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**14. ABSTRACT**

Clinically significant prostate cancer usually occurs in men who are 65 and older although precursor lesions are known to exist many years prior to cancer diagnosis. Histopathological changes referred to as Prostatic Intraepithelial Neoplasia (PIN) are considered to be the most likely precursor of prostate cancer. The mechanism(s) involved in progression of indolent to active disease remains elusive although a role for age-related increase in oxidative stress has been proposed. There are a variety of reactive oxygen species (ROS) that ultimately cause oxidative stress and any particular oxidant has not been identified as being primarily involved. We rationalized that a combination of antioxidants may be necessary to neutralize the different classes of ROS to prevent the progression of latent precursor foci to active cancer. Therefore we devised a combination of antioxidants with varied antioxidant properties to determine whether such supplementation could prevent the progression of PIN in Noble rats that are stimulated to develop PIN with hormones. Results from this study show for the first time that dietary intervention with a combination of antioxidants caused a significant decrease ($p < 0.04$) in high grade PIN formation compared to animals on control diet. Based on our data we also speculate that

**15. SUBJECT TERMS**

Oxidative Stress; prostatic intraepithelial neoplasia (PIN); antioxidants and Prevention

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## Table of Contents

**Page**

**Introduction:**

Although prostate cancer is considered to be a disease of older men, a significant number of relatively young men exhibit the earliest signs of prostate cancer. This suggests that the disease is initiated early and remains latent until some factors trigger it to become malignant. This long latency of prostate cancer progression provides an opportunity for intervention to prevent the initial disease from becoming cancerous. Since treatment options for prostate cancer are very limited for initial stages of the disease and unavailable for metastatic disease, it is imperative that other means to control the disease be vigorously tested to reduce the number of prostate cancer-related deaths in the United States.

Oxidants produced as by-products of cellular metabolism have been implicated in the genesis of prostate cancer. Oxidative stress is caused by an imbalance of cellular endogenous oxidant and antioxidant levels. Laboratory studies using different model systems indicate that oxidative stress markers increase and antioxidant enzyme levels decrease during prostate cancer progression. Oxidative stress generated by dietary fat and androgens has been implicated in prostate cancer. Further epidemiological studies with a variety of antioxidants such as selenium, tocopherols, lycopene, β-carotene etc. have been found to be effective in lowering prostate cancer risk. Although these data suggest the importance of oxidative stress and antioxidants in prostate cancer, they are flawed in that they do not add to our understanding of the nature and amounts of antioxidants that are beneficial. This is extremely important since several classes of oxidants are produced and a single antioxidant cannot quench all the different species of oxidants produced from cellular metabolism. Further, time is an extremely important factor for successful antioxidant prophylaxis. Taken together, the stage of prostate development and the kinds of antioxidants used would play a major role in determining the success of antioxidant prophylaxis. This proposal is a first step in beginning to understand whether antioxidants can prevent or delay the formation of PIN. Based on evidence presented in the literature, ***we hypothesized that a combination of antioxidants can prevent or delay the development of Prostatic Intraepithelial Neoplasia in a T/$E_2$ model of PCA by modulating the level of oxidative stress markers and endogenous antioxidant levels.*** To test our hypothesis we proposed three specific aims.

1) Determine the ability of antioxidants to prevent or delay the development of Prostatic Intraepithelial Neoplasia (PIN) and relate it to changes in T/$E_2$ in the serum and AR.

2) Determine the levels of oxidative stress markers of DNA, protein and lipids following antioxidant supplementation.

3) Determine the levels and functional ability of endogenous antioxidant components following antioxidant supplementation.

**Results:**

**Aim 1:** Determine the ability of antioxidants to prevent or delay the development of Prostatic Intraepithelial Neoplasia (PIN) and relate it to changes in $T/E_2$ in the serum and AR.

**Animal experiments:** Animal experiments were conducted in accordance with approved protocols by the institutional animal care committee. Noble rats purchased from Charles River Laboratories (Wilmington, MA) were housed under a 12 hour light-dark cycle and a temperature of $23\pm2^{o}C$ with access to food and water. At 4-6 weeks of age animals were randomized into 4 groups of 10 animals. Groups I and II animals received control diet (AIN-93G soy-free diet) with no antioxidant supplementation. All animals except group 1 were treated with testosterone and estradiol. Slow release pellets containing 240 mg testosterone propionate and 25 mg 17 β-estradiol benzoate (Innovative Research America, FL) were implanted *sc* into the flanks of the animals. Control animals received placebo pellets. Hormone stimulation lasted for 16 and 32 weeks.

Body weight changes were measured once a week during the experiment. Food cups were weighed before and after feeding to determine the amount of food consumed. All animals were weighed weekly and observed daily for signs of illness. All the animals were sacrificed at 16 weeks after initiation of hormone treatment. Animals were sacrificed by $CO_2$ asphyxiation followed by cervical dislocation. The abdominal cavity was opened and all the organs were examined for gross changes. Prostate was dissected from the rest of the genitourinary organs, weighed, cut longitudinally along the urethra, and fixed in 10% buffered formalin. Serial sections of prostate tissue were stained with H&E and evaluated based on published criteria by three pathologists.

**Histopathology of prostate lesions:** Serial sections of prostate tissue were stained with H&E and evaluated according to the criteria suggested by Leav et al (1) by three independent pathologists who were blinded to treatment status. PIN was distinguished from typical hyperplasia based on multiple layers of dysplastic epithelial cells which frequently formed alveolar or papillary structures. The increased nuclear size in addition to increased variability in nuclear shape, chromasia, nucleolar spacing, cell crowding and cytoplasmic eosinophilia which sharply contrasted with the pale stained cytoplasm of normal or hyperplastic epithelial cells were important criteria for characterizing PIN in Noble rats.

