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## Original Article

# Immunoelectron microscope localization of androgen receptors and proliferating cell nuclear antigen in the epithelial cells of albino rat ventral prostate



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## ABSTRACT

Androgen receptor (AR) and proliferating cell nuclear antigen (PCNA) play a crucial role in development and progression of various prostatic diseases including prostatic carcinoma that is a leading cause of death in males. Previous studies have evaluated the expression pattern of AR and PCNA in prostate epithelial cells using immunohistochemistry (IHC). However, this technique has limited ability to identify their precise subcellular localization. Therefore, the aim of this study was to localize, subcellularly, AR and PCNA in the secretory epithelial cells of rat ventral prostate using post embedding immunogold–electron microscopy. The ventral lobes were dissected from six adult male albino rats after being perfused with paraformaldehyde. Some specimens were immuno-labeled with AR or PCNA and others were processed for immunoelectron microscope of AR and PCNA using 15-nm gold conjugated secondary antibodies. The results showed that, by immunoperoxidase reaction, AR and PCNA were localized diffusely throughout the nuclei of the epithelial cells of prostatic acini without visible cytoplasmic expression. However, the higher resolution immuno–electron microscopy was able to detect AR and PCNA in the nucleus and some cytoplasmic organelles. In conclusion, this study emphasizes the importance of immunoelectron microscopy in precise localization of AR and PCNA at the subcellular levels in the secretory epithelial cells of the rat prostatic acini. These findings will help to further understand the mechanism of action of these receptors under normal and pathological conditions that could have future clinical application after careful human investigation.

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## 1. Introduction

The prostate gland is the largest male accessory gland that depends on different hormones such as androgens, estrogens and prolactin. The testosterone is the main

androgen that is very important for the development of prostate and maintenance of its structural and functional integrity. Subtle change in the testosterone level is usually accompanied by alterations in the growth and weight of the prostate [1]. In the cytoplasm of target cells, testosterone binds to the androgen receptor that undergoes structural change to allow nuclear translocation of the testosterone–AR complex. In the nucleus, AR bind to androgen response elements promoting transcription of androgen-responsive genes, which control a range of cellular events such as growth, differentiation and apoptosis [2].

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However, in several cell types as prostate cancer cells [3], breast cancer cells [4], osteoblasts [5] and neuronal cells [6], additional non-genomic androgen actions have been reported, including the rapid activation of kinase signaling cascades, modification of the cytoskeleton and modulation of intracellular calcium levels.

In normal adult prostate, the epithelial cells are continuously turning over. In this self-renewing condition, the rate of prostatic cell death is balanced by an equal rate of prostatic cell proliferation. As a result, neither involution nor overgrowth of the gland normally occurs [7]. Disturbance of this balance is responsible for development of severe medical conditions including prostatic carcinoma which is considered as the second leading cause of cancer-related deaths in males [8]. Therefore, it is important to study the expression of proliferation and apoptotic markers in the prostate gland as early indicators to disease development or therapeutic outcome. Among these proliferation markers is the proliferating cell nuclear antigen (PCNA), which is known as cyclin, that encircles the DNA in the nucleus. It acts as a cofactor for DNA polymerase delta which controls premitotic DNA duplication, DNA repair, replication, post-replication modifications and chromatin assembly [9]. PCNA is currently used as an objective and quantitative measure to diagnose malignancy and to assess the efficiency of cytotoxic and chemo-preventive drugs in cancer research [10,11].

The current study aimed to use immuno-electron microscopy to allocate the AR and PCNA, at the subcellular level, in the secretory epithelial cells of rat ventral prostate which could potentially be used to understand their possible role in different prostatic diseases.

## 2. Material and methods

### 2.1. The animals

This study was carried out in Glasgow University in accordance with the European Communities Council Directive (86/609/EEC) and with the terms of a project license under the UK Scientific Procedures Act (1986). Six adult male albino rats with an average body weight of 225–250 g were allowed to acclimatize for a week prior to the start of the study with a controlled environment (12 h light/12 h dark cycle) and free access to standard laboratory granulated solid Purina rodent show (CRM Diet, Labsure, Pool, UK) and tap water. Rats were deeply anesthetized with sodium pentobarbitone (30 mg/kg, intraperitoneal) [12] and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer solution (PBS) (pH 7.2). The ventral prostate lobes were dissected and immersed in the same fixative solution for 2 h at 4 °C, followed by 30% sucrose in 0.1 M PBS (w/v) until the time of processing.

