The Diagnostic Value of Seminal Proteins

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Introduction

It is well known that immediately following a sudden increase in temperature, all types of cells increase the production of a class of compounds that appears to enhance the cell's ability to recover from stress. A similar cellular response occurs with exposure to a wide variety of environmental assaults such as toxic metals, alcohols, anoxia, and many metabolic poisons. Due to the fact that so many different types of stimuli elicit the same cellular defense mechanism in virtually all organisms - from *Escherichia coli* to man - it is also commonly referred to as "the stress response" and the expressed proteins, "stress proteins" or "heat shock proteins" (HSP; 1).

The enhanced production of HSPs has been reported to occur in germ cells. Members of the HSP 70 and HSP 90 families have recently been isolated from rat spermatogenic cells,[2,3] human ejaculated spermatozoa[4] and bovine testicular tissue homogenates.[5] The purpose of this study was to identify the presence of two HSPs, HSP 70 and ubiquitin, from ejaculated bovine spermatozoa following prolonged exposure to scrotal hyperthermia.

Materials and Methods

Experimental Group

Six mature mixed breed beef bulls were used in this study. All bulls were housed indoors in individual stalls throughout the experimental period. Prior to the initiation of scrotal insulation, semen was collected by electroejaculation and all bulls were determined to have satisfactory semen quality, as evidenced by >30% individual progressive motility and >75% morphologically normal spermatozoa.

Scrotal Insulation, Semen Collection and Evaluation

Scrotal heat was retained by an insulating bag made of an inner layer of a large plastic bag surrounded by multiple layers of elastic tape which were fastened to the neck of the scrotum. Insulating bags were checked daily and adjusted as needed to ensure complete scrotal coverage at all times. During the initial 23 day period, all bulls were electroejaculated once every 3-4 days and semen evaluations performed immediately following collection according to the procedure described below. Scrotal insulation was removed on day 23. During the second 60 day period (beginning on day 24), all bulls were electroejaculated once approximately every 7 days and semen evaluations again performed.

Immediately after collection a drop of semen was diluted with 0.9% sodium chloride on a warm microscope slide and evaluated for the percentage of progressively motile spermatozoa. An additional slide was stained with eosin-nigrosin and examined at 400x magnification under oil to determine the percentage of morphologically normal spermatozoa.

Electrophoresis

Protein samples were denatured and electrophoresis was performed on sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) according to Laemmli.[6] Each ejaculate was centrifuged at 10,000 g for 30 minutes and the supernatant discarded. The sperm pellet was washed 3 times by resuspension in ice-cold Dulbecco's Phosphate Buffered Saline (PBS) and centrifuged at 10,000 g for 30 minutes. The washed sperm pellet was then stored at -20°C.

One Dimensional PAGE

A 5 µl aliquot of each thawed sperm sample was diluted in 0.5 ml of Dulbecco's PBS. Samples were microfuged for 1 minute, supernatant discarded, and the procedure repeated. Pellets were mixed with 0.05 M Trizma base containing 500 K.I.U. aprotinin/ml and sonicated over ice. Protein concentration of each sample was determined via a Coomassie Brilliant Blue-based assay.[a] The remainder of each sample was diluted with an equal volume of sodium dodecyl sulfate (SDS) sample preparation buffer containing 0.5 M Trizma base, 2% SDS, 5% 2-mercaptoethanol, 45% glycerin, and 0.001% bromophenol blue. Samples were then heated at 100°C for 2 minutes, microfuged and used immediately for electrophoresis.

[a]Bio-Rad Laboratories, Hercules, CA.

Samples containing 12µg of protein were electrophoresed in a 0.75 mm-thick 10% polyacrylamide gel. Low range molecular weight protein standards[b] were used as a marker. Electrophoresis was performed at a constant current (35 mAmp) for 1.5 - 2 hours. Gels were stained with Coomassie Brilliant Blue R-250[c] and destained in 10% acetic acid.

