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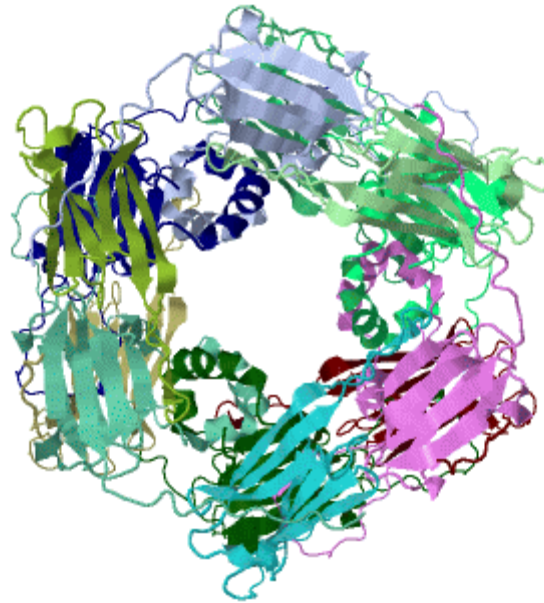
Heat Shock Proteins (HSPs) in livestock reproduction with a focus on the poultry sperm

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Heat Shock Proteins (HSPs)

- known as one of the major molecular chaperone proteins in eukaryotic cells;
- commonly divided according to their molecular weight into the HSP100 (HSPH), HSP90 (HSPC), HSP70 (HSPA), HSP60 (HSPD), HSP40 and HSP27 (HSPB) families;
- present in the cytosol and different cellular components including mitochondria, endoplasmic reticulum, and nucleus;
- have a protective function and they maintain cellular homeostasis



Jmol

- known for first time as stress-related proteins, strong evidence now refers to its protective role even under nonpathological/ stressful conditions;
- participate in the regulation of essential cell functions, such as protein translocation, refolding, assembly and the recognition, prevention of protein aggregation, renaturation of misfolded proteins, degradation of unstable proteins, etc.
- play a crucial role in regulating apoptosis

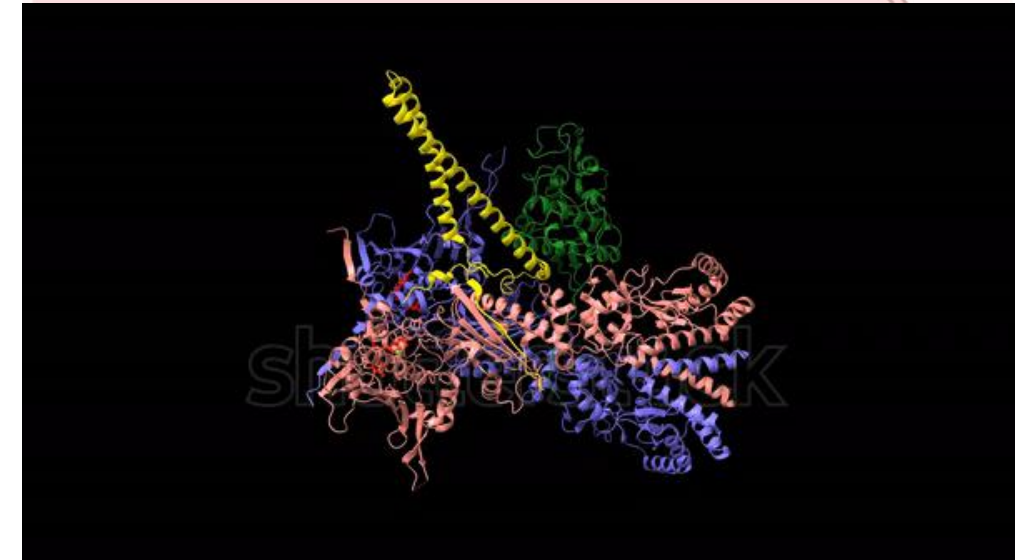
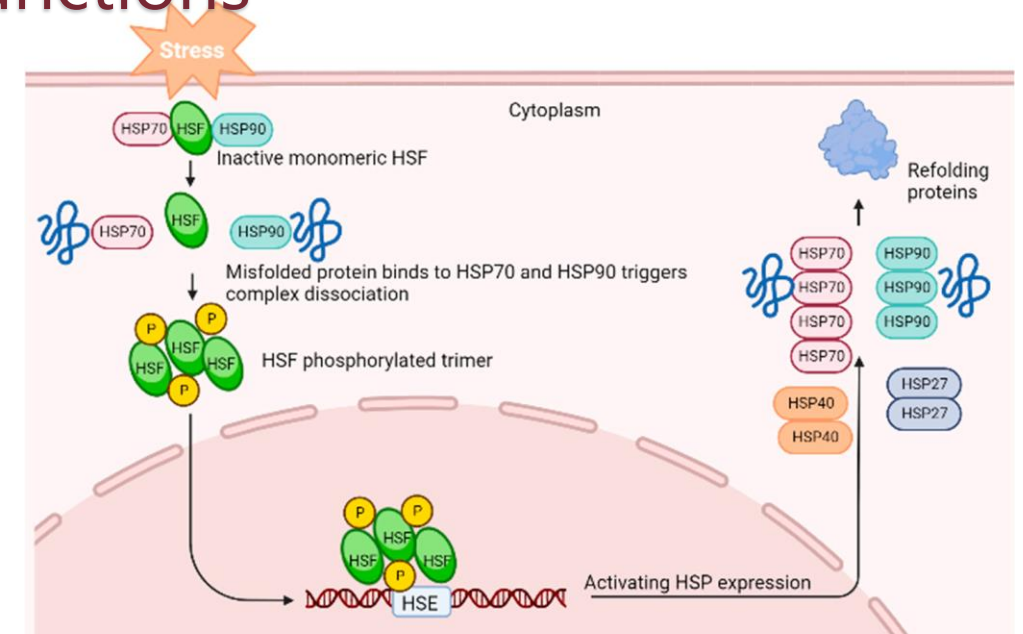
The role of HSPs in male reproductive functions

- expressed in testicular germ cells, Sertoli cells and along of the male reproductive tract
- both large and small molecular weights HSPs are isolated from different male reproductive organs;
- identified in the surface of sperm cell membrane of bull, boar, mouse, rat, stallion, dog and human.



