Immunolocalization of insulin-like growth factor-I (IGF-I) and its receptors (IGF-IR) in the equine epididymis

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Abstract. Insulin-like growth factor plays a paracrine/autocrine role in regulating testicular function in the stallion, but its presence in the equine epididymis remains unknown. The aim of this study was to test the hypothesis that insulin-like growth factor-I (IGF-I) and IGF-I receptor (IGF-IR) are localized in the caput, corpus, and cauda of the epididymis in an age-dependent manner. Immediately after castration, epididymal tissue was fixed, paraffin-embedded, and processed for immunohistochemistry (IHC). Western blot was also performed using equine epididymal extracts to verify the specificity of the antibodies against IGF-I and IGF-IR. Immunolabeling of IGF-I was observed in the cytoplasm of principal and basal cells in the caput, corpus, and cauda at the pre-pubertal (3–7 months), pubertal (12–18 months), post-pubertal (2–4 years), and adult stages (4.5–8 years). Immunolabeling of IGF-IR was observed in the cytoplasm of principal cells in all regions of the epididymis in each age group. Immunolabeling of IGF-IR was also detected in the cytoplasm of basal cells from animals of all ages. Bands observed by Western blot corresponded to the molecular weights of IGF-I and IGF-IR, ~23 kDa and 95 kDa, respectively. These results suggest that IGF-I might function as an autocrine and/or paracrine factor during the development, maintenance and/or secretions of the stallion epididymis.

Key words: Epididymis, Immunohistochemistry (IHC), Insulin-like growth factor-I (IGF-I), IGF-I receptor (IGF-IR), Stallions

The epididymis plays important roles in the transportation of sperm from the testes to the vas deferens, sperm maturation, and sperm storage. Many cell types are present in the epididymis, but epithelial cells are the principal cells that line the lumen of the epididymis [1]. The structure and function of these cells varies depending on the section of the epididymis considered, particularly between the initial segment and other segments. Principal cells are responsible for most of the proteins found in epididymal fluid [2–4]. The epididymis also contains narrow, apical, clear, and basal cells [1]. Narrow, clear, and apical cells are predominantly present in the initial segment interspersed with principal cells [5, 6]. These cell types either have little protein secretory function, or their role is unknown. Basal cells develop post-natal throughout the epididymis and vas deferens, and form a pseudo stratified epithelium with principal cells [7].

The presence of a factor and its receptor that act on the same cell or on different cells within the epididymis suggest the presence of an autocrine or paracrine system, respectively [8]. Such a localized system was reported to mediate endocrine actions in reproductive organs, and/or directly regulate cell-cell interactions [9]. Insulin-like growth factor-I (IGF-I) is a well-known paracrine/autocrine factor that plays important roles in the epididymis and testes of several species [9–15]. The localization of IGF-I in the epididymis of a post-natal mouse model suggested that the intensity of staining varied depending on the region of the epididymis and the developmental stage of the mouse [16]. The expression of IGF-I mRNA in the epididymis was also detected in rats [17, 18]. Furthermore, the localization of the IGF-I receptor (IGF-IR) in the cytoplasm and membranes of the principal cells of the epididymis in developing rats was reported [19]. The expression of IGF-I and IGF-IR in rats was highest in the distal epididymis, particularly in the cauda [20].

The structure and functions of the epididymis are highly regulated by androgens and dihydrotestosterone (DHT) [17, 21–24]. The signaling pathways activated by DHT in epididymal cells are regulated by the IGF-I system [12, 25], suggesting that it plays critical roles in the development of the epididymis. Consistent with this, IGF-I-null mice showed a disproportionately small corpus and cauda [25]. The importance of IGF-IR in the development of the epididymis was suggested by a previous study showing that changes in the expression of androgen receptor and IGF-IR in response to linuron (toxic compound, EPA toxicity class III) resulted in malformed epididymides [26].

A positive effect of IGF-I on the quality of spermatozoa has also been reported [27–29]. The administration of IGF-I using a mini-pump significantly increased total spermatozoa motility [27].
Similarly, the motility and morphology of immature spermatozoa in growth hormone (GH)-deficient dwarf (dw/dw) rats were enhanced after treatment with IGF-I [29]. Sperm longevity was also improved after the incubation of equine spermatozoa with IGF-I [28]. These studies suggest that IGF-I might be a key factor that enhances sperm motility and maturation when sperm travel from the caput to the cauda [30]. Although the IGF-I/IGF-IR system plays critical roles in the development of the epididymis as well as in the quality of spermatozoa, the localization of IGF-I and IGF-IR in the equine epididymis has not been investigated.

