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Original article

Immunolocalization of androgen and vitamin D receptors in the epididymis of mature ram (*Ovis aries*)

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ABSTRACT

This study illustrated the immunohistochemical distribution of androgen and vitamin D receptors of epididymis in 20 sexually mature ram (Rahmani breed) with average age ranged from (2–4) years and average weight ranged from (50–65kg). Androgen receptor was localized in the cytoplasm of both ciliated and non ciliated cells of efferent ductules, besides the principal cells via the entire epididymal duct. The principal cells of both corpus and proximal cauda epididymis showed the highest immunoreactivity to androgen receptors. Furthermore, vitamin D receptor was localized in the cytoplasm of all epithelium of the efferent ductules besides principal cells of all epididymal regions, however the immunoreaction was significantly higher in the efferent ductules, distal caput and distal cauda epididymis. In conclusion, these results suggest that the function of ram epididymis is regulated by both androgen and Vitamin D.

1. Introduction

Sheep are considered as the most abundant ruminant livestock species in Egypt. As they are able to produce meat and milk without consuming large amounts of feed concentrates in comparison with large ruminants (Elshazly and Youngs, 2019). Furthermore, sheep are characterized by high rates of reproductive efficiency (Hafez and Hafez, 2000).

The morphology and the functional integrity of the epididymis are regulated by androgen (Testosterone and dihydrotestosterone) (Robaire et al., 2007). Androgen plays vital role in adjustment of factors which ensure the production of physiological luminal environment which is necessary for maturation and survival of spermatozoa (Zhou et al., 2002) via maintenance of the secretory and absorptive function of the epididymal epithelium which is continually changed along the epididymal duct (Robaire et al., 2006; Sullivan et al., 2007).

1,25-dihydroxyvitamin D_3 (Vit D_3: biologically active form of Vitamin D) is a steroid hormone (Jin et al., 2015) that recently regarded a signaling molecule in regulating male reproductive biology (Jensen, 2014; de Angelis et al., 2017). Vitamin D_3 plays an eminent role for calcium homeostasis (Lips, 2006) that is critical for motility of spermatozoa, hyperactivation and acrosome reaction (Yoshida et al., 2008).

In view of this, the aim of this study is to elucidate more light and some details on the immunohistochemical localization of androgen and vitamin D receptors.

2. Material and methods

This study was carried out during Autumn season (September, October and November).

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2.1. Ethics statement

The study protocols were approved by Veterinary Cairo University institutional animal care and use committee (Vet. CU. IACUC). Protocol number: 10102019083.

2.2. Animal species

Twenty sexually mature apparently healthy ram (Rahmani breed) with an average age ranged from (2–4) years and average weight ranged from (50–65 kg) were selected after complete clinical and andrological examinations. These examinations did not reveal any pathological alteration in the testis and epididymis, and showed high semen quality. The dentation of rams was carried out according to Noden and De Lahunta (1985). After their examination, they were transported to the central abattoir in Cairo to be slaughtered.

2.3. Histological technique

Left and right epididymis of ram were collected immediately after slaughtering and transported to the Veterinary Histology Laboratory, Faculty of Veterinary Medicine, Cairo University. Each epididymis was divided into seven portions; the most proximal one represents the efferent ductules, which cut longitudinally. Distal to the latter and according to Kishore (2006), the epididymal duct was divided into six segments that crossly cut, segment I (initial duct or ascending part of the head), segment II (central caput) and segment III (distal caput) constitute the head of the epididymis; the segment IV represents the body (corpus), whereas the fifth segment (proximal cauda epididymis) was taken at the constriction between the body and tail and sixth segment (distal cauda epididymis) was taken as a transverse section at the widest part of the tail. All specimens were fixed immediately in Bouin's fixative and processed according to Bancroft and Gamble (2008).