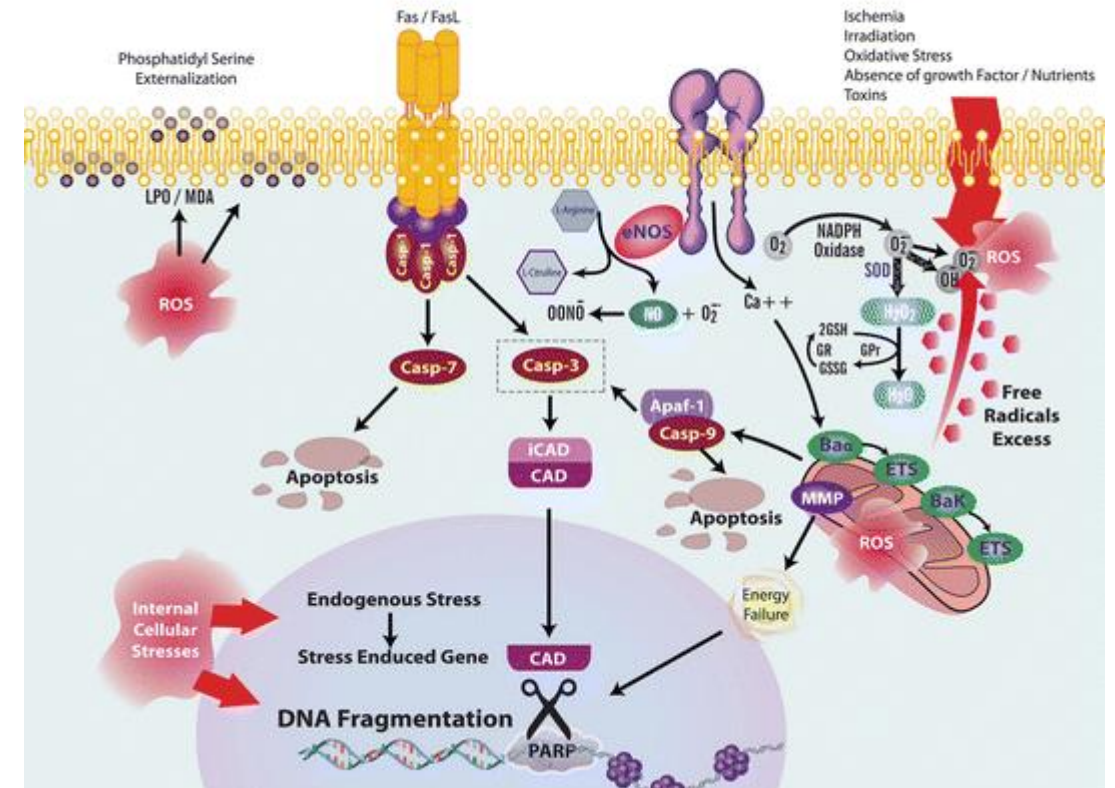
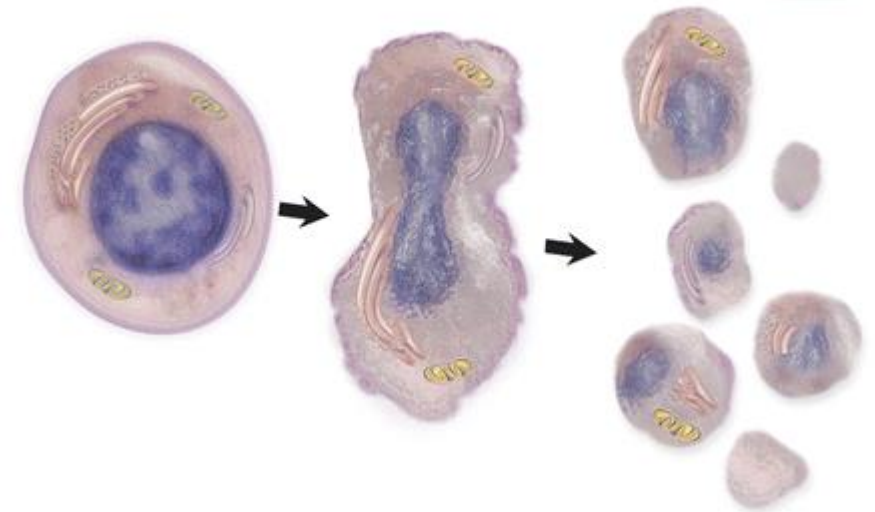
The role of HSPs in male reproductive functions

- ✓ HSP70, HSP60 and HSP90 families, are of the most important HSPs for spermatogenesis process in many species;
- ✓ involved in the different developmental stages of spermatogenesis;
- ✓ sperm cells can express HSPs pre- or post-ejaculation, indicating their role in regulating all sperm functions in both male and female reproductive tract.



HSPs and apoptosis

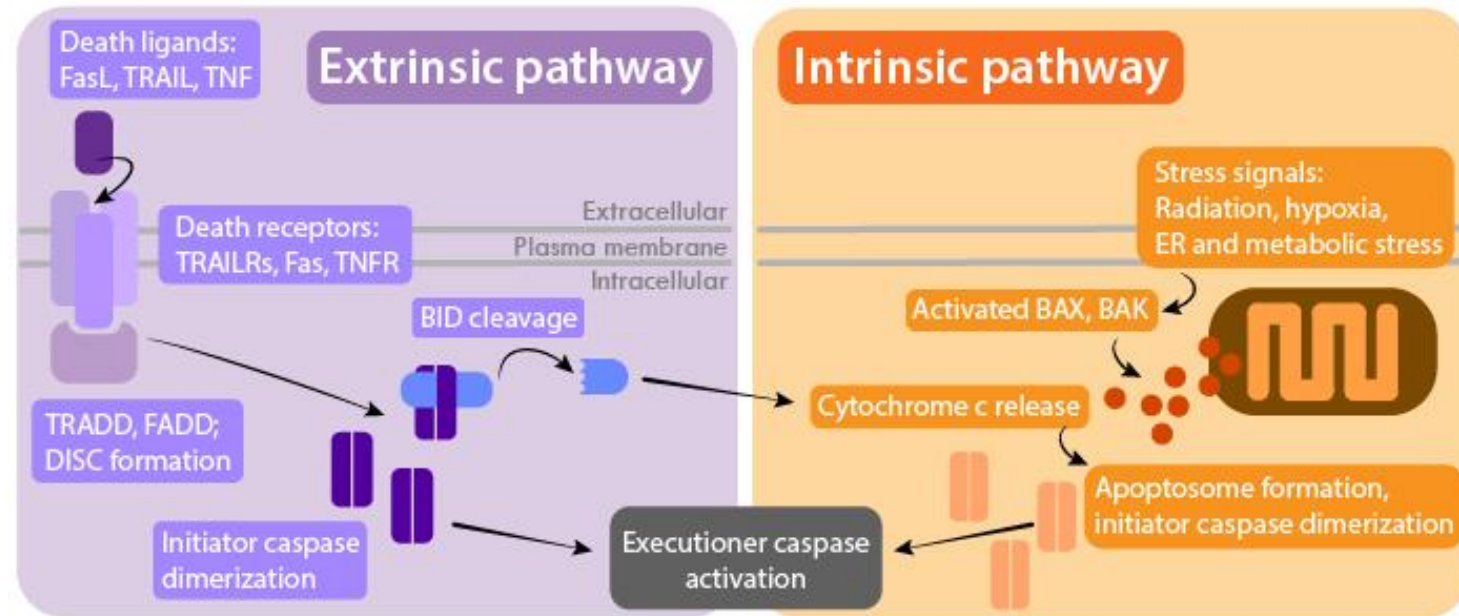
- ✓ block apoptosis by interfering with caspase activation;
- ✓ over expression of Hsp27, Hsp70, Hsp60, or Hsp90, inhibits apoptosis and prevents caspase activation in many different cellular models



Said, T., Mahfouz, R.Z., Kuznyetsova, I., Del Valle, A.P. (2015). Springer, New York, NY.