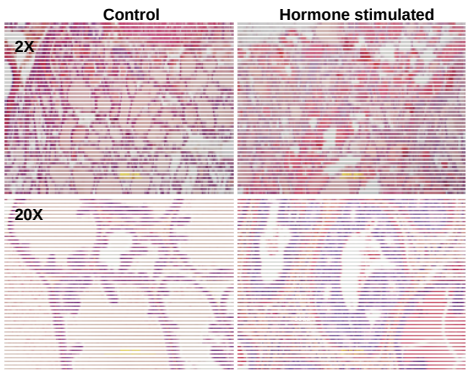


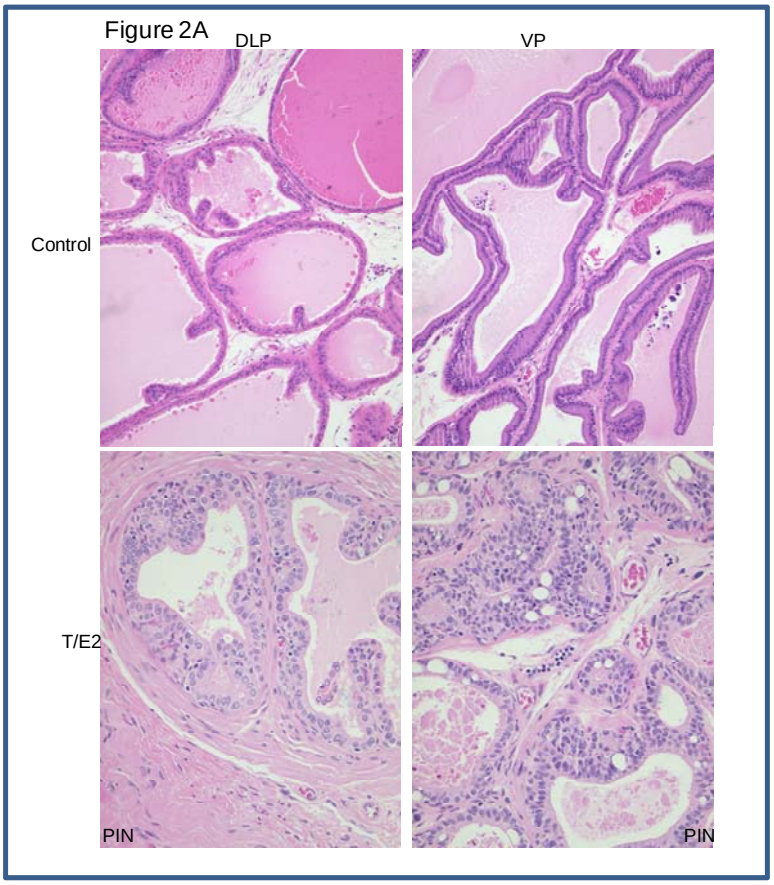

**Figure1: Histopathological analysis of prostate.** H&E evaluation was conducted by two independent pathologists. A representative picture from animals without and with hormone stimulation is shown. Low power magnification of prostate demonstrating lateral and ventral prostate architecture from control Noble rats is shown in the top left panel. Note size of the acini, consisting of secretions and thinness of epithelial lining. The stroma between acinus is less and sparsely occupied by connective tissue and cells. Higher magnification of acini shown in the bottom left panel demonstrates clean prostatic secretion in acini. Acini are lined by columnar epithelium that occasionally forming fronds.

Low power magnification of prostate demonstrating altered appearance from Hormone-stimulated Noble rats is shown in the right panel. The stroma is thickened by increased dense connective tissue and inflammatory cell infiltration. The acini are variable filled with necrotic cellular debris. In several acini the epithelium is hyperplastic. Higher magnification of acini from hormone stimulated Nobel rat prostate demonstrating a central lumen with necrotic cellular debris, sloughed cells and inflammatory cells (shown as asterisk). Inflammatory cells are trans-migrating the epithelium; The epithelial lining is thickened by reactive hyperplasia (bottom right panel).

Figure 1 shows low power magnification of prostate demonstrating lateral and ventral prostate architecture from control Noble rats. Note size of the acini, consisting of prostatic secretions and thinness of epithelial lining. The stroma between acinus is less

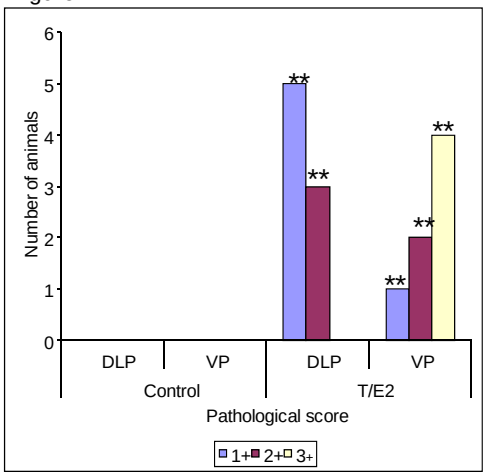
and sparsely occupied by connective tissue and cells (top left panel). Higher magnification figure demonstrating clean prostatic secretions in which acini are lined by columnar epithelium and those occasionally forming fronds are shown in bottom left panel. Top right panel shows low power magnification demonstrating altered appearance of prostate from hormone-stimulated Noble rats. The stroma is thickened by increased dense connective tissue and inflammatory cell infiltration. The acini are variable and filled with necrotic cellular debris. In several acini the epithelium is hyperplastic. Higher magnification of acini from hormone stimulated Nobel rat prostate demonstrating a central lumen with necrotic cellular debris, sloughed cells and inflammatory cells (shown as asterisk) is shown in bottom right panel. Inflammatory cells are trans-migrating the epithelium; and the epithelial lining is thickened by reactive hyperplasia (bottom right panel).

