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Functional characteristics of ram cooling-stored spermatozoa under the influence of epidermal growth factor

Alexander V. Makarevich[1], Eliska Spalekova[2], Lucia Olexikova[1], Norbert Lukac[2], Elena Kubovicova[1] and Zdena Hegedusova[3,4]

[1] *Animal Production Research Centre Nitra, 951 41 Luzianky, Slovak Republic*
[2] *Slovak Agricultural University, 949 01 Nitra, Slovak Republic*
[3] *AgriResearch Rapotin Ltd., Vyzkumniku 267, 788 13 Vikyrovice, Czech Republic*
[4] *Research Institute for Cattle Breeding Ltd. Rapotin, Vyzkumniku 267, 788 13 Vikyrovice, Czech Republic*

Abstract. The aim of the study was to examine the effects of epidermal growth factor (EGF) on ram sperm traits following hypothermic storage. Fresh ram ejaculates were diluted in Triladyl extender, pooled and divided into groups according to EGF doses added (0, 100, 200 or 400 ng/ml). Following 72–96 h storage at 4°C, the spermatozoa were stained for a plasma membrane integrity (PNA-FITC), membrane phosphatidylserine (PS) translocation (annexin V-Fluos) and apoptosis (Yo-Pro-1), and analyzed by fluorescent microscopy. Sperm motility was measured using computer-assisted sperm analysis (CASA) and sperm fertilizing ability was tested using *in vitro* penetration/fertilization test on bovine prematured oocytes. EGF increased sperm motility at all doses tested, decreased the proportion of spermatozoa with damaged plasma membrane (at 200 or 400 ng/ml), and decreased the proportion of apoptotic (Yo-Pro-1) spermatozoa when given at 200 or 400 (but not 100) ng/ml. The proportion of spermatozoa with PS translocations (8.5%) was affected by neither of the EGF concentrations tested. However, fertilizing ability of ram sperm in the *in vitro* test was not improved by EGF (200 ng/ml). In summary, EGF when given at higher concentrations improved sperm viability and motility after cooling storage, but these effects were not reflected in sperm fertilizing ability *in vitro*.

Key words: Ram — Sperm — Viability — Motility — Fertilizing ability

Abbreviations: EGF, epidermal growth factor; PI, propidium iodide; PNA, peanut agglutinin; PS, phosphatidylserine.

Introduction

Despite some progress, reached during the last two decades in the improvement of *in vitro* fertilization and artificial insemination techniques, there is a need to seek for some substances with a potential ability to stabilize sperm membranes, suppress apoptosis-like changes, stimulate sperm motility and, finally, to enhance fertilizing ability of animal and human spermatozoa. Epidermal growth factor (EGF) is one of the cytokines that plays an important role in control of reproductive functions and particularly in male fertility (Ahmad and Naz 1993). Although the EGF receptors in spermatozoa of several mammalian species have been demonstrated (Naz and Minhas 1995), the role of EGF in sperm function regulation is not ascertained (Oliva-Hernandez and Perez-Gutierrez 2008). Lack of EGF in plasma of mouse males led to the decrease in sperm concentration and motility (Liu et al. 1994), whilst the administration of exogenous EGF was useful for repair of testicular disorders (Uguralp et al. 2004; Kurokawa et al. 2005) or for the treatment of male infertility in rodents (Cheng et al. 2006). EGF regulates sperm functions (capacitation, acrosome reaction) through the activation of tyrosine kinase (Furuya et al. 1993a; Naz and Kaplan 1993) or protein kinase C (Lax et al. 1994), which are activated by specific EGF receptors demonstrated

Correspondence to: Alexander V. Makarevich, Animal Production Research Centre Nitra, Hlohovecka 2, 951 41 Luzianky, Slovak Republic
E-mail: makarevic@cvzv.sk

in several animal species (Naz and Ahmad 1992; Naz and Minhas 1995; Oliva-Hernandez and Perez-Gutierrez 2008). These observations suggest that EGF could be potentially used for the improvement of sperm characteristics in farm animal breeding and assisted reproduction.

