

Immunolocalization of heat shock protein 70 in bovine spermatozoa

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Summary. Heat shock protein 70 (HSP70) is part of a superfamily of molecular chaperones, which protect cells from chemical and heat shock. The objectives of this study were to determine the presence of HSP70 in bovine spermatozoa and its subcellular localization during different stages of spermatogenesis. Analysis of sperm proteins by Western blotting using a monoclonal antibody to the inducible form of HSP70 revealed a single immunoreactive band with an estimated molecular weight of 70 kDa in samples from 18 of 18 bulls. Using immunofluorescence microscopy and the same antibody, HSP70 was localized to the cytoplasm of prophase spermatocytes and elongating spermatids, to cytoplasmic droplets of caput epididymal spermatozoa, and to cytoplasmic droplets, acrosome, post-acrosomal region and middle piece of corpus and cauda epididymal spermatozoa. The pattern of distribution changed in freshly ejaculated spermatozoa as HSP70 was detected on the acrosome only. During capacitation and acrosome reaction, HSP70 was once again redistributed, and was localized to the equatorial segment, post-acrosomal region and middle piece. Thus, HSP70 is present in the spermatozoa of mature bulls and redistribution of the protein occurs during capacitation and the acrosome reaction.

Introduction

The mammalian heat shock protein 70 (HSP70) family consists of two main intracellular isoforms of approximately 70 kDa molecular weight, one which is constitutively expressed (HSC70) and one that is expressed at low levels in normal cells and at high levels in stressed cells (HSP70). Although these isoforms exhibit an extremely high degree of relatedness, they differ in their amino acid composition and can be identified by different monoclonal antibodies (Kerendian *et al.*, 1992; Neuer *et al.*, 1998). As chaperones, HSP70 helps maintain protein conformation, stabilize unfolded precursor proteins prior to assembly into macromolecular complexes and participate in transfer of proteins across intracellular membranes (Gething & Sambrook, 1992). During cellular stress, HSP70 synthesis is enhanced and the protein protects cells by refolding denatured protein, removing damaged proteins at degradation (Welch & Suhan, 1986) and by blocking apoptosis (Mosser *et al.*, 1997; Samali & Orrenius, 1998).

Spermatogenesis is a complex process that results in the production of a mature spermatozoon and involves a series of mitotic divisions of spermatogonia, two meiotic divisions of spermatocytes and the transformation of the spermatids into spermatozoa. Various isoforms of HSP70, including a sperm-cell specific variant in rodents (Eddy, 1999), are known to be present in a stage-specific and developmentally regulated manner during spermatogenesis in mouse, rats and man. These isoforms have been implicated in germ cell differentiation (Allen *et al.*, 1988; Zakeri *et al.*, 1988). In addition, HSP70 might be involved in mitigating effects of elevated temperatures on spermatogenesis and post-ejaculatory function of sperm (Nakamura & Hall, 1978; Meyerhoeffer

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et al., 1985) Unlike somatic cells, which are capable of synthesizing heat shock proteins in response to stress, ejaculated spermatozoa are transcriptionally inactive and their translational machinery is thought to be lost from the residual cytoplasm. Hence, stress-induced synthesis of HSPs in spermatozoa is not possible and the sperm is dependent upon pre-formed HSPs, if present, for protection from elevated temperature.

HSP70 appears to play an important role in sperm function after ejaculation and has been identified in proteins extracted from bull (Kamaruddin *et al.*, 1996) and boar sperm (Huang *et al.*, 2000), and in the seminal plasma of humans (Miller *et al.*, 1992) and bulls (Kamaruddin *et al.*, 1996). Matwee *et al.* (2001) have shown that exposure of bovine spermatozoa and oocytes to mouse anti-HSP70 during fertilization *in vitro* caused a dose-dependent reduction in fertilization. Huang *et al.* (2002) have attributed variability in fertility in a group of boars to single nucleotide polymorphisms in a promoter of HSP70. Furthermore, HSP 70 has been implicated in fertilization and embryo development in humans (Miller *et al.*, 1992; Eggert-Kruse *et al.*, 2002), rodents (Neuer *et al.*, 1998, 2000) and cattle (Matwee *et al.*, 2001). The goal of the present study was to determine the subcellular localization of inducible isoform of HSP70 on spermatozoa. To accomplish this, presence of HSP70 in bovine sperm extracts was evaluated by Western blotting while presence and localization of HSP70 on whole spermatogenic cells and ejaculated spermatozoa was determined by immunofluorescent microscopy using a mouse antiHSP70 monoclonal antibody.

