3-nitrotyrosine (3-NT) determination in tissues with prostate cancer and benign prostatic hyperplasia

Floriano-Sánchez E, Cárdenas-Rodríguez N, Castro-Marín M, Zapata-Villalba MA, Flores-Terraza JE, Torres-Salazar JJ.

ABSTRACT

Recent reports have placed prostate cancer (CaP) as the third cause of death by cancer in the world and as the principal cause in Mexico after skin cancer. The role of oxidative stress has been observed in cancer etiology. Due to the elevated incidence of prostate cancer in the Mexican population, it is of vital importance to contribute to the development of new hypotheses resulting in more complex studies and in this way increasing the probability of developing molecular markers for diagnosing cancer.

Objective: To evaluate the existence of any alteration in levels of 3-nitrotyrosine (3-NT), an oxidative stress marker, in prostate cancer and benign prostatic hyperplasia.

Materials and methods: Thirty benign prostatic hyperplasia tissue samples and forty prostate cancer tissue samples were obtained and conditions were standardized to immunohistochemically detect the presence of 3-nitrotyrosine in tissue.

Results: The percentages of immunoreactive 3-nitrotyrosine were 25.78% in prostate cancer tissue and 4.43% in benign prostatic hyperplasia tissue.

RESUMEN

Reportes recientes ubican al cáncer de próstata (CaP) en el tercer lugar mundial; en México es la principal causa de muerte después del cáncer de piel. En la etiología del cáncer se ha observado la función del estrés oxidativo en su desarrollo. Debido a la elevada incidencia del cáncer de próstata en la población mexicana, resulta de vital importancia contribuir con la generación de nuevas hipótesis que den lugar a estudios más complejos, lo cual incrementará las probabilidades de desarrollar marcadores moleculares para el diagnóstico del cáncer.

Objetivo: Evaluar la existencia de alguna alteración en los niveles de 3-nitrotirosina (3-NT), un marcador de la presencia de estrés oxidativo, en el cáncer de próstata y la hiperplasia prostática benigna.

Material y métodos: Se obtuvieron 30 y 40 muestras de hiperplasia prostática benigna y cáncer de próstata, respectivamente, y se estandarizaron las condiciones para detectar por medios inmunohistoquímicos, la presencia de 3-NT en los tejidos.

Resultados: Los porcentajes de área inmunorreactiva a 3-NT en el cáncer de próstata fueron de 25.78 ± 15.37% y 4.43 ± 2.2% en el cáncer de próstata y la hiperplasia prostática benigna, respectivamente.

1 Department of Biochemistry and Molecular Biology, Escuela Médico Militar. México City. 2 Neurochemistry Laboratory, Instituto Nacional de Pediatría. México City. 3 Urology Service, Hospital Central Militar, México City.

Corresponding author: Dr. Esaú Floriano Sánchez. Escuela Médico Militar. Cerrada de palomas S/N. Col. Lomas de San Isidro. México, D. F. Telephone: 5540-7728 Ext. 175, Fax: 52 5520-2121. Email: floriano_esau@yahoo.com
Conclusions: Densitometric data of 3-NT immunoreactivity in prostate cancer tissue were higher than those obtained in benign prostatic hyperplasia tissue, suggesting that 3-NT determination could be used as a tumor marker.

Key words: prostate cancer, benign prostatic hyperplasia, 3-nitrotyrosine, Mexico.

INTRODUCTION

Prostate cancer (CaP) is the second most common neoplasia after skin cancer in men and the second cause of death in men by cancer after lung cancer. Numerous factors are attributed to the increase of CaP. In the United States, more than 200,000 men are diagnosed with CaP annually and 30,000 die from the disease each year. After 50 years of age, CaP incidence is 3 or 4 times greater every 10 years. Afro-American men have the highest CaP incidence rate. Overall CaP incidence in this group has increased from 124 to 250 for every 100,000 men, representing an increase of 102%. Hispanic Americans have an intermediate incidence rate with 104 for every 100,000 men. Asian countries, especially Japan and China have one of the lowest CaP incidence and mortality rates in the world. In Japan from 1992 to 1995 CaP mortality incidence was 4 out of every 100,000 men. In Mexico in 1998 CaP incidence was in second place for tumors affecting men, after skin cancer. It also held second place in mortality in relation to all cancers, following cervical cancer.