IGF-I and IGF-IR have already been found in reproductive tissue of the stallion, and have been demonstrated to be involved with reproductive function. The levels of IGF-I were higher in testicular extracts from stallions younger than 2 years of age compared with those older than 2 years, suggesting that IGF-I might play a role in testicular development [31]. Recently, it was reported that IGF-I and IGF-IR were localized in equine germ cells and Leydig cells, but not in Sertoli cells [13]. The results of an in vitro study using cultured equine Leydig cells suggested that IGF-I protected Leydig cells from apoptosis [14]. In addition, IGF-I enhanced LH-induced testosterone production in stallion Leydig cells [15]. These studies support the concept that the IGF-I/IGF-IR system plays an important role in regulating reproductive function in stallions. Therefore, this study was designed to test the hypothesis that IGF-I and IGF-IR are also localized in the caput, corpus, and cauda of the equine epididymis in an age-dependent manner.

Materials and Methods

Animals

Epididymal tissues were collected from light horse breeds during routine castration procedures at the UC Davis Veterinary Medical Teaching Hospital during the breeding season (March to June) over a three-year period (2007–2010). Tissues were immediately transported to the laboratory in ice-cold Hank’s balanced salt solution (Krackeler Scientific, Albany, NY). Epididymal tissues were categorized based on the reproductive age of the stallions: pre-pubertal (3–7 months; IGF-I, n = 4 and IGF-IR, n = 5), pubertal (12–18 months; n = 5 and n = 6, respectively), post-pubertal (2–4 years; n = 7 and n = 9, respectively), and adult (4.5–8 years; n = 5 and n = 4, respectively).

Fixing epididymal tissue for IGF-I and IGF-IR

Each region of the epididymis was prepared as described previously [32]. A 1-cm³ section of epididymis was removed and fixed in 4% paraformaldehyde at 4 C overnight. Tissues were then dehydrated using increasing concentrations of ethanol (30, 50, and 70% at 4 C for 24 h each). Tissues were embedded in paraffin wax using a Vacuum Infiltration Processor (Tissue-Tek, Sakura Finetek, USA), and were then cut into 5 µm sections for immunohistochemistry (IHC) using a Historange 2218 microtome (LKB, Bromma, Sweden).

Immunohistochemistry for IGF-I and IGF-IR

Immunohistochemistry for IGF-I and IGF-IR in the epididymis was performed as described previously [13] with slight modifications. CitriSolv Hybrid (Fisherbrand, Hampton, NH, USA) was used to remove the paraffin wax, and samples were rehydrated using a series of 100, 90, and 70% ethanol baths. Unmasking solution (Vector Laboratories, Burlington, CA, USA) was then used for antigen retrieval, before endogenous peroxidase activity was blocked using 0.3% hydrogen peroxide in methanol (Fisher Scientific, Pittsburg, PA, USA). IGF-I and IGF-IR were detected in epididymal tissue using the Vectastain Elite ABC kit (Vector Laboratories). After blocking tissue with normal goat serum (ABC kit) for 30 min, tissue slides were incubated with avidin and biotin (Vector Laboratories) for 15 min each to block their native activity. Primary antibody incubations were performed using 200 µg/ml of either rabbit anti-human polyclonal IGF-I antibody (sc-9013, 1:50 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or 200 µg/ml rabbit anti-human polyclonal IGF-IR antibody (sc-713, 1:500, Santa Cruz Biotechnology) overnight with gentle rocking (Red Rocker, Hoefer Scientific Instruments, San Francisco, CA, USA). Sections were also incubated with the same concentrations of normal rabbit serum (Vector Laboratories, lot #R1007) as negative controls. Tissues were then incubated with goat anti-rabbit biotinylated secondary antibodies (ABC kit) according to the manufacturer’s instructions, followed by an avidin-biotin horseradish peroxidase complex (ABC reagent) for 45 min. Three drops of 3-amino-9-ethylcarbazol (Vector AEC Peroxidase substrate kit) were added to tissue samples, which were then incubated in the dark for ~15 min. Slides were counterstained by briefly dipping into hematoxylin (ImmunoMaster, American MasterTech Scientific, Lodi, CA, USA), and then mounted onto glass slides using FaramountTM aqueous mounting medium (Dako, Glostrup, Denmark). Slide images were captured using a QImaging MicroPublisher 3.3 digital camera with Q Capture Pro software (Q Imaging, Burnaby, BC, Canada). Equine liver was used as a positive control for IGF-I and IGF-IR expression. Two well-trained laboratory personnel assessed the staining on the slides, one of who was blinded to the age of the animals and the presence of the antibody (positive or negative control samples).

