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Arginase Distribution in Ram Spermatozoa During Epididymal Maturation

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Abstract: Polyamines play an important role in sperm motility. Extrahepatic arginase has been involved in polyamine synthesis. Our aim was to demonstrate arginase immunocytochemically and how its distribution changes during epididymal transit in ram spermatozoa. The distribution of arginase in ram spermatozoa obtained from the epididymis was analyzed by using an anti-arginase polyclonal antibody by indirect peroxidase immunocytochemical technique. Arginase from epididymal spermatozoa was purified by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and Western blot was performed on nitrocellulose paper. Its optimal pH and temperature were determined and protein concentration was also measured. In spermatozoa from the epididymal caput, arginase was found in the equatorial region and the middle piece; in spermatozoa from the epididymal body it appeared in the equatorial and acrosomal regions and the flagellum, while in spermatozoa from the epididymal tail arginase positive reaction was shown by the the acrosomal and postacrosomal regions as well as in the middle piece and flagellum. Its optimal incubation pH, time and temperature were similar to those of extrahepatic arginase. Once purified, arginase activity was 904.87 μg urea mg^{-1} protein/min. These data agree to those showing the distribution of polyamines in sperm cell and provide evidence that arginase, like other proteins, is redistributed as they are conducted trough the epididymis. It could favor polyamine synthesis in the flagellum, where they are needed by spermatozoa to induce motility and in the acrosome, where spermine has been related to stopping the untimely acrosome reaction.

Key words: Ram spermatozoa · epididymis · arginase · polyamines · maturation

INTRODUCTION

The epididymis is a highly differentiated tubule of the male reproductive system usually divided into the proximal caput, the middle body and the distal tail. Epididymal transit is necessary for spermatozoa to complete maturation, a process that enables them to successfully go through capacitation and acrosome reaction [1]. Spermatozoa from the epididymal tail are mature while those from the body and caput are not [1, 2].

It is now well known that epididymal sperm cell maturation involves structural changes and rearrangement of membrane lipids and proteins, some of these molecules are incorporated from the epididymal milieu [3, 4]. The epididymis is also a site of storage and a mean to go from the testis to the vas deferens. Those characteristics attributed to the epididymis are performed by epithelial secretion and absorption and in such a way the contents of the epididymal lumen is defined [4].

Polyamines are ubiquitous organic polycationic substances usually involved in cell replication and differentiation [5]. These molecules are synthesized from L-ornithine, a nonstandard amino acid that participates in the urea cycle, by the action of ornithine decarboxylase, the rate-limiting enzyme in the synthesis of polyamines [6]. Polyamines have been found in the spermatozoa [7, 8], seminal plasma [9] and epididymis [10, 11], as well as in other parts of the male reproductive system [12, 13]. The activities of two enzymes; ornithine decarboxylase and diamine oxidase, both involved in polyamine metabolism

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have been described in human sperm cells [12]. In spermatozoa polyamines seem to play a role in motility [8], while the biological meaning of the high content of polyamines in the epididymis is yet to be known, though quite probably they play an important role in fertility [10, 12], because their concentration seems to be regulated by androgens [11].

Arginase is the enzyme that catalyzes the hydrolysis of L-arginine to urea and ornithine [14, 15]. Arginase activity is dependent on manganese [16], favored by androgens [11, 17, 18] and controlled by competing with nitric oxide synthase for their common substrate L-arginine [19-21].

L-arginine supplementation in the culture medium protects spermatozoa against lipid peroxidation [22]. The probability exists that spermatozoa carry out this effect by synthesizing polyamines, which would require arginase activity, because in other tissues polyamines protect against lipid peroxidation [23, 24]. Besides, L-arginine is as effective as polyamines in causing an increase in sperm cell motility [25] and the blocking of arginase also stops polyamine biosynthesis in other tissues [26].

Arginase activity has been detected in spermatozoa and seminal plasma [27, 28], but its distribution in the sperm cell is not known.

In this paper we provide evidence of the presence and distribution of arginase in ram spermatozoa and how it changes during the transit of sperm cells through the epididymis.