Western Blotting / Antibody Probing

After equilibration of prestained gels and nitrocellulose paper in transfer buffer, electrophoretic transfer of gels was performed. Blots were then blocked for 1 hour in blocking solution (2.5% non-fat dried milk) and incubated with one or more of the following primary antibodies: 1) anti-Heat Shock Protein 70 (HSP 70)d monoclonal antibody, 2) anti-ubiquitin antibody.[e] Blots were rinsed with distilled water and washed for 10 minutes in Tween-20 wash solution prior to incubation with corresponding secondary antibodies. Blots were again washed and HSPs visualized by immersing blots in a 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) solution[f] and developing for 5-10 minutes. After development, all blots were reconstructed back to the original gel format.

Results

Semen Quality

A progressive deterioration in semen quality as assessed by decreased individual progressive motility (p<.003), decreased percentage of morphologically normal sperm (p<.04), and increased percentages of primary and secondary morphological abnormalities (p<.03) were observed in all 6 bulls during the experimental period (Tables 1-3). Of considerable significance is the fact that only 2 bulls became aspermic (on day 44) following 23 days of scrotal insulation, corresponding to 21 days after scrotal insulation had been removed. Bull F remained aspermic until the final day of the experimental period (day 81), at which time a low percentage (36%) of morphologically normal sperm was detected. This was not observed in bull E, which remained aspermic until completion of the study.

Table 1. Effect of prolonged scrotal insulation on gross motility.

Bull	P	1	3	7	10	13	16	20	23	30	36	44	50	56	62	71	81
A	3	5	5	5	4	5	5	4	1	2	2	2	2	2	4	2	5
B	2	4	5	3	5	3	3	4	4	2	2	1	2	2	2	3	3
C	2	5	3	4	5	4	4	4	4	3	2	3	3	4	2	4	5
D	5	4	4	5	4	3	4	2	2	1	1	1	1	1	1	2	4
E	3	4	5	4	4	5	..	5	2	1	1	..	..	..	..	1	..
F	4	3	4	5	4	4	2	2	1	1	1	..	..	..	..	1	1
				*							#						

5 = excellent
4 = good
3 = fair
2 = poor
1 = zero

P = pre-treatment
.. = missing data
* = p<.05
# = p<.10

Table 2. Effect of prolonged scrotal insulation on individual progressive motility.

Bull	P	1	3	7	10	13	16	20	23	30	36	44	50	56	62	71	81
A	3	3	4	3	4	3	3	3	2	3	2	3	2	3	3	3	4
B	4	4	4	3	3	5	4	4	3	3	2	2	3	3	4	4	3
C	4	4	3	4	4	4	5	4	4	4	3	4	4	4	4	4	4
D	4	3	4	4	3	5	4	3	2	2	2	2	2	2	2	2	4
E	5	4	3	4	4	4	..	3	2	3	2	..	..	..	..	1	..
F	4	3	4	3	3	3	3	2	2	1	1	..	..	..	..	1	1
		#		#				#	*	*	*		#				

5 = excellent
4 = good
3 = fair
2 = poor
1 = zero

P = pre-treatment
.. = missing data
* = p<.05
# = p<.10

Table 3. Effect of prolonged scrotal insulation on percentage morphologically normal sperm.

Bull	P	1	3	7	10	13	16	20	23	30	36	44	50	56	62	71	81
A	86	94	93	58	88	75	71	68	76	26	06	..	27	20	48	78	29
B	98	90	82	90	95	94	76	68	36	19	27	17	17	31	37	51	63
C	78	96	92	87	87	89	90	95	69	40	45	49	75	40	57	75	93
D	87	87	93	88	82	68	73	..	05	13	03	14	19	02	..	14	55
E	95	94	86	64	87	89	..	51	15	32	34	19	..	..	..	..	..
F	90	93	84	86	68	30	16	38	..	..	..	..	..	..	..	..	36
								*	*	*	*	*	#	*	*		

p = (pretreatment)
.. = (missing data)
* = (p<.05)
# = (p<.10)

One-Dimensional Gel Analysis

SDS-PAGE separation of sperm proteins from semen samples collected prior to scrotal insulation from 5 bulls (A, B, C, E, and F) resulted in similar protein distribution patterns based on differences in molecular weight (Figures 1 and 2). Approximately 27 sperm proteins were visualized (range, less than 20kD to greater than 116kD) in Coomassie blue-stained gels. Although pre-treatment samples from bull D also differed from this group of animals, these differences included the additional presence of 40 and 46kD proteins only.