Figure 2A




**Inflammatory and reactive changes in the prostate from hormone stimulated rats:** We used the extent of PIN for grading lesions on a scale of 1+ through 3+. Accordingly 1+ refers to minimal PIN, 2+ moderately extensive and 3+ widespread PIN. All the animals in the control group that did not receive hormones were negative for PIN in both the dorso-lateral and ventral prostate (Figure 2a). In the hormone treated group, two out of 10 animals were negative for PIN and 5 out of 10 animals showed 1+ PIN grade, and three were 2+ in the dorso-lateral lobe (Figure 2b). The results of Mann-Whitney U tests indicate that PIN grades were significantly greater for hormone treatment vs. control in the dorso-lateral prostate (p=0.0015). Interestingly, we also found higher grade PIN in the ventral prostate. Four out of 10 animals exhibited 3+ PIN; 2 animals had 2+ and 1 animal had 1+ grade of PIN in the ventral prostate (p=0.0068). Acute inflammation and prominent epithelial vacuolization accompanied more severe PIN. Acute inflammation and reactive changes were observed both in the dorso-lateral and ventral prostate of animals that were graded 2+ and 3+ PIN; such inflammatory changes were not observed in 1+

Figure 2B

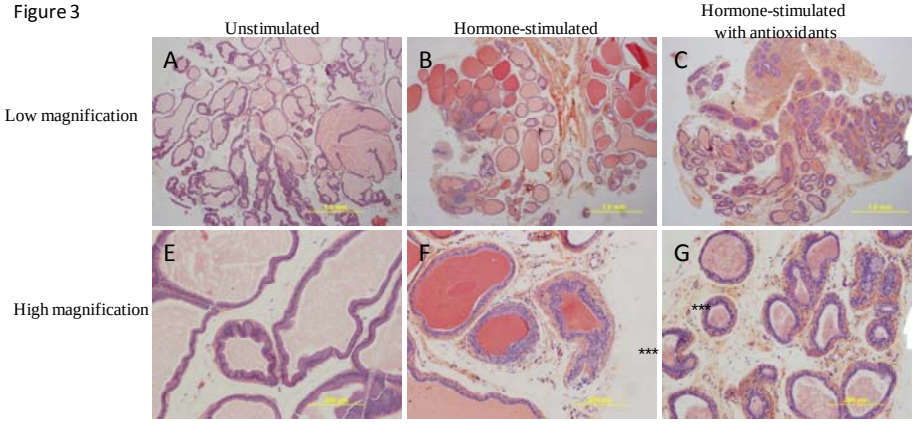


lesions (Figure 2b). A representative picture showing dorso-lateral and ventral prostate is shown in figure 2a.

**Antioxidant supplementation prevents PIN formation in hormone stimulated Noble rats:** At 4-6 weeks of age animals were randomized into 2 groups of 20 and 10 animals each. Groups I animals received control diet with no antioxidant supplementation. Group II animals received antioxidant supplemented diet (composition shown below) until hormone stimulation at 10 weeks. In addition another group of 10 animals received placebo pellets. The intervention group animals were put back on control diet prior to stimulation with hormones so that antioxidants did not modulate hormone level and or activity. Slow release pellets containing 240 mg testosterone propionate and 25 mg 17 β-estradiol benzoate (Innovative Research America, FL) were implanted *sc* into the flanks of the animals. Hormone stimulation lasted for 16 weeks. Body weight changes were measured during the experiment. Food cups were weighed before and after feeding to determine the amount of food and antioxidant consumed. All animals were weighed weekly and observed daily for signs of illness. All the animals were sacrificed at 16 weeks after initiation of hormone treatment. Animals were sacrificed by $CO_2$ asphyxiation followed by cervical dislocation. The abdominal cavity was opened and all the organs were examined for gross changes. Prostate was dissected from the rest of the genitourinary organs, weighed, cut longitudinally along the urethra, and fixed in 10% buffered formalin. Serial sections of prostate tissue were stained with H&E and evaluated based on published criteria by three pathologists.

| Antioxidant | Low dose | High dose |
|---|---|---|
| Ascorbic acid | 3 | 6 |
| Vitamin E acetate | 6 | 15 |
| Riboflavin | 0.3 | 0.6 |
| Beta carotene | 0.85 | 1.9 |
| Selenium | 0.065 | 0.125 |
| Lycopene | 0.5 | 1 |
| Lutein | 0.5 | 1 |
| Alpha Lipoic acid | 1.5 | 3 |
| Grape seed extract | 5 | 10 |
| Co-enzyme Q10 | 5 | 10 |

Figure 3




Low power magnification of prostate demonstrating architecture of lateral and dorsal prostate from Nobel Rats. Note size of acini, consisting of secretions and thinness of epithelial lining. The stroma between acinus is sparsely occupied by connective tissue and cells (A). Low power magnification of prostate demonstrating altered appearance from hormone stimulated Noble rats (B). The stroma is increased by dense connective tissue and inflammatory cell infiltration. The acini variably contain protein with increased eosinophilia; acinar walls are thickened with reactive hyperplastic epithelial cells. Low power magnification of prostate from hormone stimulated Noble rats on diet containing antioxidant supplements demonstrating appearance of acinar contents similar to the unstimulated prostate (C). The stroma contains less inflammatory components when compared to the hormone stimulated prostate. However due to possible construction the acinar walls still appear thickened.

Prostate from unstimulated, hormone stimulated and hormone stimulated with prior antioxidant supplementation shows altered pathological features. For example the acini are smaller with thin lining and the stroma between acinus is sparsely occupied by connective tissue

and cells in the prostate from unstimulated rats. Prostate from hormone stimulated rats demonstrates altered appearance with dense connective tissue and inflammatory cell infiltration into the stroma. The acini variably contain protein with increased eosinophilia; acinar walls are thickened with reactive hyperplastic epithelial cells. Interestingly prostate from hormone stimulated Noble rats on diet containing antioxidant supplements demonstrating appearance of acinar contents similar to the unstimulated prostate containing less stromal inflammatory components when compared to the hormone stimulated prostate. These changes have been shown in figure 3. We also graded these pathological changes essentially as described in figure 2. The scoring data is presented in table I. Data presented in table I show that there was a significant difference in the extent of PIN formation between animals that were on control diet compared with those on the antioxidant diet by Mann-Whitney U test ($p = 0.040$). The median H & E score for controls was 1+. Only 23% controls had a negative H & E score, while 70% of animals on antioxidant supplemented diet (high dose) had no PIN. This rate of negative scores was significantly different by Fisher's Exact Test ($p = 0.040$).