2.4. Androgen and vitamin D receptors "immunohistochemical localization" in the epididymis (Avidin biotin peroxidase complex)

Sections of (3-5μ) thick were mounted on positively charged glass slides. After processing, the slides were incubated with primary antisera to Androgen receptor (Polyclonal Rabbit Anti- Human Androgen receptor antibody, Chongqing Biospes Co., Ltd - YPA1811 at dilution 1: 400) and Vitamin D receptor (Polyclonal Rabbit Anti- Human Vitamin D Receptor antibody, Chongqing Biospes Co., Ltd - YPA1750 at dilution 1:400). The method used was outlined according to Ramos-Vara (2005). The intensity of the immunostaining reaction was evaluated according to Smolen (1990).

2.5. Statistical analysis

The results were expressed as mean ± SD. Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test (IBM SPSS statics). P-value < 0.05 was considered statistically significant.

2.6. Immunofluorescence of androgen receptor and vitamin D receptor

Mounted slides with epididymal tissue samples were examined for immunofluorescence identification of androgen and vitamin D receptors according to (Zhang et al., 1995; Vollmar et al., 1998). Tissue sections were examined and imaged using a Nikon fluorescence microscope (Model: Nikon eclipse 90i with a DS-U3 imaging system, Nikon Metrology, Inc. USA).

3. Results

3.1. Androgen receptor (AR)

Androgen receptor was localized in the cytoplasm of non ciliated and ciliated cells of the ductuli efferentes (Fig. 1A), and principal cells throughout the different epididymal segments. The intensity of reaction described in (Table 1 and Fig. 2). This positive immunoreaction to androgen receptors was prominent in both apical and basal cytoplasm of epithelial cells in efferent ductules (Fig. 1A), at basal cytoplasm of the principal cells in segment I, II and III (Fig. 1B, C, D) respectively; in apical part of principal cell cytoplasm at segment VI (Fig. 1G) and distributed via principal cell cytoplasm in segment IV and V (Fig. 1E, F) respectively.

3.2. Vitamin D receptor (VDR):

The current study revealed that all segments of epididymis displayed variable intensity of positive immunoreaction to vitamin D receptor. This reaction was exclusively cytoplasmic; in the non ciliated and ciliated cells of the ductuli efferentes, and principal cells in all segments of the epididymis (Fig. 3A,B,C,D,E,F,G, Fig. 4 and Table 2).

3.3. Androgen and vitamin D receptors' immunofluorescence

The immunofluorescence confirmed the immunoreaction obtained by immunohistochemistry within the different epididymal segments for both androgen receptor (Fig. 5A,B,C,D,E,F,G) and vitamin D receptor (Fig. 6A,B,C,D,E,F,G).

4. Discussion

This investigation showed that the cytoplasm of either ciliated or non ciliated cells of the efferent ductules exhibited moderate androgen receptor immunoreactivity. Meanwhile, Tekpetey et al. (1989) in ram reported that these cells showed faint reaction to androgen receptors. It was reported that testosterone causes a light increase in fluid reabsorption (Hansen et al., 1997). Furthermore, androgen regulates estrogen receptor's expression in non ciliated cells of efferent ductules (Goyal et al., 1998).

The current work revealed that the principal cell cytoplasm in whole epididymis displayed positive immunoreaction to androgen receptors. This finding is in contrary with the findings of Tekpetey et al., (1989) in ram; Zhou et al. (2002) in mouse; Parlevliet et al. (2006) in stallion and Kopera et al. (2009) in boar who reported that androgen receptor was expressed especially in the nuclei of the principal cells in all segments of the epididymis. The variation in the site of androgen receptors in this study besides other previous studies may be as a result of migration of androgen receptors from nuclear compartment to cytoplasmic compartment. In this respect, this study postulated that the animals might be slaughtered after 12 h of androgen hormone withdrawal since after 6–12 hours of androgen hormone withdrawal, the androgen receptors migrate to the cytoplasm and remain in a steady state until the hormone is exposed again so the receptors rapidly undergo nuclear translocation (Tyagi et al., 2000). This research revealed that the principal cells showed a regional variation in their reaction to androgen receptors. That may be reflected in its region correlated functions within the ram epididymis. This corroborates with the finding of Goyal et al. (1994) in goat who found that the response of the principal cell to androgen deprivation is varied among all epididymal regions and coincides also with Gupta et al. (1974) in rat who noticed difference in the thresholds of androgen required for maintenance of the variable parts of the epididymis. In general,