Materials and methods

SDS-PAGE and Western blotting

Preparation of sperm extract

Fresh ejaculated Holstein bull semen collected with the aid of an artificial vagina was used in this study. Semen was brought to the laboratory on ice and centrifuged at $800 \times g$ for 10 min at 4°C to separate spermatozoa from seminal plasma. The sperm pellet was washed three times in phosphate-buffered saline (PBS) at pH 7.4 and resuspended in lysis buffer containing 1.5% (w/v) Tris [hydroxymethyl]-aminomethane buffer (pH 6.8) containing 20% (w/v) sucrose, 10% (w/v) SDS, 5% (v/v) β -mercaptoethanol and 0.05% (w/v) bromophenol blue (3', 3'', 5', 5''-tetrabromophenol-sulfonephalein; Bio-Rad, Mississauga, ON, Canada) at a final concentration of 5×10^5 spermatozoa μl^{-1} . The mixture was boiled for 5 min and centrifuged at

$10\,000 \times g$ for 10 min. The supernatants or sperm extracts were stored at -80°C until further use.

Western blotting

Sperm proteins in the sperm extract were separated by SDS-PAGE using the buffer solutions of Laemmli (1970) with a 12.5% (w/v) acrylamide gel and 4.0% (w/v) acrylamide stacking gel. Each well was loaded with 20 μl of running sperm extract. Purified HSP70 and bovine HSC70 (StressGen Biotechnologies Corp., Victoria, BC, Canada) were loaded as positive and negative controls.

After electrophoresis, gels were either stained for protein visualization or transferred onto a nitrocellulose membrane for Western blot analysis. For visualization of the protein profiles, the gel was fixed in acetic acid : ethanol : H_2O (7 : 40 : 53 v/v/v), stained with 0.125% (w/v) Coomassie blue R-250 (Bio-Rad) in acetic acid : ethanol : H_2O (7 : 40 : 53 v/v/v), and destained overnight in several changes of acetic acid : ethanol : H_2O (11 : 26 : 63 v/v/v). For blotting, proteins in the polyacrylamide gel were electrophoretically transferred onto a nitrocellulose membrane (Hybond-C extra; Amersham Life Science Inc., Oakville, ON, Canada) according to Towbin *et al.* (1979). Protein transfer was carried out for 2 h at 100 V and 250 mA. Following electrophoretic transfer, the membrane was rinsed in PBS and blocked overnight in a blocking solution called T-NFM-PBS that consisted of 0.1% (v/v) Tween-20 and 5% (w/v) nonfat dry milk in PBS at 4°C . The membrane was then incubated for 3 h at room temperature with mouse anti HSP70 (SPA-810, StressGen Biotechnologies Corp.) in a solution containing 0.1% (v/v) Tween-20 and 3.0% (w/v) nonfat dry milk in PBS (T-NFM-PBS). The antibody raised against human HSP70, is specific for HSP70 and not HSC70, but cross-reacts with bovine HSP70. Afterwards, the membrane was rinsed three times in PBS containing 0.1% (v/v) Tween-20, incubated with 1 $\mu\text{g ml}^{-1}$ Protein-A horseradish peroxidase (Amersham Life Science Inc.) in T-NFM-PBS, rinsed six times and then processed for detection of immunoreactive bands using enhanced chemiluminescence detection reagents (Amersham Life Science Inc.) on Kodak Scientific Imaging Film (X-Omat AR; Eastman Kodak Company, Rochester, NY, USA).

