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Effect of insulin-like growth factor I on functional parameters of ram cooled-stored spermatozoa

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Summary

The aim of the study was to examine the effects of insulin-like growth factor I (IGF-I) on ram sperm traits after hypothermic storage. Sperm ejaculates from Lacaune rams were diluted in a Tris extender, pooled, divided into groups of IGF-I doses tested (0, 10, 100 or 200 ng.ml^{-1}) and stored (0–5°C) for 96 h. IGF-I elevated whole sperm motility as measured by a Computer-assisted Sperm Analyser (CASA) system, by 24 h (10 ng.ml^{-1}) and 48 h (200 ng.ml^{-1}) of storage, and by progressive movement on each day of storage. After 72 h the sperm samples were analysed for plasma membrane integrity (peanut agglutinin–fluorescein isothiocyanate), membrane stability (annexin V–Fluos) and apoptosis (Yo-Pro®-1) using fluorescence microscopy. The addition of IGF-I (at 100 or 200 ng.ml^{-1}) reduced the ratio of sperm with disrupted membranes and the ratio of annexin V-labelled sperm. The ratio of apoptotic sperm was reduced by IGF-I given at 10 or 100 ng.ml^{-1} compared with control. Sperm fertilizing ability, determined at 48 h by an *in vitro* fertilization (IVF) test on bovine oocytes, was increased by IGF-I given at 100 ng.ml^{-1} from 47.0 to 67.7%. In conclusion, IGF-I maintained ram sperm functions following cooling storage and its effects were reflected in sperm fertilizing ability *in vitro*.

Keywords: Fertilizing ability, IGF-I, Motility, **S**perm, Viability

Introduction

Reproduction in sheep is affected by the high sensitivity of ram sperm to cryopreservation, consequently the application of frozen–thawed semen leads to low fertility following insemination. Therefore, there is a need to optimize approaches for long-term sperm storage under non-frozen conditions, which could keep the sperm alive and suitable for fertilization (Maxwell & Salamon, 1993). Ram sperm ejaculates can be stored in a liquid state (at 0–5°C) for several days (Salamon & Maxwell, 2000). In order to maintain sperm fertilizing ability for a longer time period, the use of different extenders and additives has been tested. In particular, growth factors have been reported to increase longevity of mammalian sperm functions (Naz & Minhas, 1995).

Insulin-like growth factor I (IGF-I) is an important regulator of reproductive function (Jones & Clemmons, 1995) and is involved in the fertilization process (Henrick *et al.*, 1998). In particular, IGF-I has been reported to stimulate motility, viability, capacitation and acrosome reaction in bull (Henrick *et al.*, 1998; Lackey *et al.*, 1998) boar (Miah *et al.*, 2008), stallion (Champion *et al.*, 2002; Macpherson *et al.*, 2002), rabbit (Minelli *et al.*, 2001a,b), buffalo (Selvaraju *et al.*, 2009, 2010), rat (Vickers *et al.*, 1999) and human (Sanchez-Luengo *et al.*, 2005) spermatozoa. The IGF-I level in seminal plasma is closely associated with sperm motility, morphology and pregnancy rates in mares (Macpherson *et al.*, 2002) and sperm fertilizing ability in breeding bulls (Sauerwein *et al.*, 2000). However, the rams selected for high blood serum

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IGF-I level did not manifest improved reproductive performance (Park *et al*., 2010).

The effect of IGF-I on spermatozoa is mediated via specific receptors that have a tyrosine kinase activity and these receptors have been identified in bovine (Henrick *et al*., 1998) and human (Naz & Padman, 1999) sperm cells. The IGF-I receptor localizes to the acrosomal region of the plasma membrane of ejaculated sperm and it is supposed that IGF-I signalling may be an important factor in the capacitation and the acrosome reaction (Henrick *et al*., 1998). Therefore, it has been speculated that IGF-I can be used potentially for the improvement of traits of fresh or stored sperm in farm animal breeding and assisted reproduction. All the above-mentioned authors have studied the influence of IGF-I on fresh sperm, except for Selvaraju *et al*. (2009), who used frozen–thawed buffalo sperm. So far, no reports of the IGF-I effect on cooled-stored spermatozoa of any species, including sheep, are currently available. In this light, the investigation of the role of IGF-I in the maintainance of longevity of ram sperm function following cooling storage may be of great importance.