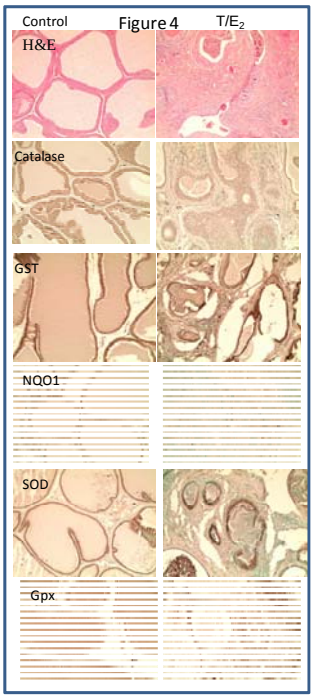
Table I: Antioxidant supplementation prevents hormone induced PIN development in Noble Rats

| Measure | Placebo control | Hormone stimulated | Anti-oxidant | p Fisher's exact t-test |
|---|---|---|---|---|
| Total | 10 | 20 | 10 | |
| Died<end of experiment | 0 | 3 | 0 | |
| No PIN | 10 | 4 | 7 | **0.04** |
| 1+ | 0 | 10 | 2 | |
| 2+ | 0 | 3 | 1 | |

**Antioxidant supplementation did not affect genitourinary weights:** Mann-Whitney U tests indicated no significant differences between controls and high dose animals for GU weights ($p = 0.125$) and SV weights ($p = 0.549$). For GU weights, the interquartile range for controls was 0.95 to 2.88 gm with a median of 1.36 gm, while the interquartile range for high dose was 0.77 to 1.92 gm with a median of 0.94 gm. For SV weights, the interquartile range for controls was 0.25 to 0.81 gm with a median of 0.34 gm, while the interquartile range for high dose was 0.23 to 0.34 gm with median of 0.28 gm. The statistical analysis was performed using Stata 10.0 (StataCorp, College Station, TX).

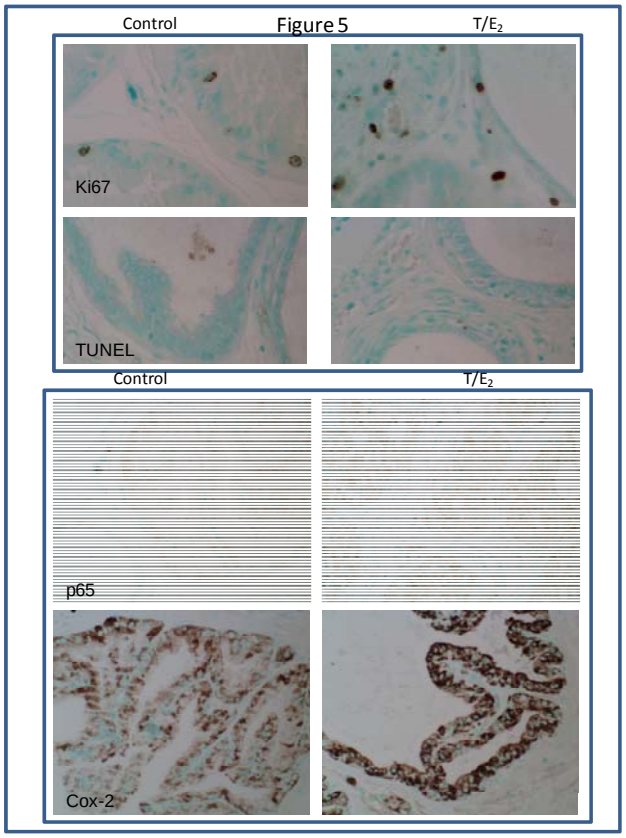
**Findings from aim 1 studies:** Overall we have accomplished the goals of specific aim 1 showing (i) antioxidant supplementation significantly reduced the development of high grade PIN. Specifically 70% of animals on antioxidant supplemented diet had no PIN whereas only 23% animals on hormone stimulated group had no PIN. This rate of negative scores was significantly different by Fisher's Exact Test ($p = 0.040$); and (ii) antioxidant supplementation had no significant effect on the wet weight of prostate or seminal vesicles ( Being submitted for publication).

**Aim 2: Determine the levels of oxidative stress markers of DNA, protein and lipids following antioxidant supplementation.**

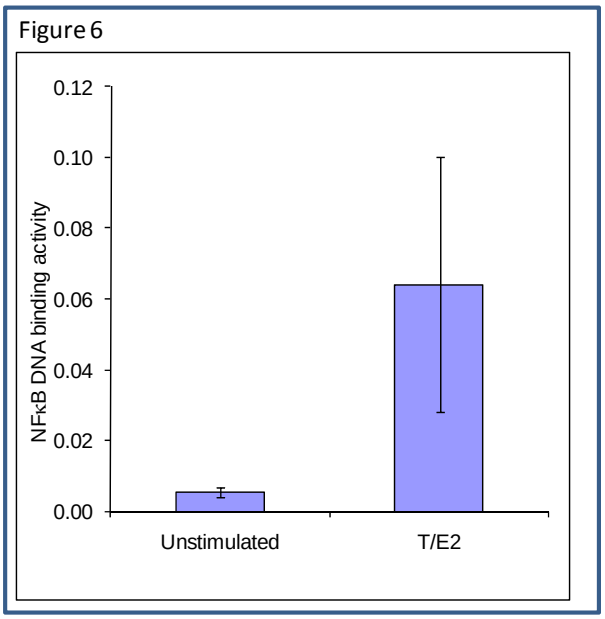



**Hormone stimulation modulates antioxidant defense enzymes in the prostate from Noble rats:** As proposed in the grant application the observed hormone induced pathological changes could be due to changes in the levels and activities of antioxidant enzymes in the prostate. In addition intracellular pro-oxidant/anti-oxidant balance has been shown to be influenced by hormone stimulation of noble rats. Further hormones may contribute to the induction of oxidative stress in rat prostate gland *in vivo* by modulating levels and activities of antioxidant enzymes . We used immunohistochemistry to analyze prostate tissue for expression of critical enzymes involved in antioxidant defense including catalase, glutathione peroxidase (Gpx), GSTpi, superoxide dismutase (SOD) and NADPH Quinone Oxidoreductase (NQO1). We did not find any significant changes in the expression of catalase, Gpx, GSTpi or SOD. However expression of NQO1 was lost in prostate from hormone stimulated rats when compared to prostate from unstimulated rats (**figure 4**).