HSPs and apoptosis

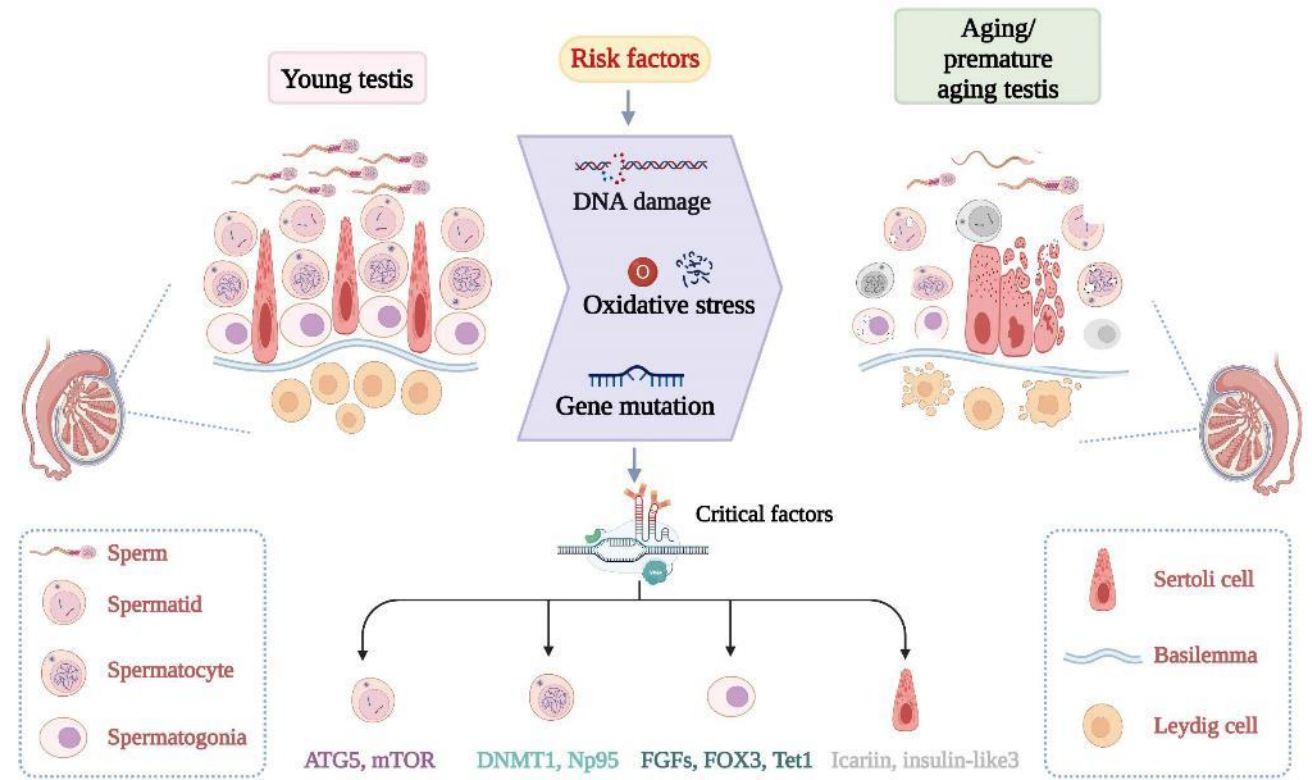
- ✓ can block both the intrinsic and the extrinsic apoptotic pathways through the interaction with keystone proteins at three points:
- ✓ (1) upstream of mitochondria, thereby modulating signaling nerve pathways;
- (2) at the mitochondrial level, controlling the release of apoptogenic particles; and
- (3) at the post-mitochondrial level, by blocking apoptosis at a later stage than any known survival enhancing drug or protein.



<https://www.biologend.com/en-us/apoptosis>

HSPs and aging

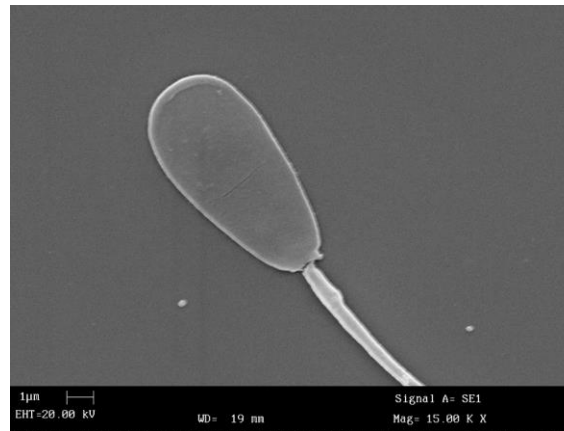
- the significant mediators of an organism's resistance to stress;
- this resistance is associated with promotion of an HSP gene, which activates the HSP expression during aging, enhancing stress resistance and extending the life span.
- During aging, HSPs show tissue and disease specific expression patterns in unstressed aging animals



- High expression of Hsp27 with its anti-oxidative potential is detected in the brains of aged and those with Alzheimer's disease.
- Hsp90 is involved in aging. Its gene and protein expression is lower in tissues and blood of aged patients and animals.
- Hsp90 is highly expressed in the testicular tissues with maturational arrest.

HSP 70/HSPA - mammals

- ✓ Hsp70 plays an important role in spermatogenesis and any decline in its levels impairs meiosis in spermatocytes and thereby significantly increases apoptosis
- ✓ HSPA2 is a major regulator of chromatin remodeling in differentiating spermatids and, in its absence, abnormal spermatozoa will be produced characterized by poorly compacted chromatin,
- ✓ regulates the expression of sperm surface receptors involved in sperm-oocyte recognition in humans, thus suggesting its vital role in fertility.