The goal of our study, therefore, was to examine the effects of IGF-I on ram sperm characteristics (motility, membrane integrity, membrane stability, apoptosis and fertilizing ability) following liquid storage under hypothermic conditions.

Materials and methods

Sperm ejaculate collection and sample preparation

Fresh ejaculates were collected from rams of the Lacaune breed with proven health and reproductive status using an artificial vagina. The rams were kept at a local farm under uniform nutritional conditions. After measuring volume, density and activity, sperm were diluted (approximately 1:3) in a Triladyl extender (Minitub Slovakia Ltd, Čeľadice, Slovak Republic) to obtain a concentration 1×10^6 sperm/ml. The ejaculates collected from three rams per each experiment (two ejaculates per each ram) were cooled to 5–7°C and transported to the laboratory in thermoboxes within 2 h. In the laboratory an aliquot of the ejaculate, intended for IGF-I experiments, was taken into separate tubes, flushed out of Triladyl extender by centrifugation at 600 *g* at laboratory temperature (20–23°C) for 7 min, and resuspended in a Tris extender (Minitub Slovakia Ltd) without egg yolk. Collected sperm samples were pooled and divided into four groups with 1 ml of the ejaculate in each. Subsequently, human recombinant IGF-I (Sigma-Aldrich Ltd, Bratislava, Slovakia) was added at concentrations of 10, 100 or 200 ng.ml^{-1}, whereas the control group did not contain IGF-I (0 ng.ml^{-1}). Then, sperm samples were stored in polystyrene tubes under cooling conditions (0–5°C in a refrigerator) for 24–96 h until analysis. Following this storage period, the sperm samples were removed from the refrigerator, washed to remove extender using centrifugation (400 *g*), resuspended in a saline solution (0.9% w/v NaCl) that contained 1% v/v fetal calf serum (saline–FCS) and analysed.

Sperm motility analysis

Sperm total motility was measured on the first (0 h), second (24 h) and third (48 h) days following the IGF-I addition using a Computer-assisted Sperm Analyser (CASA) system (Sperm Vision, Minitub Slovakia Ltd) at 0, 0.5 and 2 h after removal from storage. Spermatozoa were transferred by a pipette into a Leja counting chamber with a depth of 10 μm. The chamber was placed under a Zeiss Axioscope A.1 phase-contrast microscope (Carl Zeiss NTS GmbH, Oberkochen, Germany) with a heating plate (37°C) at ×200 magnification. The camera transferred the image to a computer, where sperm motility was measured using Sperm Vision software. In each sample at least six fields of view were counted. Of all parameters measured by CASA, only total motility and progressive movement of the sperm were evaluated in this study. The values of a total or progressive motility measured at several time points (0, 0.5 and 2 h) during the day were summarized and the average values per each day were represented graphically.

Fluorescence assays

Following 72 h of cold storage, the sperm samples were analysed for plasma membrane integrity (stained with a fluorescently labelled lectin – *Arachis hypogaea* peanut agglutinin–fluorescein isothiocyanate; PNA–FITC), membrane stability (annexin V–Fluos) and apoptosis (Yo-Pro®-1) using a fluorescence microscope.

For plasma membrane integrity the sperm samples were stained with PNA–FITC (Molecular Probes, Lucerne, Switzerland) in combination with propidium iodide (PI). The sperm samples were not fixed, allowing PNA–FITC labelling only in the spermatozoa with disrupted or otherwise damaged plasma membranes, whilst the sperm cells with intact membranes remained unstained.

