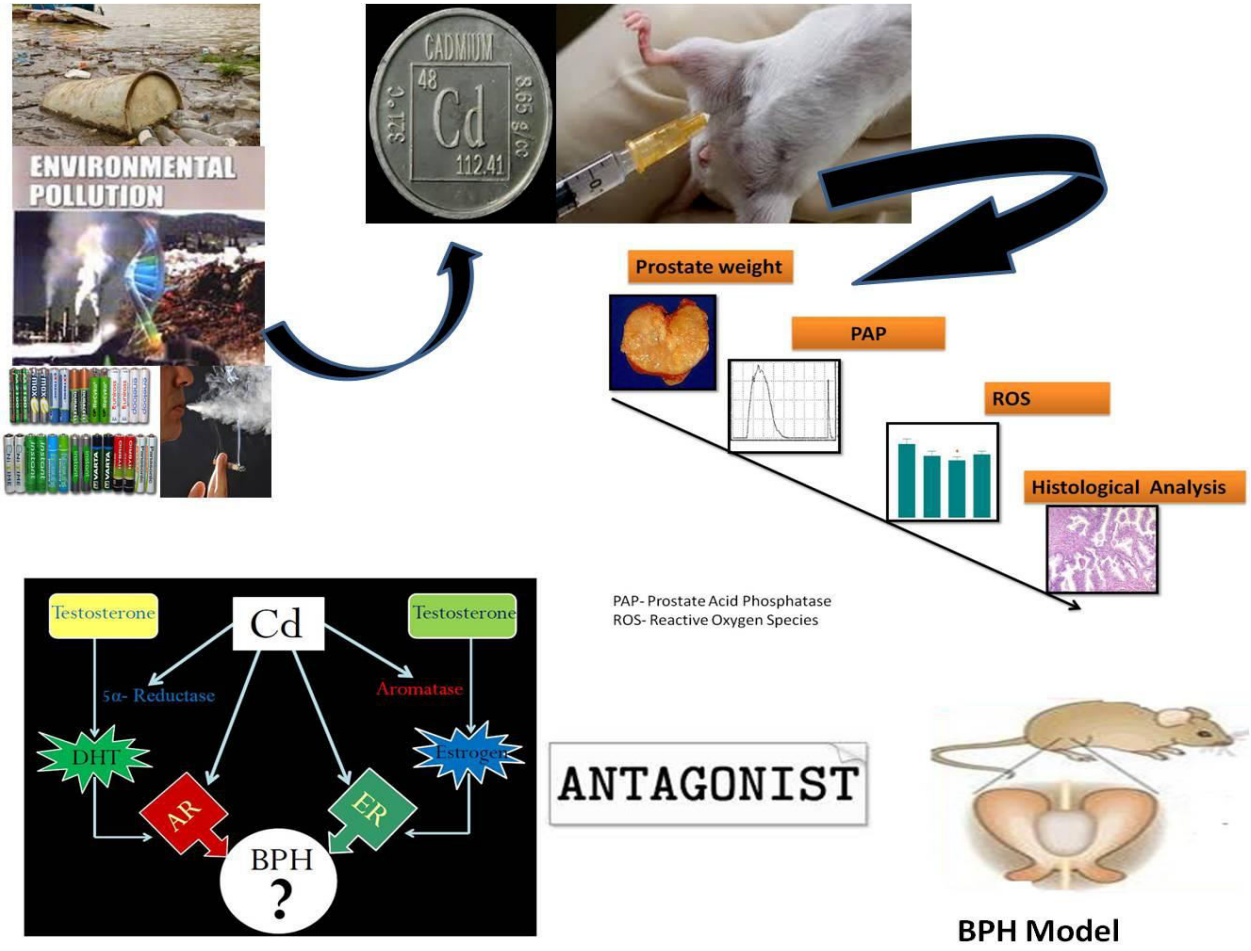
Establishment of BPH rat model and validation of progression of diseased condition in *in-vivo* model.



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A single low dose of cadmium exposure induces benign prostate hyperplasia like condition in rat: A novel benign prostate hyperplasia rodent model

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3.1 Introduction

Benign prostate hyperplasia (BPH) is a common disease of old age. It has a high public health impact and is one of the most common reasons for surgical intervention among elderly men. Anatomic or microscopic evidence of BPH is present at autopsy in approximately 55% of men aged between 60-70 years (Ziada et al. 1999). The initiating event in the development of BPH is not known; however, the nodules almost certainly result from a stromal–epithelial interaction (Cunha 2008). Despite the enormous burden of BPH on public health, its pathogenesis is incompletely understood. Hyperplastic growth in BPH has been ascribed to an imbalance between androgen/ estrogen signaling (Coffey and Walsh 1990), tissue remodeling in the aging prostate (Untergasser et al. 2005), chronic inflammation (Kramer et al. 2007), stem cell defects (Lin et al. 2007), over-expression of stromal and epithelial growth factors (Lucia and Lambert 2008), hypoxia (Berger et al. 2003), epithelial– mesenchymal transition (Alonso-Magdalena et al. 2009) and other obscure factors.

Many attempts have been made during the last decade to obtain a thorough understanding of the BPH pathogenesis. In spite of this, the etiology and pathophysiology of the disease remains unclear because of the lack of suitable animal models. Transurethral resection has been the treatment of choice from the last decade. Recently less invasive therapies such as laser prostatectomy (Chertin et al. 1999), thermotherapy (Djavan et al. 1999; Thalmann et al. 1999), and complementary medications (Cooper et al. 1999) have been introduced. Nonsurgical methods and laser treatments have been satisfactory and cost effective therapeutic options for patients. As a consequence, human BPH tissue would be unavailable for future studies. Thus, it is inevitable to develop an animal model in order to unravel the disease pathogenesis.

Spontaneous BPH is rare in species other than human. It has only been described in dogs and chimpanzees (Steiner et al. 1999). Alternatives to the study of spontaneous disease in other species are induced BPH by gene knockdown, xenograft, hormone-induced and *in vitro* models (Mahapokai et al. 2000; Oudot et al. 2012; Rick et al. 2013). A variety of growth factors, steroid hormones and proteases are involved in normal prostatic morphogenesis and function, however, their role in BPH and Prostate cancer (PCa) is poorly understood. The development of BPH in men is commonly attributed to testicular hormones and aging. The principal androgen responsible for prostate development is dihydrotestosterone (DHT). Testosterone gets converted into DHT by prostate specific enzyme 5α-reductase. 5α-Reductase occurs as 3 isoenzymes, type-

1 isoenzyme predominantly expressed in the liver and skin whereas type-2 and 3 expressed in the prostate (Steers 2001; Azzouni et al. 2012). DHT has a very high binding affinity to androgen receptors. Studies showed that hyperplasic areas usually have higher concentrations of androgen receptors as compared to normal areas (Barrack et al. 1983) with altered testosterone to DHT ratio, one of the major factor for the cause of BPH (Carson and Rittmaster 2003).

The mechanism of prostate growth is complex and heterogeneous in different species, and the testosterone-induced models of BPH show an epithelial hyperplasia (Scolnik et al. 1994). Alonso-Magdalena et al. 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). Human BPH as predominantly of epithelial origin supports the use of an androgen-induced model of BPH with predominant epithelial hyperplasia

Over all maintenance of prostate is dependent on androgens, whose withdrawal through castration demonstrates regression of the prostate gland (Coffey and Isaacs 1981). Rat and mice prostates have been documented to respond to hormone and chemical carcinogen treatment. However, only the dorso-lateral lobe of the rodent prostate is ontogenetically comparable to the human prostate. There are several factors responsible for disease pathogenesis including environmental pollutant and endocrine disruptors like pesticides, cadmium and other heavy metals. Studies suggest a potential role of cadmium (Cd) in the prostate enlargement due to androgenic and estrogenic mimicking activity (Vinceti et al. 2007); (Johnson et al. 2003). The metal binds with high affinity to the hormone-binding domain of steroid hormone receptors and activates the receptors (Stoica et al. 2000).