Conversely, EGF is known to cause a negative effect on human sperm function (Naz and Kaplan 1993), or to not affect viability and motility parameters in boar sperm (Oliva-Hernandez and Perez-Gutierrez 2008). It has been suggested that EGF effect on sperm functions *in vitro* may be dose-dependent, with a preference to higher EGF concentrations (Naz and Kaplan 1993; Naz and Minhas 1995; Oliva-Hernandez and Perez-Gutierrez 2008).

The sperm responses to exogenous EGF, reported to date, were analyzed using phase-contrast microscopy (the evaluation of sperm motility and acrosome integrity), hypoosmotic swelling (HOS) test – integrity of sperm membrane (Oliva-Hernandez and Perez-Gutierrez 2008), chlortetracycline (CTC) assay (sperm capacitation; Furuya et al. 1993a,b), acrosin distribution assay (sperm acrosome reaction; Naz and Kaplan 1993; Naz and Minhas 1995), triple staining: Trypan blue/Bismarck brown/Rose bengal (acrosomal status; Naz and Kaplan 1993), computer-assisted sperm analysis (CASA) (motility, quality of the movement, Naz and Kaplan 1993; Naz and Minhas 1995) and sperm penetration assay (sperm fertilizing ability; Naz and Kaplan 1993; Naz and Minhas 1995). However, to our knowledge, there are no reports on effects of EGF on ram sperm evaluated in relation to parameters of sperm viability and apoptosis determined by more specific and sensitive fluorescent markers. Moreover, it still remains unclear, which an EGF concentration range is more effective to maintain better quality of ram sperm during cooling storage.

The aim of the study was to examine the effects of EGF on ram sperm characteristics (motility, membrane integrity, membrane stability, apoptosis and fertilizing ability) following liquid storage under hypothermic conditions.

Materials and Methods

Semen isolation and sample preparation

Fresh ejaculates were collected from three rams of Lacaune and one of East-Friesian breeds with proven health and reproductive status using artificial vagina. The rams were kept at a local farm under uniform nutritional conditions. After measuring the volume, the density and activity the semen was diluted (1 : 3) in a Triladyl extender (Minitub Slovakia, Čeľadice, Slovak Republic) containing 20% egg yolk, lactose and 6% glycerol. The semen was cooled up to 5–7°C, transported to the laboratory, where individual ejaculates were pooled and divided into four groups with 1 ml of the semen in each. Subsequently, human recombinant EGF (Sigma-Aldrich, Bratislava, Slovakia) was added at concentrations of 100, 200 or 400 ng/ml, whereas the control group did not contain EGF. Then sperm samples were stored in polystyrene tubes at cooling conditions (4°C in a fridge) for 24–96 h until analyses. Following this storage period, the sperm samples were removed from a fridge, washed out of Triladyl using centrifugation, resuspended in a saline solution contained 1% of fetal calf serum (saline-FCS) and analyzed.

Sperm motility analysis

Sperm motility was measured after 24, 48 and 72 h since the EGF addition using CASA method (Sperm Vision, Minitüb Slovakia, Čeľadice, Slovak Republic) daily at 0, 0.5 and 2 h after removal from the storage (Lukac et al. 2010). Spermatozoa were transferred by pipette to a Makler counting chamber with depth of 10 μm. The samples were placed under an Olympus BX 51 phase-contrast microscope (Olympus, Japan) with heating plate (37°C), and the images, obtained at magnification ×200, were analyzed by Sperm Vision software. In each sample at least 6 view fields were counted. Of all parameters measured by CASA, only total sperm motility was analyzed in this study.