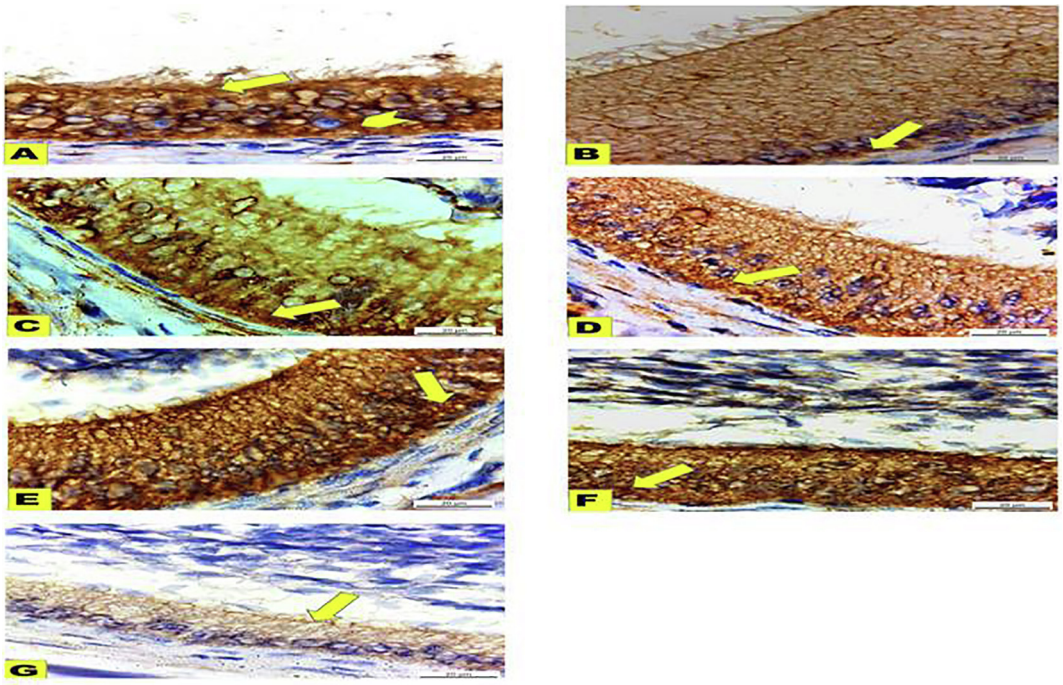

Fig. 1. Photomicrograph of a section in the epididymis of mature ram showing: Moderate immunoreaction to androgen receptors in the ciliated cells (arrow) and non ciliated cells (arrow head) of the efferent ductules (A), and in the principal cells (arrow) of segment I (B), segment II (C) and segment III (D). Strong cytoplasmic expression to androgen receptors in the principal cells (arrow) of segment IV (E) and segment V (F). Slight immunoreaction to androgen receptors in the principal cell (arrow) of segment VI (G). Androgen receptor immunohistochemistry staining. ×1000.

Table 1
. Mean value ± SD of the optical density of androgen receptors expression in different segments of the epididymis.

	Efferent Ductules	Segment I	Segment II	Segment III	Segment IV	Segment V	Segment VI
ARs	55.99 ± 2.14[a]	35.90 ± 2.47[b]	65.91 ± 3.35[c]	43.59 ± 2.54[d]	99.21 ± 1.48[e]	81.82 ± 4.55[f]	23.88 ± 2.90[g]

Tukey's post hoc test: means with different small superscript letters within the same row are significantly different at P < 0.05. Note: Androgen receptors (ARs).