To prove our hypothesis that low dose of Cd exposure induce hyperplasia like condition in rodents, we performed an experiment with different age group of Charles foster rat. A significant increase in prostate weight with characteristic histological features in five month old animals treated with single dose of 20 µg Cd /kg body weight developed BPH like condition within ten days. Cd exposure induces cell proliferation, depicted by increased prostate weight. Moreover, histological studies suggest the present condition is BPH, since the ductal morphology is maintained unlike in prostate cancer where unorganized growth in the cells is observed. Also presence of basal cells, a characteristic of BPH further strengthens the cadmium induced BPH rat model. This is the first of its kind Cd induced BPH animal model, which is, cost effective and less time consuming compared to other available models.

Cadmium is well known endocrine disruptor. In our above study we used Cd to establish BPH like condition in rodent but the molecular mechanism behind BPH induction was not known. In this context, we further aimed to find out whether cadmium binds to steroid hormone receptors and modulate the downstream signals which eventually lead to cell proliferation and BPH like condition in rat model. To reveal the precise role of Cd an experiment was performed with steroid hormone receptor antagonist in Cd induced BPH rats. The steroid hormone receptor antagonist study suggested Cd induced hyperplasia like condition by modulating steroid hormone receptor action in rats.

3.2 Materials and Methods:

3.2.1 Chemicals:

All primary mouse monoclonal antibodies and antagonists were purchased from Sigma-Aldrich, USA (Anti-Androgen Receptor cat no A9853, Anti-Vimentin cat no. C9080, Anti Ki-67 cat no. P6834, Nilutamide cat no. N8534, MPP cat no. M7068 and 4-Hydroxytamoxifen cat no. H7904) and Anti-E-Cadherin cat no.610181 from BD Biosciences, USA. RT-PCR reagents were procured from Fermentas, Germany. Cadmium acetate and sodium acetate were obtained from SISCO Pvt. Ltd. Research Laboratories, India. All the chemicals were extra pure and of analytical grade**.**

3.2.2 Animals:

Healthy adult male Charles foster rats, weighing about 250–350 g of age 5 months and 1 year were used. The animals were housed in clean polypropylene cages and kept in an air-conditioned animal house with constant 12-h light/dark cycle. Rats were allowed free access to drinking water throughout the experimental period. The animals were fed with standard rat pellet diet (Lipton India Ltd., Mumbai, India). The experiments were approved by the Institute Animal Ethical Committee (CPCSEA Reg. No. 938/a/06/CPCSEA).

3.2.3 Time-dependent Study:

To determine the optimum time of BPH development, a single intraperitoneal *(i.p)* dose of 20µg/kg body weight Cd administered (the dose used here was equivalent to the daily exposure of metal from food and drinking water as per the literature.) (Gartrell et al. 1986a; Gartrell et al. 1986b). Animals were divided into six groups, each group contained six rats. Animals were administered with a single cadmium acetate dose by *i.p.* injection with their respective controls.

Rats were sacrificed after 10^{th}, 20^{th} and 30^{th} days of experimental regime and prostate glands were surgically removed.

3.2.4 Age dependent study:

BPH is a progressive disorder of old age males. To further explore the effect of Cd, one year and five months old animals were treated with a single *i.p* dose of Cd 20µg/kg body weight along with control animals. All lobes of the prostate gland were dissected out from each group on the tenth day after Cd administration for analysis and evaluation of disease conditions.

3.2.5 Castration study:

Prostate growth is primarily dependent on androgens. The heavy metal cadmium has an androgen mimicking activity. In the present study, five months old animals were surgically castrated. After a 7-day recovery, the animals received a single *i.p* injection of Cd (20ug/kg body weight) whereas, control animals were treated with sodium acetate. After 10 days prostate gland was surgically removed to examine the androgenic activity of Cd.

3.2.6 Prostatic acid Phosphatase Analysis:

Measurement of serum PAP level indicates prostatic cell growth. Hence, blood was collected and PAP activity was estimated in serum by hydrolysed phenol method. PAP converts p-Nitrophenyl Phosphate (PNPP) into p-Nitrophenol (PNP) which can be measured at 405 nm (Bowers and McComb 1975). The addition of tartrate in the sample will lead to inhibition of prostatic acid phosphatase and by subtracting it from the total activity (without tartrate) will give the prostatic acid phosphatase activity.

3.2.7 ROS parameters:

Reactive oxygen species (ROS) are known to be the mediators of phenotypic and genotypic changes that lead to neoplasia. Hence, it is important to investigate the role of Cd in the production of ROS and its action as a potent carcinogen. For determination of ROS in Cd induced BPH like condition, prostate tissue was evaluated for reduced Glutathione (GSH) content measured by the method of Beutler and Gelbart (1985) (Beutler and Gelbart 1985). Reduced glutathione reacts with 5-5' Dithiobis (2-nitrobenzoic) acid (DTNB) to yield a yellow color, which can be measured at 412 nm. Lipid peroxidation (LPO) estimated by the method of Ohkawa et al., 1979. Lipid peroxidation leads to the formation of an endoperoxide and gives

thiobarbituric reactive substance (TBARS), which can be measured at 532 nm (Ohkawa et al. 1979).

3.2.8 Histological examination:

Prostate glands were fixed in 10% buffered formalin solution, 3µm thick tissue sections were cut and stained with hematoxylin/eosin stain. Histological observations such as number of acini and mitotic figures were quantified per 40 X objective microscopic field *(Table 3.1),* epithelial cell invaginations and basement membrane integrity were examined by Nikon TES2000 microscope (Nikon, Japan) using 20 X objective. Histology of BPH samples were evaluated by a surgical pathologist.

	Age of Animal	Control	Cd Treated(20µg/KBW)
Number of Mitotic figures/ microscopic field	1 year old animals	6.1±0.12	10.05±0.29**
	5 months old animals	5.6±0.5	10.70±0.62***
Number of Acini/ microscopic field	1 year old animals	18.9±0.54	23.2±1**
	5 months old animals	19.1±1.15	25.6±0.97***

Table 3. 1: *Histological analysis of Cd treated rat prostate gland: All values are presented as mean of six animals ± SEM, **p<0.01, ***p<0.001*

3.2.9 Antagonist studies:

The aim of this study was to determine the molecular mechanism of BPH progression due to Cd, using the steroid hormone receptor antagonist in Cd induced BPH rats. Animals were divided into nine groups and administered with a different steroid hormone receptor antagonist along with Cd (20µg/kg body weight). AR antagonist nilutamide :10mg/kg/day *i.p* (Horsmans et al. 1991; Huang et al. 2008), ERα antagonist methyl piperidine pyrazole: 50ug/kg body weight/day *i.p* (Davis et al. 2008) and ERβ antagonist 4-hydroxytamoxifen :1mg/kg/day administered sub-cutaneously (Reed et al. 2005) everyday till 10 days (required time period for BPH development) as per available literatures. Animals were sacrificed after 10^{th} day. The weight of the dissected prostate was noted and the tissues were subjected to histological examination, biochemical analysis, gene expression and immuno-histochemistry studies.

3.2.10 Relative gene expression studies:

Total RNA was isolated from freshly removed complete prostate gland and re-suspended in RNA stabilizing solution procured from Amresco laboratories. RNA samples (n=3) were quantified by spectrophotometer at 260 nm. Complementary DNA (cDNA) was synthesised by reverse transcriptase (RT) using 1µg RNA (Fermentas First stand cDNA synthesis kit). After

reverse transcription, cDNA samples were amplified by RT-PCR using gene specific primers for AR, ERα, ERβ and 5 α reductase (type -2) genes. GAPDH was used as an endogenous control. (*Table3.2*). Reactions were carried out in an Eppendorf Gradient PCR. The PCR products were electrophoresed on an ethidium bromide stained 2% agarose gel in TAE buffer. Gels were photographed by Gel documentation unit from UVITEC Cambridge alliance 4.7 and densitometrical analysis were carried out using Image J software.

