Chapter II

Protective Effects of Black Tea Extract on Testosterone Induced Oxidative Damage in Prostate
Chapter 2

Protective Effects of Black Tea Extract on Testosterone Induced Oxidative Damage in Prostate.

This chapter describes our studies to evaluate the role of androgens on oxidative stress in prostate of Wistar rats and its modulation by supplementation of aqueous tea extract (ATE). A detail description of our study is as follows:

2.1 Introduction and Rationale

Persistent generation of reactive oxygen species (ROS) such as superoxide (\(O_2^\cdot\)), hydrogen peroxide (\(H_2O_2\)), and hydroxyl radical (\('OH\)) is an inevitable consequence of mitochondrial respiration in aerobic organisms. It is known that low levels of ROS are required in the regulation of broad range of normal cellular responses, including proliferation and cell survival (Martindale & Holbrook, 2002; Burdon, 1995; Davies, 1999) via oxidative modifications of redox-sensitive transcripitive factors and intermediate signaling molecules. In contrast, induction of high levels of ROS subjects the cells to a state of oxidative stress (OS), which may damage cellular DNA, proteins and lipids and result in cell-cycle arrest, cellular senescence and cell death (Martindale & Holbrook, 2002).

The ROS are known to play a major role in either the initiation or progression of carcinogenesis by inducing oxidative stress (Sun, 1990). Peroxides and \(O_2^\cdot\) produce cytotoxicity/genotoxicity in cellular system (Perchellet & Perchellet, 1989). The source of \(H_2O_2\) in tissues is mainly through superoxide dismutase (SOD) medicated dismutation of \(O_2^\cdot\) generated in the tissues by endogenous enzyme system as well as by non-enzymatic pathways (Perchellet & Perchellet, 1989). In addition, the highly reactive hydroxyl radical (\(OH\)), generated from \(H_2O_2\) is known to damage DNA to produce the pathological alteration (Perchellet & Perchellet, 1989). A number of studies have suggested a link between oxidative stress i.e. an increase in prooxidant(s) and tumor development in many tissues (Katiyar & Mukhtar, 1996; Fieshner & Koltz, 1999; Cerruti, 1985). Studies have shown that following administration of chemopreventive agents, levels of antioxidant enzymes such as glutathione
peroxidase (GPx) and catalase (CAT) and phase II detoxifying enzymes, glutathione-s-transferase (GST) and quinone reductase (QR), are modulated (Laskin et al., 1992; Katiyar & Mukhtar, 1996; Fleshner & Koltz, 1999; Cerruti, 1985; Wilding, 1995).

Based on the recent studies, the roles of prostate hormonal environment and diet and nutrition have emerged as two major direction of research focus (Ho et al., 2004). Epidemiological studies and laboratory observation suggest an association between diet and levels of androgens in the body that may actually alter prostate cancer risk (Ripple et al., 1997). Androgens appear to play an important role in prostate carcinogenesis but their precise role is not clear. Higher concentration of androgen is associated with a shift in the prooxidant-antioxidant balance of the prostate towards more oxidative stress (Ripple et al., 1997a & b). Occasionally, these changes result in oxidant stress-mediated stimuli to specific changes of gene expression thereby resulting in dysregulated cell growth and therefore tumor development in prostate, and androgens play a major role in either the initiation or progression of CaP by inducing oxidative stress (Ripple et al., 1999; Tam et al., 2003a). ROS such as superoxide radicals, \( \text{H}_2\text{O}_2 \) and \( \cdot \text{OH} \) are shown to cause lipid peroxidation, thus altering the activity of sulfhydryl (SH-) depending enzymes and of damaging DNA and other critical cellular organelles. ROS associated oxidative damage is well documented in human prostate cancer (Ripple et al., 1997a; Tam et al., 2003b; Murakoshi et al., 1997) and down modulation of antioxidant enzymes in human prostate carcinoma cell lines viz. DU145 and LNCaP (Ripple et al., 1997a; Ripple et al., 1997b; Ripple et al., 1999). In addition, alterations in the redox balance of the cells have been shown to affect the activation of certain transcription factor and thus can control gene expression.