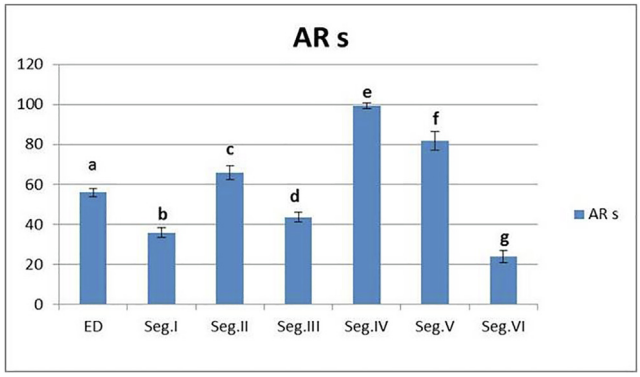

Fig. 2. Mean value ± SD of the optical density of androgen receptors expression in different segments of the epididymis. The different small letters on the graph shows the different significance at P < 0.05. Note: Androgen receptors (ARs), Efferent Ductules (ED), Segment (Seg).

androgen considered a key regulator of many function of the epididymis (Pearl et al., 2007). In addition, it is critical for maintenance of normal epithelial morphology (Ezer and Robaire, 2002) since, the height of the epithelium is greatly reduced in androgen deprived epididymis as a result of cytoplasmic regression and degradation of the nuclei (Smithwick and Young, 2001). In this consideration, androgen is essential for normal secretory function (Robaire and Hermo, 1988; Robaire and Viger, 1995) which in turn important for maturation of the spermatozoa (Ezer and Robaire, 2002). Moreover, androgen acts as a regulator of the expression of many proteins involved in the formation of adhering and tight junctions that are essential for the intact blood epididymal barrier (Cyr et al., 2002). However, Zhou et al. (2002) in mouse; Bilinska et al. (2005) in stallion and Kopera et al. (2009) in boar reported that all epididymal segments showed the same intense reaction to androgen receptor.

This study revealed that the reaction to androgen receptor was intense in corpus and proximal part of cauda epididymis of mature ram. The high level of androgen receptor expression in segment IV of ram corresponds with the regional localization of 5 α reductase enzyme (Amann, 1987) that converts testosterone into dihydrotestosterone which reflects the importance of this region in sperm maturation (Fournier_Delpech et al., 1983; Amann, 1987). In this respect, Cohen et al. (1981) mentioned that the dihydrotestosterone is critical for sperms to acquire their fertilizing potential. This may be related to regulation of proteins secretion which involved in sperm membrane remodeling to allow binding to zona pellucida (Pearl et al., 2007). In addition, androgen is important for management of sperm motility via acidification of luminal fluid in body of the epididymis as it could maintain the usual expression of carbonic anhydrase 2 and 4 isoforms (Kaunisto et al., 1999). Furthermore, the proximal part of cauda epididymis requires high levels of androgen receptors which coincides with the finding of Lindsey and Wilkinson (1996) in rat who reported that androgen, in this region, induces the expression of Pem homeobox gene. This transcription factor may regulate the

