

## The effects of prepubertal epididymal ligation upon androgen receptor distribution in the rat caput epididymis

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**ABSTRACT:** The aim of the present study was to investigate the androgen receptor (AR) distribution in epididymal cells of developing rats and the effects of prepubertal epididymal obstruction upon AR distribution in the rat caput epididymis. At 15 days of age, the young rats were divided at random into groups for epididymal ligation or sham operation. In the ligation group the corpus epididymides were ligated bilaterally; in the sham group only laparotomy was performed. Both groups were sacrificed at 21, 56, 90, 120 days. The epididymes were removed, fixed in Bouin's fixative and embedded in paraffin wax. The tissues were sectioned at 5 µm and stained using the microwave stimulated antigen retrieval technique for immunohistochemistry. The features of the immunohistochemical staining of caput epididymal cells for the AR were similar across both groups. The operation did not affect AR distribution in caput epididymis. Positive immunohistochemical staining for the AR appeared in nuclei but not in the cytoplasm of caput epididymal cells at all ages beginning from 21 to 120 days old. The staining intensity of AR-positive cells did not change depending on age. In the caput epididymis, immunostainable AR were found in tubular epithelial cells (principal cells, basal cells and apical cells) and interstitial stromal cells (peritubular smooth muscle cells). There were no significant histological alterations in epididymal epithelium.

**Keywords:** androgen receptor; immunohistochemistry; epididymal ligation; epididymis; rat

The mammalian epididymis is a complex organ, whose many functions such as absorption of testicular fluid or secretion of proteins, promote the maturation and storage of spermatozoa produced in the testis (Ungefroren et al., 1997). The epididymis is histologically divided into three major parts: the caput, the corpus and the cauda. The caput epididymis is formed of efferent ducts and an epididymal duct. The structure of the ductus epididymis is comprised of highly coiled ducts which have a big lumen and a thick wall and lie embedded in collagenous connective tissue. The epithelium of the epididymis is composed of principal, basal, apical and clear cells (predominant in the tail of the epididymis; Ungefroren et al., 1997; Abraham and Kierszenbaum, 2007; Moonjit and Suwanpugdee, 2007).

Early studies established that the epididymis requires androgens for its prenatal and postnatal differentiation (Brooks, 1979; Orgebin-Christ et al., 1996) and maintenance of epithelial structure (Maneenly, 1959). Androgens reach the epididymis via the blood supply and intraluminal fluid (Turner et al., 1984). Androgen effects are principally mediated by the androgen receptor, a member of the steroid hormone receptor superfamily (Carson-Jurica et al., 1990). This nuclear transcription factor, on binding to androgen, becomes competent for binding DNA and of stimulating androgen-dependent gene transcription (Zho et al., 1994; Bardin et al., 1996). The presence of androgen receptors has been demonstrated in epididymal cells from several species using biochemical (Tindall et al., 1975; Carreau et al., 1984a,b; Tekpetey et al.,

1989; Takeda et al., 1990; Paris et al., 1994; Zhou et al., 2002) as well as molecular genetic approaches (Cooke et al., 1991; Blok et al., 1992; Viger and Robaire, 1995).

Obstruction of the male ducts has frequently been studied at the ductuli efferentes. Ligation of the efferent ducts has been shown to have no effect on the androgen receptor concentration, suggesting an exclusive dependence of this concentration upon the androgens reaching the epididymis via the blood supply rather than androgens arriving at the epididymis with the intraluminal fluid (Pujol and Bayard, 1979).

Ligation of the ductus deferens during vasectomy may affect the microenvironment of the epididymis through mechanical pressure on the epithelial cells, affecting their function directly, or by causing cessation of flow in the tubule, thereby decreasing delivery of required testicular factors and androgens (Johnson and Howards, 1975). In the same way, ligation of the corpus epididymis may result in the same altered androgen receptor distribution in the caput epididymis. The aims of the present study were to determine AR distribution in the epididymal cells of developing rats and to establish whether prepubertal epididymal obstruction affects androgen receptor distribution in the rat caput epididymis.