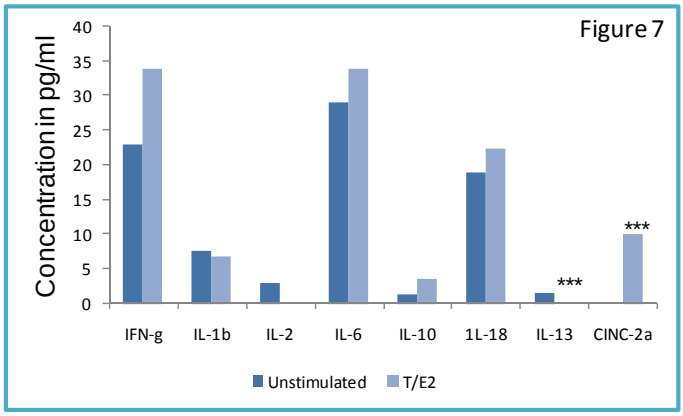
**Hormone stimulation modulates proliferation, apoptosis and inflammation associated Cox-2 in the prostate from Noble rats:** Additionally the observed development of PIN could be due to increased proliferation, decreased apoptosis or alteration in the expression of inflammation associated genes such as NFκB and downstream effectors such as Cox-2. In an attempt to decipher the mechanism of hormone-induced inflammation and PIN development, we evaluated the expression of Ki67 (proliferation indicator), apoptosis (TUNEL staining), p65 (NFκB signaling molecule) and Cox-2 (a downstream target of NFκB involved in inflammation) using immunohistochemical analysis in three representative prostate samples from each group. As shown in figure **5** expression of Ki67 was higher in the prostate from hormone stimulated rats compared to placebo group animals. We also found that Ki67 was higher in the hormone treated animals with inflammation including stromal part. There was no significant change in apoptosis compared with unstimulated animals suggesting that the balance between proliferation and apoptosis is disrupted upon hormone-stimulation.



Expression of both p65 and Cox-2, an inflammation associated gene, was very strong in the dorsolateral and ventral prostate from hormone stimulated rats. In contrast, prostate tissue of unstimulated rats showed low level of Cox-2 immunoreactivity (figure **5**). It is well established that Cox-2 is regulated by transcription factor NFκB. NFκB has been shown to regulate a wide variety of genes involved in cell proliferation, survival, migration, tumorigenesis, and metastasis. Accordingly we observed significantly higher levels of NFκB DNA binding activity in extracts prepared from prostate tissue of hormone treated animals compared to the placebo group (figure **6**). These data implicate a potential role for activation of NFκB signaling pathway and its downstream target Cox-2 in response to hormone stimulation in the Noble rat model.




**Modulation of chemokines in hormone-stimulated Noble rats:** Since chemokine network is activated by inflammation we investigated the presence of various cytokines and chemokines in serum from these animals using multiplex immunoassay. Analysis of these data indicated non-significant alterations in the serum levels of most of the cytokines and chemokines measured in response to hormone stimulation. However, we saw significant (10-fold; p=0.001) upregulation of cytokine-induced neutrophil chemoattractant-2α (CINC-2α) in serum from animals stimulated with hormones (figure **7**).



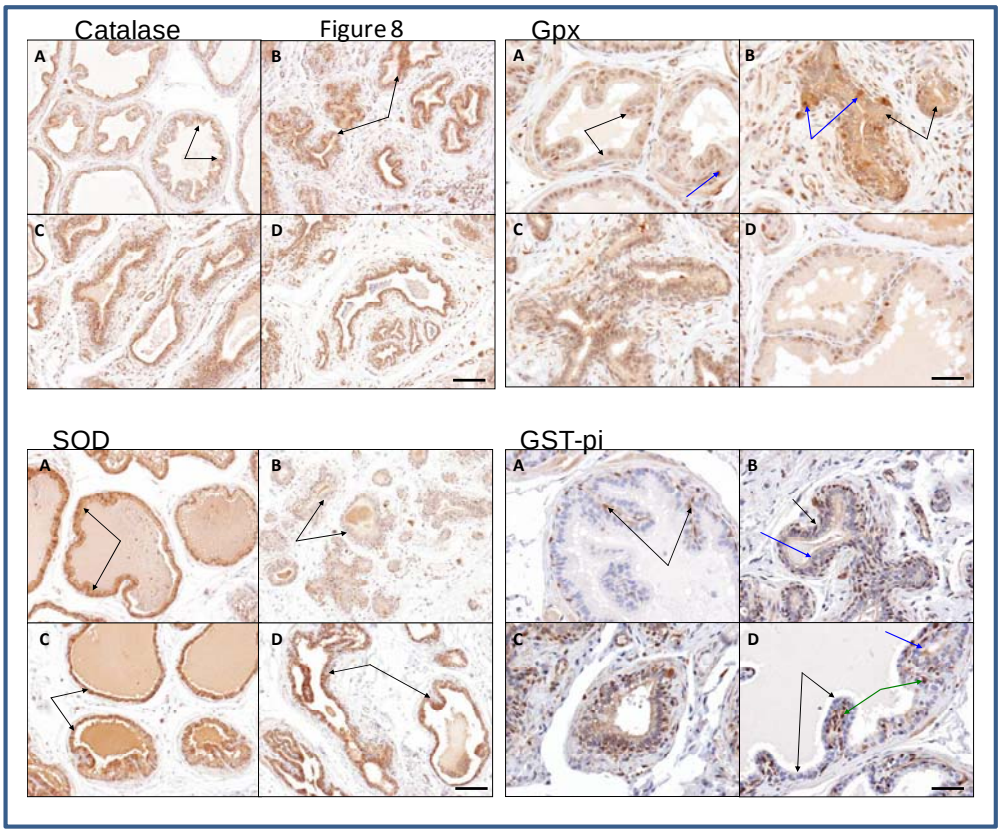

CINC-2α is a counterpart of the human growth regulated oncogene product (GRO) that has been shown to be vital for cell survival and malignant transformation and activated by RAS. GRO-1 has also been shown to be present in higher levels in serum samples from women with ovarian cancer. Both protein and mRNA levels of CINC-2α is upregulated in gastric mucosa cells in response to TNFα. Although we do not know how CINC-2α is involved in prostate carcinogenesis these studies implicate a potential role for CINC-2α in inflammation-mediated prostate tumorigenesis.