Fluorescent assays

All fluorescent assays were performed immediately following 72–96 h storage of semen at 4°C. For plasma membrane integrity the sperm samples were stained with fluorescently-labelled lectin – *Arachis hypogaea* (PNA-FITC; Molecular Probes, Lucerne, Switzerland) in combination with propidium iodide (PI). The sperm samples were unfixed, what enabled the PNA-FITC labelling only in spermatozoa with disrupted or otherwise permeated plasma membrane, whilst spermatozoa with intact membranes remained unstained.

Following washing in saline-FCS, the sperm suspension was incubated in a staining solution containing 20 μmol/l of PNA-FITC and 5 μg/ml PI in saline-FCS for 20 min at room temperature. After the incubation, sperm samples were washed in saline and, following centrifugation, 4 μl of sperm suspension were placed onto microslide, gently mixed with 4 μl of Vectashield mounting medium with DAPI (H-1200, Vector Laboratories, Burlingame, CA, USA), a blue-fluorescent DNA stain which marks nucleoplasma of all sperm cells in samples. The obtained drops were flattened with a coverslip and immediately observed under a Leica fluorescent microscope (Mikro, Bratislava, Slovak Republic) with respective bandwidth filters for green, red and blue fluorescence.

Membrane phosphatidylserine (PS) translocation (membrane destabilization) was detected after staining with fluorescently-labelled annexin V using an Annexin-V-Fluos

staining kit (Roche Slovakia, Bratislava, Slovak Republic). Semen samples were washed in a binding buffer (supplied with a kit) and after centrifugation 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 the staining the sperm samples were washed out of unbound annexin V in a binding buffer, and drop of the semen suspension (4 µl) was placed onto microslide, mixed with 4 µl of Vectashield containing DAPI and flattened with a coverslip. The staining was immediately checked under a Leica fluorescent microscope using 488 nm wave-length filter. The spermatozoa with disordered PS asymmetry exhibited green fluorescence, whilst the spermatozoa with intact membrane were unstained.

For detection of apoptosis the sperm were stained with a Yo-Pro-1 specific green fluorochrome (Molecular Probes, Lucerne, Switzerland) in combination with PI for the identification of dead sperm cells. Following washing in saline-FCS the sperm suspension was incubated in a staining solution: saline-FCS consisted of 5 µmol/l Yo-Pro-1 and 5 µg/ml of PI. After 20 min staining at room temperature, the sperm samples were washed in a saline-FCS solution and 4 µl of sperm suspension were placed onto microslide, mixed with 4 µl of Vectashield with DAPI and the resulted drop was flattened with a coverslip. The preparations were immediately evaluated under a Leica fluorescent microscope with special filters for green, red and blue fluorescence. The green fluoresced sperm cells were regarded as apoptotic ones. The cells colored pink were considered as dead or necrotic.

Analysis of sperm penetrating/fertilizing ability

Sperm penetration/fertilization test was done using bovine prematured oocytes isolated from ovaries of cows provided by a local slaughterhouse. 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, fetal calf serum (BioWhittaker) and gentamycine, as described by Makarevich and Markkula (2002). Following maturation period, the oocytes were stripped of the cumulus cells using hyaluronidase treatment and placed into fertilization drop (Fert-TALP medium with 10 µg/ml heparin and either 0 or 200 ng/ml EGF) under mineral oil. The sperm suspension was washed out of Triladyl in Sperm-TALP medium, resuspended in a fresh Fert-TALP medium containing above mentioned additions and placed into a fertilization drop up to final concentration of 2–3 × 10^6 sperm/ml. Fertilization was performed in the incubator at 38.5°C in a humidified atmosphere with 5% CO_2 during 20 h. Subsequently, the presumptive zygotes were cleaned off the excessive sperm and the rest of cumulus cells by vortexing and transferred into B2 culture medium (CCD Laboratories, Vernouillet, France) onto culture dish with previously prepared monolayer of BRL (Buffalo rat liver) cells. The zygotes were cultured at 38.5°C in a humidified atmosphere with 5% CO_2 until evaluation. Following 5 days the zygotes and embryos were fixed in 3.7% formalin, mixed in a drop of Vectashield with DAPI, mounted between microslide and coverslip and inspected under a Leica fluorescent microscope with respective UV filter.