Following washing in saline–FCS and centrifugation at 600 *g* for 7 min, the sperm suspension was incubated in a staining solution that contained 20 μmol.l^{-1} of PNA–FITC and 5 μg.ml^{-1} PI in saline–FCS for 20 min at room temperature. After incubation, sperm samples were washed in saline, centrifuged at 600 *g*

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for 7 min and then 4 μl of the sperm suspension were placed onto a microscope slide, and gently mixed with 4 μl of Vectashield mounting medium that contained 4′,6-diamidino-2-phenylindole (DAPI; H-1200, Vector Laboratories Inc., Burlingame, CA, USA), a blue-fluorescent DNA stain that marks nucleoplasma of all sperm cells in samples. The droplets obtained were flattened with a coverslip and observed immediately under a Leica inverted fluorescence microscope with respective bandwidth filters for green, red and blue fluorescence.

Membrane phosphatidylserine (PS) translocation (membrane stability) was detected after staining with fluorescently labelled annexin V using an annexin V–Fluos staining kit (Roche Slovakia Ltd, Bratislava, Slovak Republic). Semen samples were washed in the binding buffer (supplied with a kit), centrifuged, and then the semen suspension (5 μl) was mixed with 100 μl of annexin V–Fluos working solution and incubated for 20 min at 37°C. Following staining, unbound annexin V was diluted in the binding buffer and after centrifugation (400 *g*) a droplet of semen suspension (4 μl) was placed onto a microscope slide, mixed with 4 μl of Vectashield that contained DAPI and flattened with a coverslip. Staining with annexin V was immediately examined under a Leica fluorescence microscope using a 488 nm wave-length filter. The sperm with disordered asymmetry of membrane PS exhibited green fluorescence, whilst the sperm with intact membranes were unstained.

For the detection of apoptosis, sperm were stained with a Yo-Pro®-1 specific green fluorochrome (Molecular Probes, Lucerne, Switzerland) in combination with PI for identification of dead sperm cells. Following washing in saline–FCS, the sperm suspension was incubated in a staining solution: saline–FCS with 5 $\mu mol.l^{-1}$ Yo-Pro®-1 and 5 $\mu g.ml^{-1}$ of PI. As before, after 20 min staining at room temperature, the sperm samples were washed in the saline–FCS solution and 4 μl of sperm suspension were placed onto a microscope slide, mixed with 4 μl of Vectashield that contained DAPI and the droplet was flattened with a coverslip. The preparations were evaluated immediately under a Leica fluorescence microscope with special filters for green, red and blue fluorescence. The green fluorescing sperm cells were regarded as apoptotic cells. The cells coloured pink were considered as dead or necrotic cells.

Determination of staining rate for fluorescent markers described above was carried out by counting the stained sperm and total sperm number under a relative ultraviolet (UV) light filter. Sperm images were made and the sperm number was counted using a computer monitor. Experiments were repeated five times. In each experiment, about 8–10 microscopic view fields per each group were photographed. In total, more than 1500 spermatozoa per each group were counted.

Analysis of sperm penetrating/fertilizing ability

Sperm penetration/fertilization ability was determined at 48 h of cold sperm storage by an *in vitro* fertilization (IVF) test on bovine pre-matured oocytes isolated from ovaries of cows that had been provided by a local slaughterhouse. The ability of bovine zygotes with zona pellucida to be fertilized by ram sperm has been validated previously (Garcia-Alvarez *et al.*, 2009). Briefly, isolated oocytes were matured during 24 h incubation in maturation medium 199 with glutaMAX (Gibco Invitrogen, Auckland, New Zealand) supplemented with gonadotropins (FSH/LH 1/1 I.U., Pluset, Lab. Calier, Barcelona, Spain), sodium pyruvate, FCS (BioWhittaker, Verviers, Belgium) and gentamicin (Makarevich & Markkula, 2002). Following the maturation period, the oocytes were stripped of the cumulus cells using vortexing and placed into a fertilization drop (Fert-TALP medium with 10 $\mu g.ml^{-1}$ heparin and either 0 or 100 $ng.ml^{-1}$ IGF-I) under mineral oil. The sperm suspension was washed in Sperm-TALP medium to remove Triladyl, resuspended in a fresh Fert-TALP medium that contained the above-mentioned additions and placed into a fertilization droplet to a final concentration of 2×10^6 sperm/ml. Fertilization was performed in the incubator at 38.5°C in a humidified atmosphere of 5% CO_2 in air for 20 h. Subsequently, the presumptive zygotes were cleaned of the excessive sperm and the rest of cumulus cells by a vortexing and transferred into B2 INRA culture medium (CCD Laboratories, Vernouillet, France) on a culture dish with a previously prepared monolayer of buffalo rat liver (BRL) cells. The zygotes were cultured at 38.5°C in a humidified atmosphere with 5% CO_2 in air until evaluation. After 5 days the zygotes and embryos were fixed in 3.7% w/v formalin, mixed in a droplet of Vectashield with DAPI, mounted between microslide and coverslip and inspected under a Leica fluorescence microscope with the respective UV filter.