**Findings from aim 2 studies:** Overall conclusions from experiments proposed in aim 2 are (i) prostate from hormone stimulated Noble rats showed marked decrease in the expression of NADPH Quinone Oxidoreductase (NQO1) with no significant change in the expression of glutathione peroxidase (Gpx), glutathione S-transferase pi (GSTpi), superoxide dismutase (SOD) or catalase; (ii) prostate from hormone stimulated rats showed very strong expression of p65, Cox-2 and NFκB DNA binding activity; (iii) cytokine-induced neutrophil chemoattractant-2α (CINC-2α) was significantly upregulated by more than 10-fold (p=0.001) in serum from animals stimulated with hormones. NQO1 is an important biotransformation enzyme that has been implicated in protecting cells from oxidative stress and against carcinogenesis. Consistent with this

loss of NQO1 under hormone stimulated conditions may sensitize the prostatic epithelium to the observed pathological changes. However detailed studies need to be done to demonstrate the role of NQO1 in prostate tumorigenesis. Although further studies are required, we speculate that activation of NFκB/ CINC-2α/Cox-2 along with modulation of antioxidant defense mechanisms may create a pro-inflammatory environment suitable for tumor growth and survival. ***These results are in (Translational Oncology).***

**Aim 3: Determine the levels and functional ability of endogenous antioxidant components following antioxidant supplementation.**

After establishing that antioxidant supplementation prevents extent of PIN development we investigated if the observed pathological changes correlate with expression of some of the antioxidant defense enzymes. We used immunohistochemistry to address this. Prostate and bladder tissue (non specific control) from 3 rats from each group was used in immunohistochemical analysis. The prostate tissue collected was fixed in 10% neutral buffered formalin and processed using standard histological techniques and stained with hematoxylin and eosin. Serial sections were subjected to immunohistochemical procedures for catalase (Abcam 1877), NFκB p65 (Abcam 31481), Gpx-1 (Abcam 16798), c-fos (Abcam 7963) and superoxide dismutase (Abcam 13498) and GST-π (MBL 312). The prostate tissues were given a diagnosis based on the changes present on H&E slides. Immunohistochemical staining intensity was assessed using the scoring scale (0, no notable change; trace, minimal intensity staining barely detectable, 1+ mild intensity staining, 2+ moderate intensity staining and 3+ marked intensity staining). The location (nuclear or cytoplasmic) and the approximate % of the cell type that was stained by the different scores were also assessed.



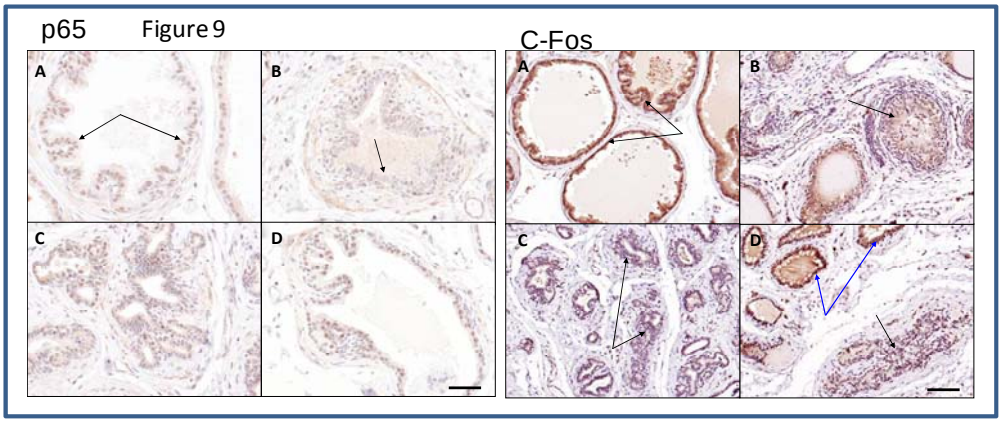

**Catalase:** Staining of the catalase antibody is similar between groups. The prostatic epithelium is 2+ cytoplasmic in 100% of cells, and occurs near the apical border. Smooth muscle around acini and blood vessels are 1+ cytoplasmic catalase positive. The catalase Ab also stains cytoplasmic regions of all endothelial cells

(2+) and most inflammatory cells (2+), when present. The positive control prostate sample has a similar pattern, and the negative control tissue has no appreciable Ab staining.

**Glutathione Peroxidase 1 (Gpx1):** Staining of the Gpx1 antibody in the prostate occurs in a similar pattern across treatment groups, except for the presence of increased numbers of intensely staining (2+ cytoplasmic) macrophage-like cells within the epithelial layer of some dysplastic acini. Otherwise staining of this Ab is generally 1+ cytoplasmic in smooth muscle, 1-2+ cytoplasmic in prostatic epithelium, 1+ cytoplasmic in endothelium, and 2+ in most inflammatory cells, when present. The positive control sample of rat liver exhibits 2-3+ cytoplasmic and nuclear staining in all hepatocytes, 2+ cytoplasmic in macrophages, and 2+ cytoplasmic in bile duct epithelial cells. The negative control tissue has no appreciable Ab staining.

**Superoxide Dismutase (SOD)*:*** Staining of the SOD antibody in the prostatic epithelium is typically cytoplasmic and appears to be less intense in the hormone stimulated animals compared to the placebo control and antioxidant supplementation group of animals. The positive control sample of rat lung tissue is 2+ cytoplasmic positive in 100% of bronchiolar epithelial cells, 1+ cytoplasmic positive in 100% of endothelial cells, and 1+ cytoplasmic positive in 100% of alveolar and tissue macrophages. The negative control samples are negative.

**GST-π*:*** Staining of the GST-π antibody in the prostatic epithelium is typically 2+ cytoplasmic in the placebo controls, but only appears to stain a subset of basally located cells that may represent the macrophage-like cell that resides here. The staining population represents approximately 10% of the total prostatic epithelium. Acinar epithelial cells are typically negative in the naïve controls. This is in contrast to hormone stimulated group of animals where there are increased numbers of these 2+ staining basally-located epithelial cells, and increased numbers of 1+ cytoplasmic staining acinar epithelial cells. This pattern generally holds for antioxidant supplementation group, except in areas where acinar epithelium appears more morphologically normal. In these areas the epithelial staining pattern is more similar to that of naïve controls. The negative control sample is negative.