Indirect immunofluorescence studies of spermatogenic cells and spermatozoa

Fresh semen samples ($n = 8$ Holstein bulls) were centrifuged twice at $300 \times g$ for 10 min and the sperm pellets were diluted in Hepes-buffered Tyrode's albumin-lactate-pyruvate medium (HE-PES-TALP) supplemented with 0.05 mg ml^{-1}

gentamicin (Parrish *et al.*, 1988). The centrifugation procedure was repeated and the pellet was resuspended to a concentration of about 1×10^6 sperm ml^{-1} . Sperm were smeared and air-dried on cleaned poly-L-lysine-coated slides. Slides were fixed for 5 min in absolute methanol at -20°C , air dried, and kept at -20°C for further studies.

Bovine testes ($n = 6$) were obtained from an abattoir. The tunica albuginea was sliced open and sections from the testes and the three parts of epididymis (caput, corpus and cauda) were removed and suspended separately into a container containing Sperm TALP. Each tissue was sliced and minced, resuspended in Sperm TALP and filtered through gauze. The filtered spermatogenic cells were centrifuged, smeared onto slides, air-dried and fixed as described above.

To determine the pattern of HSP70 in capacitated and acrosome-reacted sperm, fresh spermatozoa were induced to capacitate and undergo acrosome reaction according to the method described by Parrish *et al.* (1988). For capacitation, fresh semen was diluted 1 : 4 in equilibrated Sperm TALP, centrifuged at $300 \times g$ for 10 min at room temperature, and resuspended at a concentration of 20×10^6 sperm ml^{-1} in Sperm TALP containing $10 \mu\text{g ml}^{-1}$ heparin (Sigma, Oakville, ON, Canada). For the acrosome reaction, the heparin-treated spermatozoa were incubated for 4 h in 5% CO_2 at 38.5°C . At the end of the 4-h period, $100 \mu\text{g ml}^{-1}$ of egg yolk lysophosphatidylcholine (LPC; Sigma) was added to sperm, gently mixed and then incubated for another 15 min in 5% CO_2 at 38.5°C . Smears of pre-capacitated, capacitated and acrosome-reacted sperm from eight bulls were fixed as described above to determine the patterns of HSP70 distribution in these germ cells.

The procedure for indirect immunofluorescence was based on the one previously described by Baccetti *et al.* (1989) with some modifications. Slides of ejaculated sperm and spermatogenic cells were rehydrated twice for 5 min in PBS. Non-specific binding sites were blocked with 10% (v/v) normal goat serum (Jackson Immunoresearch Laboratory, Inc., West Grove, PA, USA) in PBS for 30 min. Slides were incubated with anti-HSP70 in PBS containing 5% bovine serum albumin (w/v), covered with parafilm and incubated overnight in a humid chamber at 4°C . Slides were washed three times in PBS before incubating with goat anti-mouse-FITC (GAM-FITC; Jackson Immunoresearch Laboratory, Inc.), incubated in the dark for 2 h at room temperature and subsequently washed three times in PBS. A mixture of anti-fade mounting medium consisting of 1,4-diazobicyclo-[2.2.2]octane (Sigma) in glycerol-PBS and $1.25 \mu\text{g ml}^{-1}$ propidium iodide to counterstain the

nucleus was layered onto the slides. Slides were mounted with coverslips, sealed and observed under a Leitz Aristoplan fluorescence microscope (Leica Microsystems, Richard Hill, ON, Canada), using a dual rhodamine-FITC filter (excitation wavelength = 490–520 nm and emission wavelength = 505 nm) at $1000\times$ magnification. A minimum of 200 spermatozoa per slide were observed in each study. Negative control slides included those incubated with (i) anti-HSP70 without GAM-FITC (Jackson Immunoresearch Laboratory Inc.) or (ii) GAM-FITC without anti-HSP70.

Acrosome integrity

The integrity of the acrosome of sperm from the eight Holstein bulls (above) was determined using *Pisum sativum* agglutinin conjugated with FITC (PSA-FITC; Sigma) based on the method by Cross *et al.* (1986) with some modifications. Smears of pre-capacitated, capacitated and acrosome-reacted sperms were air-dried and fixed for 5 min in absolute methanol at -20°C . The slides were incubated with PSA-FITC ($40 \mu\text{g ml}^{-1}$ in PBS) for 20 min at room temperature, rinsed in PBS for 15 min, and mounted and examined as described for immunofluorescence. A minimum of 200 spermatozoa was evaluated for each slide.