Following 23 days of scrotal insulation the migratory patterns of samples taken from 3 bulls (A, B, and C) were unchanged with respect to the original protein separation and remained so for the duration of the experimental period. Changes in the patterns of sperm proteins occurred between pre- and post-treatment

[b]Bio-Rad Laboratories, Hercules, CA.
[c]Bio-Rad Laboratories, Hercules, CA.
[d]Sigma Chemical Company, St. Louis, MO.
[e]Sigma Chemical Company, St. Louis, MO.
[f]Sigma Chemical Company, St. Louis, MO.

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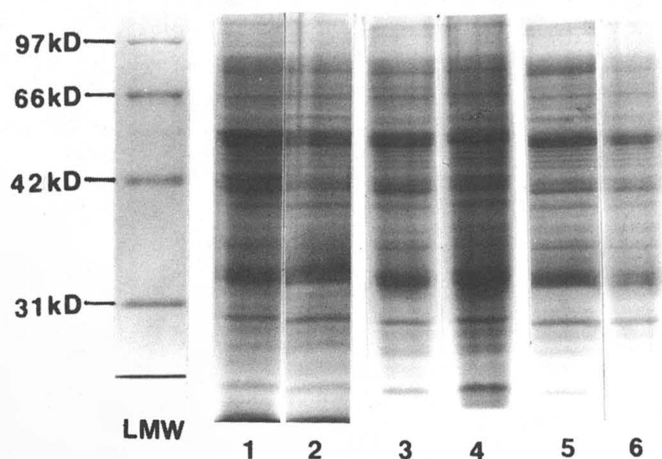

Figure 1. SDS-PAGE separation of sperm proteins. Lanes 1 and 2, bull A (pre- and post-treatment; respectively); lanes 3 and 4, bull B (pre/post); lanes 5 and 6, bull C (pre/post).

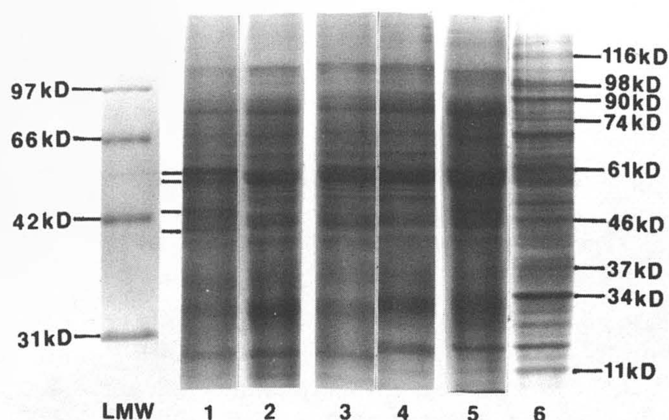

Figure 2. SDS-PAGE separation of sperm proteins. Lanes 1 and 2, bull D (pre- and post-treatment; respectively); lanes 3 and 4, bull E (pre/post); lanes 5 and 6, bull F (pre/post).

samples from 3 animals (D, E, and F). These changes were detected as early as 3 days following the application of scrotal insulation and remained so throughout the experimental period. For all 3 bulls, changes between pre- and post-treatment samples included the appearance of new protein bands and the disappearance of other specific bands, as well as detectable changes in relative quantities of certain proteins. In bull F new protein bands at 11, 34, 37, 46, 61, 74, 90, 98, and 116kD were detected in post-treatment samples, while 45, 47, and 54kD protein bands disappeared. Decreased concentrations of 33 and 81kD proteins were also observed post-treatment.

Considerably fewer changes were detected in post-treatment samples from the remaining two bulls. The most significant changes in bull D included the disappearance of 40 and 40kD proteins, increased concentrations of 56kD, and decreased concentrations of 58kD proteins. No protein changes were detected on Coomassie blue-stained gels for 30 days following the application of scrotal insulation for bull E. At this time the most significant changes included the appearance of 44, 97, 102, 125, 135, 145, 155kD proteins, the disappearance of a 60kD protein, and increased concentration of a 70kD protein (Figure 3).