## MATERIAL AND METHODS

Wistar rat pups (thirty two in number) were obtained by purchasing mother rats with litters of 12-day old young. The animals were kept in an air-conditioned room with a 12 : 12 hour light/dark cycle and given food and water *ad libitum*. When the pups reached 15 days of age, they were divided at random into groups for epididymal ligation or sham operation.

### Surgical procedures and fixation

All 15-day old rats were anaesthetized by an intraperitoneal injection with 50 mg/kg ketamine under local antiseptic conditions. In the ligation group, after small vertical midline abdominal incisions, the testis and epididymes were bilaterally exposed, then the narrowest region of each epididymis, approximately at the midpoint of the corpus, was ligated twice with 6–0 silk. The same incision

and procedure was performed in the sham group except for the ligation of the epididymides. After operation, the pups were returned to their mothers, who suckled them and cared for them normally until the pups were weaned at the age of 21 days. Pups were observed at weekly intervals postoperatively to ensure that the testes descended normally.

Five rats in the epididymal ligation group and three sham-operated rats were killed at 21, 56, 90, and 120 days. Before being killed all male pups were anaesthetized with ethyl ether. The caput epididymides were taken out rapidly and fixed in Bouin's fixative for 36 h at 4 °C and then dehydrated in ethanol, cleared in xylene, and embedded in paraffin wax.

### Immunohistochemistry

5 µm thick sections were cut, mounted on poly-L-lysine-coated slides and heated in an oven at 60 °C for 1 h to promote adherence to the slide. The sections were dewaxed in xylene and then rehydrated in descending grades of ethanol. Endogenous peroxidase was blocked by 15 min incubation in 3% H<sub>2</sub>O<sub>2</sub> in methanol.

An antigen retrieval step was performed by heating the sections, immersed in 0.01M citrate buffer at pH 6.0, four times for 5 min in a 600 W microwave oven. After heating, the material was left to cool down to room temperature, after which the slides were washed in phosphate buffered saline (PBS).

Immunohistochemistry procedures were performed using the PG21 antibody. Its use as a valid immunological probe for the AR in a variety of species, including human and rat, has been previously established (Prins et al., 1991; Suarez-Quian et al., 1996, 1997, 1998).

Immunocytochemistry was performed the using avidin-biotin (ABC) technique. After antigen retrieval, sections were incubated with 10% nonimmune goat serum for 10 min at room temperature to block nonspecific binding. Later, sections were incubated (4 °C, 16–20 h) in a humidified chamber with the AR-specific antibody PG21 [Rabbit Anti-Rat/Human Androgen Receptor Polyclonal Antiserum (Millipore Corporation)] diluted 1 : 50 in PBS. Sections were rinsed with PBS and were subsequently incubated with biotinylated goat anti-rabbit antibody (Zymed 50-235Z) for 30 min, streptavidin horseradish peroxidase (HRPO

Table 1. A comparison of immunolocalization of the AR in the caput epididymis of rats with that of published data in other species\*

Epithelial cells	This study	Human		Mouse	Rat		Ram	Goat
		Ruizeveld de Winter et al., 1991	Ungefrören et al., 1997	Zhou et al., 2002	Takeda et al., 1990	Yamashita, 2004	Tekpetey et al., 1989	Goyal et al., 1997a
Principal cells	++/-	++	++	++	++/+	++	++	++
Basal cells	+	-	++	++	++/+	++	NE	+
Apical cells	+	NE	NE	++	++/+	+	NE	+
Interstitial stromal cells								
Peritubular smooth muscle cells	+/-	++/-	++/-	+/-	+/-	+	+/-	+

\*Symbols are as follows: ++ = strong, + = moderate, - = negative, ++/-, +/- = variable, NE = not examined

conjugate, SA 1007, CALTAG) for 30 min, and AEC (3-amino-9-ethyl-carbazole) for 10 min at room temperature. Between incubations, sections were rinsed three times with PBS. Sections incubated without the primary antibody but with PBS were used as negative controls (Figure 9). Counterstaining was performed with Mayer's hematoxylin (3 min). After counterstaining, sections were rinsed in distilled water and coverslips mounted with mounting medium. Stained tissues were examined with an Olympus BX-51 photomicroscope and photographs were taken.