Statistical analyses

Analysis of variance using the General Linear Models (GLM) procedure of SAS (Statistical Analysis System Inc., Version 6.12, Cary, NC, USA) was carried out on arcsine transformed data to test whether the proportion of spermatozoa showing the different distribution patterns of HSP70 fluorescence varied with physiological states. When differences were found, Duncan's multiple range test was used to ascertain the differences among the treatment means. Differences were considered to be statistically significant at $P < 0.05$. The values reported in the text and tables are mean \pm standard error of mean (SEM).

Results

SDS-PAGE and Western blot analysis of bovine sperm proteins

Sperm protein extracts separated by SDS-PAGE under reducing conditions contained nine major protein bands of molecular weights 3, 6, 8, 15, 28, 32, 44, 70 and 87 kDa. Numerous other less intense bands were also observed (Fig. 1a). The pattern was essentially similar for ejaculates from each of three different bulls tested. When the protein extracts

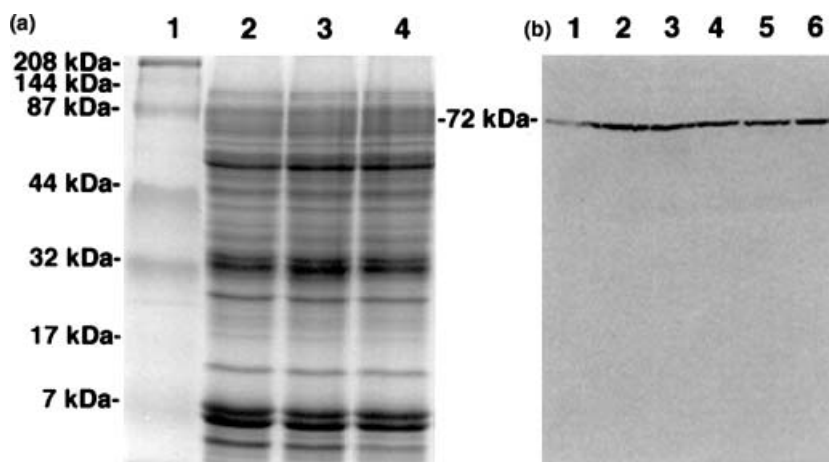


Figure 1. (a) Sperm protein extracts of three bulls separated by SDS-PAGE (12.5% w/v acrylamide] under reducing conditions (lanes 2–4). Protein bands were identified by Coomassie blue staining. Lane 1 contained pre-stained standards. (b) Western blot of sperm protein extracts of six bulls using mAb to the inducible form of HSP70.

were transferred to a nitrocellulose membrane and immunoprobed with anti HSP70, a distinct single band characteristic of HSP70 was observed on the immunoblot (Fig. 1b). This band did not cross react with antibody against HSC70 (result not shown).

Indirect immunofluorescence of spermatogenic cells and ejaculated spermatozoa

In testicular smears immunostained with anti-HSP70, green granular fluorescent signals indicative of HSP70 were confined to the cytoplasm of meiotic and post-meiotic germ cells while spermatogonia, Sertoli cells, Leydig cells and stroma were nonfluorescent. In prophase spermatocytes, the cytoplasm displayed intense green signals whereas in spermatids in different stages of spermiogenesis, the fluorescent signals delineated the cytoplasm in the process of being caudally displaced (Fig. 2a–c). The condensed and elongated spermatid nucleus generally displayed no fluorescence whereas weak signals were seen in the neck region and residual cytoplasm of tubular spermatozoa.

In spermatozoa recovered from the caput epididymidis, signals were noted only in the cytoplasmic droplets (Fig. 2d). For spermatozoa collected from the corpus epididymidis, in contrast, fluorescent signals were noted in globules of different size and contour located in the acrosomal and post-acrosomal regions and middle piece as well as in cytoplasmic droplets (Fig. 2e). More intense fluorescent signals for HSP70 were noted in the acrosomal region at the base of the sperm head and in the post-acrosomal region of spermatozoa collected from cauda epididymidis (Fig. 2f).