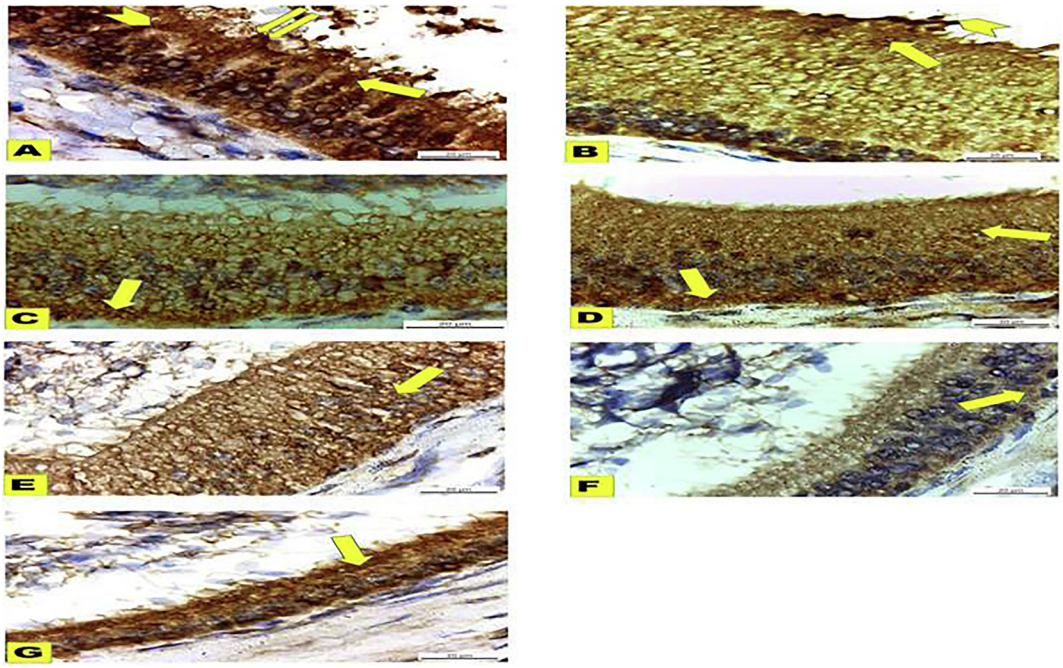

Fig. 3. Photomicrograph of a section in the epididymis of mature ram showing: High level of expression of VDR in ciliated cells (arrow) and non ciliated cells (arrow head) of ductuli efferentes (A), and principal cells (arrow) of segment III (D) and segment VI (G). Moderate immunoreaction to VDR in the principal cells (arrow) in segment I (B), segment II (C) and segment IV (E). Weak immunoreaction to VDR in the principal cell (arrow) of segment V (F). Notice: (A) a positive reaction of apical blebs of non ciliated cells of efferent ductules (double arrow). (B) a positive reaction of apical protrusion of principal cell in segment I (arrow head). VDR immunohistochemistry staining. ×1000.

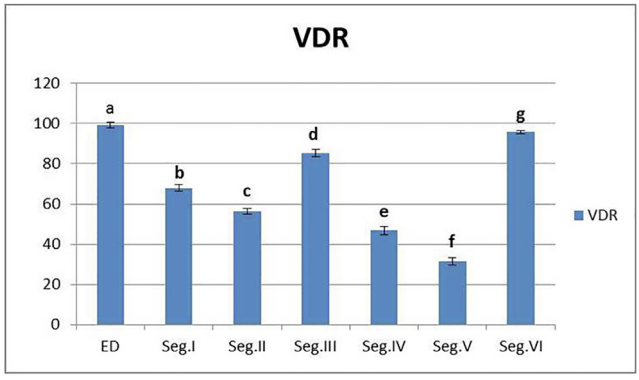

Fig. 4. Mean value ± SD of the optical density of Vitamin D receptors in the principal cell of different segments of the epididymis. The different small letters on the graph shows the different significance at P < 0.05. Note: Vitamin D receptor (VDR), Efferent Ductules (ED), Segment (Seg).

transcription of many genes which are participating in sperm gain of forward motility and ability to fertilize an ovum. Moreover, androgen controls the uptake of carnitine by the epithelial cells of cauda epididymis (Bohmer and Hansson, 1975) that acts as a sperm protective agent (Jeulin and Lewin, 1996).