Immunohistochemically stained sections were reviewed by two independent observers. Only nuclear staining was regarded as positive AR immu-

noreactivity. The degree of immunostaining was designated by semi quantitative analysis as strong (++), moderate (+), negative (-) or variable (++/-, +/-) immunoreactivity (Table 1). Nuclei were declared negative if receptor staining intensity did not differ visibly from that of negative control sections on a within-slide basis.

## RESULTS

To determine AR distribution in the caput epididymal cells of both the ligated and sham-operated groups of rats, we performed immunohistochemical

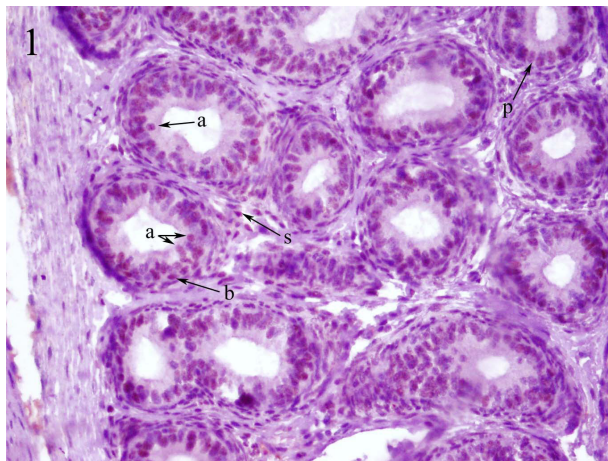


Figure 1. Immunohistochemical localization of the AR in the caput epididymal cells of ligated rats (21 days old); a = apical cell, b = basal cell, p = indicates principal cell, s = other stromal cell. Positive immunohistochemical staining for the AR appeared in nuclei but not in the cytoplasm of caput epididymal cells; 400×

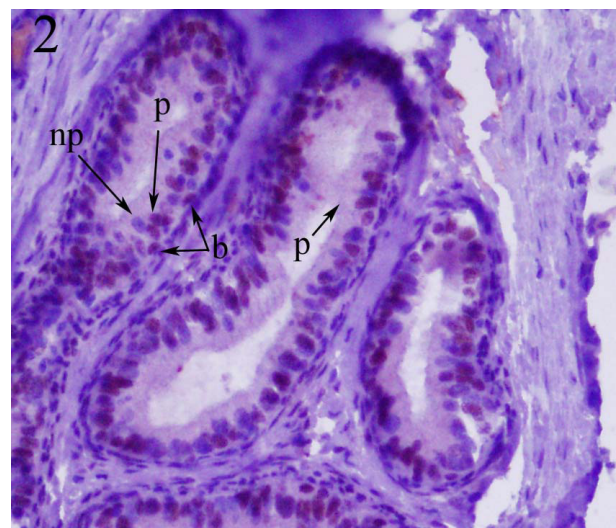


Figure 2. Immunohistochemical localization of the AR in the caput epididymal cells of sham-operated rats (21 days old); b = basal cell, p = indicates principal cell, np = negative principal cell; 400×

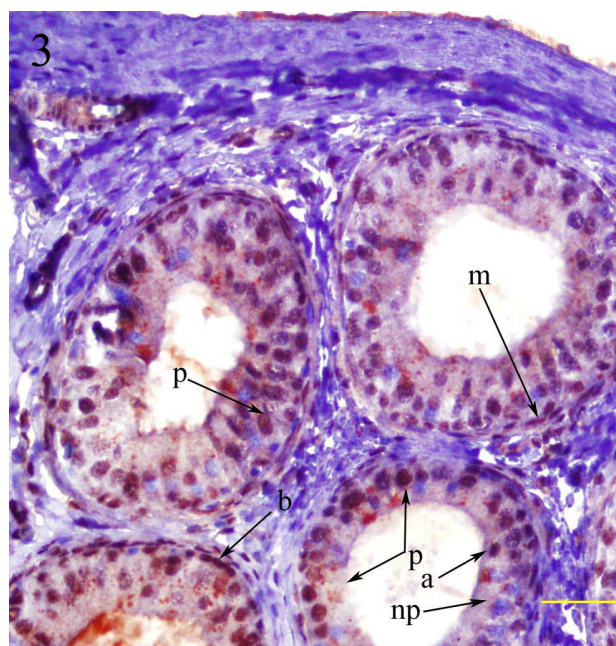


Figure 3. Immunohistochemical localization of the AR in the caput epididymal cells of ligated rats (56 days old); a = apical cell, b = basal cell, m = peritubular smooth muscle cell, p = indicates principal cell, np = negative principal cell; 400×