Fixed fresh ejaculated bovine spermatozoa immunostained with anti-HSP70 produced green granular fluorescence on the acrosome (Fig. 2g). Fluorescence signals were absent in the post-acrosomal region, middle piece and the cyto-

plasmic droplet. Negative control slides incubated only with the GAM-FITC showed no fluorescent signal on the spermatozoa. Unfixed slides stained with antibody showed less intense fluorescent signals on the acrosome, equatorial segment, post-acrosomal region and middle piece.

Indirect immunofluorescence of capacitated and acrosome-reacted spermatozoa

The fluorescent pattern of sperm induced to capacitate in a heparin-containing medium and those subjected to acrosome reaction in the presence of egg white LPC differed from that noted in untreated spermatozoa. As with fresh spermatozoa, fluorescent signals were localized in the acrosome of pre-capacitated (untreated) spermatozoa. Spermatozoa exposed to heparin for capacitation, however, exhibited intense fluorescent signals in the acrosomal and post-acrosomal regions, equatorial segment and, in some cases, in the middle piece (Fig. 2h). On the contrary, spermatozoa induced to undergo the acrosome reaction by exposure to LPC exhibited several distinct patterns that probably reflect variation in the stages of acrosome reaction experienced by individual spermatozoa at the time of analysis (Fig. 2i). A majority of spermatozoa showed no fluorescent signals on the head. Others, in which the acrosome was in the process of being detached from the head, displayed signals with exclusively on the acrosome, on the equatorial segment, on the post-acrosomal region or on all these three domains. The middle piece of acrosome-reacted sperm generally did not exhibit fluorescence. In spermatozoa that had lost their acrosome, fluorescent signals were confined to the equatorial segment. The proportions of spermatozoa displaying specific pattern of signal localization in response to different treatments are summarized in Table 1. Almost 80% of untreated spermatozoa