The zygotes and embryos were evaluated as follows: penetrated eggs – at least one sperm under *zona pellucida* or 1 pronucleus were present; fertilized eggs – at least two pronuclei were present; cleavaged embryos – two or more blastomeres with visible nuclei were present; non-penetrated oocytes – with no spermatozoa inside. Penetrating ability was calculated as the sum of penetrated oocytes, fertilized eggs and cleavaged embryos. Fertilizing ability was calculated as the sum of fertilized eggs and cleavaged embryos.

Statistical processing

Staining rate for fluorescent markers was done by counting of stained or total sperm number under a relative UV filter. The sperm images were made and a number of sperm was counted from a PC monitor. The experiments were performed in five repeats. In each experiment, about 8 microscopic views *per* each group were photographed. Totally, more than 1000 spermatozoa *per* each group were counted. The results of sperm motility were summarized and the average values obtained from several measurements were statistically evaluated as below. Comparisons of arithmetic means between groups of EGF concentrations were performed by a one-way ANOVA with fixed effects. The elementary contrast between mean values of fluorescent assays was evaluated by a Bonferroni test. The data of penetration/fertilization test were analyzed using Chi-square test. Since percentage values have been used in the study, all the values were subjected to the arcsin transformation in order to eliminate any abnormality of the distribution and/or non-homogeneity of the data. The statistical analysis was performed with original data using the Statistix analytical software (Analytical Software, version 8.0. Tallahassee, USA).

Results

An average value of sperm motility, determined by CASA, *prior* to put in the experiment was about 94–96%. After liquid storage under cooling conditions during 24, 48 or 72 h the average values of sperm motilities were 91, 74 and 65%, respectively. Following 24 or 72 h of liquid storage, no effect of EGF at any concentration on sperm motility was

noted. A stimulatory effect of EGF on this parameter was manifested by 48 h of storage at the concentrations of 200 or 400 ng/ml (Fig. 1).

Integrity of sperm plasma membrane was determined by triple staining with fluorochromes PNA-FITC, PI and DAPI. This staining enabled to distinguish among three populations of spermatozoa: the sperm with disrupted plasma membrane was green-labelled in the anterior region of the sperm head; the dead sperm was red-labelled either through a whole sperm head or in the posterior region; the rest of spermatozoa were stained blue and represented live intact sperm. About 41% of a whole sperm population was labeled with PNA-FITC, indicating disruption of the sperm plasma membrane The proportion of membrane-damaged spermatozoa was lowered by EGF ($p < 0.05$) given at 200 or 400 ng/ml (Fig. 2A). The percentage of necrotic/PNA-positive spermatozoa (PNA^+/PI^+; 7.9% in control) was significantly ($p < 0.05$) lowered by any of the EGF concentrations (4.52, 3.89 and 4.3% for 100, 200 or 400 ng/ml, respectively).

Using Yo-Pro-1/PI/DAPI, three populations of sperm cells were visible: the apoptotic sperm cells (green or yellow stained), the dead (necrotic) sperm cells (coloured red) and live intact spermatozoa (coloured dark-blue). Yo-Pro 1 stained entire the sperm head, whilst PI stained either whole the sperm head or only their post-acrosomal part. EGF suppressed Yo-Pro-1-detected apoptosis in spermatozoa ($p < 0.05$) when given at doses of 200 or 400 ng/ml ($p < 0.05$). At the dose of 100 ng/ml it showed no effect (Fig. 2B). The percentage of spermatozoa showing signs of both apoptosis and necrosis ($Yo\text{-}Pro1^+/PI^+$) in the control group was 6.03 ± 1.43%. This parameter was not significantly changed by any of EGF concentrations tested: 4.87 ± 0.77, 5.0 ± 1.55 and 5.75 ± 1.09% for 100, 200 and 400 ng/ml EGF, respectively.