examination of paraffin-embedded sections from Day 21 to Day 120 old rats. In Table 1 the results of this examination are summarized and compared with those reported previously in other species.

In sham-operated rats immunohistochemical staining for the AR could be detected in the caput epididymis. Positive immunohistochemical stain-

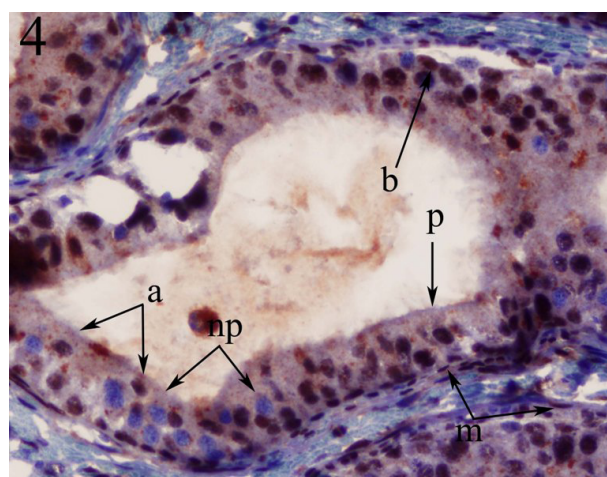


Figure 4. Immunohistochemical localization of the AR in the caput epididymal cells of sham-operated rats (56 days old); a = apical cell, b = basal cell, m = peritubular smooth muscle cell, p = indicates principal cell, np = negative principal cell; 800×

ing for the AR appeared in nuclei but not in the cytoplasm of caput epididymal cells at all ages beginning from 21 to 120 days old (Figure 2, 4, 6, 8). The staining intensity of AR-positive cells did not change depending on age. In the caput epididymis, immunostainable ARs were found in tubular epithelial cells and interstitial stromal cells. The AR staining in the epithelial cells appeared to be stronger than in the stromal cells. Epididymal epithelium consists of two major cell types (principal cells and basal cells) and one minor cell type

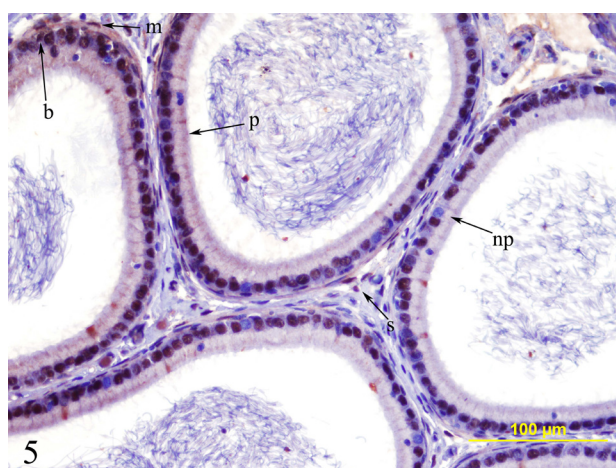


Figure 5. Immunohistochemical localization of the AR in the caput epididymal cells of ligated rats (90 days old); b = basal cell, m = peritubular smooth muscle cell, p = indicates principal cell, s = other stromal cell, np = negative principal cell; 400×