### 2.2. Immunohistochemistry

Pieces of prostatic ventral lobes were rinsed with 0.1 M PBS and dehydrated through an ascending grades of ethanol then were cleared by amyl acetate using automatic tissue processor (Reichert-Jung, Germany). The tissues were imbedded in paraplast and 5- $\mu$ m sections were cut

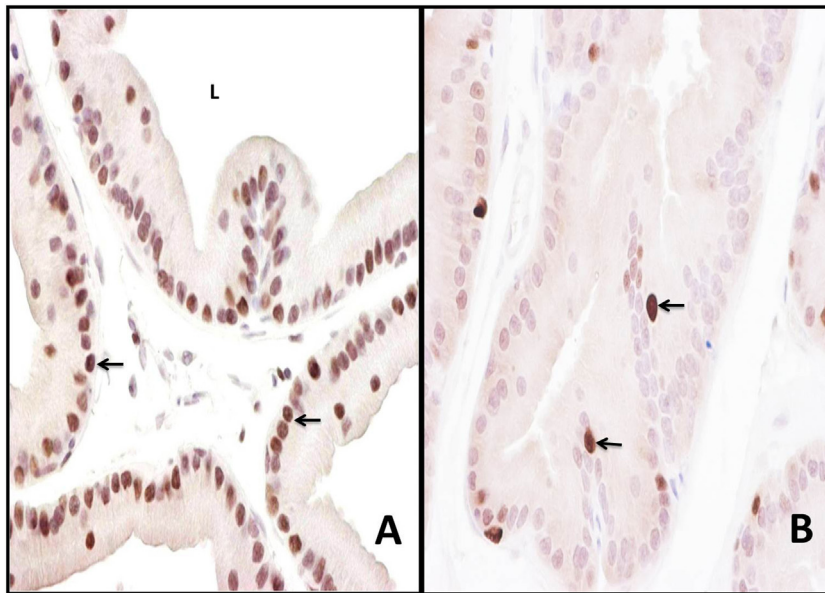
using microtome and were collected on coated slides for immunohistochemistry. Antigen retrieval was performed using 0.1 M sodium citrate buffer (pH 6.0) for 5 min in pressure cooker and the endogenous peroxidase was blocked by 3% hydrogen peroxide (VWR) in methanol for 30 min. After that, the sections were treated with 5% bovine serum albumin (Sigma) and 20% normal goat serum (Sigma) in 0.05 M tris-buffer saline as a blocking buffer for 1 h at room temperature and incubated overnight at 4 °C with the primary antibodies; mouse anti-PCNA (Abcam-29) and rabbit anti-AR (Santa Cruz Biotechnology-816) diluted in a blocking solution (1:1000 and 1:100 respectively). Sections were then washed in 1% TBS-tween-20 and TBS, incubated for 1 h at room temperature with goat anti-mouse and goat anti-rabbit biotinylated secondary antibodies (Jackson) diluted (1:200) in the blocking buffer solution. Avidin-Biotin-Complex (Vectastain ABC kit, Elite-PK-6100 Vector Labs) was added to the sections for 30 min followed by washing in TBS. The 3,3-diaminobenzidine (DAB) was used (Vector Laboratories) with Mayer's hematoxylin as a counter stain before dehydrating the sections and mounting with DPX to be imaged using CCD camera (Axiocam HRc, Zeiss, Germany) mounted on the light microscope (Eclipse 800, Nikon, Japan). To test for any non-specific reaction, the primary antibody was replaced by blocking solution in the negative control sections.