The proportion of spermatozoa with PS translocations (annexin V-FITC) in the control sample was approximately 8.9% of a whole sperm population. This value was not affected by any of the EGF concentrations tested (Fig. 2C). In most of spermatozoa the annexin V positivity was localized in the acrosomal part of sperm head and tail (63–74%) with no differences among EGF concentrations. The rest of the annexin V positivity was localized in the post-acrosomal region, equatorial segment and head-tail connection (Fig. 3). The spermatozoa with the labelling in at least one of the above-mentioned sperm compartments were counted as an annexin V-positive.

For the *in vitro* fertilization test, the EGF concentration of 200 ng/ml was chosen, as it was stimulating in most of the parameters tested in this study. At comparison of penetrating ability of ram sperm no significant difference between the control (53%) and the EGF group (about 55%) was found (Table 1). Of all penetrated oocytes, about 33% of oocytes in the control and 30% in the EGF group were fertilized and subsequently developed until 2–8 cell stage embryos. Therefore, no effect of EGF on fertilizing ability of ram sperm was demonstrated.

Discussion

Reported effects of EGF on sperm of different mammalian species vary among studies. For example, EGF showed no

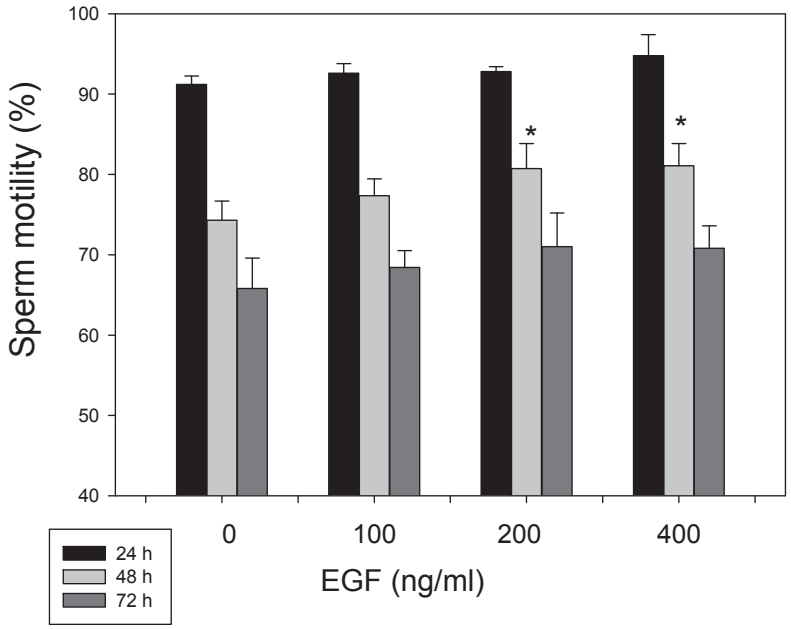

Figure 1. Effect of EGF on motility of chilled ram sperm.

effect on freshly isolated boar sperm (Oliva-Hernandez and Perez-Gutierrez 2008), manifested inhibiting effect on fresh human sperm (Naz and Kaplan 1993) and stimulating effect for mouse, human (Furuya et al. 1993a,b) and bovine (Lax et al. 1994) sperm functions *in vitro*. The absence of EGF effect on boar sperm function may be explained by the fact, that in this species not all the sperm cells can bear EGF receptors (Oliva-Hernandez and Perez-Gutierrez 2008). This phenomenon has not been observed in other mammalian sperm; however, it may not be excluded in case of ram sperm. For the evaluation of EGF effect on cooling stored ram semen we chose the analysis of motility, some fluorescent markers of viability and apoptosis, as well as the test of fertilization ability under *in vitro* conditions.