Gene Name	Primer Sequence	Annealing Temperature	Product size
Estrogen Receptor-α (ERα)NM_012689.1	Fw: 5'CCTTCTAGACCCTTCAGTGAAGCC-3' Rv: 5'ACATGTCAAAGATCTCCACCATGCC-3'	59.3	287bp
Estrogen Receptor-β (ERβ)NM_012754.1	Fw:5'AAAGCCAAGAGAAACGGTGGGCAT-3' Rv:5'GCCAATCATGTGCACCAGTTCCT-3'	57.7	204bp
Androgen Receptor (AR)NM_012502.1	Fw:5'ATCGAGGAGCGTTCCAGAATCTG-3' Rv:5'ATATGGTCGAATTGCCCCCTAGG-3'	58	630bp
5α-Reductase type-2 NM_022711.4	Fw: 5' ATCCTGTGCTTAGGGAAAC 3' Rv: 5' CATACGTAAACAAGCCACC 3'	54.5	496bp
GAPDH NM_017008.4	FW: 5'CAAGGTCATCCATGACAACTTTG3' RW: 5'GTCCACCACCCTGTTGCTGTAG 3'	58	496bp

Table 3. 2*: Reverse Transcriptase-PCR Primers Sequence and Annealing Temperature*

3.2.11 Immuno-histochemistry:

Tissue sections (3µm) were deparaffinised and rehydrated using standard protocols and incubated overnight with primary mouse monoclonal antibodies at 4^0C. Sections were then rinsed twice with washing buffer (1:10 dilution of blocking buffer in PBS) followed by 1 hour incubation with secondary antibodies conjugated with FITC and TRITC fluorophores (Sigma Aldrich, USA) in the dark at room temperature. For negative controls, the primary antibodies were omitted. Tissue sections were mounted with mounting medium containing 4'6'-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma Aldrich, USA). The expression of antigens in tissue sections were assessed by immunofluorescence method. Images were captured by confocal microscope LSM710 (Carl Zeiss, Germany) using 63X objective.

3.2.12 Statistical Analysis:

The values were represented as Mean ± SEM at n=6 animals. The values were accepted as significant at $P \leq 0.05$ Newman-keuls post hoc one way ANOVA (analysis of variance) and t-test by using Prism software version 5.0.

3.3 Results:

A positive co-relation between cadmium concentration and severity of BPH disease in Indian human population has been depicted in our previous lab studies (Pandya et al. 2013) supported by other reports (Lee et al. 2009). It has also been reported that cadmium has a potent androgen and estrogen like activity in the prostate gland (Martin et al. 2002). Thus, the goal of the present study was to ascertain whether the metal binds with steroid hormone receptors in the rat prostate, inducing hyperplasia like condition.

3.3.1 Time Dependent Study:

To establish BPH like condition, a single dose of cadmium 20 µg/kg body weight (~108 nmol/kg) was administered (intra-peritoneal) to the 5 months old animals. The dose used was 1/500 of LD50 of the metal, equivalent to the daily exposure from food and drinking water (Gartrell et al. 1986a; Gartrell et al. 1986b). To determine the optimal time of BPH development, a time dependent exposure of Cd (20 µg/kg body weight) was performed for 10, 20 and 30 days *(Fig.3.1)*. Significant increase in prostate weight of animals was observed within 10 days after a single dose of Cd exposure, indicating the development of BPH like condition.

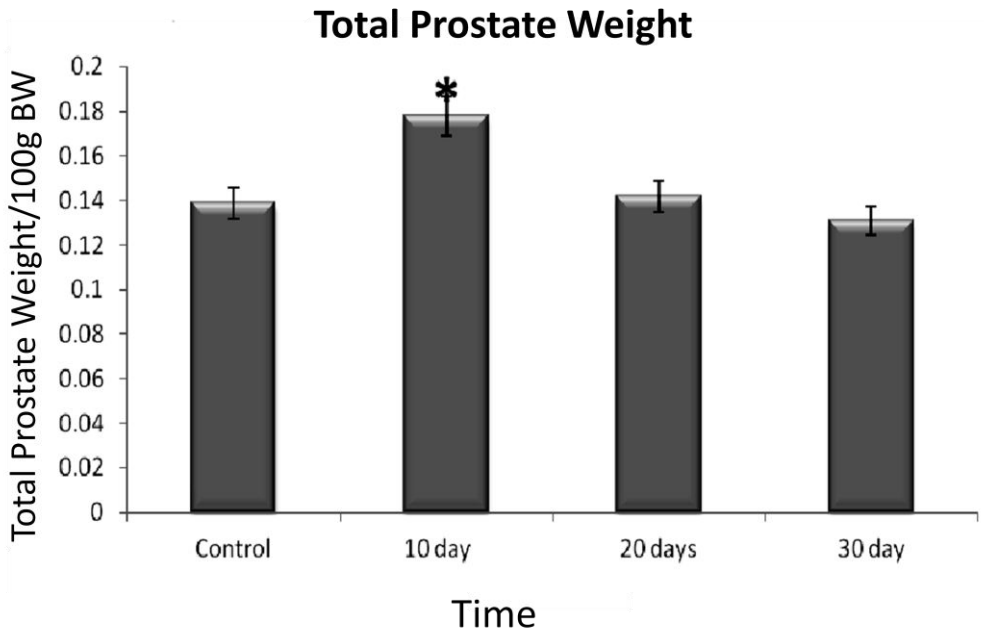

Figure 3. 1*: Time dependent effect of a single dose of cadmium on rat prostate. The results represent the mean of six animals ± SEM, *p<0.05, 20µg/kgBw Cd vs control.*

3.3.2 Age Dependent Study:

As BPH is an age dependent pathology, we further explored the effect of 20µg/kg body weight Cd dose in 1 year aged animals and compared with 5 months old animals. Though 20µg/kg body weight dose of Cd significantly induced prostate weight in both the age groups, the increase was significantly higher in 5 month old Cd treated as compared to the 1 year old animals with their respective controls *(Fig. 3.2)*.

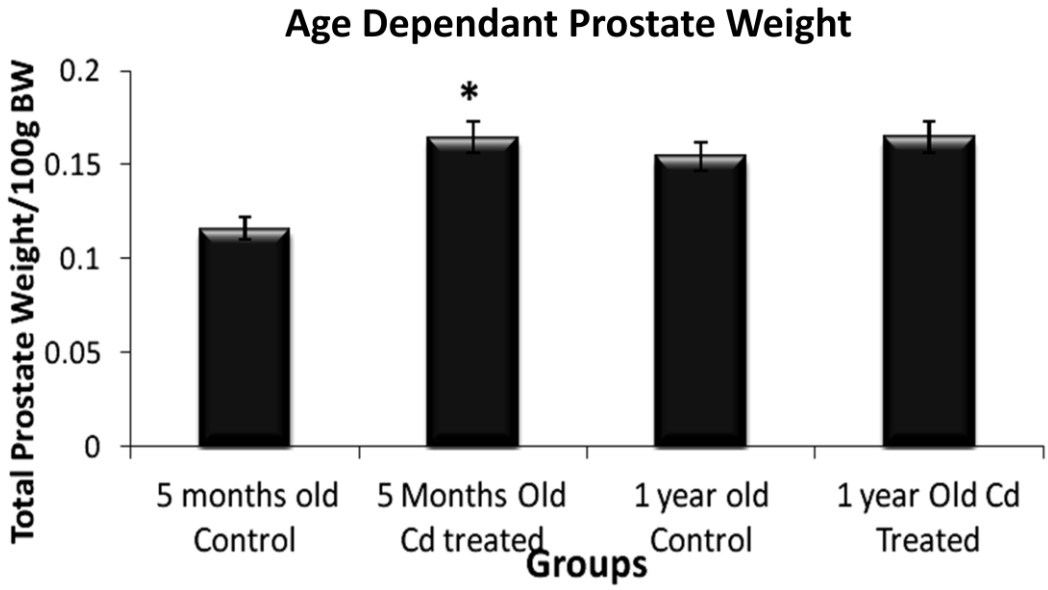

Figure 3. 2*: Age dependent effect of a single dose of cadmium on rat prostate. The results represent the mean of six animals ± SEM, *p<0.05, 20µg /kgBw Cd vs control.*

Oxidative stress has been implicated in pathogenesis of several diseases. Previous studies of our lab and other groups showed Cadmium as an inducer of oxidative stress and potent clinical and biochemical environmental toxicant for BPH pathogenesis (Aryal et al. 2007; Pandya et al. 2013). Cd treated animals demonstrated significant decrease in Glutathione (GSH) and increase in lipid peroxidation (LPO) level *(Fig. 3.3 a, b)* which, further supported our previous results and role of cadmium as an oxidative stress inducer causing BPH like condition.