The zygotes and embryos were evaluated as follows: (i) penetrated eggs – at least one sperm under the *zona pellucida* or one pronucleus were present; (ii) fertilized eggs – at least two pronuclei were present; (iii) cleaved embryos – two or more blastomeres with visible nuclei were present; and (iv) non-penetrated oocytes – with no spermatozoa inside.

Statistics

Comparisons of arithmetic means between groups of IGF-I concentrations were performed by a one-way analysis of variance (ANOVA) with fixed effects. The elementary contrast between mean values of

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fluorescence assays was evaluated by Bonferroni test. The results of total or progressive motility, obtained from several measurements, were statistically processed by repeated-measure ANOVA. The penetration/fertilization test was repeated four times, and the obtained data were analysed using the chi-squared test. As percentage values were used in the study, all the values were subjected to arcsin transformation in order to eliminate any abnormality of the distribution and/or non-homogeneity of the data. Statistical analysis was performed with original data using Statistix analytical software (Version 8.0; Anonymous, 2001).

Results

The initial motility of spermatozoa, determined by CASA prior to start of the experiment, was in the range of 90–92%. After liquid storage under cooling conditions the average values (composed of measurements at 0, 0.5 and 2 h) of total sperm motility in the control group were 89, 75.5 and 71.9% for first, second and third days of storage, respectively. The progressive movement in the control group was 83.4, 69.2 and 66.7% for first, second and third days of storage, respectively. The effect of IGF-I measured shortly after addition (0 h) only slightly increased (200 ng.ml^{-1}) total sperm motility, whilst these parameter values increased significantly on the second (10 ng.ml^{-1}) and third (200 ng.ml^{-1}) days of storage (Fig. 1*A*). The stimulatory effect of IGF-I on progressive movement was manifested on each day of sperm storage (Fig. 1*B*).

The integrity of the sperm plasma membrane was determined by triple staining with fluorochromes PNA–FITC, PI and DAPI. This staining enabled us to distinguish among the three populations of spermatozoa: (i) the sperm with disrupted plasma membrane were green-labelled in the anterior region of the sperm head; (ii) the dead sperm were red-labelled either through a whole sperm head or in the posterior region; and (iii) the rest of the spermatozoa were stained blue and represented live intact spermatozoa. About 23% of whole sperm populations in the control group were labelled with PNA–FITC, which indicated disruption of the sperm plasma membrane. The proportion of membrane-damaged spermatozoa was lowered by IGF-I ($P < 0.05$) given at 100 or 200 ng.ml^{-1} (Table 1). The percentage of necrotic/PNA-positive spermatozoa (PNA+/PI+; 5.5% in control) was not significantly changed by addition of IGF-I at any concentration.

Using Yo-Pro®-1/PI/DAPI staining, three populations of sperm cells were visible: (i) the apoptotic sperm cells (green and/or yellow stained); (ii) the dead (necrotic) sperm cells (coloured red); and (iii) live intact spermatozoa (coloured dark-blue). Yo-Pro®-1 stained the whole sperm head, whilst PI stained either the whole sperm head or only their post-acrosomal part. The addition of IGF-I suppressed the occurrence of the Yo-Pro®-1-detected apoptosis in spermatozoa, when given at 10 or 100 ng.ml^{-1} (Table 1). The ratio of both apoptotic/necrotic (Yo-Pro1+/PI+) spermatozoa in the control group was about 8%. This parameter was significantly changed by IGF-I given at each concentration tested.