MATERIALS AND METHODS

Reagents: Butanedione monoxime and thiosemicarbazide were purchased from Aldrich Chemical Company; L-arginine, glycine, urea and magnesium chloride were purchased from Merck. Folin and Ciocalteu's phenol reagent, serum albumin, Tris-HCl and molecular weight markers for sodium dodecyl sulphate gel electrophoresis (MW-SDS-70L) were purchased from Sigma Chemical Co. (St. Louis Mo. USA); arginase was purchased from Nutritional Biochemicals Corporation (Cleveland Ohio, USA); Goat anti-rabbit IgG was purchased from Zymed (San Francisco, California, USA). All other chemicals were of analytical grade.

Biological material: Ram testes were obtained from a nearby slaughterhouse and carried on ice to the laboratory where they were processed within the first hour after animals were butchered. Epididymides were excised and the epididymal caput, body and tail were sectioned in 0.1 M phosphate-buffered saline pH 7.4, minced and filtered separately trough gauze and then seen under light microscopy. No other cells were present in significant numbers.

Protein determination: Diluted sperm cells 1:200 were counted by using the Neubauer chamber and protein concentration was determined by the method of Lowry *et al.* [29]. Briefly, a 10 million spermatozoa sample was poured in 0.5 ml of a 1 M NaOH solution and incubated during 30 min at 37°C, then a 0.2 ml sample was taken to 0.5 ml with double-distilled water, 2 ml of alkaline cupper solution was added and left at room temperature for 10 min, after this 0.2 ml of the Folin and Ciocalteu's phenol reagent was added, mixed, left at room temperature during 20 min, centrifuged during 5 min at 2000 rpm and absorbance registered at 550 nm using a DU-64 spectrophotometer (Beckman Instruments, Inc., Brea CA).

Arginase activity: Arginase activity was determined by a colorimetric reaction according to Kung *et al.* [30], which measures the content of urea formed. Briefly, a 10 million spermatozoa sample was added to 500 µl arginase buffer (0.02 M $MnCl_2$, 0.04 M Tris and 0.154 mM NaCl), incubated for 1 h at 55°C, then 0.1 ml of a 0.14 M L-arginine solution was added and kept at 37°C during 15 min. Samples were heated in boiling water during 7 min to stop the reaction. Then 0.1 ml aliquots were taken to 1 ml with double-distilled water, 1 ml monoxime-thiosemicarbazide and 2 ml $H_2SO_4/FeCl_3$ were added, then heated at 92°C during 25 min and absorbance registered at 530 nm.

Arginase isolation: Arginase isolation as well as determination of the optimal pH and temperature where performed according to the Schimke method [31].

Antibody induction: Polyclonal anti-arginase antibodies were developed according to standard protocols by using bovine liver arginase. For this, arginase was injected to rabbits at 0, 15 and 30 days. Blood was drawn 38 days later and antibody titration carried out by enzyme-linked immunosorbent assay [32].

Immunocytochemical analysis: With the serum obtained, immunocytochemical detection of arginase was carried out in sperm cells. For this, ram spermatozoa were washed three times with phosphate-buffered saline and incubated