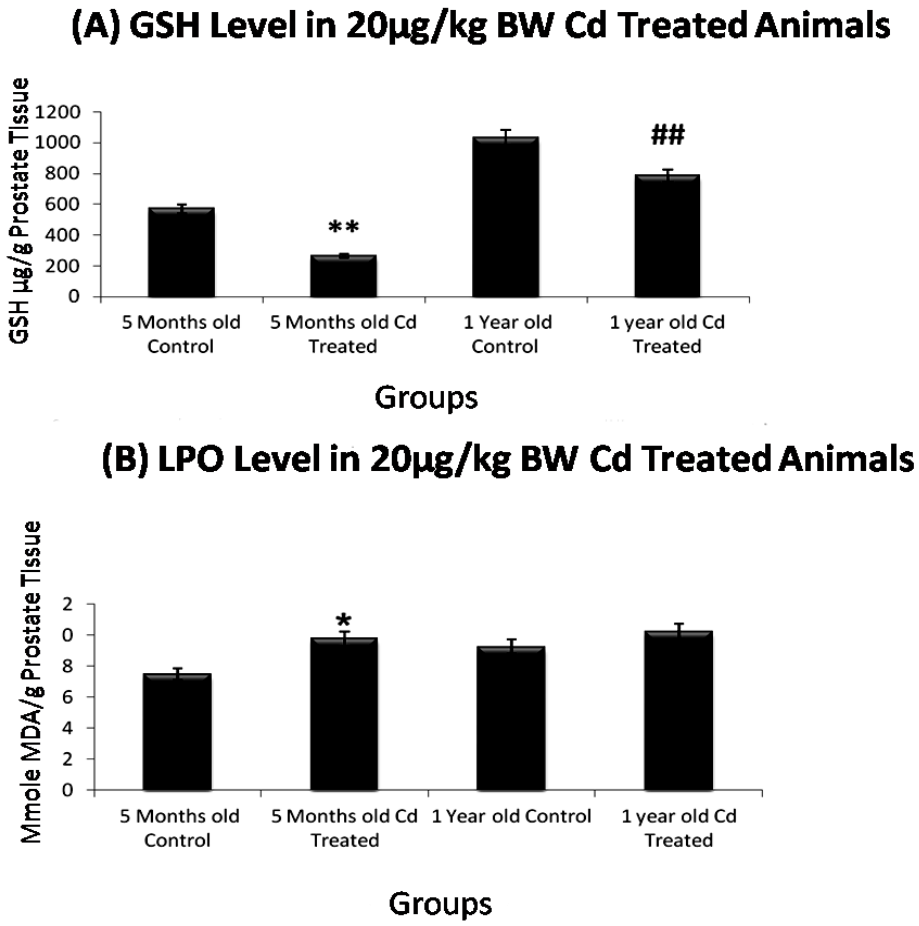

Figure 3. 3*: Age dependent effect of a single dose of cadmium at GSH and LPO level in rat prostate. The results represent the mean of six animals ± SEM, (a) **p<0.01, 5 months old Cd treated vs 5 months old control, ##p<0.01, 1 year old Cd treated vs 1 year old control ,(b) *p<0.05, 5 months old Cd treated vs 5 months old control.*

3.3.3 Confirmation of BPH model by histological studies:

A single 20 µg/kg body weight cadmium dose was selected for the development of BPH rat model in 5 month old animal, which was supported by histological observations. The histology reveals a significant increase in the number of acini and mitotic figures in 5 months old Cd treated group *(Table 3.1)* as compared with 1 year old animal *(Fig 3.4)*. As evident from *fig 3.5* normal prostate is characterized by compound tubular alveolar glands with presence of basement membrane. The cell lining of the duct is columnar to cuboidal with basally located nuclei that are round to oval in shape. The alveolar portions of gland contain primary and secondary infoldings of secretory epithelium that project into the alveolar lumen and the alveoli separated by a delicate fibrous connective tissue stroma with an increased number and irregular acinar growth

pattern. Each of the lobule is larger and has more elaborate branching than in the normal gland. In addition, the size of the secretory epithelial cells is increased principally due to an increase in the amount of cytoplasm. The amount of stroma is relatively less than normal gland, and the basement membrane appears somewhat attenuated. Further, basal cells proliferation was observed by immuno-histochemisty using anti Ki-67 antibody which is a marker of epithelial proliferation *(Fig.3.5).*

3.3.4 Mechanism of cadmium in prostate hyperplasia induction:

To determine whether cadmium mimics the androgenic response in animals, the effects of the metal along with the wet weight of the prostate was tested in 5 month old castrated animals. Results demonstrated statistical difference in an average weight of the prostate *(Fig. 3.6)*. Previous lab data suggests that Cd mimics the action of steroid hormone, however, its action via binding to steroid hormone receptors was not well defined. To answer this question, an experiment was designed where animals were treated with steroid hormone receptor antagonists.

The animals received antagonists namely Nilutamide, Methyl piperidino pyrazole and 4-hydroxytamoxifen till 10 days. First, rats were treated with steroid hormone receptor blocker and then Cd was administered after three hours of AR antagonist and 30 minutes of ER antagonists treatment (Harris et al. 1993; Kim et al. 2010) to block the availability of steroid hormone receptors for Cd. Increase in wet weight of the prostate and prostatic acid phosphatase activity in antagonist treated group were blocked *(Fig. 3.7 a, b)*. Further histological examination of the prostate from antagonists treated group exhibited large and regular acini with no epithelial infolding in AR and ERα antagonists group. Whereas, epithelial infoldings were observed in ERβ antagonist group. *(Fig. 3.8)*

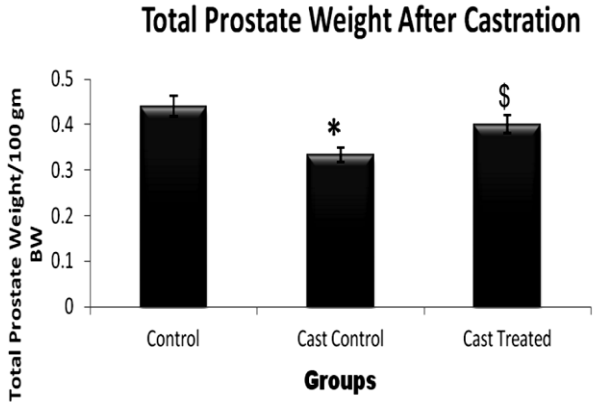

Figure 3. 4*: Effect of a single dose of cadmium on (5 month old) castrated rat prostate. The results represent the mean of six animals ± SEM, *p<0.05, castration control vs control, $ p<0.05, castration treated vs castration control.*

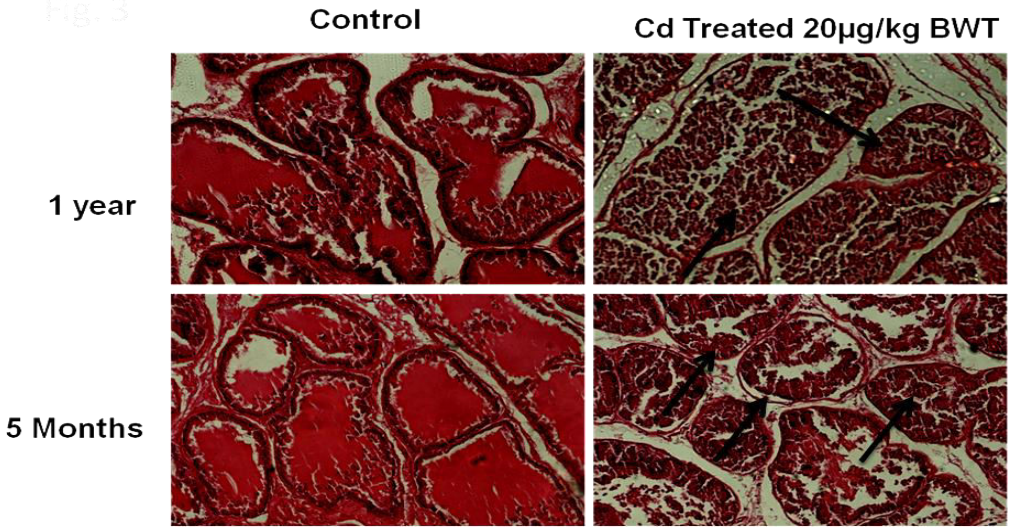

Figure 3. 5*: Age dependent histological changes of a single dose of cadmium in rat prostate. Sections were stained by hematoxylin/eosin staining. Images were captured by light microscope depicting epithelial infolding and acinar growth pattern using 20 x objective.*