Following hypothermic storage about 9.6% of the whole sperm population manifested destabilization of the membrane PS asymmetry (annexin V–Fluos labelling). This value was lowered significantly in the presence of 100 ng.ml^{-1} IGF-I, whilst the effects of IGF-I given at other doses tested were not significant (Table 1). In most spermatozoa (about 75%) annexin V positivity was localized to the acrosomal part of the sperm head and the tail. The remaining annexin V positivity was localized to the post-acrosomal region, equatorial segment and the head-to-tail junction. The percentage of dead sperm (AnV+/PI+; 6.20%) in the control group was lower than that of annexin V-labelled sperm (9.59%). The ratio of dead sperm was lowered by the addition of IGF-I at the highest concentration (Table 1).

Fertilizing ability of ram spermatozoa following 48 h of cooling storage with or without IGF-I was evaluated in the *in vitro* penetration/fertilization test using bovine pre-matured oocytes. IGF-I, given at 100 ng.ml^{-1}, improved sperm penetrating ability (penetrated oocytes and fertilized zygotes) by 15% and fertilizing capacity (cleaved embryos) by 20% (Table 2).

Discussion

IGF-I is a cytokine that is involved in regulation of reproductive function (Jones & Clemmons, 1995). As specific IGF-I receptors are present on spermatozoa (Henrick *et al.*, 1998), IGF-I may be assumed to be a possible regulator of sperm function.

To evaluate any IGF-I effect on cooling-stored ram spermatozoa, we analysed motility, fluorescent markers of viability and apoptosis, and assessed *in vitro* fertilization (IVF) ability. Sperm motility is an important parameter of sperm quality. Sperm motility during sperm liquid storage can be maintained for several days with only a moderate decrease after approximately 6 days of storage (Waterhouse *et al.*, 2004; Trzcinska *et al.*, 2008). In our study, motility of the sperm samples was measured after 48 h of storage at the latest (third day) and during this period these