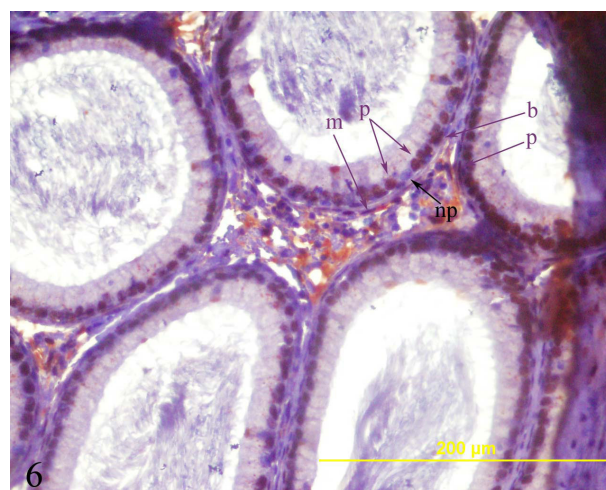


Figure 6. Immunohistochemical localization of the AR in the caput epididymal cells of sham-operated rats (90 days old); b = basal cell, m = peritubular smooth muscle cell, p = indicates principal cell, np = negative principal cell; 400×

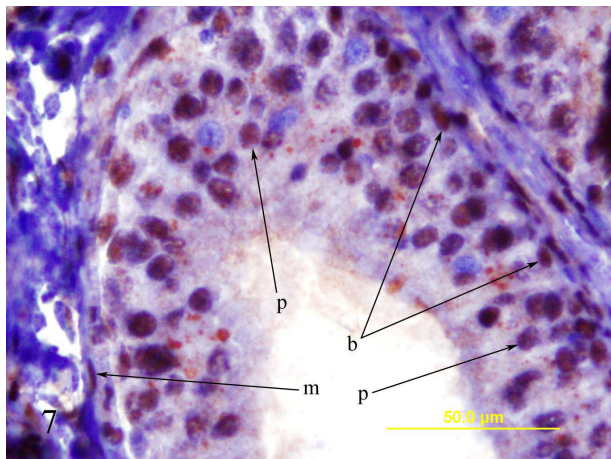


Figure 7. Immunohistochemical localization of the AR in the caput epididymal cells of ligated rats (120 days old); b = basal cell, m = peritubular smooth muscle cell, p = indicates principal cell; 1000×

(apical cells). Staining intensity was stronger in principal cells than in basal cells and apical cells. Nearly all principal cells were AR-positive but some were AR-negative. In the stromal area, AR positivity was observed in peritubular smooth muscle cells and some other stromal cells. Most stromal cells were moderately positive to negative for AR. The observed histological changes are presented in detail in Table 1.

In the ligation group there were no significant histological alterations in epididymal epithelium. Ligation of the corpus epididymis led to cessation of the seminal fluid and sperm flow and a slight dilatation of the tubules (56, 90 and 120 day-old ligated rats). In the testes the initial histological changes in ligated animals observed at 56 days included an increased diameter of the seminiferous



Figure 8. Immunohistochemical localization of the AR in the caput epididymal cells of sham-operated rats (120 days old); b = basal cell, m = peritubular smooth muscle cell, p = indicates principal cell, np = negative principal cell; 400×

tubule and thickness of the basal membrane, decreased thickness of the germinal epithelium, depletion of spermatids and presence of multinucleated spermatids. At 90 and 120 days in the ligation group germ cells were greatly reduced in number and seminiferous epithelium was composed mainly of Sertoli cells.

In ligated rats, the features of immunohistochemical staining of caput epididymal cells for the AR were similar to sham-operated rats at all ages. The ligation of the corpus epididymis did not affect immunostaining properties of the caput epididymis (Figure 1, 3, 5, 7). The negative control is depicted in Figure 9.

## DISCUSSION

In the present study, immunohistochemistry was used to investigate specific cellular localization of the AR in the caput epididymis of developing rats and the effects of prepubertal epididymal obstruction on the distribution of the AR in the caput epididymal cells of rats.