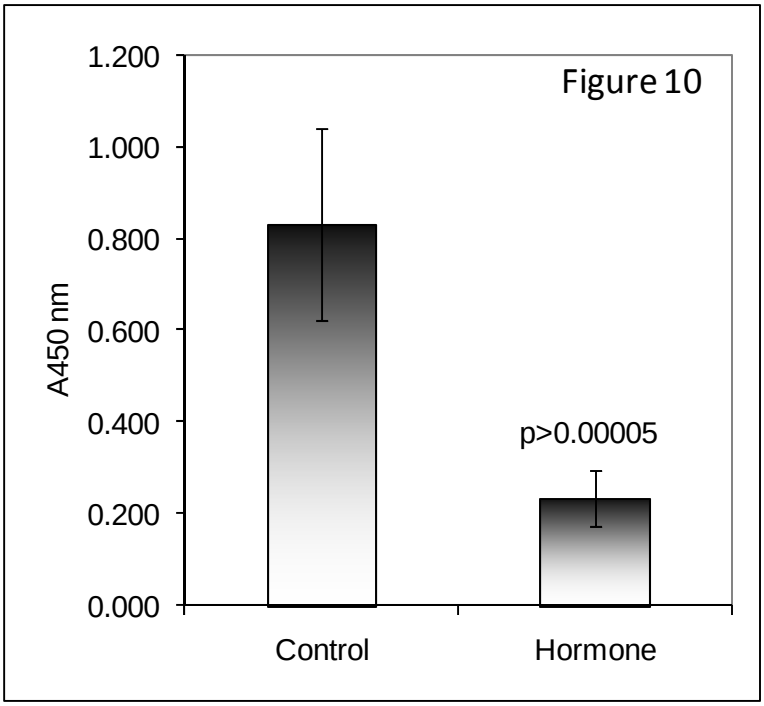

**NFκB p65*:*** Staining of the p65 antibody in the placebo prostatic epithelium is 1+ in most nuclei, and trace in the cytoplasm. In hormone stimulated animals there is less 1+ nuclear staining in the prostatic epithelium, most of which is dysplastic. In antioxidant supplementation group the prostatic epithelium has 1+ nuclear staining in more of the acinar epithelial cells, similar to the placebo control group. This does not appear to differ between dysplastic and more normal acini in antioxidant supplementation groups. Otherwise the staining is similar between groups. Staining of this Ab is generally 1+ cytoplasmic in smooth muscle and 1-2+ cytoplasmic in most inflammatory cells, when present. The Ab is not obviously expressed in endothelial cells in any group. The

positive control human prostate sample has 1-2+ cytoplasmic staining in all prostatic epithelium. The negative control tissue has no appreciable Ab staining.

**c-Fos*:*** Staining of the c-Fos antibody in the prostatic epithelium is typically cytoplasmic and appears to be less intense in hormone stimulated animals compared to placebo control group. Prostate epithelium from antioxidant supplementation group has generally less intense staining than hormone stimulated prostatic epithelium. The positive control sample of human spinal cord is 1-2+ cytoplasmic positive in neuronal cell bodies. The negative control samples are negative.

**Findings from aim 3 studies:** Immunohistochemical staining for NFκB p65, c-fos, superoxide dismutase and GSTπ are altered in prostate tissue by hormonal therapy compared to placebo controls and these alterations tend towards being similar to placebo controls in the antioxidant therapy group. In addition there are increased numbers of Gpx-1 positive macrophage like cells in the epithelial layer of most dysplastic or PIN acini regardless of treatment compared to placebo controls. The catalase antibody is positive in all prostatic epithelium and the pattern of staining is similar between all groups. These results are being prepared for publication.

**Identification of Preprohaptoglobin in serum from hormone stimulated rats:** We used Rapid Analysis Meso-Resolution (RAMR) protein profiling to identify PIN associated proteins in the serum from rats. Serum samples containing 600 μg total proteins was mixed in a final volume of 100 μl equilibrium buffer and passed through chromatographic resin. The flow-through and two column volumes were collected as fraction 1. Following a second wash with equilibration buffer, elution buffer was applied and mixed, then eluted. This eluted fraction was then applied to the 2nd chromatographic resin equilibrated with the buffer used to elute from the 1st column. The procedure was repeated with the 2nd resin. The flow-through from the second resin was designated fraction 2, and the elution fraction from this column was designated fraction 3. Both fractions 2 and 3 were desalted and concentrated using Ultra 4 centrifugal concentrators with a 5 Kd exclusion limit. Total protein was determined for each fraction by the method of Bradford. 3 μg of protein from each fraction in duplicate was applied to 12% SDS-PAGE gel. Following electrophoresis gels were stained using Sypro Ruby protein gel stain solution and images were captured using a Gene Flash image capture system and images were analyzed using Phoretix 2D software. These data indicated that the hormone treatment resulted in global changes in protein abundance, whereas relatively small set of proteins differed following antioxidant supplementation. ***Interestingly several proteins following antioxidant supplementation seemed to revert back to the levels found in placebo control animals.***



We selected 37 kDa protein showing more than 2-fold difference in the SYPRO Ruby stained gel for further characterization. These protein spots were digested and analyzed by mass spectrometry. Using peptide search engine Mascot we identified Preprohaptoglobin as a protein that is modulated following hormone treatment. Literature search revealed that haptoglobin, a byproduct of Preprohaptoglobin has been shown to be involved in protection of tissue damage from hemoglobin induced oxidative stress and renal toxicity (6). Studies also show association of haptoglobin polymorphisms with diabetic nephropathy, hypertension and proetinuria. We measured serum levels of Preprohaptoglobin using ELISA based assay (Immunology consultants Lab Inc., Newberg, OR). As shown in figure 10 serum levels of Preprohaptoglobin decreased in hormone stimulated rats showing the possible utility of this as a potential prognostic marker of prostate cancer progression.