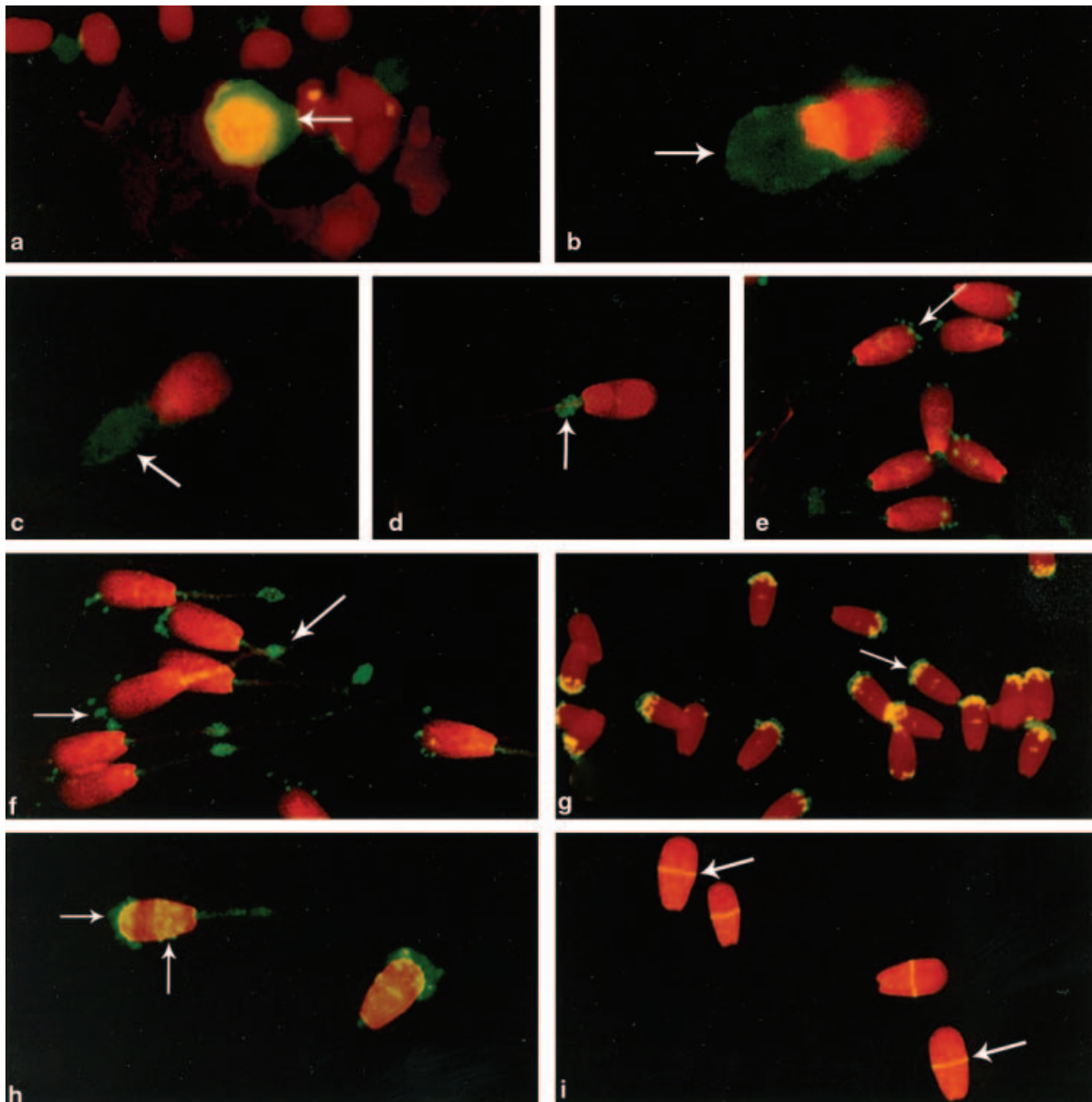


Figure 2. Localization of HSP70 on bovine spermatozoa using mouse anti-HSP70 mAb as primary antibody and goat anti-mouse-FITC as secondary antibody (a–h), and acrosomal membrane integrity of spermatozoa as determined by *Pisum sativum*-FITC staining (i–l). Green fluorescence indicates positive immunostaining revealed by fluorescein isothiocyanate, red fluorescence indicates nuclear staining with propidium iodide and yellow fluorescence indicates overlapping of green and red fluorescence. (a) Pachytene spermatocyte. (b,c) Elongating spermatid. (d) Caput epididymal spermatozoa. (e) Corpus epididymal spermatozoa. (f) Cauda epididymal spermatozoa. (g) Fresh ejaculated spermatozoa. (h) Capacitated spermatozoa. (i) Acrosome-reacted spermatozoa.

showed fluorescent signals on the acrosome, equatorial segment, post-acrosomal region and middle piece compared with less than 10% in heparin–LPC-treated spermatozoa. Accordingly, the absence of fluorescent signals was detected in 11 and 61% of untreated and heparin–LPC-treated spermatozoa, respectively. The percentage of sperm with fluorescences on detaching acrosome was about 20% in heparin–LPC-treated spermatozoa compared with less than 10% in untreated and heparin-treated spermatozoa.

The percentage of spermatozoa displaying intact acrosomes was lower ($P < 0.01$) in sperm exposed to heparin for capacitation ($65.7 \pm 4.09\%$) than for untreated sperm ($97.1 \pm 4.08\%$). A high incidence of vesiculation of the acrosomal membrane was noted in samples of sperm exposed to heparin. Treatment of capacitated sperm with LPC for 15 min significantly reduced the number of sperm with intact acrosome to $29.0 \pm 3.33\%$ ($P < 0.01$).