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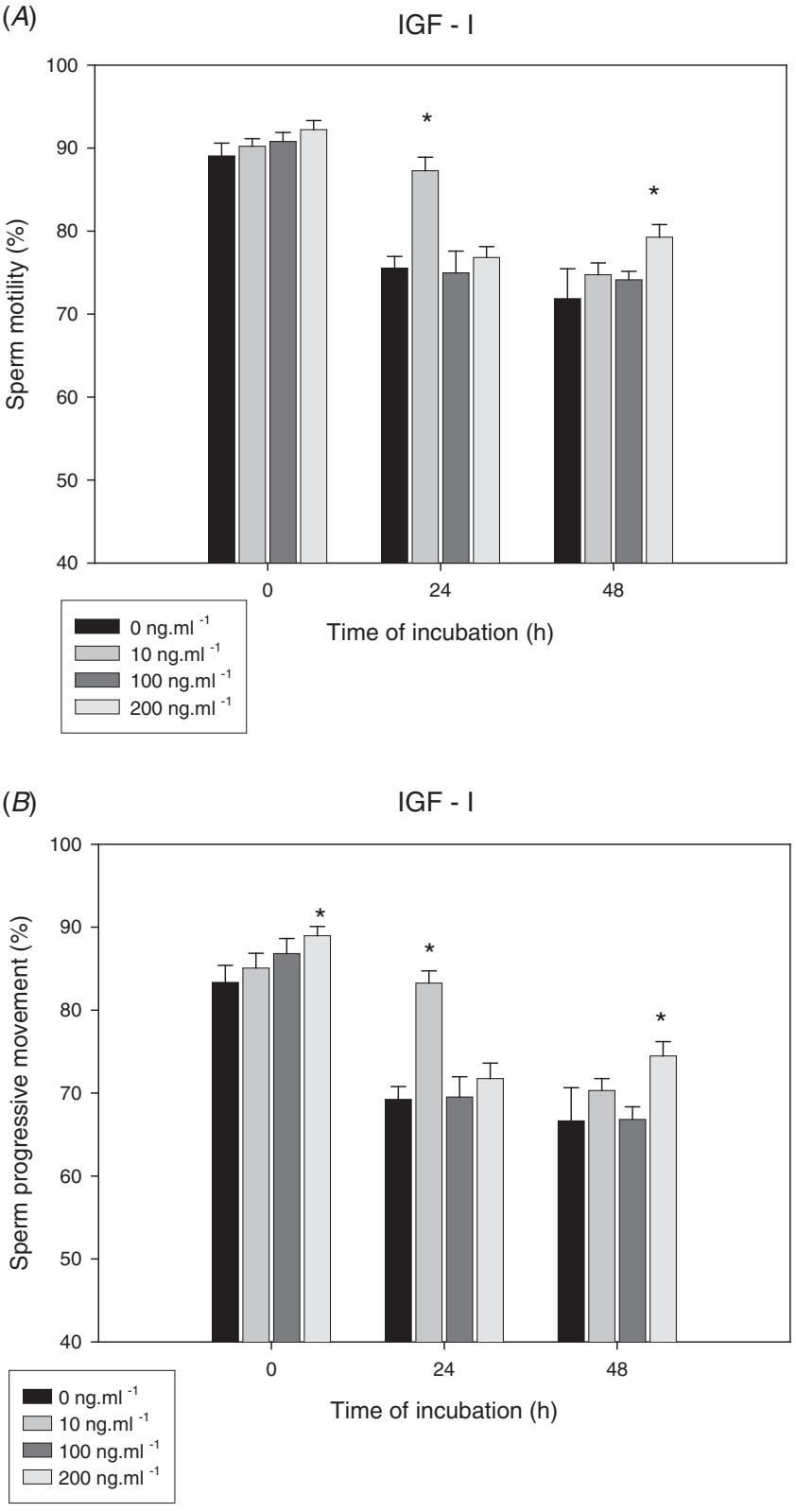

Figure 1 Effect of IGF-I. (*A*) On a total motility of ram spermatozoa. (*B*) On progressive movement of ram spermatozoa. *Significant difference at $P < 0.05$ compared with control (0 ng.ml^{-1} IGF-I).

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Table 1 Effect of IGF-I on membrane characteristics and apoptosis in ram sperm

	IGF-I concentration, ng.ml^{-1} (%, mean ± S.E.M.)			
Parameter	0	10	100	200
Plasma membrane integrity				
PNA–FITC+	23.03 ± 2.72	21.49 ± 2.66	15.95 ± 1.07[a]	16.50 ± 1.97[a]
PNA+/PI+	5.55 ± 1.74	3.84 ± 0.72	5.03 ± 0.84	4.27 ± 1.12
Apoptosis				
Yo-Pro®-1+	9.10 ± 2.07	6.03 ± 0.78[a]	4.88 ± 0.98[a]	6.39 ± 0.78
Yo-Pro+/PI+	8.12 ± 1.01	5.09 ± 0.59[a]	3.04 ± 0.47[a]	3.01 ± 0.76[a]
Membrane stability				
Annexin V–FITC+	9.59 ± 1.64	6.82 ± 1.13	5.02 ± 1.98[a]	6.77 ± 1.18
AnV+/PI+	6.29 ± 0.92	4.09 ± 1.36	4.91 ± 1.02	3.60 ± 0.99[a]

[a]Significant difference at $P < 0.05$ compared wth control (0 ng.ml^{-1} IGF-I).