CaP etiology is not completely understood. Presently, risk factors are considered to be the age of individual, androgen production and metabolism, geographic area/ethnicity, dietary habits and family history.

Benign prostatic hyperplasia (BPH) is histologically defined as a disease characterized by an increase in epithelial and stromal cells in the periurethral area of the prostate. It is a pathological process that contributes to the development of lower urinary tract symptoms in older men. Principal complications in BPH patients are complaints of the lower urinary tract such as nocturia, straining, reduction in strength and caliber of urinary stream and incomplete voiding sensation of the bladder.

BPH is the most common benign tumor in men and its incidence is age-related. BPH prevalence is age-dependent and the onset of its development is after 40 years of age. Its prevalence is 50% at around 60 years of age and 90% at 85 years of age. Although clinical evidence of the disease appears less frequently, lower urinary tract symptoms are also age-related. Approximately 50% of men histologically diagnosed with BPH present with moderate to severe lower urinary tract symptoms.

Like CaP, BPH is age-related and requires androgens in order to develop and grow. Unlike CaP, BPH is a benign lesion that rarely progresses into a malignant neoplasm. Another difference from CaP is the fact that there is absolutely no evidence of clonal activity in BPH. Only some genetic changes suggesting genomic stability have been found. Morphologic degrees in the nucleus that are characteristic of malignant neoplasms have not been detected in BPH. Varying stromal growths are often present in hyperplastic pathologies and it has been suggested that the secretion of growth factors by the mesenchyme could have an effect on adjacent epithelial cells and contribute to their developing hyperplasia.

It has been estimated that diet can be a contributing factor in 35% of all human cancers. There is consistent epidemiological evidence that low intake of antioxidants or low blood level of antioxidants can increase the risk of presenting with cancer. Free radicals are the principal components of the action mechanisms of smoking as well as chronic inflammation - two of the principal causes of cancer.
The critical role of oxidative damage has recently been associated with various clinical anomalies, including malignant diseases. Reactive oxygen species (ROS) can cause DNA oxidation and protein damage, damage to tumor suppressing genes and an increase in proto-oncogene expression. Cancer demonstrates a pro-oxidative change in the oxide-reduction state.

Nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) activity is one of the most important endogenous sources of ROS in the organism. This enzymatic system integrates with various proteins distributed between the cytoplasm and plasmatic membrane. During leukocyte activation the components located in the cytosol have to emigrate to the plasmatic membrane where the assembling of the function complex that makes up the active enzymatic system takes place.\(^\text{10}\)

The presence of oxygen is a vital requisite for the destruction and digestion of pathogenic agents by phagocytes but it is not necessary for phagocytosis itself. NADPH oxidase catalyzes the transference of an electron from NADPH to \(O_2\) by forming radical superoxide. Superoxide is rapidly converted into hydrogen peroxide, radical hydroxyl and hypochlorous acid.\(^\text{11}\)

Polymorphonuclear leukocyte activation is a characteristic event of the inflammatory process. There is an increase in nitric oxide (NO)\(^\text{-}\) caused by inducible nitric oxide synthase (iNOS) induction that takes place simultaneously with the inflammatory process. NO superproduction determines its reaction, mediated by diffusion with the superoxide anion, forming peroxynitrite (ONOO\(^-\)), a highly reactive species capable of oxidizing and nitrating cellular and tissular components such as tyrosine residues of cellular and plasmatic proteins. Structural and functional damage at the mitochondrial level that becomes irreversible due to oxidation and nitration of mitochondrial components is well-documented. ONOO\(^-\) also oxidizes and produces a decline in endogenous antioxidants such as ascorbate, glutathione and superoxide dismutase. The presence of ONOO\(^-\) and other reactive species derived from nitrogen (NRS) such as nitrogen dioxide (NO\(^2\)), extends the classic concept of oxidative stress to nitrosative stress.\(^\text{12}\)