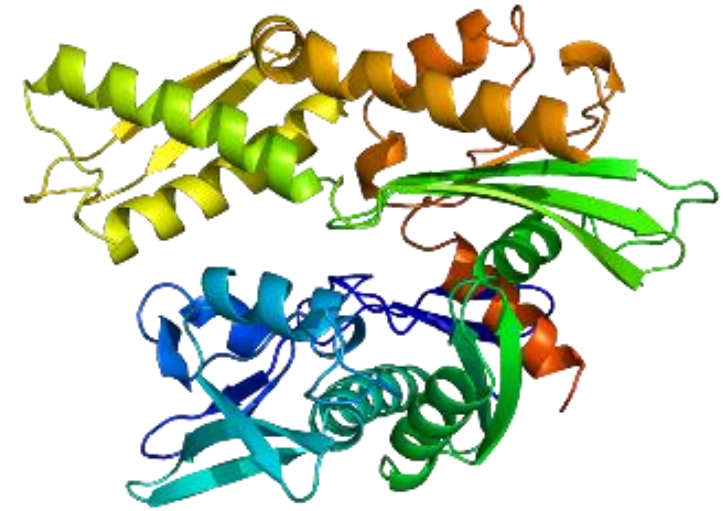


- ✓ In bull sperm is relocated from the acrosome to the equatorial segment, post-acrosomal region and midpiece during capacitation and the acrosome reaction,
- ✓ In boar sperm it is both relocated and translocated from the inner to the outer leaflet of the plasma membrane following acrosome reaction,
- ✓ Stallion spermatozoa present a post-acrosomal its localization in a thick band,
- ✓ In canine spermatozoa shows immunoreactivity in the neck of cells,
- ✓ In both epididymal and ejaculated feline spermatozoa, Hsp70 immunoreactivity is present diffusely in the sub-acrosomal region and in most part of midpiece

Kamaruddin et al., 2004; Spinaci et al., 2005; Volpe, et al. 2008. *Reprod Domest Anim*, ;43(4):385-392

HSP 70/HSPA - poultry

✓ While HSPA2 was reported to be down-regulated in mammalian male germ cells in response to heat stress, this gene was up-regulated in chicken



✓ In chicken sperm HSPA2 and HSPA8 gene expressions were significantly downregulated by freezing-thawing. The result indicates that HSPA2 and HSPA8 may play a critical role in rooster sperm cryopreservation.

HSP 60/HSPD

- ✓ Mammalian Hsp60 is mostly contained within the mitochondrial matrix and participates in the folding of mitochondrial proteins, thus facilitating the proteolytic degradation of misfolded or denatured proteins in an ATP-dependent manner; it acts in association with Hsp10, that regulates the chaperone function of Hsp60 during substrate binding and ATPase activity
- ✓ present in human seminal fluid and in rat and human testis, localized only in the somatic type of mitochondria in both spermatogonia and primary spermatocytes
- ✓ Hsp60 has been found in mature spermatozoa in bovine, stallion, boar, mouse, dog and cat

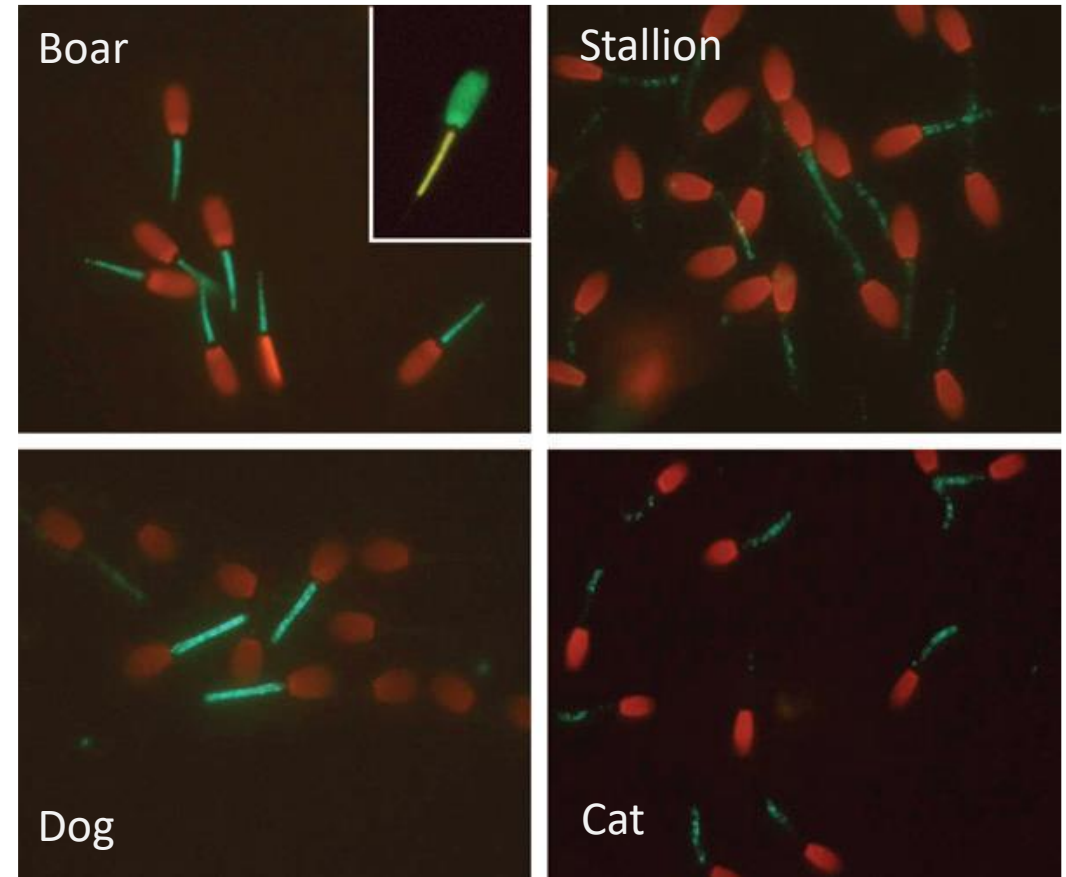
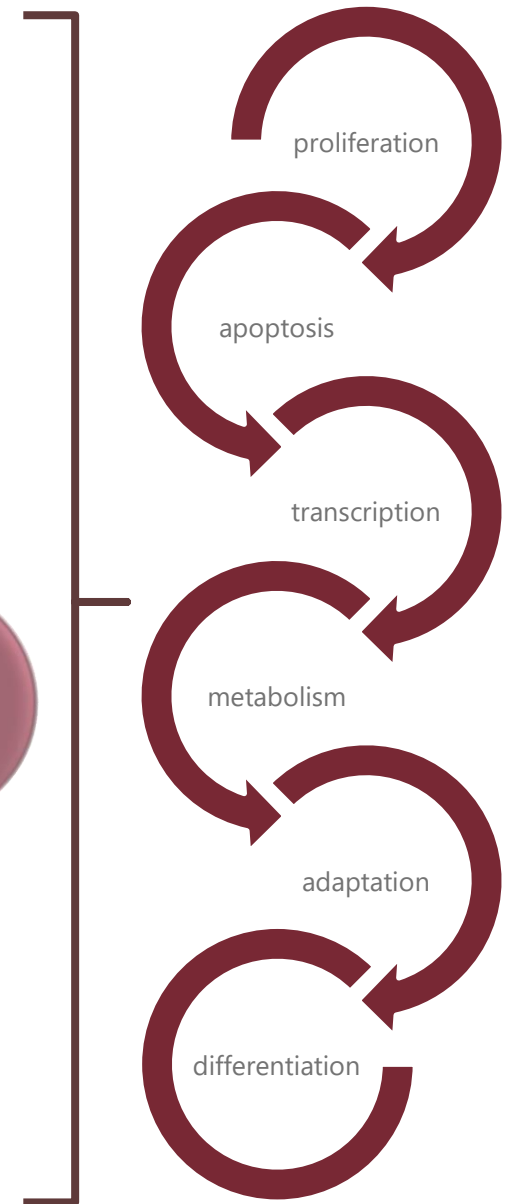
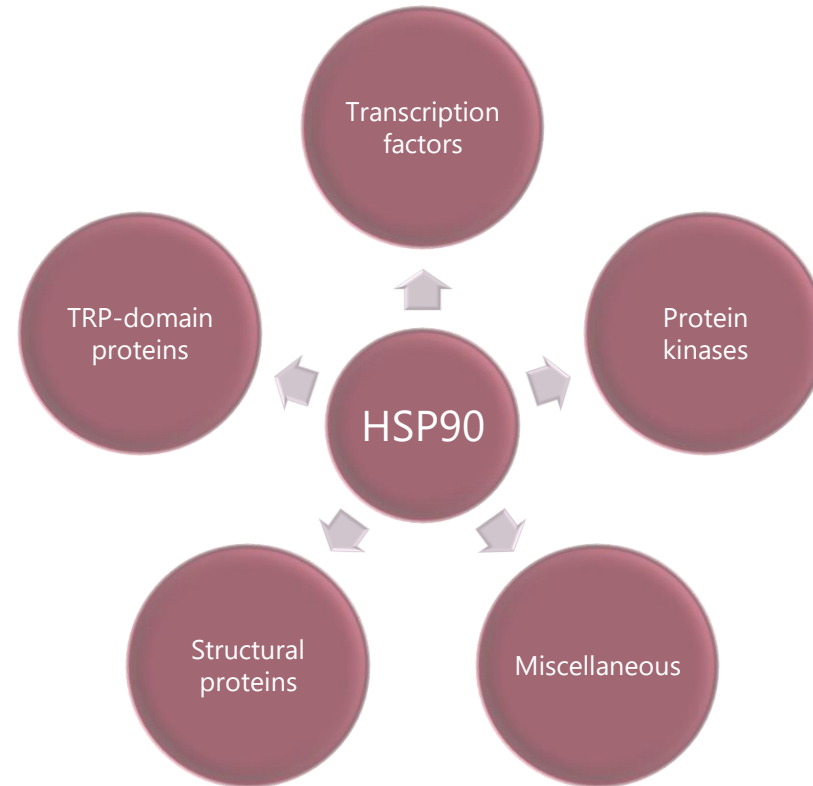
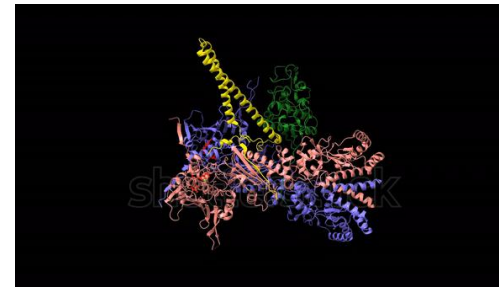