Table 2 Effect of IGF-I on penetrating/fertilizing capacity of ram sperm

Parameter	Control (none)	IGF-I (100 ng/ml) [a]
Total no. oocytes, *n*	102	93
Unfertilized oocytes, *n* (%)	48 (47.06)	30 (32.26)
Penetrated and fertilized zygotes, *n* (%)	54 (52.94)	63 (67.74)
Cleaved embryos from Total no., *n* (%)	48 (47.06)	63 (67.74)

[a]Difference is significant compared with control at $P < 0.05$ (chi-squared test).

parameter values decreased from 90% (start of cooling storage) to 66%, which is still an acceptable number for this species. This finding indicated that the basic parameters for sperm samples (initial quality of ejaculates, semen extender, storage conditions) were satisfactory. In our tests, the stimulating effect of IGF-I addition on total motility was manifested by 24 and 48 h of sperm storage, whilst on the first day (0 h) there were no significant differences when compared with the control. This observation is in agreement with the data reported for stallion spermatozoa (Champion *et al.*, 2002); whereas, in buffalo, the addition of IGF-I had already increased sperm motility at 30 to 60 min post-treatment (Selvaraju *et al.*, 2009). Previously, the addition of IGF-I was also reported to improve motility and quality of movement in stallion, rabbit, rat, bull, buffalo and boar spermatozoa (Henrick *et al.*, 1998; Vickers *et al.*, 1999; Minelli *et al.*, 2001b; Champion *et al.*, 2002; Miah *et al.*, 2008; Selvaraju *et al.*, 2009). However, any effect of IGF-I on motility of ram sperm has not been reported.

It is supposed that the effects of IGF-I on spermatozoa are mediated via ligand–receptor binding (Henrick *et al.*, 1998; Macpherson *et al.*, 2002). However, to date, IGF-I receptors have been found on the acrosomal part or the equatorial segment, but not on the flagella part of the sperm cell. In an earlier study Henrick *et al.* (1998) classified IGF-I as a chemokine factor that is involved directly in regulation of sperm movement; IGF-I can stimulate flagellar motion and thrust of sperm and thereby increase straight-line velocity (VSL; Henrick *et al.*, 1998). These authors assumed that IGF-I can maintain sperm motility via two possible ways: (i) energy metabolism (glucose uptake, lactate production, pyruvate dehydrogenase activity etc.); and/or (ii) the antioxidant effect of IGF-I. This antioxidant property of IGF-I can explain some of the positive effects of IGF-I on sperm viability parameters observed in our study.

The evaluation of the ejaculate on the basis of sperm motility may not always reflect its fertilizing ability. For instance, sperm with low motility can have a high potential for insemination due to the low content of apoptotic and/or necrotic spermatozoa (Trzcinska *et al.*, 2008). The presence of apoptotic spermatozoa in fresh semen could be one of the reasons for poor fertility of bulls (Anzar *et al.*, 2002) and infertility in man (Marchetti *et al.*, 2002). Therefore, sperm motility tests should be complementary to a determination of apoptotic or necrotic changes.

We determined apoptosis occurrence in ram sperm using Yo-Pro®-1, a fluorescent marker of apoptosis, in combination with PI. Apoptotic cells are permeable to Yo-Pro®-1 green fluorochrome and impermeable to PI. Thus, the use of combined Yo-Pro®-1 and PI dyes may provide a sensitive indicator of apoptosis. This technique has been validated previously on boar (Peña *et al.*, 2005; Trzcinska *et al.*, 2008), ram (Garcia-Alvarez *et al.*, 2009) and bull (Martin *et al.*, 2004) spermatozoa and was shown to be inexpensive, quick and easy to perform. In our study, about 9% of sperm cells in the control group were labelled as apoptotic

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(Yo-Pro®-1 positive), and about 8% of cells were labelled as both apoptotic and dead. IGF-I was shown to be a potent inhibitor (at each concentration tested) of apoptosis/necrosis in ram sperm populations during hypothermic storage.