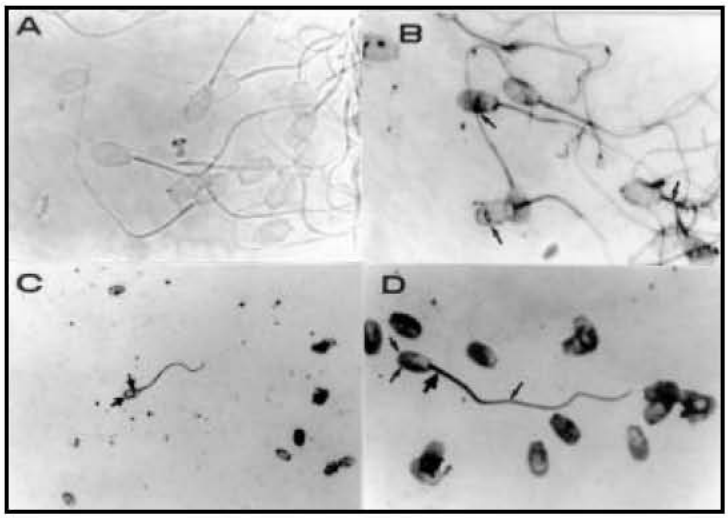

Fig. 1: Immune detection of arginase in epididymal ram spermatozoa stained with diaminobenzidine. A) Control spermatozoa from whole epididymis without exposure to arginase antibodies. B) Spermatozoa from the epididymal caput stain positive for arginase in the equatorial region and the middle piece. C) Spermatozoa from the epididymal body show a positive reaction for arginase in the flagellum, the equatorial and acrosomal regions. D) Spermatozoa from the epididymal tail stain positive for arginase in the flagellum, middle piece and the acrosomal and postacrosomal regions

Table 1: Arginase distribution in sperm cells obtained from ram epididymis

Epididymal segment	Regional localization of positive immunoreactivity for arginase in spermatozoa
Caput	Equatorial, middle piece
Body	Acrosome, equatorial, flagellum
Tail	Acrosome, postacrosomal, middle piece, flagellum

during 20 min in 0.074% phosphate-buffered saline-ammonium chloride. Membrane permeabilization was carried out by incubating in acetone during 20 min at -20°C, washed and non-specific protein binding blocked with albumin in phosphate-buffered saline. Anti-arginase antibody diluted 1:50 was added and then incubated during 12 h at 4°C in a humid chamber. Goat anti-rabbit IgG coupled to peroxidase was added. Peroxidase was developed with diaminobenzidine, slides were mounted with 1:1 phosphate-buffered saline:glycerol and seen under light microscopy.

In order to characterize sperm arginase, sodium dodecyl sulphate-polyacrylamide gel electrophoresis [33] was carried out with spermatozoa from the epididymis without considering the region they came from. For this, spermatozoa were homogenized using a PCU-Polytron homogenizer and 40 µg protein was added in each line. Gels were transferred to nitrocellulose paper and Western blotting was also performed to detect arginase [34].

RESULTS

Using the indirect peroxidase antiperoxidase immunocytochemical stain, arginase was localized in the equatorial region and middle piece of sperm cells from the epididymal caput, in the acrosomal and equatorial regions and the flagellum of sperm cells from the epididymal body, while the acrosomal and postacrosomal regions as well as the middle piece and flagellum also showed positive reaction in spermatozoa from the epididymal tail (Fig. 1 and Table 1).

The presence of arginase was determined by enzyme-linked immunosorbent assay. A decreasing optical density in the immunoassay directly proportional to enzyme concentration was observed as the dilution increased (Fig. 2).

Specific activity of purified arginase from ram epididymal spermatozoa increased as the total protein concentration diminished, until reaching the highest value for the enzyme activity of 904.87 µg urea mg^{-1} protein/min. The optimal incubation pH and temperature were 10 and 55°C, respectively, while the required time to reach its maximal activity ranged between 50 and 70 min (Fig. 3).

Electrophoresis of a homogenate from epididymal ram spermatozoa without considering any regionalization,