Western blot

Western blot was used to evaluate the specificity of the polyclonal antibodies used for IHC in equine tissue. Western blot for IGF-I and IGF-IR in the epididymis was performed as described previously [13] with slight modifications. Briefly, equine epididymal and liver tissues were snap frozen in liquid nitrogen and used for Western blot. Samples were thawed at room temperature, and then homogenized in 0.5 ml of phosphate-buffered saline (PBS) for ~2 min (Barnant Mixer, series 10, Chicago, IL, USA). The protein concentration of each sample was measured using a Bradford Bio-Rad Total Protein assay (Bio-Rad Laboratories, Hercules, CA, USA). The homogenized tissues were then diluted to 4 mg/ml with PBS, and sample preparation buffer was added (0.5 M Tris-HCl [pH 6.8], glycerol [0.1% w/v], 10% [w/v] SDS, 2-β-mercaptoethanol [0.05% w/v], and bromophenol blue in distilled water). Samples containing 25–30 µg protein were heated in a 95 C water bath for 8–10 min, and then separated on a 15% SDS-polyacrylamide gel using a Mini-Protein II system (Bio-Rad). A standard was also added to each gel (10 µl, Kaleidoscope pre-stained standard; Bio-Rad). Proteins were then transferred to an Immobilon-P membrane (Millipore, Bedford, MA, USA). After blocking with 10% heat-treated donkey serum (Donkey Jack, UC Davis Horse Barn, Davis, CA, USA), membranes were then incubated with rabbit anti-human IGF-1 (sc-9013, 1:50) or
rabbit anti-human IGF-IR antibodies (sc-713, 1:500) overnight at 4°C. For negative controls, membranes were incubated with the same concentrations of normal rabbit serum overnight at 4°C. Membranes were subsequently incubated with donkey anti-rabbit horseradish peroxidase at a 1:60,000 dilution for 45 min at room temperature, and bands were visualized using chemiluminescence (Western Lighting Plus-ECL, PerkinElmer, Shelton, CT, USA) with Kodak Scientific Imaging film (X-Omat, Blue XB-1, PerkinElmer).

**Results**

**Localization of IGF-I in the epididymis**

Staining of IGF-I was observed in the epididymis of stallions at all age groups (Fig. 1). Specifically, IGF-I was detected in the cytoplasm of both principal and basal cells in the caput, corpus, and cauda of animals at the pre-pubertal, pubertal, post-pubertal, and adult stages (Fig. 1A–L). Staining of IGF-I appeared to be more intense in the corpus and cauda of pubertal animals (Fig. 1E and F) compared with sections from animals of other ages. Immunolabeling was also observed near the apical membrane of principal cells at all stages and sections (Fig. 1). No staining was observed in negative control samples that had been treated with normal rabbit serum instead of primary antibody (lower right insets).

**Localization of IGF-IR in the epididymis**

Staining of IGF-IR was observed in the epididymis of stallions from all age groups (Fig. 2). In pre-pubertal animals, IGF-IR was detected in the cytoplasm, of principal cells, and in the cytoplasm of basal cells (Fig. 2A–C). There appeared to be more intense staining of IGF-IR in the cytoplasm of both principal and basal cells in the corpus and cauda compared with the caput. In pubertal, post-pubertal, and adult animals, immunolabeling was also observed in the cytoplasm of principal cells in the caput, corpus, and cauda (Fig 2D–L). In the basal cells, immunolabeling was present in the cytoplasm (Fig 2D–L). No staining was observed in negative control tissue samples that had been treated with normal rabbit serum instead of primary antibody (Fig. 2 insets).

**Specificity of the IGF-I and IGF-IR antibodies**

Western blot was performed using equine epididymal tissue samples to verify the specificity of the antibodies against IGF-I and IGF-IR. The protein band corresponding to IGF-I was observed at ~25 kDa (Fig. 3A), which is close to the reported molecular weight of IGF-I [13, 33, 34]. The band corresponding to IGF-IR was observed at ~95 kDa (Fig. 3A), which is consistent with the reported molecular weight for IGF-IR [13, 33].