Fig. 1. Representative fluorescent micrographs of heat shock protein 60 (Hsp60) immunolocalization on sperm cell. Green fluorescence indicates positive immunostaining revealed by FITC-conjugated antibodies, red fluorescence indicates nuclear staining with PI. (a) Boar spermatozoa (in the insert JC-1 orange fluorescence indicates mitochondria position in sperm midpiece); (b) stallion spermatozoa; (c) dog spermatozoa; (d) cat spermatozoa

Volpe S, Galeati G, Bernardini C, et al. Comparative immunolocalization of heat shock proteins (Hsp)-60, -70, -90 in boar, stallion, dog and cat spermatozoa. *Reprod Domest Anim.* 2008;43(4):385-392.

HSP 90/HSPC

- is a highly efficient, ATP-dependent molecular chaperone involved in the maturation and stabilisation of a wide-range of proteins in both physiological and stress conditions being an important hub in the protein network that maintains cellular homeostasis and function.
- Hsp90 belongs to a family of proteins known as "chaperones," which are solely dedicated to helping other proteins (client proteins) correct folding, function and stability.
- Hsp90 participates in many cellular processes including cell cycle control, cell survival, hormone and other signaling transduction pathways, often acting as hormone receptors and is considered to be key player in maintaining cellular homeostasis and adaptive response to stress



HSP 90/HSPC

- In pig, Hsp90 has been clearly demonstrated to play a crucial role in regulating sperm motility (Huang et al. 2000).
- Tail localization of Hsp90 in mature fresh semen of bull, stallion, boar, mouse, dog and cat may be essential for the signaling events associated with capacitation and its presence could play a role in mediating the fertilizing ability of the spermatozoon.
- In chicken sperm the expression of Hsp90 was higher in fresh sperm than in frozen–thawed sperm. (Qi et al. 2020)