A sperm plasma membrane with normal integrity and function is a prerequisite for successful fertilization. It is hypothesized that membrane phosphatidylserine (PS) translocation may indicate the cells with altered membrane function that will eventually undergo necrosis (Januskauskas *et al.*, 2003). Such PS translocation in mature spermatozoa (Peňa *et al.*, 2005) may be considered as an apoptosis predictor. Annexin V/PI staining has been used successfully for detection of early changes in the membrane of boar sperm (Peňa *et al.*, 2003), whilst in bovine spermatozoa this assay failed to discriminate objectively apoptotic population from non-apoptotic ones (Martin *et al.*, 2004). In our study, annexin V labelling detected about 9.6% of spermatozoa with PS membrane destabilization, and this percentage was reduced significantly with all IGF-I concentrations tested. However, we cannot clearly state whether these annexin V-labelled spermatozoa were really apoptotic. Several authors have suggested that annexin V-labelled changes in sperm are related to the capacitation and the acrosome reaction process rather than to apoptosis (Martin *et al.*, 2004; Kurz *et al.*, 2005). This statement is in concert with our observations, in which for most spermatozoa the annexin V positivity localized to the acrosomal part of the sperm head – the region where the above-mentioned processes occur. All together, these facts can indicate indirectly that the annexin V-labelled spermatozoa are not necessarily apoptotic. However, we may hypothesize that IGF-I in our study prevented spermatozoa from membrane destabilization during cooling storage.

For analysis of plasma membrane integrity we stained native (unfixed) semen preparations with fluorescently labelled PNA in order to visualize only those spermatozoa with damaged plasma membrane irrespective of acrosome integrity. About 23% of the sperm cells were permeable to PNA (had compromised plasma membrane in the sperm head region). This value is much higher than the percentages of apoptotic (Yo-Pro®-1), dead (PI) or membrane destabilized (annexin V) sperm cells measured in our study. Moreover, the motility of spermatozoa during the storage was maintained at a relatively high level (from 90 to 66%). Therefore, it seems that the loss of integrity in the acrosomal part of the plasma membrane does not substantially affect the motility of spermatozoa. IGF-I, added at higher doses, lowered the number of sperm with membrane disturbance and this finding confirmed the membrane stabilizing action of this growth factor.

Because there is a wide variability from study to study in sperm quality and fertilizing capacity, a need for effective methods that give reliable prediction of sperm fertility is still necessary. Testing individual male fertility by artificial insemination is expensive and labour intensive. The most suitable method to assess fertility may be an *in vitro* fertilization (IVF) test. This procedure evaluates spermatozoan–oocyte interactions that occur during *in vitro* fertilization, and allows the determination of different endpoints at early stages of embryo development. In our study we used a heterologous system for testing ram sperm fertility on bovine pre-matured oocytes with intact zona pellucida. The addition of IGF-I improved the penetration/fertilization rate from 53 to 68%, and the cleavage rate from 47 to 67% compared with control, respectively. The *in vitro* fertilization test to evaluate IGF-I effects on sperm fertilizing ability has been reported for buffalo sperm (Selvaraju *et al.*, 2010), in which the addition of IGF-I increased embryo cleavage rate from 45% (in the control group) up to 57%. This study supports our results regarding the stimulatory effect of IGF-I on the ability of sperm to fertilize ova. Co-incubation of ram spermatozoa with bovine oocytes with intact zona pellucida in the heterologous IVF test has been reported previously as a useful technique to predict the *in vivo* fertility of rams (Garcia-Alvarez *et al.*, 2009). However, these authors did not find any correlation between male fertility and sperm functional parameters (motility, viability and apoptosis) measured in laboratory tests. In the above cited study, frozen–thawed spermatozoa were analysed, a factor that might explain the absence of any correlation between fertility and functional parameters; we cannot exclude that for fresh semen correlation values could be different. Moreover, when the IVF assay is used to test sperm fertilizing ability, it should be considered that batch-to-batch variability in quality of bovine oocytes recovered from slaughterhouse-derived ovaries could substantially affect the results of this evaluation. Therefore, female gamete quality should be taken into account.

Taken together our observations indicate that IGF-I as an additive to sperm extender may be effective in the improvement of several functional characteristics of ram cooling stored spermatozoa (motility, membrane integrity, apoptosis). These IGF-I effects were reflected in sperm fertilizing ability *in vitro*.

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