Table 1. Percentage of spermatozoa showing specific pattern of HSP70 fluorescence following treatments to induce capacitation and acrosome reaction

Treatment	Distribution patterns of fluorescent signals on spermatozoa			
	Acrosome, ES, PAR and MP	Detaching acrosome, ES, PAR and MP	ES only	Absence of fluorescence on sperm head and MP
No treatment	78.85 ± 3.5 ^a	5.44 ± 2.9 ^a	5.91 ± 4.5 ^a	10.81 ± 6.4 ^a
Heparin-treated	14.49 ± 3.1 ^b	9.00 ± 2.6 ^a	25.66 ± 4.0 ^b	50.85 ± 5.7 ^b
Heparin-LPC-treated	5.35 ± 2.8 ^c	17.55 ± 2.4 ^b	15.93 ± 3.6 ^{ab}	61.46 ± 5.2 ^b

No treatment, control; heparin-treated, capacitated; heparin-LPC-treated, acrosome-reacted; ES, equatorial segment; PAR, post-acrosomal region; MP, middle piece.
Percentage ± SEM with different superscripts within column are significantly different ($P < 0.05$) from each other and within a given distribution.

Discussion

This investigation has shown that HSP70 is present in bovine spermatogonia, spermatids and spermatozoa, and that intracellular localization of this molecular chaperone changes during spermatogenesis, after ejaculation, and as a result of capacitation and acrosome reaction. These findings are based on Western blot analysis and immunocytochemistry using a commercially available monoclonal antibody that has previously been shown to detect the inducible form of bovine HSP70, but not the constitutive (HSC70) isoform (Kerendian *et al.*, 1992). A testis-specific variation of HSP 70 exists in mice (Eddy, 1999) but it is not known whether the HSP70 antibody used here recognizes that particular protein. Detection of HSP70 in bovine spermatozoa is in agreement with previous reports of multiple isoforms of HSP70 and HSP90 in extracts of ejaculated human spermatozoa (Miller *et al.*, 1992).

Analysis by indirect immunofluorescence indicates that HSP70 is present during meiotic prophase in pachytene spermatocytes and in spermatids undergoing spermiogenesis. The strong fluorescent signals in the cytoplasm of bovine spermatids at different phases of spermiogenesis suggest that during post-meiotic differentiation, when the transformation of round spermatids to highly polarized tubular spermatozoa takes place, HSP70 is confined to the cytoplasmic droplets. In previous studies in mice, HSP70 was not detected in meiotic germ cells unless they were exposed to heat stress (Allen *et al.*, 1988), although the constitutively expressed HSC70 was present in the cytoplasm of pachytene spermatocytes, round spermatids, residual bodies of elongating spermatids and at the post-acrosomal region of the condensing spermatids (Mackawa *et al.*, 1989). The difference between the present findings on bovine germ cells and previous observation on mouse germ cells cannot be attributed to the cross reaction of the mouse HSP70 with

HSC70 in bovine germ cell cytoplasm as the HSP70 antibody did not bind to the constitutively expressed HSC70 in Western blot analysis.

The present study showed that HSP70 exhibits a remarkable shift in intracellular location at two different stages in spermatozoal development. The first shift occurs during transit of spermatozoa from seminiferous tubules through the epididymis while the second is detected during the physiological changes associated with capacitation and acrosome reaction. Thus, fluorescent signals detected only in the cytoplasmic droplets of caput epididymal spermatozoa were replaced by granular fluorescent signals in the cytoplasmic droplets, middle piece and the acrosomal region in spermatozoa from the corpus and cauda regions of the epididymus. There are at least three possible mechanisms responsible for the appearance of additional fluorescent signals on spermatozoa as they move through the corpus and caudal epididymidis. The HSP70 in spermatozoa in the process of ductal transit may be synthesized as a precursor protein in the acrosome, equatorial segment, post-acrosomal region and middle piece during spermatogenesis in the elongating spermatids. Additionally, sperm plasma membrane remodelling during epididymal transit might redistribute the HSP70 to other domains as has been reported for other spermatozoal surface proteins (Myles & Primakoff, 1984; Jones *et al.*, 1990). Finally, HSP70 could be acquired by sperm as a result of uptake of secreted HSP70 of epididymal origin.