**Reportable outcomes:**

- Prostate form hormone stimulated Noble rats showed marked decrease in the expression of NADPH Quinone Oxidoreductase (NQO1) with no significant change in the expression of glutathione peroxidase (Gpx), glutathione S-transferase pi (GSTpi), superoxide dismutase (SOD) or catalase.
- Prostate from hormone stimulated rats showed very strong expression of p65, Cox-2 and NFκB DNA binding activity.
- In addition, cytokine-induced neutrophil chemoattractant-2α (CINC-2α) was significantly upregulated by more than 10-fold (p=0.001) in serum from animals stimulated with hormones. Although further studies are required, we speculate that activation of NFκB/ CINC-2α/Cox-2 along with modulation of antioxidant defense mechanisms may create a pro-inflammatory environment suitable for tumor growth and survival. ***These results have been accepted for publication in Translational Oncology.***

- Antioxidant supplementation significantly reduced the development of high grade PIN. Specifically 70% of animals on antioxidant supplemented diet (high dose) had no PIN whereas only 23% animals on hormone stimulated group had no PIN. This rate of negative scores was significantly different by Fisher's Exact Test (p = 0.040).
- Antioxidant supplementation had no significant effect on the wet weight of GU or seminal vesicles.
- Immunohistochemical staining for NFκB p65, c-fos, superoxide dismutase and GSTπ are altered in prostate tissue by hormonal therapy compared to placebo controls. Such alterations tend towards being more similar to placebo controls in the antioxidant therapy group.
- In addition there are increased numbers of Gpx-1 positive macrophage like cells in the epithelial layer of most dysplastic or PIN acini regardless of treatment compared to placebo controls.
- NFκB p65, c-fos, superoxide dismutase and GSTπ may play a significant role in mediating Antioxidant effects in Noble Rats. ***These results are being communicated for publication.***

- Hormone treatment resulted in global changes in protein abundance in the serum using Rapid Analysis Meso-Resolution (RAMR) protein profiling, whereas relatively small set of proteins differed following antioxidant supplementation.
- Several proteins following antioxidant supplementation seemed to revert back to the levels found in placebo control animals.

- Identified Preprohaptoglobin as one such protein.
- Serum levels of Preprohaptoglobin decreased in hormone stimulated rats in an independent ELISA assay showing the possible utility of this as a potential prognostic marker of prostate cancer progression.
- Although no studies have been conducted in prostate carcinogenesis with regard to Preprohaptoglobin, it has been shown to be involved in oxidative stress.
- Novel finding of identification of Preprohaptoglobin may mediate inhibition of PIN development in hormone stimulated Noble rats. ***These results are being prepared for communication.***

**Conclusions:** Primary management of prostate cancer for a majority of patients consists of radical surgery or radiation therapy. Although this is adequate for disease control in some patients a significant number of patients relapse and ultimately develop metastatic disease. There are limited treatment options for patients who have undergone primary therapy with curative intent. Early initiation of hormonal ablation is associated with significant morbidity and effect on quality of life including hot flashes, loss of libido, decreased muscle mass, and osteoporosis with long term use. Since PIN precedes prostate cancer delaying the progression of PIN or reversing HGPIN to LGPIN serves as an excellent mechanism to ensure quality of life for elderly men. Several lines of evidence suggest a beneficial role for vitamin consumption against prostate cancer. In this context Meyer and colleagues have shown that supplementation with nutritional doses of vitamin C, vitamin E, β-carotene, selenium and zinc daily for 8 years significantly reduced the rate of prostate cancer development in men with normal PSA (< 3ng/ml; 2). The α-tocopherol, β-carotene (ATBC) cancer prevention trial in Finland found that consumption of vitamin E reduced clinical prostate carcinoma by 32% and prostate cancer mortality by 41% and no effect of vitamin E on latent prostate cancer (3). The double-blinded selenium chemoprevention trial by Clark and colleagues originally directed towards high-risk skin cancer patients found that selenium reduced prostate carcinoma risk significantly (4-5). However SELECT trial concluded that selenium or vitamin E alone or in combination did not prevent prostate cancer (7). Therefore our data showing efficacy of antioxidant in preventing PIN are highly significant. While these studies suggest a role for antioxidant vitamin supplementation in the development of prostate carcinoma they do not shed any light regarding their effectiveness in preventing the progression of early PIN lesions towards clinically significant prostate cancer.

Studies conducted during this funding period clearly suggest that (i) reduced number of animals on antioxidant supplemented diet develop PIN following hormone stimulation compared to animals on regular diet; (ii) suggest that the observed pathological changes are associated with changes in the expression of NFκB p65, SOD, c-fos and GSTπ; (iii) suggest potential role for Preprohaptoglobin in preventing the development of PIN in hormone stimulated Noble Rats. At this time we do not know whether this supplementation has resulted in delaying the progression of LGPIN to HGPIN or whether HGPIN formation has been completely suppressed in these animals. We stopped dietary antioxidant supplementation before inducing the animals with hormones to ensure that both the control and antioxidant groups received hormone stimulation under the same conditions. Yet a vast majority of the animals in the special diet group did not develop HGPIN suggesting that the antioxidants modified the prostate environment in a way to prevent the progression of LGPIN to HGPIN upon hormone stimulation. Our results also suggest that antioxidant intervention enabled the

environment not only to remove damaged cells through induction of apoptosis but also suppressed hormone-induced proliferation of prostatic epithelial cells. In addition it may modulate inflammatory signaling molecules including NFκB and Cox-2. The levels of testosterone or the ratio of testosterone to estradiol at the end of the study was not significantly different between animals on control vs. special diet (data not shown). None of the animals in any group were found to have gross abnormalities in kidney, bladder, seminal vesicle, prostate and liver. The data from this study clearly demonstrate the importance of an antioxidant combination in preventing the progression of precursor LGPIN to HGPIN in the noble rat model.

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**Manuscripts accepted:**


Ghosh, R., Schoolfield, J., Yeh, I-Tien., Smith, ML.,Hursting, SD., Chan, DC., Lucia, MS and Kumar, AP. 2009. Loss of NADPH Quinone Oxidoreductase (NQO1) in the prostate and enhanced serum levels of cytokine-induced neutrophil chemoattractant-2α (CINC-2α) in hormone stimulated Noble rats: potential role in PIN development. Translational Oncology (In press)


**Manuscripts under preparation/submitted:**

- Ghosh, R et al. Antioxidant combination blocks progression of prostatic intraepithelial neoplasia hormone stimulated Noble rats.
- Ghosh, R et al. Preprohaptoglobin as a potential prognostic marker for prostate cancer.