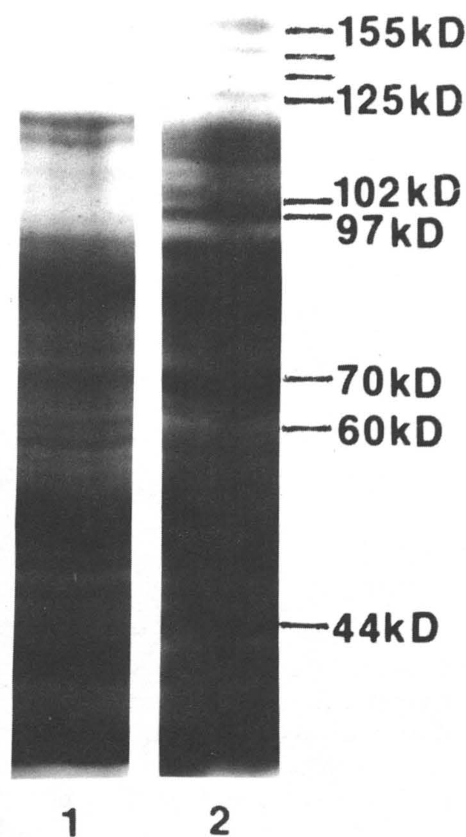

Figure 3. SDS-PAGE separation of sperm proteins. Lanes 1 and 2, bull E (after 13 and 30 days of scrotal insulation, respectively).

Composite Western blot analysis identified the presence of HSP 70 in 2 of the 3 bulls whose sperm samples showed changes in protein composition and distribution following scrotal insulation (E and F). In bull F a corresponding protein band (74kD) was observed in the Coomassie blue-stained gel from the post-treatment sample as compared to its absence in the pre-treatment sample. Greater sensitivity of the Western blot, however, revealed the presence of HSP 70 in both pre- and post-treatment samples (Figure 4). HSP 70, absent on Western blots performed on pre-treatment samples obtained from bull E, was subsequently detected in post-treatment samples beginning 20 days after the initiation of scrotal insulation, the reaction becoming more intense later in the experimental period (Figure 5).

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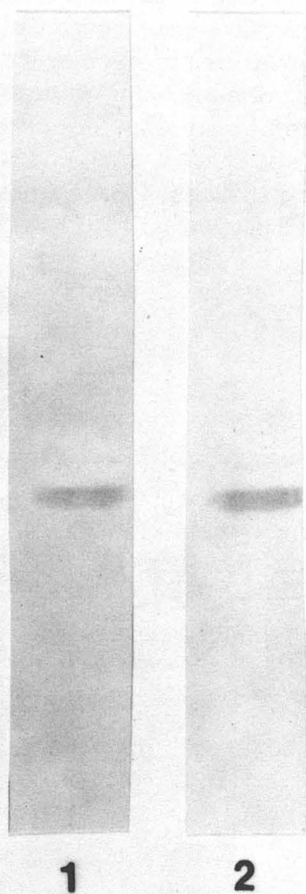

Figure 4. Western blot of sperm protein sample from bull F. Primary antibody: anti-Heat Shock Protein 70 (HSP 70) monoclonal antibody. Lanes 1 and 2, pre- and post-treatment samples, respectively.