On the other hand, this study revealed that the principal cells of the distal part of cauda epididymis were faintly reacted to androgen receptor similar to that mentioned by Ungefroren et al. (1997) in human as the mitochondrial enzymes in the epithelium of the tail of epididymis require little amount of androgen to express their maximal activity (Brooks, 1979). Androgen, in this region, regulates the secretion of proteins which are essential for protection and storage of the spermatozoa (Pearl et al., 2007).

This investigation showed that the principal cells of the first three segments of the epididymal duct had variable degree of moderate reaction to androgen receptor. The initial segment needs androgen for regulation of expression of some genes in the principal cell (Krutskikh et al., 2011). In addition, androgen maintains the claudin-1, which is an element of tight junction in the first segment of the epididymis (Gregory et al., 2001). In the caput, androgen has a great role in the transcriptional activation of HE6 gene (ADGRG2) (Yang et al., 2018). It was reported that ADGRG2 gene (Adhesion G-Protein-Coupled receptor G2) encodes an epididymis -selective transmembrane receptor which is essential for the function of the epididymis (Patat et al., 2016) and maturation of the spermatozoa (Yang et al., 2018). This function confirmed especially in the light of recent studies in Hemizygous mutant male mice with disrupted HE6 gene (Human Epididymal Protein 6) where the fertility of this mice reduced as a result of mis regulation of fluid reabsorption so the spermatozoa can't migrate via the epididymis (Davies et al., 2004).

Furthermore, many members of beta - defensin family, in the caput epididymis, are regulated by androgen (Hu et al., 2014). This family plays a significant role in protecting the epididymis against a wide range of pathogens as E-coli, Staph -aureus and C. albicans (Yenugu et al., 2004; Diao et al., 2007; Zhao et al., 2011).

Table 2
. Mean value ± SD of optical density of vitamin D receptors in the principal cell of different segments of the epididymis.

	Efferent Ductules	Segment I	Segment II	Segment III	Segment IV	Segment V	Segment VI
VDR	99.27 ± 1.47[a]	67.86 ± 1.58[b]	56.24 ± 1.52[c]	85.25 ± 1.69[d]	46.81 ± 2.14[e]	31.51 ± 1.73[f]	95.77 ± 0.92 [g]

Tukey's post hoc test: means with different small superscript letters within the same row are significantly different at P < 0.05. Note: Vitamin D receptor (VDR).