Because of the reasons described above, we determined the role of androgens in oxidative stress in prostate of Wistar rats and its modulation by supplementation of ATE.
2.2 Materials and methods

Chemicals

Testosterone, Phenozone methosulfate (PMS), 1-chloro-2,4-dinitrobenzene (CDNB), nicotinamide adenine dinucleotide phosphate reduced (NADPH), 2-thiobarbituric acid (TBA) nitro blue tetrazolium (NBT), nicotinamide adenine dinucleotide reduced (NADH), reduced glutathione (GSH) & oxidised glutathione (GSSG) were obtained from Sigma chemical company (USA) rest of the chemicals were of analytical grade of purity and procured locally.

Preparation of Aqueous Tea Extract (ATE)

Dry black tea leaves procured locally were suspended in appropriate concentration in hot water (70-80°C) for 15 min and allowed to cool to room temperature. 0.5, 1.0 and 1.5 % (w/v) ATE was prepared similarly. The extracted solutions were filtered through double-layered muslin cloth. The extracts were prepared fresh on daily basis.

Animal and Treatment

Male Wistar rats (~ 225 g b.w.) were obtained from ITRC animal colony and acclimatized for one week. They were randomly divided into six groups, each containing eight animals. Animals were kept under standard conditions (25 ± 2°C, relative humidity 57 ± 2 % and 12/12 hours light/dark phase) and were fed with synthetic pellet diet (Ashinwad, Chandigarh, India). Rats in groups I and II were fed with normal drinking water whereas animals in groups III, IV and V were given 0.5, 1.0 and 1.5% (w/v) ATE respectively, animals of group VI served as vehicle control and were given the 1.5% (w/v) ATE as sole source of drinking fluid ad libitum. The feeding regimen was followed for 15 days and on 16th to 20th days, rats in group II, III, IV and V received subcutaneous injections of testosterone (5mg/kg body weight dissolved in 100% ethanol and was diluted to 0.5 ml with corn oil). Group I and VI animals were injected with corn oil as vehicle control. Animals from all the groups were examined every week for gross morphological changes and body weight changes and fluid consumption during
Twenty-Four hours after final testosterone administration, all of the animals were sacrificed humanely by cervical dislocation and prostate tissues from each animal were excised and immediately snap frozen in liquid nitrogen and stored at –80°C until further use. For further experiments, the tissue was removed washed with ice cold saline and homogenized in ice cold phosphate buffer (pH 7.4) containing 0.15 M KCl and S-9 fraction was taken as the enzyme source.

Biochemical estimations to assess oxidative stress

The activity of CAT was analyzed according to the method of Sinha (1972) using H₂O₂ as substrate. In brief, the reaction in a final volume of 3 ml consisted of phosphate buffer (pH 7.0), 0.2 M H₂O₂ and enzyme protein (25-30 mg/ml). The enzyme activity was measured following the disappearance of H₂O₂ at 570 nm using a spectrophotometer and was expressed as μ moles/min/mg protein.

SOD was analyzed as per the protocol of Kakkar et al. (1984) the assay mixture in a final volume of 3 ml contained 0.052 M Sodium pyrophosphate buffer (pH 8.3), 186 μM PMS, 300 μM NBT, 780 μM NADH, sonicated enzyme source and water. The reaction was initiated by addition of NADH followed by incubation at 37°C for 90 seconds. After the incubation, the reaction was stopped by addition of 1.0 ml of glacial acetic acid and the contents were shaken rigorously with 4 ml of n-butanol, allowed to stand for 10 minutes, centrifuged and butanol layer was separated. The color intensity of chromogen in butanol was measured against butanol using a spectrophotometer. A reaction mixture devoid of enzyme served as control. The SOD activity was expressed as specific activity in units per milligram protein. As the assay was done in 90 seconds, the factor 2/3 was applied for calculating units. One unit was equivalent to enzyme concentration required to inhibit the optical density (at 560 nm) of chromogen formation by 50% in 1 min.