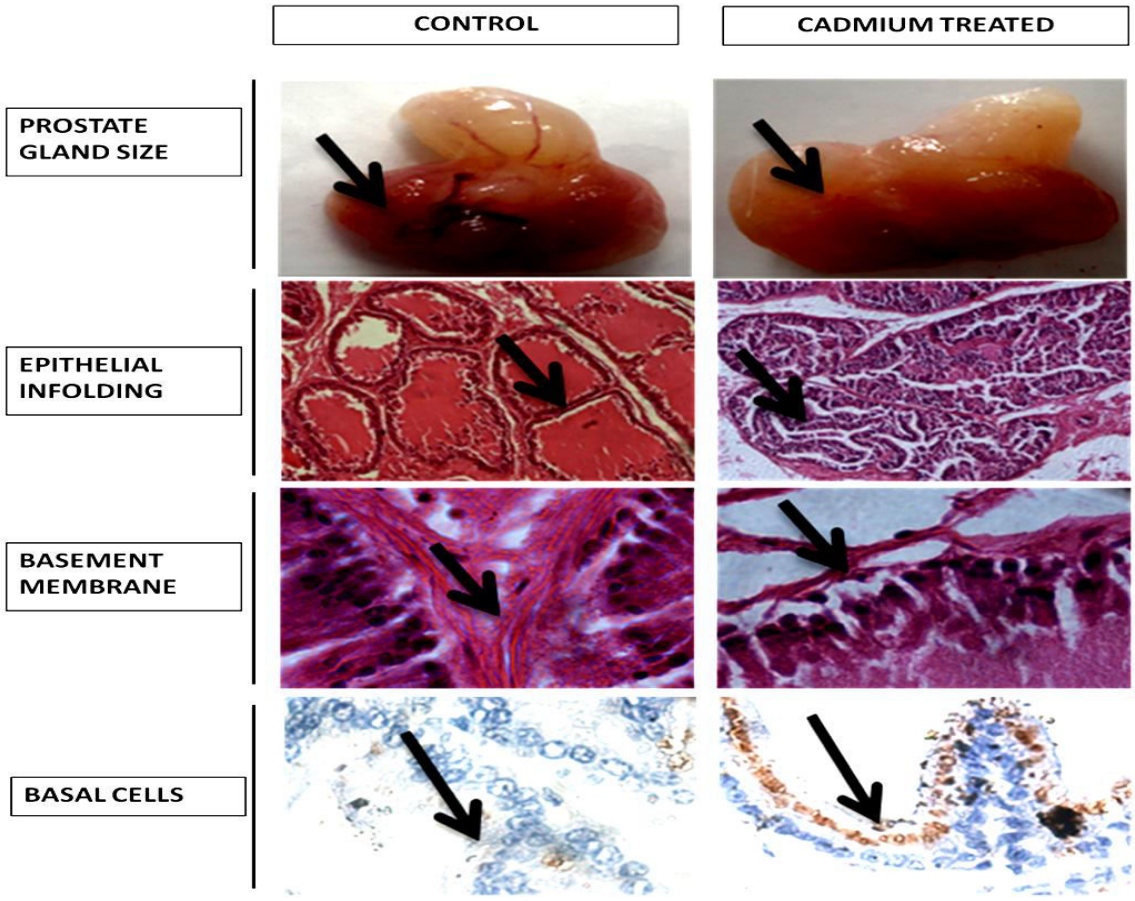

Figure 3. 6: *Histological evaluation of a single dose of cadmium in 5 month old rat prostate. Sections were stained by haematoxylin/eosin staining. Images were captured by light microscope using 20 x objective.*

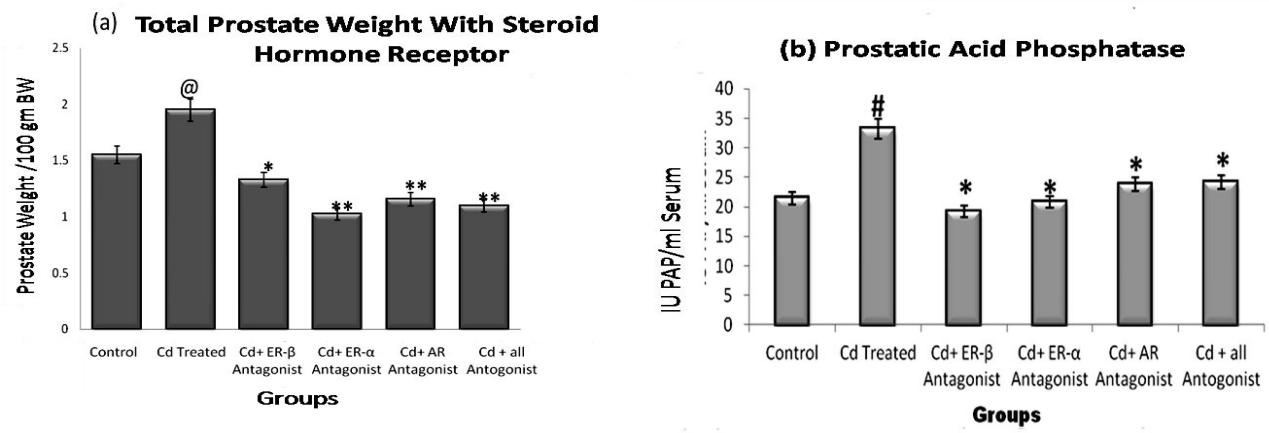

Figure 3. 7: *Effect of a single dose of cadmium along with steroid hormone receptor antagonist on prostate weight and prostatic acid phosphatase activity. The results represent the mean of six animals ± SEM, (a) @p<0.05, Cd treated vs control. @ p<0.05, Cd treated vs control,*p<0.05, Cd treated vs ER- β. **p<0.01, Cd treated vs ER- α, AR and all antagonist. (b) #p<0.05, Cd treated vs control. *p<0.05, cd treated vs ERα,β, AR and all antagonist*