Immunohistochemistry using the IGF-I antibody was also performed in horse liver tissue as a positive control (Fig 3B). Staining was observed in the cytoplasm of liver cells, but labeling was not observed in the tissues without the presence of IGF-I antibody (negative control).

**Discussion**

The IGF-I/IGF-IR system plays an important role in the development and function of the testes and epididymes of several species, including the horse [10, 13–15, 26, 35–38]. In this study, the localization of IGF-I and IGF-IR in the caput, corpus, and cauda of equine epididymides from different age group animals was assessed using IHC. In pre-pubertal horses, IGF-I and IGF-IR immunolabeling was detected in the epididymis. During early development, the IGF-I system might induce the proliferation of immature epididymal cells [35, 37]. Interestingly, the staining intensity of both IGF-I and IGF-IR appeared to be higher in the corpus and cauda of pubertal and pre-pubertal animals, respectively, suggesting that their expression varies depending on the region and age of the epididymis. The staining pattern of IGF-I and IGF-IR was reported previously in the rat epididymis, where the expression was highest in the distal epididymis, particularly the cauda. Differences in the timing and region of IGF-I and IGF-IR expression might be associated with testosterone secretion [39]. Testosterone (or DHT) and the IGF-I autocrine/paracrine system might play critical roles in epididymal development and the secretion of factors that are important for sperm maturation [39]. In the present study, IGF-I was localized in the cytoplasm of both principal and basal cells in each epididymal segment. The intense immunolabeling observed in principal cells toward the lumen area suggests that IGF-I from epididymal cells was secreted into the lumen of the epididymis. A similar pattern of immunolabeling of IGF-I was also observed in basal cells, suggesting that both basal and principal cells are the sources of IGF-I in the epididymis.

The localization of IGF-I and IGF-IR in both cell types in the epididymis suggests that IGF-I might function in an autocrine and/or paracrine manner in the equine epididymis. A previous morphological study suggested that basal cells are capable of secreting factors that might regulate the function of principal or other cells [40]. Factors secreted from basal cells can play important roles in regulating the transport of electrolytes and water from the principal cells via the secretion of transient receptor potential channel proteins and cyclooxygenase-1 [41]. These studies suggest that basal cells might be important paracrine regulators of adjacent cells such as principal cells [40, 41].

It has previously been suggested that IGF-I may be involved in regulating the expression of several genes in response to DHT in the epididymis [12]. Several molecules expressed in the mouse epididymis have been reported to play roles in the maturation, motility, and storage of sperm [2–4, 42, 43]. Principal cells are responsible for the largest portion of protein secretion in the epididymis [2–4]. These secreted proteins bind to sperm, and enhance the capacity for sperm-egg binding and fertility. In addition, proteins secreted from the cauda appear to be important for sperm storage [4]. Recently, a synergistic effect of IGF-I/IGF-IR and estradiol (E2) was proposed to be responsible for the growth of breast cancer cells [44]. Consistent with this, Yu et al. [45] revealed that IGF-I and E2 stimulate the translocation of estrogen receptor (ER) from the nucleus to the cytoplasm, where the ER interacts with IGF-IR to stimulate cell growth signaling pathways. It was demonstrated previously that ERα is also localized in the equine epididymis, and estradiol was present in all three epididymal regions [13]. The results of these studies support a concept whereby IGF-I and IGF-IR that are localized in the epididymis might interact with the ER system to stimulate the growth of equine epididymal cells. In addition, a study by Henderson et al. [17] suggested that sex steroids and IGF-I become down-regulated
following the inhibition of 5α-reductase, which leads to altered epididymal gene expression and function, particularly in the corpus and the cauda. Taken together, these studies suggest that IGF-1 might be involved in the pathways that are regulated by steroids during epididymal growth and sperm maturation.

In conclusion, this study revealed that IGF-I and IGF-IR are present in the principal and basal cells of the equine epididymis at all reproductive stages. The IGF-I system, including IGF-IR, appears to play a time-dependent role in the development and maintenance of different regions of the stallion epididymis, particularly the corpus and cauda. They may also play a role in sperm maturation, motility, and storage. Nevertheless, further studies are warranted to elucidate the function of IGF-I and its receptor in both principal and basal cells, including its potential interaction with DHT and estrogen during epididymal growth and sperm maturation, particularly during puberty, in the corpus and cauda.
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