The remodelling and redistribution of sperm proteins during epididymal maturation may be essential for the development of fertilizing potential which is acquired as spermatozoa pass through the epididymis. Sperm maturation takes place in the corpus and cauda regions of the epididymides where the spermatozoa begin to display forward motility and acquire the potential to bind to and fuse with the plasma membrane of the oocyte and eventually to fertilize the oocyte (Moore, 1996). The negative surface charge of spermatozoa increases

during this process of maturation when compositional changes of the plasma membrane take place through the incorporation of proteins, sugars and lipids secreted by the epididymis under the control of androgens (Orgebin-Crist *et al.*, 1975). The presence of HSP70 on the acrosomal region may be required to stabilize proteins of the sperm plasma membrane.

The second shift in intracellular localization of HSP70 was observed following the exposure of fresh ejaculated spermatozoa to heparin. This phenomenon is likely the result of membrane remodelling or alterations during capacitation-acrosome reaction. In fresh ejaculated spermatozoa, fluorescent signals were confined to the acrosome of fixed spermatozoa whereas in unfixed spermatozoa the acrosome, equatorial segment, post-acrosomal region and middle piece showed positive signals. The presence of fluorescent signal in unfixed fresh ejaculated spermatozoa indicates that HSP70 may exist in extra-membranous and transmembranous forms in bovine spermatozoa. Indeed, Western blot analysis indicated that HSP70 can be found in membrane extracts of bovine sperm (data not shown).

The apparent redistribution of fluorescent signals in capacitated spermatozoa (to the equatorial segment, post-acrosomal region and middle piece) requires further investigation. It would appear that the antigens present in the plasma membrane overlying the equatorial segment, post-acrosomal region and middle piece are made accessible to the HSP70 antibody when remodelling of sperm plasma membrane occurs by removal or unmasking of sperm surface components (Benoff *et al.*, 1993). The inaccessibility of the HSP70 epitopes (recognized by the HSP70 antibody) prior to capacitation could explain why signals were not detected in domains other than those in the acrosomal region.

The results of our study using the acrosome content binding PSA-FITC stain suggest that the distribution of HSP70 on spermatozoa parallel the changes in the integrity of the acrosome membrane. Samples of fresh ejaculated bovine spermatozoa that show fluorescent signals for HSP70 on the acrosomal cap also display intense PSA-FITC signals indicative of an intact acrosome membrane (Scully *et al.*, 1987). Disrupted and patch-like PSA-FITC fluorescent signals indicating fusion of the outer acrosomal membrane and the plasma membrane noted in spermatozoa exposed to LPC suggest that acrosomal HSP70 antigens undergo remodelling, unmasking or relocalization. The loss of HSP70 from the acrosomal regions, post-acrosomal regions and middle piece, and its presence as a fluorescent band in the equatorial segment indicate the loss of acrosomal cap upon completion

of the fusion of the plasma membrane with the outer membrane, during acrosome reaction. A similar pattern was also seen in spermatozoa stained with PSA-FITC. Fluorescent staining of the equatorial segments has been attributed to the presence of remnants of the outer acrosomal membrane (Scully *et al.*, 1987). However, in our study, the total percentage of acrosome-reacted spermatozoa with HSP70 on the equatorial segment was about 10% higher than that in acrosome intact spermatozoa exposed to PSA-FITC, indicating that the antigens may be bound to the inner acrosomal membrane.

In summary, the inducible form of HSP70 is present in bovine spermatozoa but its location shifts according to the stages of spermatogenesis, sperm maturation and the post-ejaculatory physiological changes. It is abundant in the cytoplasm of pachytene spermatocytes and elongating spermatids, and in cytoplasmic droplets of caput epididymal sperm. In spermatozoa from the corpus and cauda epididymis, HSP70 becomes more widely distributed and is observed in cytoplasmic droplets, acrosome, post-acrosomal region and middle piece. In ejaculated spermatozoa, distribution of HSP70 becomes more focused, being detected on the acrosome, and acrosome reaction leads to the equatorial segment, post-acrosomal region and middle piece. The post-ejaculatory changes in distribution point to the fluidity and post-translational modification of membrane proteins and suggest a possible role for HSP70 in fertilization.

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