### 2.3. Immuno-electron microscopy

Small pieces of ventral prostate were washed thoroughly with 0.1 M PBS and dehydrated in graded ethanol, then were infiltrated with 2:1 LR white acrylic resin (medium grade) (London Resin Company/Hampshire, UK) diluted in 70% ethanol for 1 h followed by further infiltration in 4 changes of pure LR white resin. The specimens were polymerized at 55 °C for 48 h in the bottom of closed gelatin capsules filled with fresh LR white resin. Ultrathin sections (70 nm) were picked on dull (bright, coated) sides of 200 mesh nickel grids and were processed for post-embedding immunogold labeling. After incubation on drops of blocking buffer (5% bovine serum albumen, 20% normal goat serum and 0.1% Triton-X100 in TBS) for 1 h to block nonspecific binding, the sections were incubated with primary antibodies (mouse anti-PCNA and Rabbit anti-AR) diluted 1:10 in blocking buffer in a humidified chamber overnight at 4 °C. The ultrathin sections were washed on frequent drops of TBS with 0.1% Triton-X100, before applying the secondary antibodies; 15-nm gold labeled (goat anti-mouse) and (goat anti-rabbit) (British Bio Cell International, Cardiff, UK) diluted (1:25) in blocking buffer for 2 h at room temperature. The grids were counterstained with uranyl acetate and lead citrate for examination and photography with transmission electron microscope. The specificity of the immunoreactions was assessed by omitting the primary antibodies from the labeling protocol [13].

## 3. Results

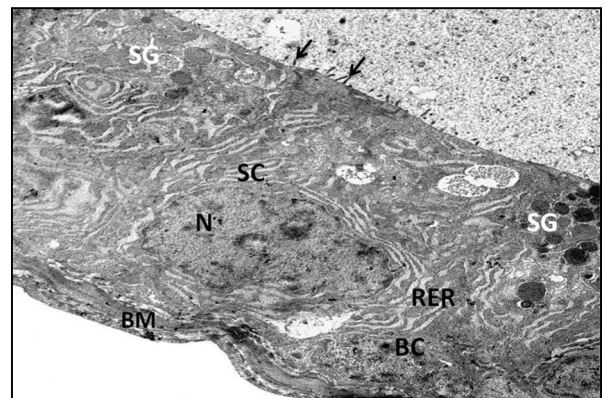
Immunohistochemistry of AR and PCNA in the prostatic acini of the rat ventral prostate showed a positive



**Fig. 1.** Immunohistochemistry of AR and PCNA in prostatic acini of rat ventral prostate. A: nuclear expression of AR (arrow). B: nuclear expression of PCNA (arrow) in few secretory epithelial cells of prostatic acini. L, lumen; S, stroma ( $\times 400$ ).

nuclear immunoreactivity in the secretory epithelial cells with no cytoplasmic expression. The majority of the secretory cells were immune-positive for AR, while few cells were immune-positive for PCNA (Fig. 1). The specificity of the antibody reaction was verified by the absence of immunostaining in the negative control sections either by the light or the electron microscopes. Electron microscope imaging showed that the prostatic acini were lined with secretory columnar epithelial cells which were intermingled with basal flat cells. These secretory cells were characterized by basally located oval shaped nuclei (showing homogenous nuclear chromatin with sites of higher condensation near the nuclear periphery) with clearly visible nuclear envelope and nucleolus. Whereas, their cytoplasm was composed of; granular endoplasmic reticulum that was formed of numerous flattened and parallel cisterns located in the perinuclear and basal regions of the cell, rounded secretory granules observed in the apical cytoplasm with multiple microvilli covered the apical surface facing the lumen, while the basal surface rested on the basement membrane (Fig. 2).