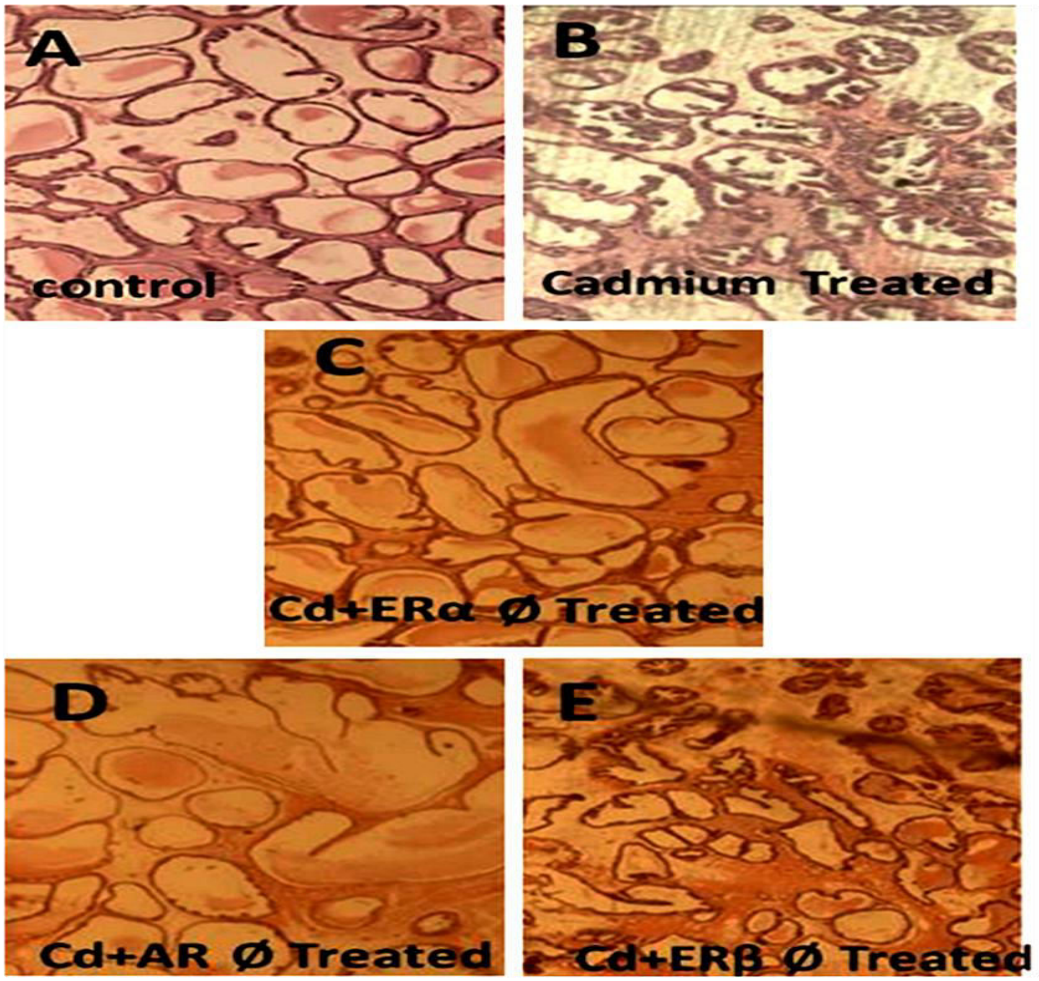

Figure 3. 8: *Histological changes in rat prostate of a single dose of cadmium treated group along with antagonists. Sections were stained by hematoxylin/eosin staining . Images were captured by light microscope showing epithelial infolding and acinar growth pattern using 20 x objective. φ=antagonist*

3.3.5 Relative gene expression and Immunohistochemistry:

The metal has an ability to bind to steroid hormone receptors and stimulate proliferation. To shed light into the gene expressions underlying the response of Cd treatment, steroid hormone receptor antagonist groups along with Cd treated animals were examined for gene expression profile (AR, ERα, ERβ and 5 α reductase type -2) by RT-PCR method *(Fig. 3.9).*

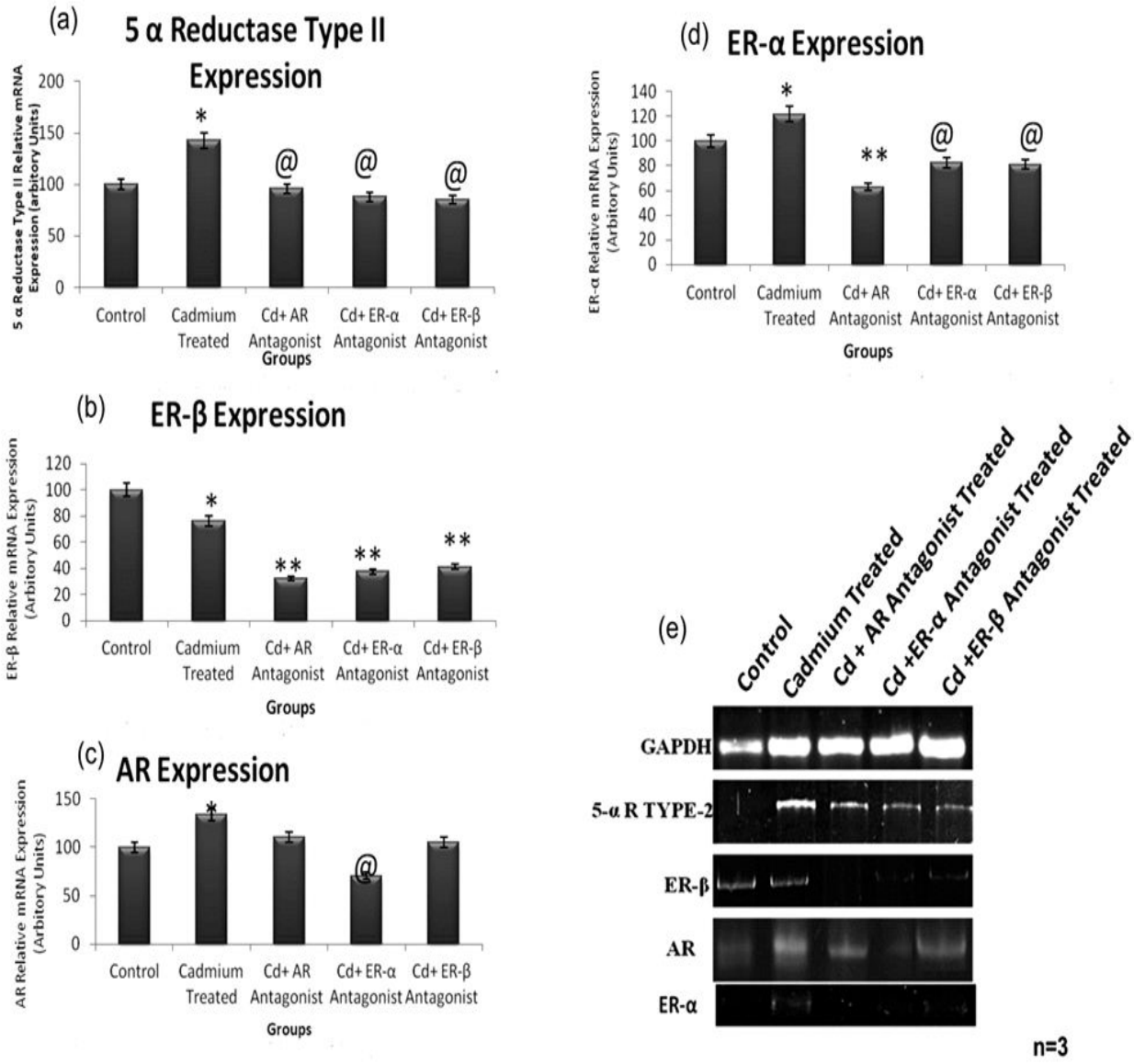

Figure 3. 9*: Effect of a single dose of cadmium on prostatic genes expression profile in presence of antagonists. The results represent the mean of three animals ± SEM, (a) *p<0.05, Cd vs Control, @p<0.05, Cd vs AR,ERα,β-Antagonist. (b) *p<0.05, Cd vs Control, **p<0.01, Cd vs AR,ERα,β-Antagonist .(c) *p<0.05, Cd vs Control, @p<0.05, Cd vs ERα-Antagonist. (d) *p<0.05, Cd vs Control, **p< 0.01 Cd vs AR antagonist, @p<0.05, Cd vs ERα,β-Antagonist.(e) Gel electrophoresis bands.*

The relative expression of 5α Reductase type II, an enzyme responsible for conversion of testosterone into DHT showed significant decreased expressions in all antagonists groups when compared with Cadmium treated group, indicating decreased conversion of testosterone to DHT and hence less proliferation. Cadmium has an ability to transactivate AR. Increased expression of AR and ERα along with decreased expression of ERβ were observed in the cadmium treated group compared with control.

To further substantiate the action of Cd via steroid receptor, immuno-histochemistry was performed. Cadmium treated group exhibited a higher Ki-67 index, which clearly indicated epithelial cell proliferation than in the normal section of the gland, whereas, AR and ERα antagonists group showed no proliferation. Similarly, when the sections were stained with anti AR antibody, Cd treated group showed a significant increase in expression of AR compared to antagonists group *(Fig.3.10)*. ERβ treated group showed Ki-67 and AR positive staining. Weak E-cadherin and abundant expression of vimentin in the Cd treated group were observed and compared with control group which further provided information about EMT (epithelial to mesenchymal transformation) in the BPH pathogenesis *(Fig. 3.11)*.