GST was analyzed as per the protocol of Habig et al. (1974) the assay mixture in a final volume of 3 ml contained 0.2 M Phosphate buffer (pH 6.5), GSH, CDNB, enzyme and water. The reaction was initiated by addition of CDNB.
The difference in optical density per 30 sec was measured at 340 nm for 3 min against a reference cuvette devoid of enzyme. The activity was expressed in 
moles/min/mg protein.

GR activity was measured by the protocol of Carlberg & Mannervik (1985), briefly the assay mixture in a final volume of 3.0 ml contained 0.067 M phosphate buffer (pH 6.6), NADPH, 7.5x 10^-3 GSSG (pH adjusted to 6.6 with 1 N NaOH) enzyme and water. The reaction was initiated with enzyme preparation. The difference in optical density per 30 sec was measured for 3 min at 340 nm against a reference cuvette devoid of GSSG and NADPH.

Lipid peroxidation was analyzed by the method of Chan et al. (1998). The reaction mixture in a final volume of 1 ml contained 0.1 M phosphate buffer (pH 7.4) 100 mM Ascorbic acid and 100 mM Ferric chloride. The reaction mixture was incubated at 37 °C for 1 hr in water bath, reaction stopped by addition of 10% TCA followed by 1 ml of 0.67% TBA. The mixture was placed in boiling water bath for 20 min and immediately shifted to crushed ice bath for 10 min. The mixture was centrifuged at 2500xg for 10 min. The amount of Malondialdehyde (MDA) formed was assayed by measuring OD of supernatant at 535 nm against a blank devoid of enzyme. The activity was expressed as n moles of MDA formed/hr/g of tissue at 37 °C using molar extinction coefficient of 1.56x10^5 M^-1 cm^-1. The protein content of the tissue was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Statistical Analysis

Significant difference of variance in antioxidant level data between positive control (Group II) and experimental groups (Groups III–V) was analyzed using student t-test and p<0.05 was considered to be significant.

2.3 Results

Little is known about the roles of androgens in the regulation of redox state in the prostate. In this study, our data demonstrated that subcutaneous administration of Testosterone resulted in a significant increase in the oxidative stress in prostate of Wistar rats (Figures 1-5). The administration of ATE was
Testosterone administration was found to induce the levels of GST up to 73%. The administration of ATE (0.5, 1.0 and 1.5%) effectively inhibited androgen induced GST activity by 22, 28 and 47%, respectively (Figure 1). Similarly, testosterone-mediated increase in CAT activity was found to be significantly inhibited (59, 67 and 75% by 0.5, 1.0 & 1.5% ATE administration respectively) (Figure 2). The LPO level (as evident from TBARS analysis) were found to be significantly induced by testosterone (56 fold); whereas, ATE administration resulted in 86, 91 and 98% inhibition of LPO formation at 0.5, 1.0 and 1.5% dose of ATE, respectively (Figure 3).

Testosterone administration was found to cause a 150% increase in the activity of enzyme GR in the rat prostate. A significant decrease in the induced levels of GR was found to occur at all the dosages of ATE tested (Figure 4).

Similarly, the levels of enzyme SOD was also found to be elevated up to an extent of 100% over untreated controls (Group I) following testosterone administration (Group II). ATE supplementation was found to inhibit the SOD activity in dose dependent manner (Figure 5).