Oxidative and nitrosative stress perpetuate the inflammatory process by different mechanisms. ROS and NRS have chemotactic effects favoring neutrophil recruitment. In addition, oxidative stress mediating molecules become intracellular messengers for transducing inflammatory process signals. ROS intracellular sources include complexes I and II of the electronic transport mitochondrial chain, peroxisomes, mono- and dioxygenase enzymatic systems and polymorphonuclear leukocytes. In the last few years it has been demonstrated that vascular cells under different physiopathological stimuli produce ROS and that the principal source of free radicals in these cells is a vascular oxidase that utilizes NADH and NADPH as reductive substrates for monoelectronic transference to molecular oxygen and the concomitant production of superoxide. In many respects, this vascular oxidase is similar to neutrophil NADPH oxidase even though their activation mechanisms are different. A particularly important aspect of NADH/NADPH vascular oxidase is that its activity is stimulated by angiotensin II, cytokines and oxidated lipoproteins through the stimulation of said vascular oxidase. The short half-life of NO\(^-\) and the existing delicate balance between its synthesis, utilization by normal biological targets or contrastingly, by reactions that mediate its decomposition, inactivation or secondary transformation into NRS, result in situations in which its availability is diminished, thus favoring and creating tissue damaging processes.\(^\text{12}\)

NO\(^-\) is a gas that is mildly soluble in some solvents and can spread fairly easily within biological membranes, but its solubility in water is low.\(^\text{13}\) NO\(^-\) is formed by the enzymatic conversion of the amino acid L-arginine (levogyre isomer) into L-citrulline through the action of nitric oxide synthase (NOS). However, it can also be formed from nitrites in oxygenated water. NO\(^-\) is also produced by the endothelium or by platelets and is synthesized in central nervous system neurons where it acts as a neurotransmitter.\(^\text{13}\)

In the peripheral nervous system NO\(^-\) is a mediator liberated by a wide nerve network previously known as non-adrenergic or non-cholinergic. These nerves mediate certain forms of neurogenic vasodilation and regulate certain gastrointestinal, respiratory and genitourinary functions. NO\(^-\) is also created in large quantities during host defense mechanisms. Such NO\(^-\) creation was first observed in activated macrophages that contribute to tumor cell and invading microorganism toxicity.\(^\text{13}\)

NO\(^-\) can also react with radical superoxide to produce ONOO\(^-\), which in turn can nitrate tyrosine, forming 3-nitrotyrosine. NOS is an enzyme that contains a hem group with a sequence similar to that of cytochrome P450 reductase. NOS forms homodimers that catalyze L-citrulline and NO\(^-\) formation from L-arginine utilizing oxygen and NADPH as substrates and flavin adenine mononucleotide (FMN), flavin adenine dinucleotide (FAD), tetrahydrobioperin (H4B), hem, Ca\(^2+\)/calmodulin and Zinc2+ as co-factors. NOS is expressed in almost all mammalian cells, both normal and neoplastic.\(^\text{13}\)
It is believed that high NO- levels may be cytostatic or cytotoxic for certain tumor cells and that in certain places where NO- levels are low, tumor growth could be promoted. 14

NO- and its metabolites interact with ROS to create potent nitrosative agents that lead to 3-nitrotyrosine formation. This is one of the well-known chemical modifications that take place during oxidative/nitrosative stress.16

NO- modulates ROS levels partly through its reaction with the superoxide anion. Proteins involved in the response to cellular stress are also important in oxidative damage modulation. Each component of the antioxidant system is specifically located in subcellular compartments.15

Because ROS, RNS and NO- or its metabolites can bring about peroxynitrite formation and thus protein nitration and because the altered cellular oxidoreduction state can promote neoplastic cellular state, the objective of the present work was to study the degree of protein nitration in CaP and BPH.

■ MATERIALS AND METHODS

Study subjects: Samples were collected from December 2006 to April 2008 from patients listed in the surgery register of the urology service of the Hospital Central Militar that met all inclusion, exclusion and elimination criteria.

1. Inclusion criteria: (a) patients diagnosed with CaP and lower urinary tract obstructive syndrome, and (b) patients diagnosed with BPH and indicated for transurethral resection of the prostate (TURP) or radical prostatectomy.