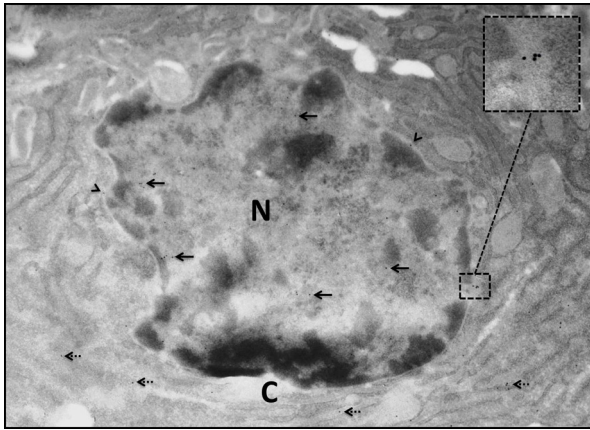
Immunoelectron microscopy of androgen receptor revealed the presence of gold particles-labeled AR in the nuclei as well as in the cytoplasm of the secretory epithelial cells. AR was distributed in all nuclear compartments including; the nuclear membrane, the nuclear pore, the nucleoli and over the chromatin (peripheral condensed and extended chromatin) with different distribution patterns. The gold particles were either freely distributed or aggregated together in the periphery of the nucleus (Figs. 3 and 4). In the cytoplasm, the gold particles were scattered near the nuclear membrane, mainly close to the rough endoplasmic reticulum as well as over the mitochondrial matrix and membranes (Fig. 5).



**Fig. 2.** Electron photomicrograph of a part of a rat ventral prostatic acinus showing secretory columnar epithelial cells (SC) and basal flat cells (BC), both resting on the basement membrane (BM). The secretory epithelial cell shows; basal nucleus (N), parallel cisternae of rough endoplasmic reticulum (RER), apical secretory granules (SG) of varying electron density and apical microvilli (arrow) are projecting in the lumen. L, lumen; S, stroma (TEM,  $\times 2000$ ).

Furthermore, immune-electron microscopy of PCNA showed subcellular localization of gold particles-labeled PCNA in nuclear and different cytosolic structures. Within the nucleus, the gold particles were scattered over the nuclear membrane, nuclear pores, peripheral heterochromatin and the nucleolus. Whilst, in the cytoplasm, the gold particles were dispersed in-between the cisternae of rough endoplasmic reticulum, in the mitochondria and in the apical cytoplasm in-between the secretory granules (Fig. 6).





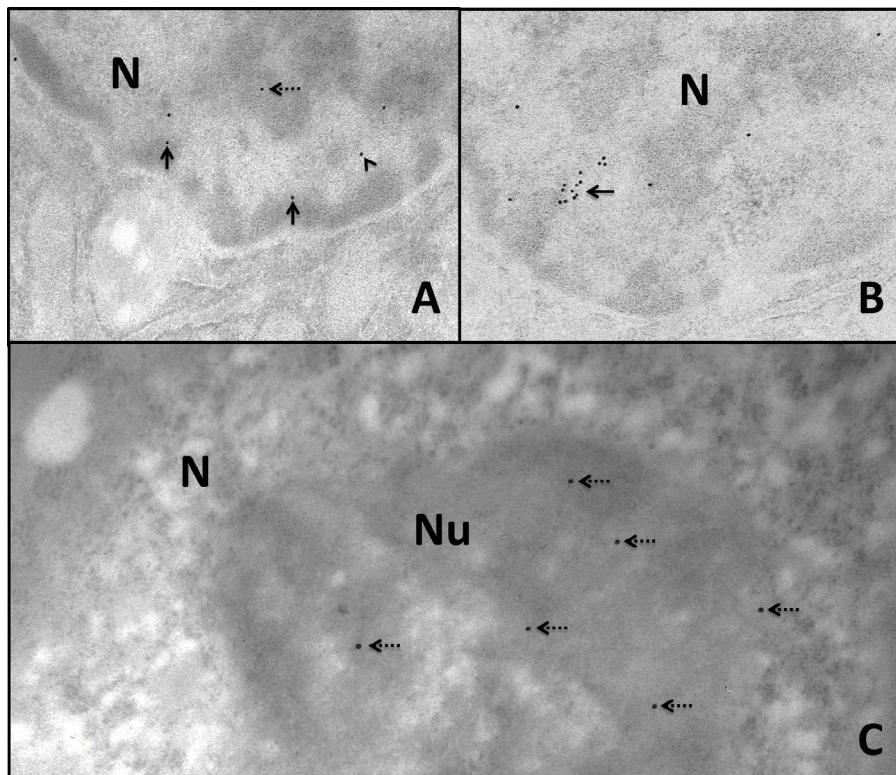
**Fig. 3.** Immunoelectron photomicrograph of AR in a secretory epithelial cell of a rat ventral prostatic acinus showing immunogold particles distributed in the nucleus (N) (arrow), nuclear membrane (arrow head) and in the nuclear pore (inset). AR is also detected in the cytoplasm (C) (dashed arrow) close to the rough endoplasmic reticulum. (TEM,  $\times 13\,000$  – inset,  $\times 33\,000$ ).