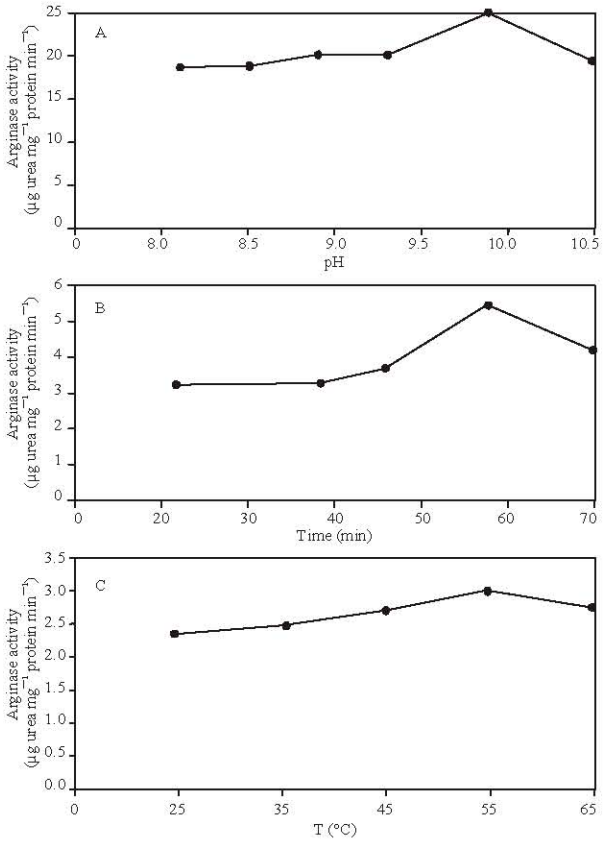

Fig. 2: Antibody quantitation against arginase from epididymal ram spermatozoa by enzyme-linked immunosorbent assay

showed a molecular weight for epididymal arginase similar to liver bovine arginase (Fig. 4). Western blot also showed arginase was present in epididymal ram spermatozoa.

DISCUSSION

Previously, the arginase activity in sperm cells isolated from ram epididymis was reported by our laboratory [27]. In this study, we are informing on the distribution of this enzyme in ram epididymal spermatozoa. As it was described above, arginase has been related to polyamine formation in extrahepatic tissues [14]. The fact that sperm cells present arginase gives meaning to previous results that polyamines increase sperm cell motility [8, 25] and that this semen quality parameter also increases with L-arginine *in vivo* [35] and *in vitro* [25, 36] administration. We consider that spermatozoa may synthesize polyamines and that these substances are not, as was thought many years ago [37],

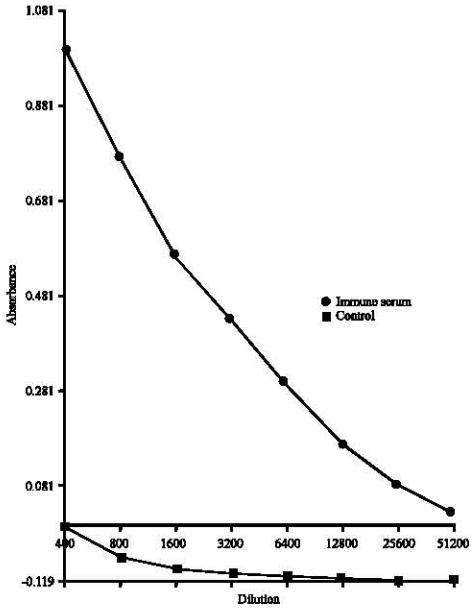

Fig. 3: Optimal incubation pH, time and temperature for arginase of epididymal ram spermatozoa. A) The optimal incubation pH was 10. B) The optimal incubation time was 60 min. C) The optimal incubation temperature was 55°C

a biochemical vestige of evolution. However, our data do not show whether polyamines present in spermatozoa are only produced inside the sperm cell or are also taken up from epididymal fluid before being in contact with seminal plasma which contains polyamines formed mainly in the prostate gland [12, 13]. This aspect remains to be studied.

On the other hand, the regional distribution of arginase in ram spermatozoa positively correlates with the degree of maturation reached, concentrating these substances more in the acrosome besides the flagellum, as the germinal cells advance through the epididymis. These data indicate that arginase could be used by sperm cells in promoting motility and preventing premature acrosome reaction. These data could also indicate from which part of the epididymis the spermatozoa come from, a fact that, if also shown by human spermatozoa, could bear clinical application.

Arginase activity has been shown to depend on the concentration of androgens in several tissues [18], including the male accessory sex glands [11, 17]. Further research should be conducted in order to show whether this is also true in spermatozoa.

It is known that the polyamine concentration in semen positively correlates with sperm motility [11] and