2. Exclusion criteria: (a) patients that did not give their consent to participate in the procedure, and (b) patients that were not TURP candidates.

3. Elimination criteria: (a) insufficient tissue quantity, and (b) tissue that underwent degradation during transport.

The quantity of tissue necessary for the study was 1-5 gr. Samples were obtained immediately after surgery. Maximum transport time was 1 hour from the time of obtaining the sample to its arrival at the Molecular Biology Laboratory of the Escuela Médico Militar. Samples were kept at a temperature of -70°C in a Revco® ultra-low temperature freezer (Legaci ULT2186 3-35 Dupont SUVA Refrigerants).

Immunohistochemistry: By means of light microscopy, CaP and BPH tissue samples were fixed by formalin immersion (pH=7.4) and embedded in paraffin. For histological analysis tissue sections (3 mm) were stained with hematoxylin and eosin (H&E). Tissue sections were stained with periodic acid-Schiff (PAS) to identify polysaccharides, mucopolysaccharides and glycoproteins of the cell membrane. Slices were incubated with periodic acid for 5 minutes and washed with distilled water. They were then incubated with Schiff reagent for 5 minutes and counterstained with hematoxylin for 30 seconds. The histological profile of 5 randomly selected fields (3 samples per patient) was registered using KS-300 software (Carl Zeiss, Jena, Germany), obtaining the percentage of damaged area with histopathological alterations (400x magnification). For immunohistochemistry, tissue sections (3 mm) were deparaffinized and heated to unmask antigenic sites. Endogenous peroxidase activity was blocked with 0.03% of H2O2 in absolute methanol. Tissue sections were incubated overnight at 4°C at a 1:200 dilution of monoclonal antibody against 3-NT in TRIS solution. Primary antibody was removed and sections were washed with TRIS twice. Slices were incubated at a 1:500 dilution of rabbit polyclonal antibody as a secondary antibody and washed twice with TRIS. Bonded antibodies were detected with the avidin-biotin complex (Vectastain ABC-kit) and diaminobenzidine as a substrate. After repeated washes with TRIS, slices were counterstained with hematoxylin. Immunostaining was comparable since all slices were incubated under the same conditions with the same antibody concentration and at the same time. All immunohistochemistry was examined under an Axiovert 200M optical microscope (Carl Zeiss, Jena, Germany). For the automatized densitometric analysis positive area percentage (brown color) was determined using a KS-300 3.0 computerized image analyzer (Carl Zeiss, Jena, Germany). This equipment automatically detects positive areas, determining their percentage by field. Five random fields were studied at a 100x magnification (1,584,000 m² total area). Results were expressed as percentage.

Data analysis: 3-NT immunoreactivity data were analyzed using the Student t test and data were expressed as mean ± standard deviation. CaP and BPH groups were compared and statistically significant difference was considered when P< 0.05. Statistical tests were applied using the Graph Prisma Version 3.32 program.

■ RESULTS

Biological samples were obtained from a total of 70 patients. Forty of those patients (57.14%) were diagnosed with CaP and 30 (42.85%) with BPH.
D 3-nitrotyrosine (3-NT) determination in tissues with prostate cancer and benign prostatic hyperplasia could promote local NO· creation. Other studies have demonstrated that protein nitration is increased in some tumors when compared with tumor-free areas.¹⁴

There is evidence that ROS and RNS alterations promote neoplastic growth since these reactive species are involved in signal transduction. This has important implications in biological function regulation and it has been suggested that NO· and protein nitration may have significant effects on tumor development and progression. On the other hand, in different pathological entities such as cancer, expression of the three isoforms of NO· synthases (inducible: iNOS, endothelial: eNOS and neuronal: nNOS) is increased. In 1998 Klotz T. Blochw et al suggested that iNOS increases NO·, playing an important role in tumor development and angiogenesis. These synthases have not been sufficiently studied in CaP. These enzyme concentrations appear to be low in benign prostate tissue and Blochhw et al conclude that epithelial iNOS expression can be used as a specific marker for CaP since to a certain extent NO· plays an important part in disease development. In 1999 Gradini R. et al lent support to this conclusion when they found an increase in iNOS expression in BPH tissue that was not observed in normal prostate tissue.¹⁶

In another study, Marangoni K. et al found that eNOS expression could play an important role in the carcinogenic process in prostatic tissue. NO· production by this enzyme can promote cancer progression by regulating selective proliferation of tumor cells by means of angiogenic stimulation in the organ.¹⁷ These results lend support to the findings of the present study in which differential concentration levels were observed, given that there was greater endothelial (glandular) cell nitration than in other areas of cancerous tissue and the difference was greater in BPH tissue when compared with CaP tissue. 3-NT concentration was 6-8 times higher in CaP.