2.4 Discussion

Persistent generation of ROS such as $O_2^-$, $H_2O_2$ and $OH$ is an inevitable consequence of mitochondrial respiration in aerobic organisms. It is now known that low levels of ROS are required in the regulation of a broad range of normal cellular responses, including proliferation and cell-survival. Induction of CaP by androgen is well documented (Ripple et al., 1997a & b; 1999). Limited treatment options and diagnostic approaches as well as poor treatment success rate make CaP, one of the leading causes of death. In this scenario, mechanism based approaches are needed to develop novel strategies for the treatment. Chemoprevention by naturally occurring compounds such as a commonly consumed beverage tea appears to be a practical approach to fight with prostate carcinogenesis (Sohal & Weindruch, 1996). In this study, we evaluated whether
Figure 1: A dose dependent inhibition of testosterone induced GST activity in rat prostate tissue by the administration of 0.5%, 1.0% and 1.5% ATE. Vertical axis represents the enzyme activity and horizontal axis shows different control and treated groups. Values are expressed as mean ± SE, of eight animals. * Represent significant ($p \leq 0.05$) suppression over controls (Group I and Group II) and ** represent significant ($p \leq 0.05$) induction in testosterone induced GST activity by ATE administration.
Figure 2: A dose dependent inhibition of testosterone induced catalase activity in rat prostate tissue by the administration of 0.5%, 1.0% and 1.5% ATE. Vertical axis represents the enzyme activity and horizontal axis shows different control and treated groups. Values are expressed as mean ± SE, of eight animals. * Represent significant (p ≤ 0.05) suppression over controls (Group I and Group II) and ** represent significant (p ≤ 0.05) induction in testosterone induced catalase activity by ATE administration.
Figure 3: A dose dependent inhibition of testosterone induced lipid peroxidation (TBARS) activity in rat prostate tissue by the administration of 0.5%, 1.0% and 1.5% ATE. Vertical axis represents the activity and horizontal axis shows different control and treated groups. Values are expressed as mean ± SE, of eight animals. * Represent significant (p< 0.05) suppression over controls (Group I and Group II) and ** represent significant (p≤ 0.05) induction in testosterone induced lipid peroxidation by ATE administration.
Figure 4: A dose dependent inhibition of testosterone induced glutathione reductase activity in rat prostate tissue by the administration of 0.5%, 1.0% and 1.5% ATE. Vertical axis represents the enzyme activity and horizontal axis shows different control and treated groups. Values are expressed as mean ± SE, of eight animals. * represent significant ($p < 0.05$) suppression over controls (Group I and Group II) and ** represent significant ($p \leq 0.05$) induction in testosterone induced glutathione reductase activity by ATE administration.
Figure 5: A dose dependent inhibition of testosterone induced superoxide dismutase activity in rat prostate tissue by the administration of 0.5\%, 1.0\% and 1.5\% ATE. Vertical axis represents the enzyme activity and horizontal axis shows different control and treated groups. Values are expressed as mean ± SE, of eight animals. * Represent significant (p≤ 0.05) suppression over controls (Group I and Group II) and ** represent significant (p≤ 0.05) induction in testosterone induced superoxide dismutase activity by ATE administration.
or not i) testosterone treatment causes oxidative damage in prostate and ii) tea polyphenols inhibit the development of CaP via decreasing testosterone-mediated oxidative damage in prostate.

Extensive studies have verified cancer chemopreventive effects of tea against many tumor bioassay systems (Siddiqui et al., 2004; Mukhtar & Ahmad, 2000; Yang, 1999; Shukla & Taneja, 2002; Javed & Shukla, 2000). In these studies tea has been shown to offer protection against all stages of multistage carcinogenesis that includes initiation, promotion and progression. A tea constituent EGCG has been shown to induce apoptosis in human prostate carcinoma cells DU145 (Ahmad et al., 1997). The present study supports our hypothesis that testosterone mediated induction of oxidative stress is an important contributor of development of CaP, and black tea is capable of inhibiting the development of prostate carcinogenesis by targeting at oxidative stress.

Androgens are essential for normal prostate physiology and have been shown to play a key role in the development and pathogenesis of CaP (Wilding, 1995). Thus we analyzed the effect of testosterone and ATE on modulations in oxidative enzyme system. The results of the present investigation have shown a significant increase in the levels of antioxidant enzymes in response to testosterone and a dose dependent inhibition by ATE (Figures 1-5).

These findings may open novel prospective in cancer chemoprevention, while mechanism of antioxidant activity of aqueous tea extract is not very clear, various factors seem to contribute to it. Taken together this study has demonstrated that ATE inhibits the androgen mediated oxidative injury in prostate. These data imply that antioxidant enzymes can be used as a target for studies on prevention of CaP and that aqueous tea merits further investigations for developing strategies against prostate carcinogenesis.
References


Chapter-II