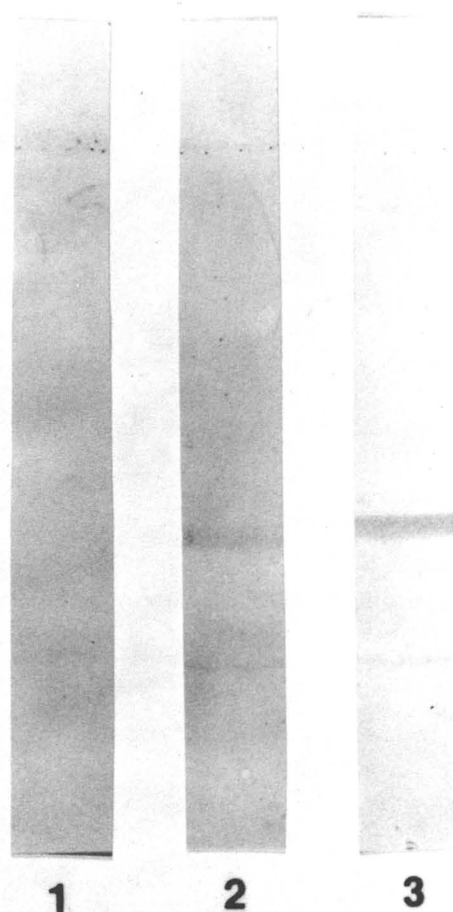

Figure 5. Western blot of sperm protein sample from bull E. Primary antibody: anti-Heat Shock Protein 70 (HSP 70) monoclonal antibody. Lanes 1, 2, and 3: 7, 20, and 30 days post-treatment, respectively).

Incubation of blots derived from pre- and post-treatment sperm samples from all bulls with the antibiquitin antibody revealed considerable variations in detection of protein bands within and among bulls. One or more proteins with a molecular weight in the range of 62-66kD were consistently present in all bulls in both pre- and post-treatment samples (Figure 6).

Discussion

Spermatogenesis is a process which has been shown to be adversely affected by alteration of the complex testicular thermoregulatory mechanism.[7] The epithelium of the seminiferous tubules is extremely sensitive to the influence of an elevation in intrascrotal temperature.[7] When elevations in intrascrotal temperature are artificially induced via scrotal insulation, the resulting spermatogenic dysfunction produces a decrease in fertility.[8] The enhanced expression of heat shock proteins in cells of all living organisms following temperature elevation is a phenomenon which has also been found to occur in germ cells.[9] Although an original hypothesis suggested that the increased susceptibility of the seminiferous epithelium to temperature elevations may result from an inherent lack or low production of HSPs,[2] members of the HSP 70 and HSP 90 families have recently been observed in rat spermatogenic cells, [2,3] human ejaculated spermatozoa,[4] and bovine testicular tissue homogenates.[5] In the present study we report the identification of HSP 70 and ubiquitin from both normal and heat-stressed ejaculated bovine spermatozoa.

SDS-PAGE separation of bovine spermatozoal proteins identified approximately 27 distinct sperm proteins in pre-treatment samples, with some variation between samples obtained from different bulls. Twenty-three days of scrotal insulation resulted in changes (detectable as early as 3 days following the application of scrotal insulation) in the migratory patterns of spermatozoal proteins from samples obtained from 3 of 6 bulls. Of these 3 bulls, immunoblotting techniques specifically identified one of the proteins as HSP 70 in bulls E and

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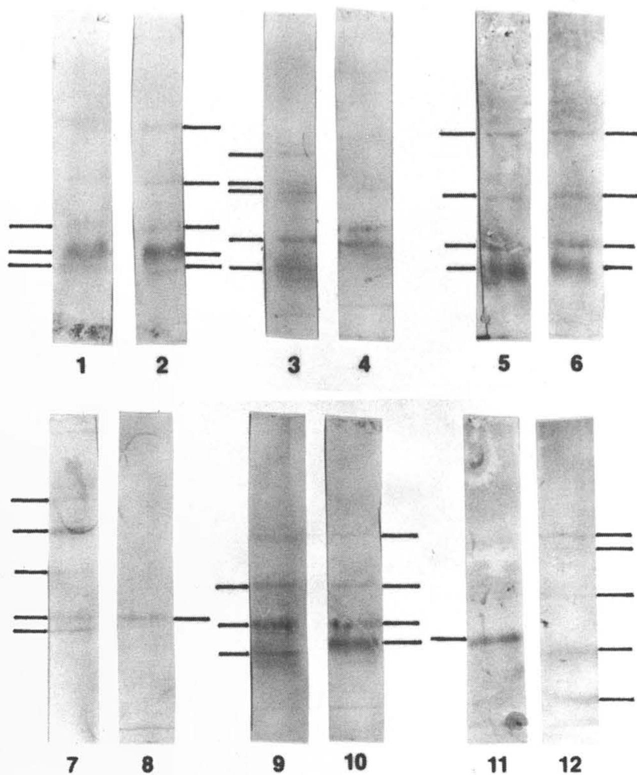

Figure 6. Western blot analysis of sperm protein sample. Primary antibody: anti-Ubiquitin antibody. Lanes 1 and 2, bull B; lanes 3 and 4, bull A; lanes 5 and 6, bull C; lanes 7 and 8 bull F; lanes 9 and 10, bull D; lanes 11 and 12, bull E. All pairs shown are pre- and post-treatment samples, respectively. Lines indicate a positive reaction.