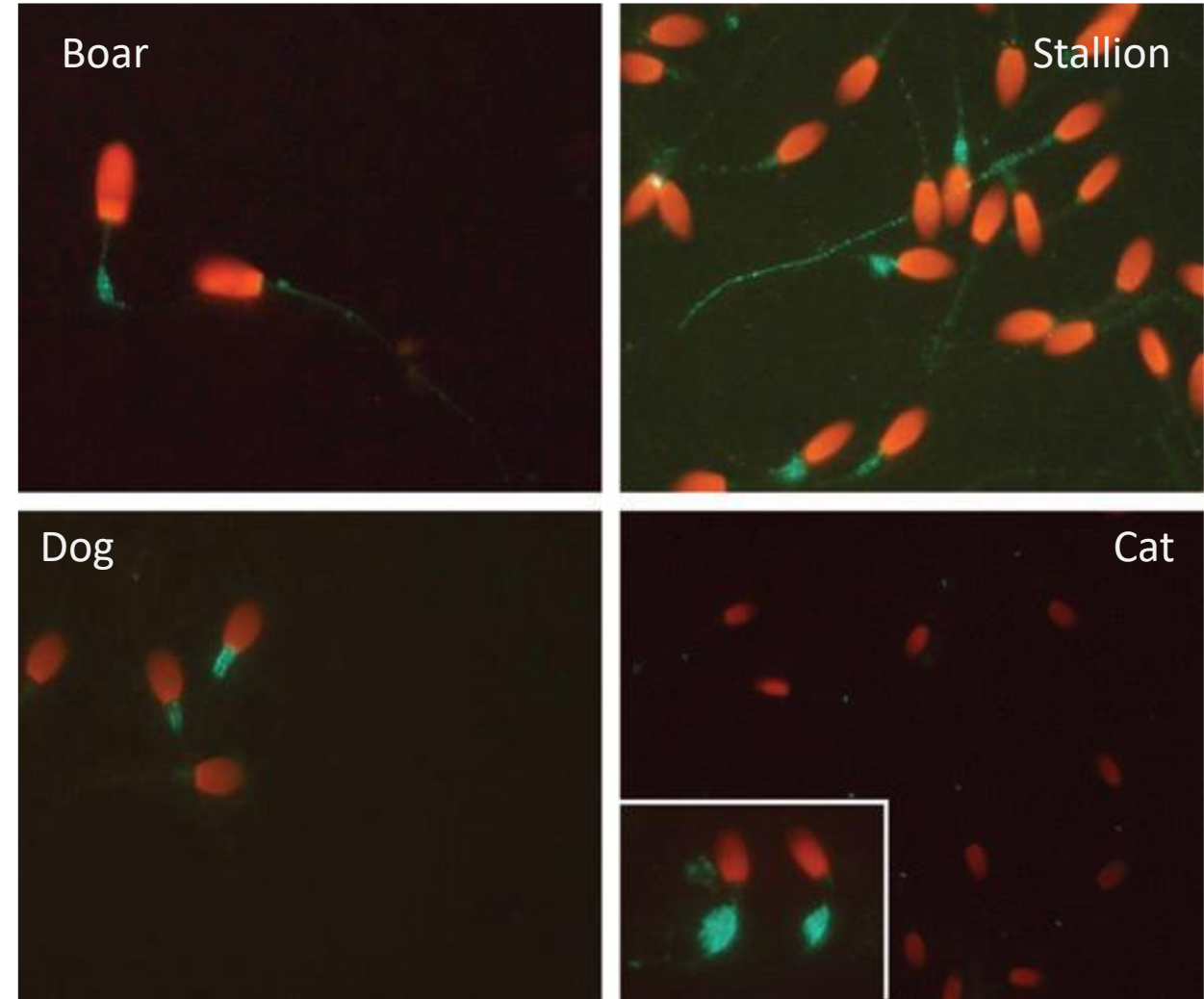
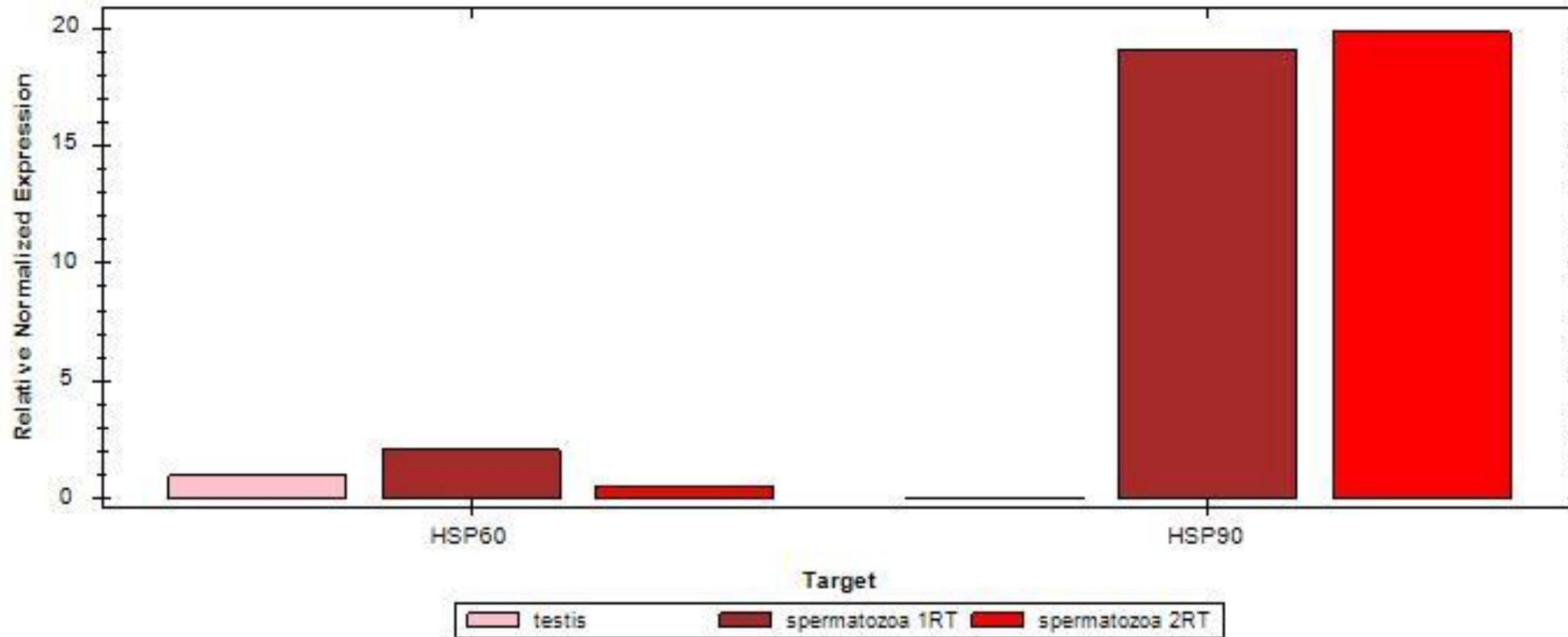


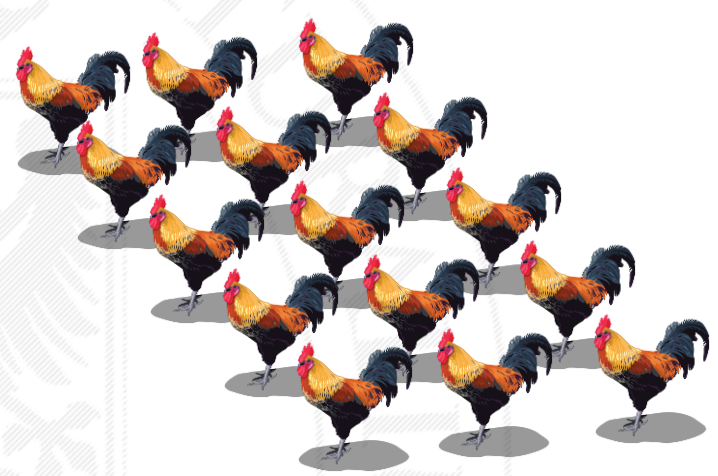
Fig. 3. Representative fluorescent micrographs of heat shock protein 90 (Hsp90) immunolocalization on sperm cell. (a) Boar spermatozoa; (b) stallion spermatozoa; (c) dog spermatozoa; (d) cat spermatozoa (the insert shows the strong immunoreactivity of the cytoplasmic droplets)

Volpe S, Galeati G, Bernardini C, et al. Comparative immunolocalization of heat shock proteins (Hsp)-60, -70, -90 in boar, stallion, dog and cat spermatozoa. *Reprod Domest Anim.* 2008;43(4):385-392.