In sham-operated rats a positive reaction was visible in the nuclei but not in the cytoplasm of caput epididymal cells. The nuclear immunolocalization of this receptor is in agreement with previous studies in humans (Ruizeveld de Winter et al., 1991; Kimura et al., 1993; Janssen et al., 1994), monkeys (West and Brenner, 1990), rats (Bremner et al., 1994; Trybek et al., 2005) and mice (Iguchi et al., 1991), and supports the concept that ligand-de-

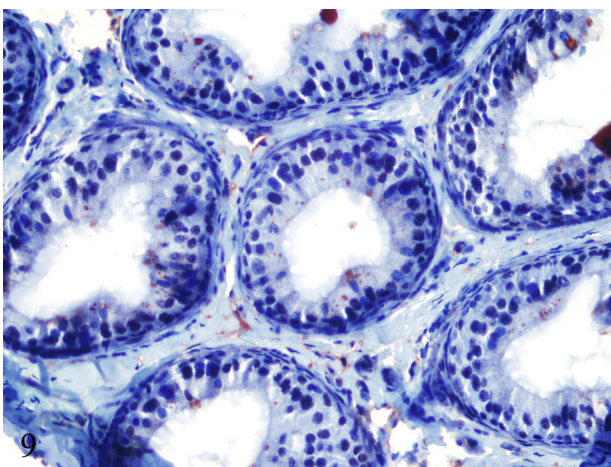


Figure 9. Negative control; 400×

pendent regulators are mainly found in the nuclei of steroid-sensitive target cells (Malayer and Gorski, 1993). We report that the AR was present at all ages in the caput epididymal cells of developing rats. Studies describing the development of the AR at the cellular and subcellular level in caput epididymis have been limited to a few species. Parlevliet et al. (2006) reported the presence of the AR in all four age groups and no change in receptor localization in caput epididymal cells in developing stallions. Similarly Goyal et al. (1997b) observed staining for the AR in the I–V region of goat epididymis which did not change in intensity with age. However Carreau et al., (1984a,b) described AR levels in the epididymis of sheep as being low or undetectable in infantile males followed by an increase during adulthood.

In this study AR-positive staining was detected in epididymal epithelial cells and interstitial stromal cells; moreover, the AR staining intensity in the epithelial cells appeared to be stronger than in the stromal cells. These findings are consistent with the results of previous reports (Sar et al., 1990; Takeda et al., 1990; Goyal et al., 1997a; Zhu et al., 2000). In this study, the staining features of epididymal epithelial cells differed. Staining intensity was stronger in principal cells than in basal cells and apical cells. This result is in agreement with a previous study (Goyal et al., 1997a). The present study showed that most principal cells are AR-positive but that some are negative. To our knowledge, this is the first study in caput epididymis that reports AR negativity for principal cells. Data are accumulating regarding AR localization in principal cells (Sar et al., 1990; Takeda et al., 1989; Ruizeveld de Winter et al., 1991; Goyal et al., 1997a,b; 1998). We confirm earlier results and, in addition, observed some AR-negative cells.

In the stromal area, some of the peritubular smooth muscle cells and some other stromal cells were AR-positive. These results are in agreement with those reported for humans (Sar et al., 1990; Ruizeveld de Winter et al., 1991), rats (Takeda et al., 1990), and monkeys (Roselli et al., 1991; Goyal et al., 1997a; Zhu et al., 2000).

In ligated rats histological changes were not detected in the epididymal epithelium and neither caput lumen diameters nor epithelial height were significantly altered by ligation. These findings are consistent with the results of previous reports (Flickinger et al., 1990; 1995; Turner et al., 1999, 2000). Also the immunohistochemical staining of

caput epididymal cells for the AR was similar to sham-operated rats at all ages. This observation, namely, that the ligation of the corpus epididymis in prepubertal rats did not alter the AR immunostaining of caput epididymal cells, suggests that mechanical pressure on the epithelial cells and cessation of flow in the tubule do not affect AR distribution in the caput epididymis. Previous studies (Pujol and Bayard, 1979; Goyal et al., 1998) reported that ligation of the efferent ductules did not affect the biochemical concentration of AR in the epididymis of rats and goats. Goyal et al. (1998) reported that orchidectomy, regardless of the region or the cell type, caused a severe reduction in AR immunostaining of epididymis which was restored to the intact level with testosterone treatment. These facts, together with the present results, support the notion that circulating androgen alone, without any input from luminal androgen or other rete fluid contents can regulate expression of the androgen receptor.

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