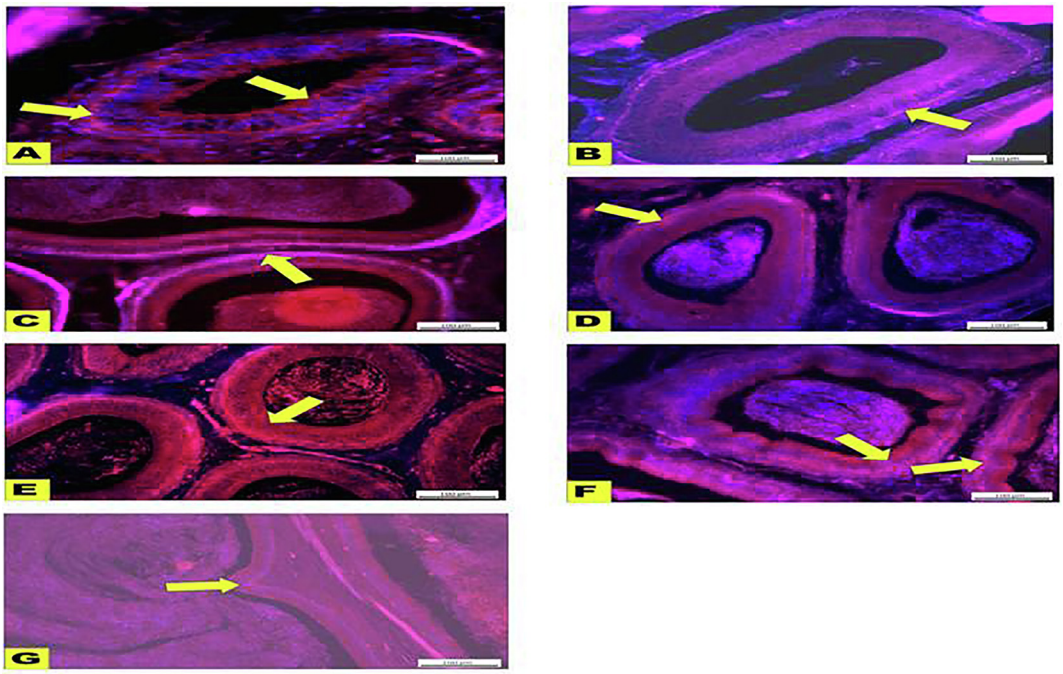

Fig. 5. Fluorescence micrograph of the epididymis of mature ram showing: Moderate androgen receptors immunofluorescence reactivity (arrow) in efferent ductules epithelium (A), and principal cells in segment I (B), segment II (C) and segment III (D). Intense androgen receptors immunoflurescence reactivity (arrow) in the principal cells of segment IV (E) and V (F). Faint androgen receptors immunofluorescence reactivity (arrow) in the principal cells of segment VI (G). The picture expressed the nuclei of the principal cells in a blue color (DAPI staining) and the immunofluorescence reactivity with red color. ×200.

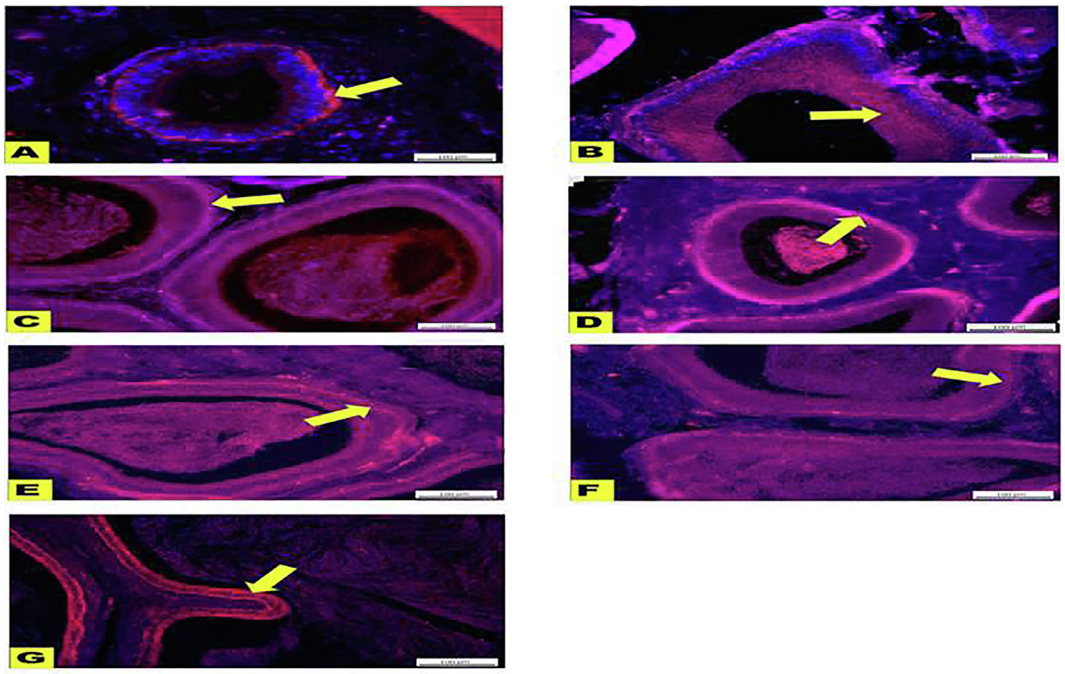

Fig. 6. Fluorescence micrograph of the epididymis of mature ram showing: Strong vitamin D receptors immunofluorescence reactivity (arrow) in epithellium of the efferent ductules (A), and principal cells in segment III (D) and segment VI (G). Moderate vitamin D receptors immunoflurescence reactivity (arrow) in the principal cells of segment I (B), segment II (C) and segment IV (E). Faint vitamin D receptors immunofluorescence reactivity (arrow) in the principal cells of segment V (F). The picture expressed the nuclei of the principal cells in a blue colour (DAPI staining) and the immunoflurescence reactivity with red colour. ×200.