Gene expression of HSP60 and HSP90 in chicken testes and spermatozoa



The effect of semen cryopreservation on the expression of Heat Shock Proteins in chicken spermatozoa



semen



EK diluent



EK control



EK + NAC 5 mM



EK + SOD 200 IU/ml

Semen cryopreservation – pellet method

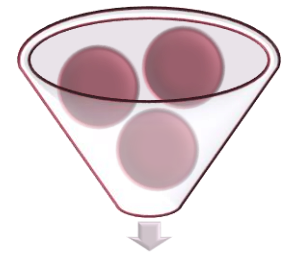
6% DMA



Freezing

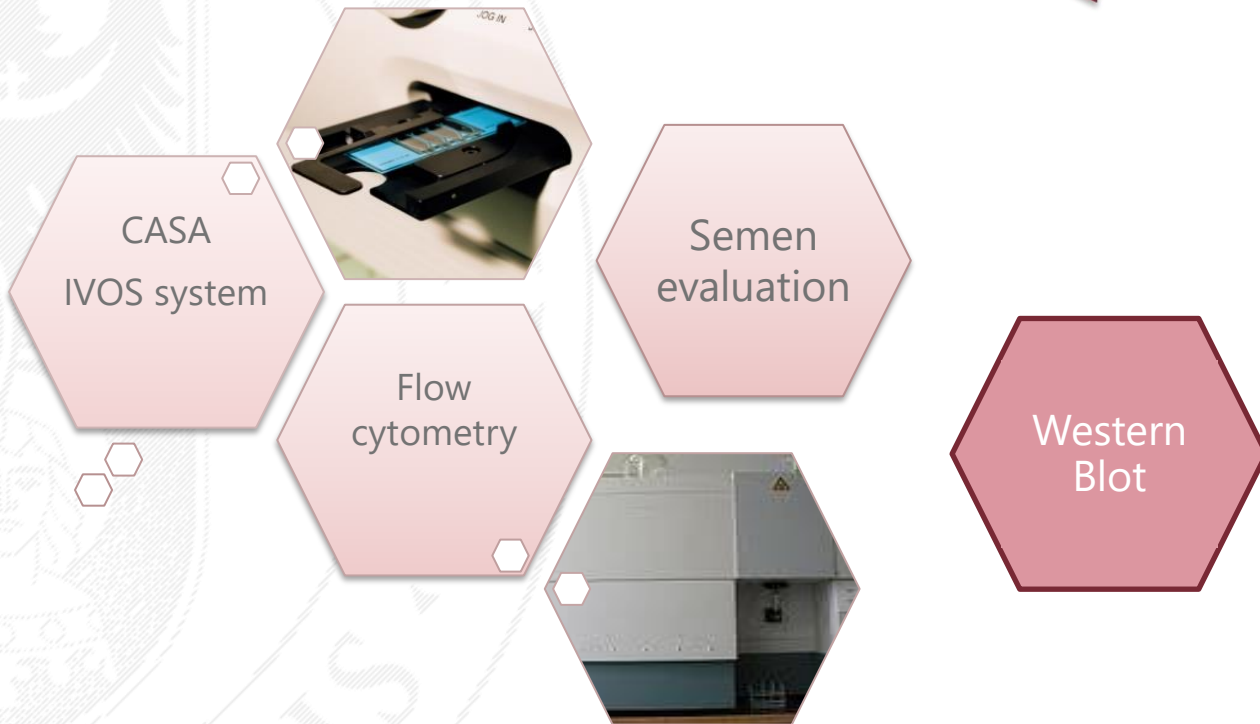


Thawing





Material & Methods





Material & Methods

Centrifugation

- 3 times
- 600 x g; 10 min
- In PBS



Storage

- 80°C



Disintegration

- Pellets (200×10^6 spermatozoa)



Buffer

- (5x Laemmli Sample Buffer with 5% 2-mercaptoethanol)



Heating

- at 100°C for 5 min
- Vortexing**
- 30 sec



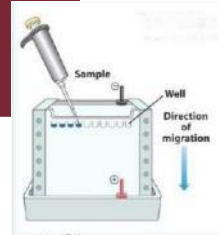
Centrifugation

- 10 min at 10 000 x g



SDS-PAGE gel electrophoresis

- 10% polyacrylamide gels
- ~ 100×10^6 spermatozoa per lane)



Trans Blot Turbo,
Biorad semi-dry
chamber

- the transfer conditions:
25V const., 1.5mA, 35 min

Incubation

- Pierce Western Blot Signal Enhancer (Thermo Scientific)

Blocking

- Western Dot blocking buffer (Invitrogen; the blocking conditions: 180 min. at 37°C)





Material & Methods

First antibodies:

mouse monoclonal antibodies to:

Hsp60 clone LK-1 (Enzo ADI-SPA-806),

Hsp70 clone C92F3A-5 (Enzo ADI-SPA-810),

Hsp90 clone AC88 (Enzo ADI-SPA-830),

GAPDH (GenTex GTX 30666)

Second antibody:

goat anti-mouse IgG HRP-conjugate (Sigma Aldrich A-9044) (dilution 1:100 000)

Incubation

- First antibodies
- (dilution 1:2000) for overnight at 4°C

Washing

- 3 times
- 5 min TBST

Incubation

- Second antibody
- dilution 1:100 000

Visualisation

- 3 times
- 5 min TBST

ChemiDoc XRS+
Instruments
(BioRad)

Western Blot normalization using a single protein (housekeeping protein) and total protein was made using Image Lab™ software (BioRad).

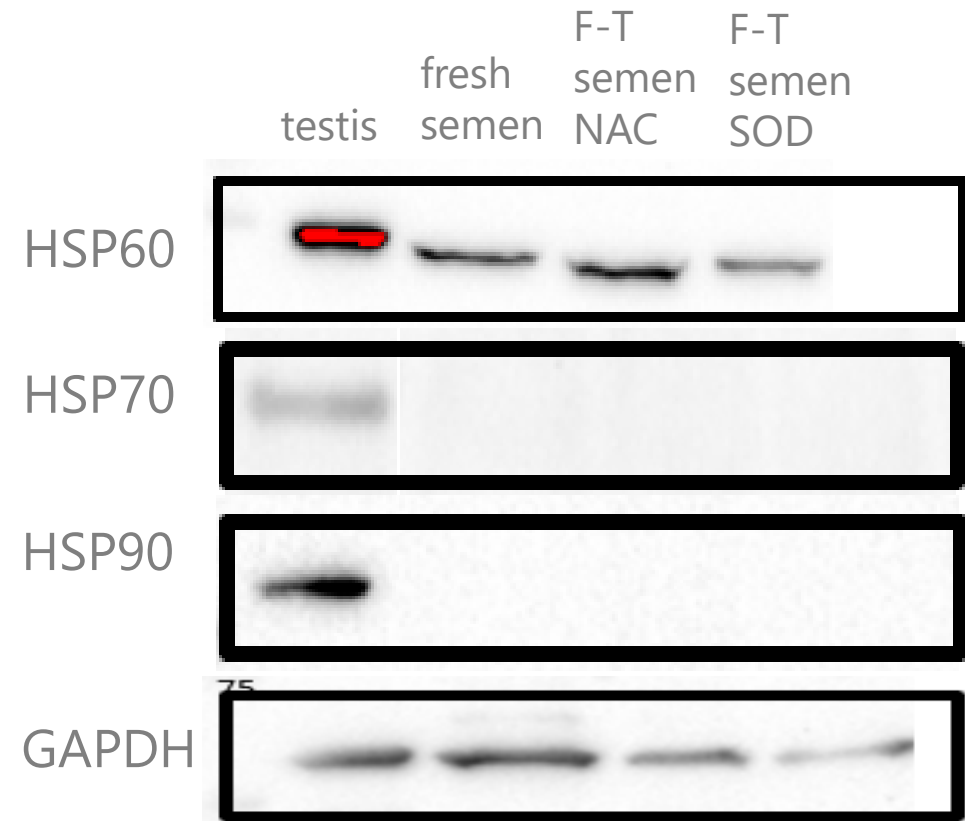


Results

As a positive controls the chicken testis lysate was used.