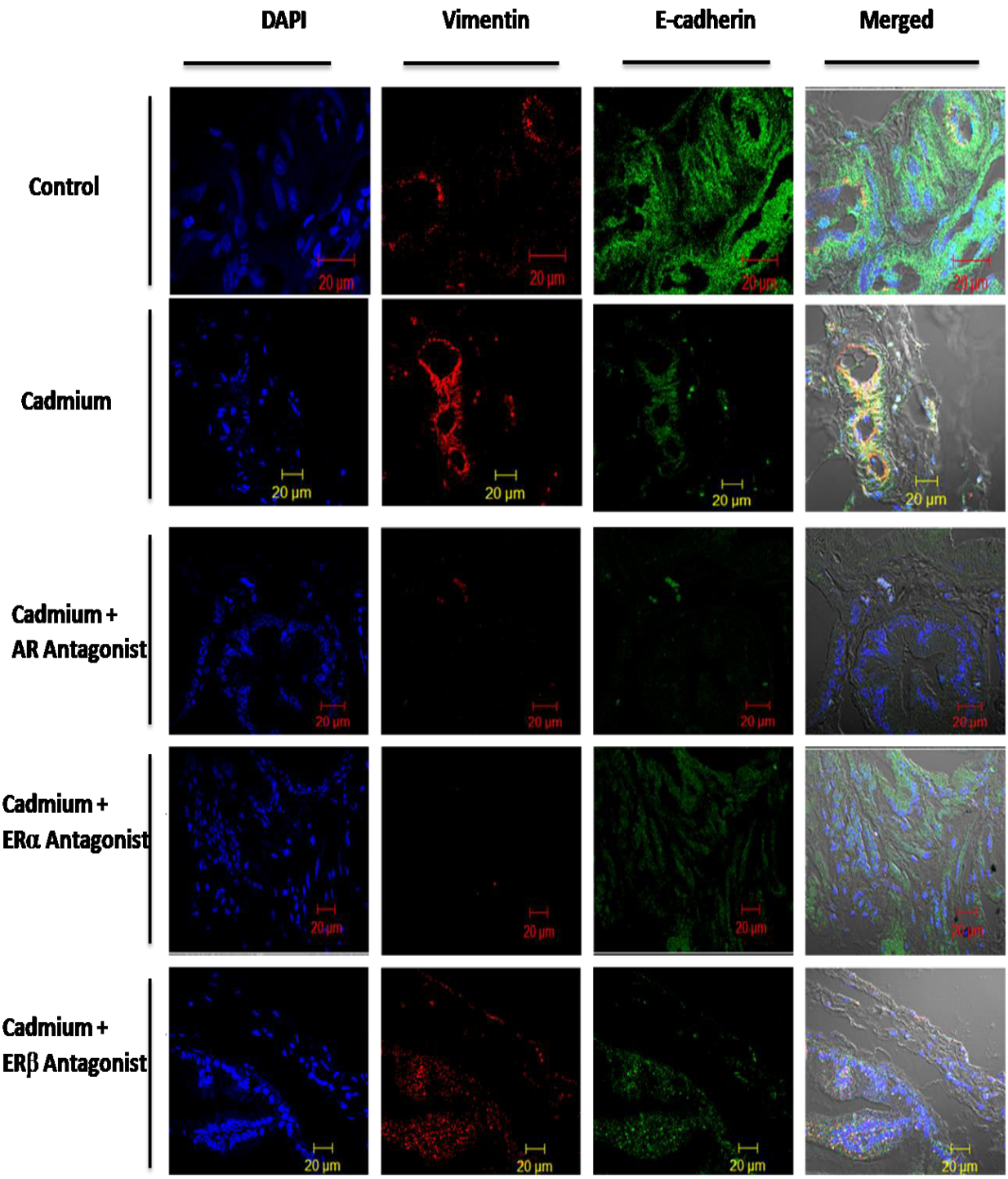

***Figure 3. 10**: Effect* of a single dose of cadmium on expression profile of vimentin and e-cadherin on rat prostate in the presence of antagonists by immunofluorescence method. Tissue sections were stained with secondary antibodies conjugated to CY5 (vimentin) and FITC (e-cadherin) fluorophores along with DAPI for nuclear staining. Images were captured by confocal microscope LSM 710 (Carl Zeiss, Germany) using 63x objective.

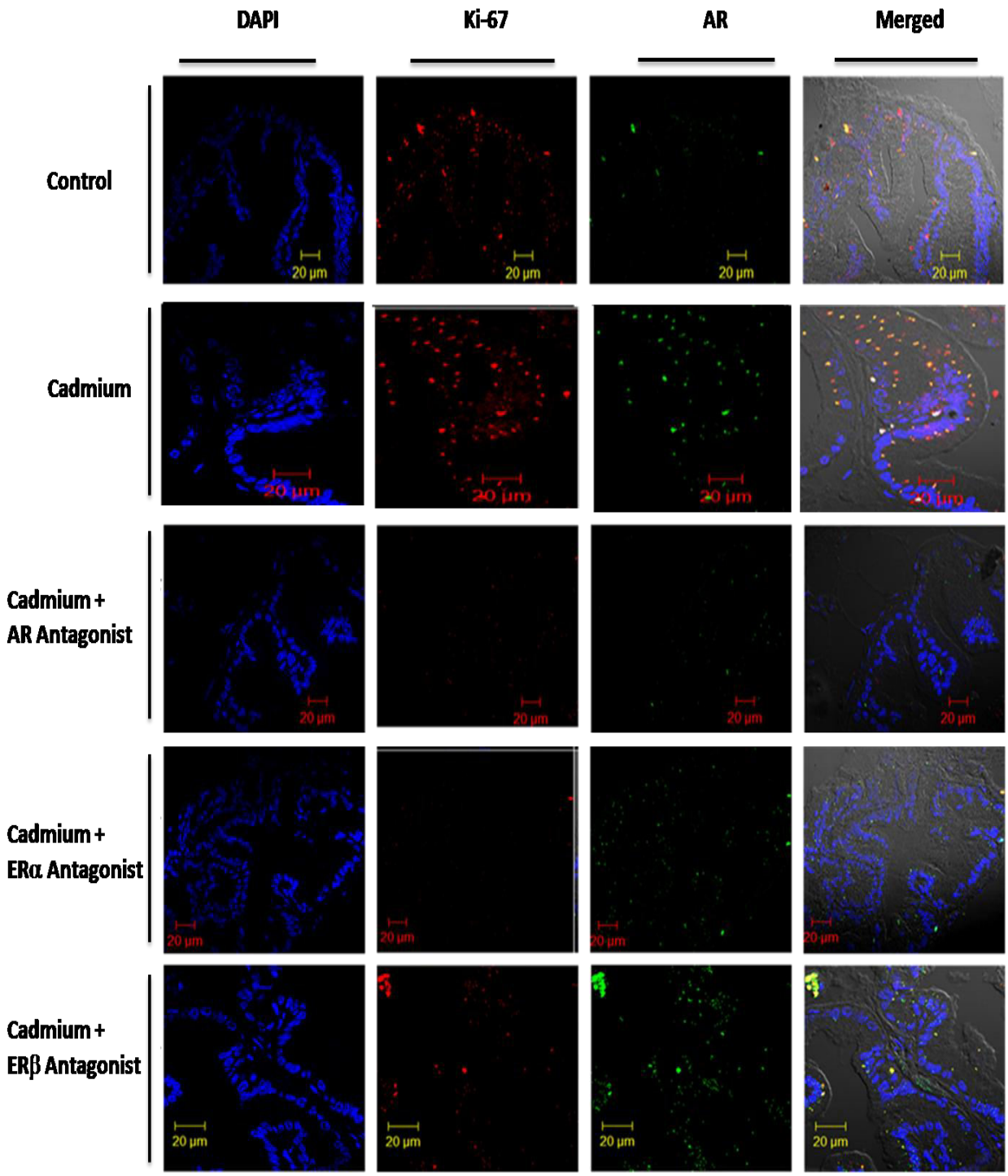

Figure 3. 11: Effect *of a single dose of cadmium on expression profile of ki-67 and AR on rat prostate in the presence of antagonists by immunofluorescence method. Tissue sections were stained with secondary antibodies conjugated to FITC (AR) and TRITC (ki-67) fluorophores along with DAPI for nuclear staining. Images were captured by confocal microscope LSM 710 (Carl Zeiss, Germany) using 63x objective.*

3.4 Discussion

The pathogenesis of BPH remains very elusive in prostate biology till date. Many attempts have been made during the last decades to understand the pathophysiology of the disease. In this context several *in vitro* and *in vivo* animal models have been developed for studying BPH (Mahapokai et al. 2000; Hieble 2011; Oudot et al. 2012). Rat prostate has been documented to respond for hormone and other chemical treatments such as citral, exogeneous tesosteron, DHT and estradiol (Scolnik et al. 1994; Constantinou and Omata 1996) Similarly, Lee *et al* also developed BPH rat model by combined administration of DHT and adenoreceptor antagonist prazosin, subcutaneously for 14 days (Lee et al. 1998). Beside these steroid hormone and other chemical induced BPH rat model recently several transgenic and knockout animal models were also developed to study the pathogenesis of BPH such as liver X receptor (LXR)(is a ligand-activated transcription factor) knockout mouse, overexpression of keratinocyte derived chemokine, the murine analog of the chemokine IL-8, and prolactin overexpressing rodent models (Wennbo et al. 1997; Kim et al. 2009; Schauer et al. 2009). However, above mentioned animal models are costly, time consuming and required transgenic or knockout species.