F. Interestingly, this HSP was found in both pre- and post-treatment samples in bull F, but only in post-treatment samples from bull E. Ubiquitin was also identified via immunoblotting as a spermatozoal protein component of both pre- and post-treatment samples taken from all 6 bulls.

There is currently much debate over the exact function of heat shock proteins. The expression of HSPs in normal, unstressed cells, as well as those that have been traumatized, suggests a role in essential metabolic pathways in addition to cellular defense. The participation of HSPs in the maturation of cellular proteins is thought to involve the stabilization of adequately folded proteins as well as the identification and removal of denatured proteins from the traumatized cell.[1]

Of the apparently numerous different pathways involved in cellular protein degradation, a complex nonlysosomal process involves the conjugation of proteins selectively committed to degradation to ubiquitin.[10] Ubiquitin is also thought to be involved in processes such as DNA repair, cell cycle progression, the modification of histones and of receptors, and the heat shock response.[11] Protein denaturation occurs as a result of heat stress and the removal of protein irreversibly damaged in this process is necessary. Ubiquitin conjugation may be involved in the removal of these damaged proteins.[13] It has been suggested that inactivation or overloading of the ubiquitin system may lead to the subsequent induction of heat shock proteins.[14] Considerable variation in the identification of ubiquitinated protein bands in both pre- and post-treatment samples from all 6 bulls supports the involvement of ubiquitin in the removal of proteins of various molecular weights.

On the other hand, the strong correlation between the induction of HSPs and cellular thermotolerance implies that HSPs impart some degree of protection towards various forms of stress; among those, heat.[14,15] Miller[4] proposed that spermatozoa may be "'pre-prepared' for adverse environmental conditions by virtue of their existing complement of HSPs."

To our knowledge, this is the first report of the identification of heat shock proteins, specifically HSP 70 and ubiquitin, from both normal and heat-stressed ejaculated bovine spermatozoa. Previous studies[16] involving SDS-PAGE separation of spermatozoal proteins from bulls subjected to prolonged scrotal insulation (20 days) described differences in protein migratory patterns following heat stress. Although all 5 bulls in this study became aspermic, these protein changes were most noticeable in those 2 bulls that eventually regained normal sperm motility and morphology following the insulation period. Of the two bulls which became aspermic in the present study, only bull F showed evidence of regaining the potential to produce normal spermatozoa. Whether or not the same would have eventually occurred in the future with bull E is impossible to determine. In both studies, reversibility of the artificially induced testicular degeneration may be dictated by the potential that exists for members of the HSP 70 and/or other HSP families to be expressed or synthesized.

The fact that only two bulls (E and F) in the present study became aspermic following 23 days of scrotal insulation is in distinct contrast to the previously mentioned report by Wolfe *et al*[16] in which aspermia was achieved in all 5 experimental bulls after 20 days of scrotal insulation. Interestingly, these two bulls were also the only 2 from whose semen HSP 70 was identified. In previous studies on the expression of HSP by isolated mouse spermatogenic cells,[3] one form of HSP 70 (P70) was expressed in a stage-specific manner (predominately in pachytene spermatocytes which are particularly sensitive to heat) during cell differentiation, whereas HSP 70 was synthesized in response to stress in all spermatogenic cell populations examined. Due to the fact that synthesis of P70 in pachytene spermatocytes correlated with an apparent increased sensitivity to heat stress, it

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has been speculated that this HSP 70-like protein may perhaps interfere with the function of other members of the HSP 70 family, thus rendering these stages of spermatogenesis particularly sensitive to heat and other environmental stresses.[3] An exact understanding of the function of HSPs in cells in which they are expressed is vital to the understanding of the relationship between thermosensitivity and the presence of HSP family members.[17]

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