There was no evidence of Hsp70 and 90 proteins expression in the analyzed rooster sperm.

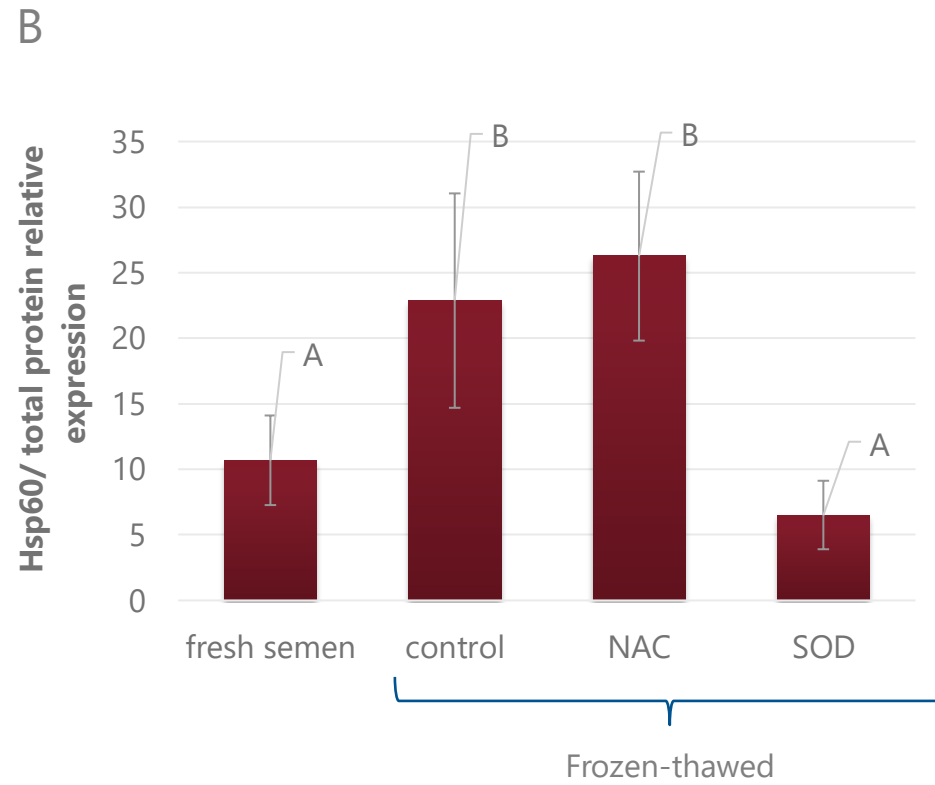
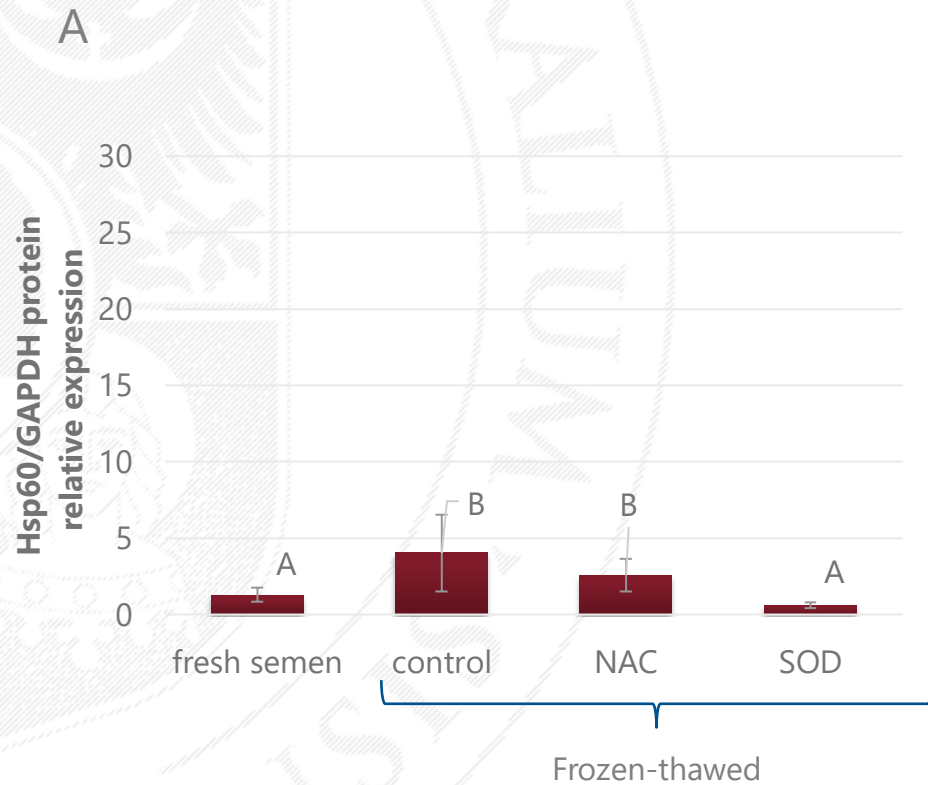
There was no evidence of Hsp60, 70 and 90 proteins expression in the analyzed chicken seminal plasma.





Results

The relative level of Hsp60 protein expression in sperm from fresh and cryopreserved semen normalized by A) GAPDH protein expression B) total protein expression



Values with different superscripts differ significantly A, B - $P < 0.01$



Conclusions

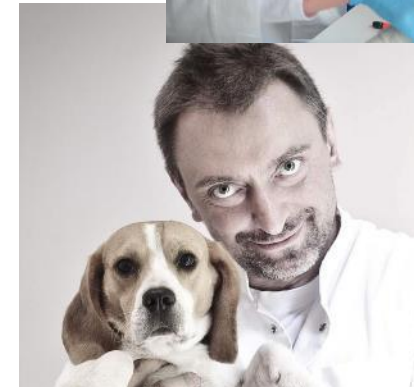
The chicken semen cryopreservation causes a significant increase of the expression of Hsp60.

The addition of SOD to chicken semen before freezing does not change the level of expression of the Hsp60 compared to fresh semen, which may indicate an important role of this antioxidant in reducing changes related to cryopreservation.

The use of the above-mentioned method of separation and identification of proteins in chicken sperm (both fresh and cryopreserved) made it possible to detect only Hsp60. No Hsp70 and 90 have been identified.



Thanks to:
Prof. Wojciech Nizański
Dr Joanna Bajzert
Skarlet Napierkowska DVM
Jagoda Nestorowicz



Wroclaw University of Environmental
and Life Science