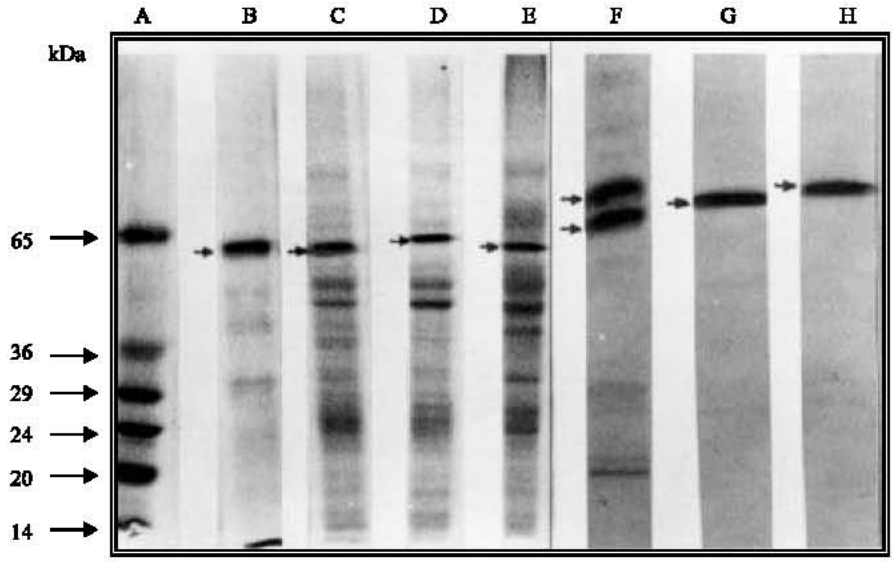

Fig. 4: Polyacrylamide gel electrophoretic (A-E) and Western blot (F-H) patterns of arginase from epididymal ram spermatozoa. A) Molecular weight markers. B) Arginase from bovine liver. C) Proteins from epididymal caput spermatozoa. D) Proteins from epididymal body spermatozoa. E) Proteins from epididymal tail spermatozoa. F) Arginase from bovine liver. G) Proteins from epididymal caput spermatozoa. H) Proteins from epididymal tail spermatozoa. A semiquantitative comparison of arginase content in epididymal regions showed the following tapering order: tail (++++), head (+++) and body (+)

that the oxidation of polyamines by diamine oxidase can turn this high concentration into a deleterious effect on fertility [38, 39], as oxidized polyamines attack spermatozoa.

In the central nervous system the regeneration is favored by an increase in cyclic adenosine monophosphate, which is in turn related to an up-regulation of arginase, with the subsequent synthesis of polyamines [26]. Furthermore, in tumor cells cyclic adenosine monophosphate increases both activity and expression of arginase [40]. It has also been published that polyamines increase intracellular cyclic adenosine monophosphate [41]. It is of interest to notice that increased sperm motility is also related to an increase in cyclic adenosine monophosphate [1, 42] and that nitric oxide synthase, the enzyme that synthesizes nitric oxide from L-arginine is present in spermatozoa and regulated by cyclic adenosine monophosphate as well [43]. It could be interesting to study the relationship of these two enzymes in the spermatozoa and their possible interaction in increasing sperm cell motility.

It has recently been shown that arginase can regulate the activity of nitric oxide synthase by competing for the same substrate arginine in several organs [19-21]. Since both polyamines and nitric oxide are related to an increase in sperm cell motility, it would be interesting to investigate a possible co-regulation of these enzymes in spermatozoa, because arginase is regulated by androgens [17, 18], nitric oxide by estrogens [44, 45] and aromatase, the enzyme that converts testosterone to estradiol, is present in spermatozoa [46, 47], no doubt the study of the possible co-regulation of both enzymes in spermatozoa would be interesting to be undertaken.

In conclusion, our findings are that arginase is present in ram epididymal spermatozoa and that its distribution changes as the sperm cells traverse the epididymis. We hypothesize that the presence of arginase in sperm cells of the epididymis is required to provide ornithine, the precursor of polyamine biosynthesis, before reaching the seminal fluid, but it remains to be demonstrated. The presence of arginase in spermatozoa may be of clinical relevance, since L-arginine increases sperm motility, as it has been demonstrated in sperm cells obtained from diabetic patients [25], which is in accordance to the fact that extrahepatic arginase-/-mice show a reduction in fertility [48] and agree to the polyamine distribution in ram spermatozoa [7] and to the fact that it has been suggested that spermine prevents premature capacitation and acrosome reaction [49]. Finally, we consider that more research is urged in order

to know the molecular mechanisms that regulate or co-regulate arginase expression and activity in sperm cells as well.

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