#### 4. Discussion

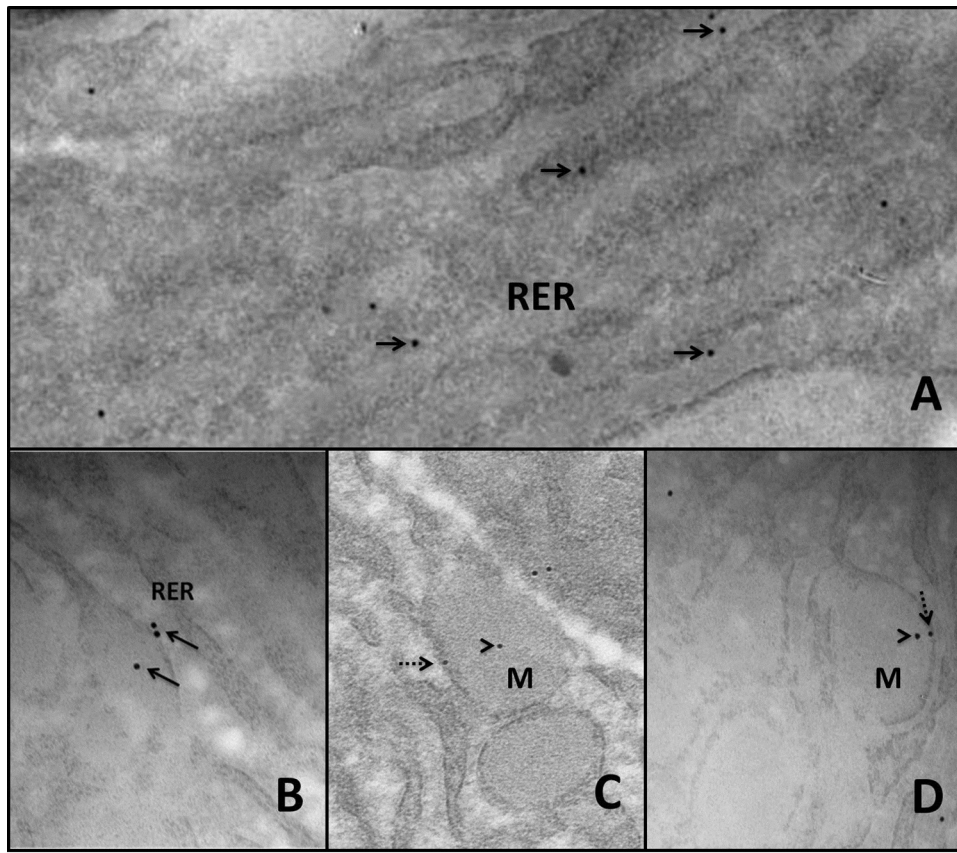
Immuno-electron microscopy means using antibodies for intracellular localization of particular protein under electron microscopy. This can be technically challenging,

expensive and requires hard optimization of tissue fixation and processing methods. However, the use of the secondary antibodies conjugated with gold particles allows high resolution detection and localization of antigen in the cells and tissues which offers additional insights into the structure–function relationships. The two most widely used techniques for immune-electron microscopy are; the pre-embedding and the post-embedding immunogold labeling. Although the sensitivity of the post-embedding methods is thought to be much lower than that of the pre-embedding labeling, post-embedding labeling is more practical and economic, as the resin-embedded materials can be reused for testing various antibodies with different dilutions [14]. Additionally, LR-white resin was used in this study as an embedding medium because it is hydrophilic (Acrylic) resin, ready to be used, less toxic, more permeable to aqueous solutions. It is also polymerized under low temperature or even under UV light that allows preservation of the ultrastructure and maintains the immunogenicity of the cells with clear localization of antibodies signals [15]. Moreover, using 15 nm gold particles is optimum for the post-embedding immunogold labeling to be visualized under transmission electron microscopy with no need for silver enhancement technique [16].