To study the pathogenesis of BPH, spontaneous and hormone-induced models are more desirable (Hieble 2011; Oudot et al. 2012; Rick et al. 2013). Hormone-induced spontaneous BPH model in the dogs and chimpanzees are more readily available, but ethical and financial matters need to be considered. However, rat and human prostate differ markedly including differences in the gross and microanatomy that have implications for pathological interpretation in clinicopathologic characteristics of human prostatic disorders(Price 1963; Shappell et al. 2003) Yet, the rodents and human prostate have many anatomical similarities such as development of the gland in the form of lobular glands from the Wolffian ducts and the urogenital sinuses. Both species have androgen-sensitive organs and distinctly differentiated epithelial cells with similar functions. The rat Dorso-Lateral Prostate has been documented to be the most homologous to the human Peripheral Zone. These similarities help to support the rat models for the study of molecular alterations in the development and progression of Prostatic enlargement (Price 1963).

To the best of our knowledge, the present work depicts the development of cadmium induced rat model for the first time, which is cost effective, less time consuming and aids in revealing the mystery of pathogenesis of BPH with great ease, compared to other available models. This model had also showed a broad spectrum of histopathological lesions corresponding from normal

to hyperplasia progression and is useful for understanding disease pathogenesis and drug discovery.

The present study suggests that cadmium has significant potential as an inducer of prostate hyperplasia in Charles foster rats. A significant increase in prostate weight with characteristic histological features in five month old animals treated with a single *i.p.* dose of 20 µg cadmium/kg body weight developed BPH like condition within ten days time compared with 1 year old animals.

The metal- binding protein, metallothionein (MT), is thought to be involved in detoxification of various metal toxicities including, Cd. It has also been reported that, MT is poorly expressed in ventral prostate whereas, high basal expression has been observed in dorsolateral prostate of rats (Coogan et al. 1995), suggesting, protective role of MT in later time period of cadmium dose (20 and 30 days) as demonstrated in our results.

Cadmium exposure induces cell proliferation, depicted by increased prostate weight. Previous reports suggested that the old age rats were more resistant to cadmium induced toxicity compared with young age rats (Yamano et al. 2000) which, supported our results of less weight gain in 1 year old rats.. The current findings suggest that, a single dose of Cd causes 1.62 fold increases in the prostate weight compared to control, which is in concordance to earlier reports by Martin *et. al*, 2002 (Martin et al. 2002). Several studies reported the induction of prostate carcinoma by administration of Cd, however the doses of Cd used were much higher than that used in the present study (Waalkes et al. 1992; Benbrahim-Tallaa et al. 2007). Moreover, histological studies suggest that in BPH, the ductal morphology is maintained, unlike in prostate cancer where unorganized growth is observed. Also the presence of basal cells, a characteristic of BPH further strengthens the cadmium induced BPH condition in the present study. It was reported earlier that epithelial cells originate from basal cells and play important role in prostate development and exhibit higher proliferation in BPH like condition, whereas, the basal cells are absent in adenocarcinoma of the prostate (Totten et al. 1953)

The overall maintenance of prostate is dependent on androgens, and the prostate demonstrates regression after withdrawal of androgen, such as castration (Coffey and Isaacs 1981; Benbrahim-Tallaa et al. 2007). In present study also a decrease in prostate weight was observed in castrated

group of animals, supporting androgen mimicking activity of cadmium, which was ameliorated with Cd treatment suggesting cadmium induces a hyperplasia like condition.

Further ability of antagonists to block these effects suggests that the effects of cadmium are mediated through the steroid hormone receptor. In antagonist experiment, the effects from prostate weight and PAP activity were more significant in the group treated with AR and ERα receptor antagonist along with Cd as compared to ERβ receptor antagonist, providing the fact that Cadmium would probably mediate its effect by binding to the ERα and AR with more affinity than with ERβ receptor. Previous studies also support that cadmium binds to hormone-binding domain of ERα and AR with high affinity and activate receptors (Stoica et al. 2000) (Martin et al. 2002) thus, supporting our results. Moreover, histological observations demonstrated larger acini and no epithelial infoldings in AR and ERα antagonists group compared to Cadmium treated group. Whereas, ERβ antagonist treated group showed epithelial infoldings, indicating that cadmium treatment blocked anti-proliferation activity of ERβ and induced hyperplasia of the gland. Further suggesting that Cd effect is mediated through AR and ERα receptors, causing hyperplasia like condition.

The gene expression studies were carried out to study the expression levels of the receptors and 5α Reductase type II enzyme. 5α Reductase type II, enzyme is responsible for conversation of testosterone into DHT. Available literature indicates that the expression of 5α-R2 increase in BPH condition and decrease in PCa (Thomas et al. 2008). Elevated level of transcriptional activity of the enzyme was noted in Cd treated group and hence more DHT production confirmed androgen mimicking activity of cadmium. Our results showed decreased expression of the same in all antagonists groups compared to cadmium treated group, indicating decreased conversion of testosterone to DHT and hence less proliferation.

AR mRNA levels are regulated by androgens and other steroid hormones (Gelmann 2002). Increased AR mRNA expression in cadmium treaded group and decreased expression in antagonist treated group, suggests that Cd mediated it's action through AR as reported earlier and modulate the mRNA expression (Martin et al. 2002). Moreover, it is also known that ERα is the dominant ER form mediating the effects of early estrogen exposure on the prostate gland (Prins et al. 2001). It has been observed that ER is auto-regulated by estrogen (Barton and Shapiro 1988). Stoica *et al.,* 2000 suggested that Cd interacts with the hormone-binding domain of the receptor and activate ERα (Stoica et al. 2000). ERα m RNA expression was significantly

high in Cd treated group compared with control while in other groups it was very less. Similarly, other reports also were unable to detect ERα expression in normal rat prostate tissue (Pelletier et al. 2000). The primary function of ERβ is suppressing proliferation and promoting differentiation of prostatic cells. Decrease in ERβ expression is reported in BPH (Gabal et al. 2007). We have also noticed a decrease in expression of ERβ in the cadmium treated group which further strengthen the fact that cadmium induces a BPH like condition. Similarly, a study of human breast cancer patients previously treated with estrogen antagonist tamoxifen, had reduced ERβ level compared with healthy, age matched controls (Cohen et al. 1997) further supporting our observations.

In the present study, less E-cadherin expression and abundant Ki-67 were observed in Cd treated group. The epithelial characteristics are lost due to high proliferative capability and high vimentin expression indicating possible EMT transition in BPH pathogenesis. During EMT the epithelial cells lose their polarity, stability and become more fibroblast-like cells. The features with parallel loss of epithelial marker and gaining mesenchymal phenotype which would further, alter key signalling pathways responsible for the disease pathogenesis (Alonso-Magdalena et al. 2009).

The steroid hormone receptor antagonist study suggested Cd induced hyperplasia like condition is by activating the androgen receptor and estrogen receptor alpha action and suppressing estrogen receptor beta action in rats. Therefore, we report for the first time a cost effective and less time consuming rat model of BPH by using low level of Cadmium which strongly suggests its co-relation to the pathogenesis of human BPH. Therefore, Cd causes BPH like condition upon binding to AR and ERα receptors which in turn control 5α reductase type 2 enzyme expression, epithelial growth, differentiation, function and epithelial-stromal cross talk.

The experimental model used here is a potentially valuable tool for investigating the respective roles of the epithelial and stromal hormone receptors and for its applicability in the study of the genesis of human BPH, which would be helpful to understand disease pathogenesis and progression and further designing appropriate therapeutics interventions.

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