Díaz Arce observed a relation between the number of mutations and RNS concentrations, lending support to the above conclusions.¹⁸ RNS can directly modify DNA through oxidation and deamination or base methylation processes and can indirectly modify it by suppressing DNA-repairing enzyme activity. Oxidation and deamination of nitrogenated DNA bases can cause transversions or transitions in such a way that these mechanisms can cause mutations in DNA, sequences that can participate in oncogene activation or suppressor gene inhibition of tumors or both, favoring tumor development or progression.¹⁸

In 2007 Cronauer et al demonstrated that chronic inflammation increased the risk of developing different

---

**DISCUSSION**

In the present study, protein damage (protein nitration) was determined and analyzed in prostate cancer (CaP) and benign prostatic hyperplasia (BPH) tissue. To ensure adequate methodology the tumor focus was first determined as well as BPH characteristic areas using monoclonal antibody against 3-NT to quantify 3-NT concentration (immunohistochemistry) in CaP and BPH tissue.

In previous studies, Masrri et al verified alterations for NO· and its metabolites in lung cancer and showed that NO·, nitrites and nitrotyrosine are elevated in patients with lung cancer. This is consistent with findings in the present study in which there was greater protein nitration in CaP tissue than in BPH tissue. Glandular tissue was the histological area (tumor focus in CaP) analyzed and there was an obvious increase in immunoreactivity as can be seen in Image 2. Masrri et al stated that the 3-NT increase was limited to the tumor and thus to the tumor process and that acidosis together with the greatly reduced tumor environment

---

**Image 1.** 3-NT determination in CaP and BPH. 3-NT immunoreactive area percentage values by field in CaP and BPH are represented on the y-axis and CaP and BPH study groups are represented on the x-axis. The graph shows that the marked area (immunoreactive) for 3-NT is much greater in CaP tissue when compared with BPH tissue. CaP, n=40 and BPH, n=30; P<0.001. Values are represented as mean ± standard deviation.

---

The percentage of area immunoreactive for 3-NT was 25.78 % in CaP and 4.43 % in BPH, (P<0.001) (Images 1 and 2).
types of cancer, including prostate cancer. Their study showed that iNOS intervenes in the development of and increase in the inflammatory process, given that NO- production is increased by this enzyme, paving the way for the presence of acute or chronic inflammation, including the presence of autoimmune diseases or tumorigenesis. They observed that in relation to tumor development, there was an increase in protein nitration that could be influential in an increase in acute as well as in chronic inflammatory processes. These processes may participate in neoplasia etiopathogenesis. However, in the literature, the role of iNOS activity is not well understood in the majority of diseases and data produced in different studies is still not consistent.

In this same study, through CaP biopsy and immunohistochemistry, iNOS protein expression in tumor cells was found to have a solid connection with 3-NT concentration, indicating that it plays an important role in tumor process, development and progression. In the present study there was an obvious increase in protein nitration in the tumor tissues studied. That increase was even more apparent in glandular tissue, making the findings of the present evaluation consistent with those in the literature. Cronauer et al have suggested that iNOS intratumoral activity favors cell development and that these cells are capable of proliferating independently, thus promoting the development of neoplastic cells.

Lastly, S. Baltaci et al reported that NO- increase produced by iNOS could be involved in tumor growth in prostate cancer by at least two mechanisms: angiogenesis stimulation and increased mutations in DNA through the direct action of free radicals.

**CONCLUSIONS**

Protein nitration, and consequently its alteration, was much more apparent in prostate cancer tissue than in benign prostatic hyperplasia tissue when tissues from patients presenting with these diseases were compared.

**BIBLIOGRAPHY**