In this study, prostatic tissues were first immunostained with anti-AR and anti-PCNA antibodies using immunohistochemistry techniques in order to test the specificity of



**Fig. 4.** Immunoelectron photomicrograph of AR in a secretory epithelial cell of a rat ventral prostatic acinus showing the different distribution patterns of gold particles-labeled AR in the nucleus (N) of secretory epithelial cell. A: the immunogold particles are broadly distributed in the peripheral heterochromatin (arrow), euchromatin (arrow head) and nucleolar associated chromatin (dashed arrow). B: the immunogold particles are aggregated in the periphery of the nucleus (arrow). C: the gold particles are scattered across the nucleolus (Nu) (dashed arrow). (A & B:  $\times 10\,000$ ; C:  $\times 33\,000$ ).



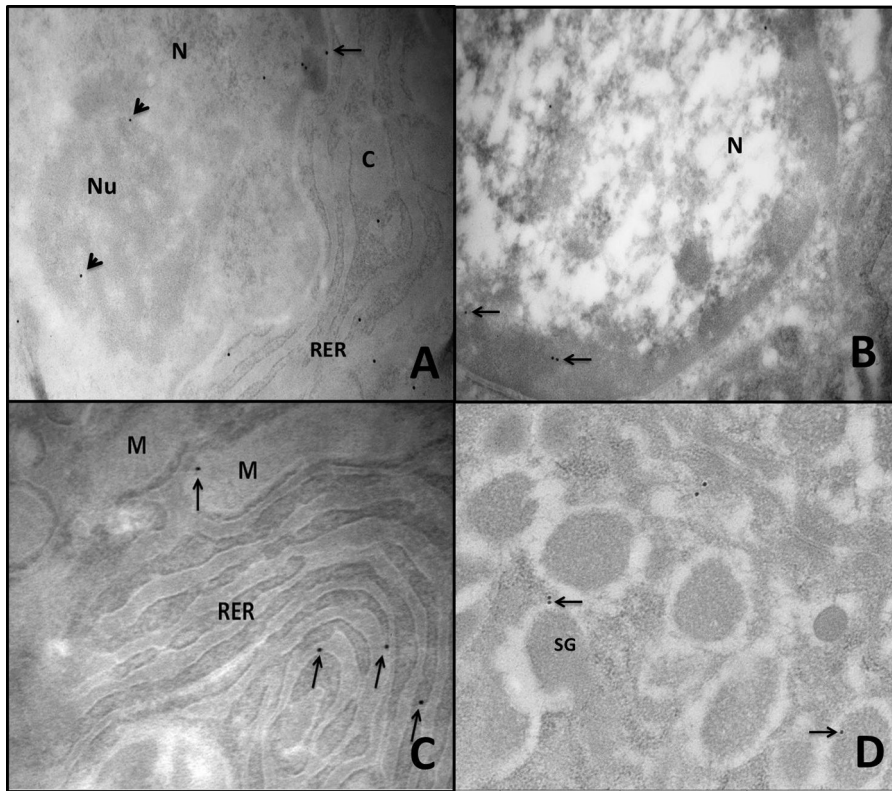
**Fig. 5.** Immunoelectron photomicrograph of AR in a secretory epithelial cell of a rat ventral prostatic acinus showing the different distribution patterns of gold particles-labeled AR in the cytoplasm of secretory epithelial cell. A & B: the gold particles are distributed over the rough endoplasmic reticulum (RER) (arrow). C & D: the gold particles are localized inside the mitochondria (M) (arrow head) as well as on the mitochondrial membrane (dashed arrow). (A & B:  $\times 66\,000$ ; C & D:  $\times 33\,000$ ).