The current study revealed that the efferent ductules exhibited strong positive cytoplasmic reaction to vitamin D receptor. On the other hand, Ford et al. (2014) in golden Syrian hamster reported that the non ciliated cells nuclei exhibited positive reaction to vitamin D receptor. Vitamin D has a great role in reabsorption of fluid (Yao et al., 2018). As, it regulates the estrogen signaling in the male genital tract (Boisen et al., 2018) through controlling the expression of aromatase gene (Kinuta et al., 2000) which converts testosterone and androstenedione into estradiol and estrone respectively (Miller and Auchus, 2011).

This study showed that all epididymal segments possessed positive cytoplasmic immunoreaction to vitamin D receptor. As the spermatozoa of ram have no vitamin D receptor so, the effect of Vitamin D might be mediated via the epididymis (Jin et al., 2015). Vitamin D may modulate the transfer of calcium ions across the epithelial cells in the ejaculatory tract (Blomberg Jensen et al., 2010). Accurate regulation of intraluminal calcium concentration in the epididymal duct is critical for production of spermatozoa ready for fertilization (Weissgerber et al., 2011). This investigation revealed that the reaction to vitamin D receptor was intense in the principal cells of segment VI and III but those of segment V displayed weak cytoplasmic reaction. In contrary, Jin et al. (2015) in ram don't detect any significant regional difference in the reaction of the principal cells to vitamin D receptor. The high expression of VDR in segment VI, in this study, might reflect that this region requires abundant Vitamin D. In this respect, Yao et al. (2018) hypothesized that deficiency of vitamin D receptor in epididymis of Hu sheep results in decreasing the number or motility of spermatozoa, which in turn leads to infertility. As Vitamin D induces the motility of the spermatozoa by enhancing the activation of the spermatozoa mitochondrial respiratory chain to supply ATP via cAMP/PKA pathway, and the increase of intracellular Ca^{2+} concentration (Jueraitetibaike et al., 2019). Vitamin D receptor regulates TRPV6 (Transient Receptor Potential Vanilloid 6) which is, a membrane calcium channel, localized specially in the epididymis (Boisen et al., 2018). The great role of this channel in reabsorption of calcium ions from the luminal fluid is clearly demonstrated in TRPV6 $^{D541A/\ D541A}$ pore mutant mice, which suffered from impaired reabsorption of calcium ions from the luminal fluid in the distal part of cauda epididymis. That results in a rise in the concentration of calcium around the spermatozoa that leads to decreased motility and viability of the spermatozoa which in turn results in impaired fertility (Weissgerber et al., 2011). Boisen et al. (2018) added that the calcium concentration in the epididymal luminal fluid of the tail of epididymis is about 12.5% that of the caput epididymis which correlates with the finding of White and Aitken (1989) in hamster who observed that the cytoplasmic calcium content in spermatozoa obtained from the cauda epididymis is lower than in sperm from the caput epididymis. Therefore, TRPV6 is likely to be a critical determinant for the acquiring of fertilization competence by sperm (Weissgerber et al., 2011).

5. Conclusion

This study analyzed the different regional expression of androgen and vitamin D receptors in the epididymis reflecting that the cellular responsiveness to androgen and vitamin D receptors is varied based on the requirement of each segment for androgen and or Vitamin D. In addition, the marked expression of vitamin D receptor in the distal cauda epididymis suggests that Vitamin D may be important for motility of spermatozoa, which in turn influence efficiency of sheep fertility.

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