The motility of sperm in the ejaculate is the most important parameter of sperm quality. Sperm motility during liquid storage can be maintained for several days with 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 at latest by 72 h of storage and this parameter was declined from 96% (start of storage) to 65%, which is adequate to an average value for this species. This indicates that the basal milieu for ram semen samples (initial quality of ejaculates, semen extender, storage conditions) was satisfactory. The sperm in our tests responded to EGF additions (at 200 and 400 ng/ml) by a significant increase in total motility, especially when EGF was added to the sperm after 2-day storage. EGF was also reported to improve quality of movement in boar spermatozoa (Oliva-Hernandez and Perez-Gutierrez 2008). An exact mechanism by which EGF influences sperm motility is not ascertained. It is supposed that this effect is mediated *via* Ras or Rho components of ERK pathway, which are localized in flagellum of hamster (NagDas et al. 2002) and

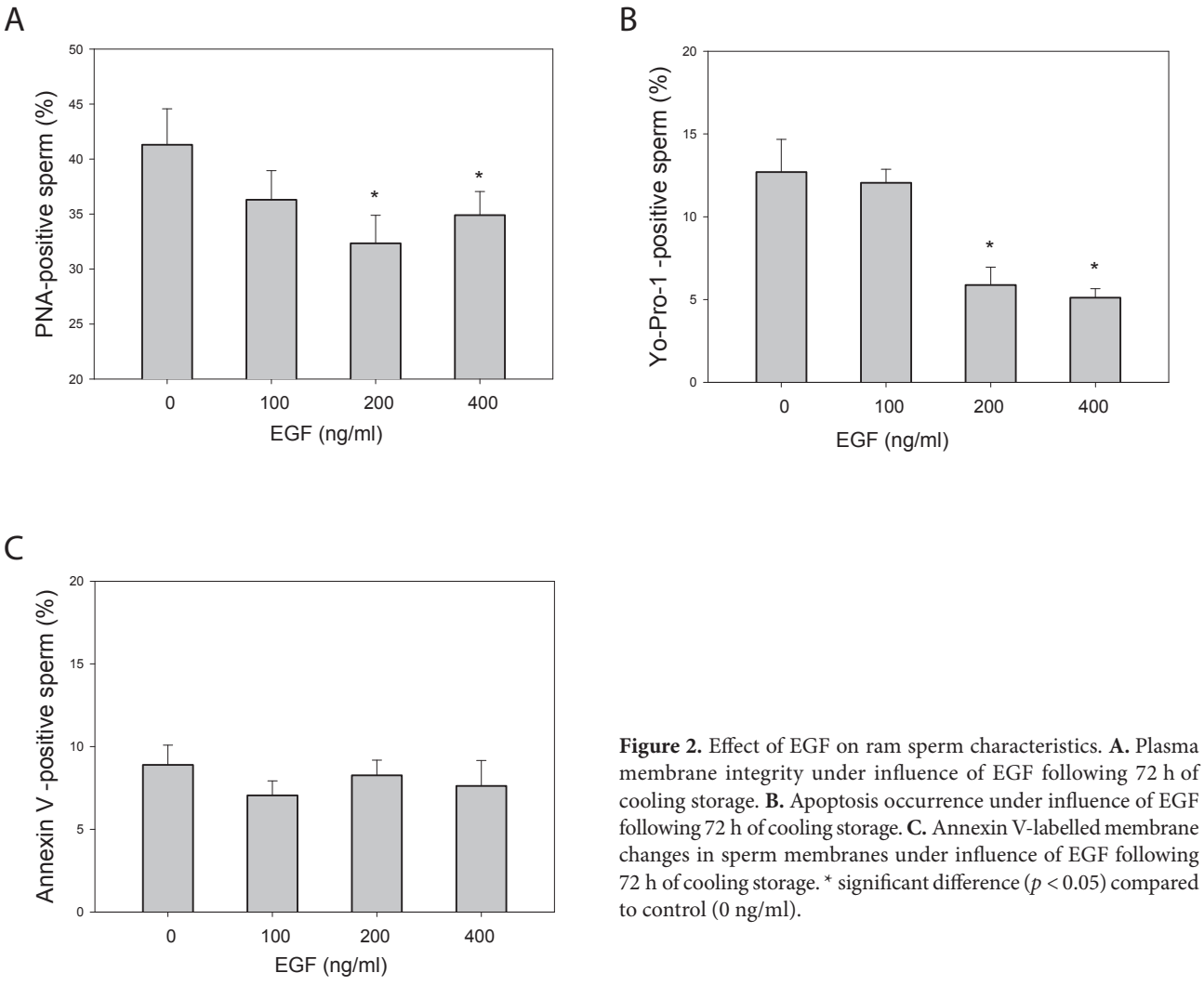

Figure 2. Effect of EGF on ram sperm characteristics. **A.** Plasma membrane integrity under influence of EGF following 72 h of cooling storage. **B.** Apoptosis occurrence under influence of EGF following 72 h of cooling storage. **C.** Annexin V-labelled membrane changes in sperm membranes under influence of EGF following 72 h of cooling storage. * significant difference ($p < 0.05$) compared to control (0 ng/ml).

Table 1. Effect of EGF on penetrating/fertilizing ability of ram sperm (Chi-square test)

	n (%)	
	Control	+ EGF (200 ng/ml)
Total no. oocytes	180	165
Penetrated oocytes	36 (20.00)	42 (25.45)
Fertilized eggs	37 (20.55)	23 (13.94)
Cleavaged embryos	23 (12.78)	27 (16.36)
Non-penetrated oocytes	84 (46.67)	73 (44.24)

bovine (Fujita et al. 2000), respectively, and are involved in sperm motility regulation.