the primary antibodies and to optimize dilution of antibodies for immune-electron microscopy which is usually 1/10 of that used for immunohistochemistry. Moreover, to show the expression pattern of these proteins at the light microscope level. Immunohistochemistry revealed that AR and PCNA are predominantly localized in the nuclei of the secretory epithelial cells. However, the immunoelectron microscope staining showed their existence in the nucleus as well in the cytoplasm of the secretory epithelial cells. This was in agreement with Zhuang et al. [17], who used pre-embedding immunogold-silver enhanced methods for subcellular localization of androgen receptors in both ventral prostate and seminal vesicle. They also showed that most of the silver enhanced gold particles were located over the cell nuclei with the most intense labeling in heterochromatin. Similarly, Soeffing and Timms [18] located AR in the cytoplasm and nuclei of the prostate secretory and basal epithelial cells. The cytoplasmic and the nuclear localization of AR was proposed to be the result of its cytoplasmic-to-nuclear relocalization which depend on the testosterone. This was supported by remaining of AR in the cytoplasm with export of unloaded AR from the nucleus to the cytoplasm, where it is transcriptionally inactivated, in absence of testosterone. These findings can

help understanding the potential biological role of androgen receptors in the cytoplasm [2,19,20].

PCNA was originally identified as a nuclear antigen that is expressed in the cells during DNA synthesis phase of the cell cycle. In this study, PCNA was detected in the nuclei of the secretory epithelial cells by using immunohistochemistry and immuno-electron microscopy. This was in settlement with Baran et al. [21] who localized PCNA throughout the pronuclei of fertilized mouse eggs using post-embedding immunogold methods on ultrathin cryosections. Furthermore, in this study, immunogold labeling of PCNA revealed its distribution in the cytoplasm of the secretory epithelial cells. This was identified also by Grzanka et al. [22], who revealed positive nuclear (mainly in the heterochromatin) staining for PCNA in biopsies taken from patient with laryngeal squamous cell carcinoma with no expression was detected in the nucleoli. The same authors showed that gold particles-labeled PCNA was detected throughout the cytoplasm, mainly on rough endoplasmic reticulum and ribosomes. Similar results were reported by Bouayad et al. [23], who showed the cytoplasmic localization of PCNA in neutrophils which was proposed to be the result of its nuclear-to-cytoplasmic relocalization during its differentiation to inhibit neutrophil





**Fig. 6.** Immunoelectron photomicrograph of PCNA in a secretory epithelial cell of a rat ventral prostatic acinus showing the different distribution patterns of gold particles-labeled PCNA in the secretory epithelial cell. A: the gold particles are detected in the nucleus (N), mainly in the nuclear pore (arrow), the nucleolus (Nu) (arrow head). B: Immunogold particles are detected on the peripheral heterochromatin. C: In the cytoplasm, the gold particles (arrow) are distributed over the rough endoplasmic reticulum (RER), and the mitochondria (M). D: The immunogold particles (arrow) are present in the apical cytoplasm between the secretory granules (SG). (A, B & D:  $\times 33\,000$ ; C,  $\times 46\,000$ ).

apoptosis. The same expression pattern was reported in the fibroblast in which a selective delocalization of nuclear PCNA to cytosol was observed after serum starvation, suggesting that PCNA nuclear-to-cytoplasmic relocalization might play a role in different cells differentiation. Additionally, Naryzhny and Lee [24] revealed that, a substantial amount of PCNA in the cytoplasm is associated with several cytoplasmic oncoproteins, such as elongation factors, malate dehydrogenase and regulators of glycolysis pathway.

## 5. Conclusion

This study showed that regular immunohistochemistry techniques may not be sufficient for precise subcellular localization of AR and PCNA as well as this study highlights the importance and feasibility of immunogold electron microscope as a reliable alternative. The immunohistochemistry was able to detect AR and PCNA in the nucleus only, but the immunogold electron microscope showed not only additional cytoplasmic expression of both receptors but also showed their subnuclear and subcytosolic localization in the secretory epithelial cells of the prostatic acini. The exact localization of receptor site will ultimately help understanding its exact mechanism of action and its dynamic inside the cells during healthy and diseased

condition. Therefore, these findings could potentially help in early disease diagnosis, mentoring therapeutic outcome and could have future clinical application after careful investigation.

## Conflict of interest

None declared.

## Acknowledgements

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