The evaluation of semen on the basis of sperm motility is not always sufficient for sperm selection, because sperm samples with low sperm motility can have a high potential for insemination due to the low content of apoptotic and/or necrotic spermatozoa (Trzcinska et al. 2008). Research conducted to date indicates that the presence of apoptotic spermatozoa in fresh semen could be one of the reasons for poor fertility of breeding bulls (Anzar et al. 2002) and for male infertility in humans (Marchetti et al. 2002). Therefore, sperm motility evaluation should be combined with a determination of apoptotic or necrotic changes.

Apoptosis detection using Yo-Pro-1 staining was previously tested on somatic cells (Idziorek et al. 1995) and later on boar (Peňa et al. 2005; Trzcinska et al. 2008) and bull (Martin et al. 2004) spermatozoa and showed to be inexpensive, quick and easy to perform. In our study, about 13% of sperm cells were labeled by Yo-Pro-1 as apoptotic, whilst only about 6% of cells were stained by PI as dead ones. This may indicate that Yo-Pro-1 either alone or with PI is more specific dye to mark subtle changes in sperm than PI alone. Moreover, the rate of apoptotic (Yo-Pro-1) cells was lowered by EGF, however, a similar effect was not observed in the PI stained sperm.

A sperm plasma membrane with normal integrity and functions is a prerequisite for successful fertilization. There are numerous staining techniques for determination of membrane status (Gillian et al. 2005), in particular, various fluorescently labeled lectins (Flesch et al. 1998; Jankovičová et al. 2008), SYBR-14 (Garner and Johnson 1995) or membrane destabilization marker – annexin V (Peňa et al. 2003). It is hypothesized that the incidence of cells with PS translocation may indicate the cells with altered membrane function that will eventually undergo necrosis (Januskauskas et al. 2003). Such PS translocation may be occurred in mature spermatozoa (Peňa et al. 2005) and mostly is considered as an apoptosis harbinger. However, annexin V-FITC assay failed to discriminate objectively apoptotic population from non-apoptotic ones in bovine sperm cells (Martin et al. 2004). In our study, annexin V-labelling detected about 9% of spermatozoa with PS membrane destabilization. This value is lower than that of Yo-Pro-1-detected apoptotic cells (about 13%). Moreover, the percentage of annexin V-positive spermatozoa, unlike to Yo-Pro-1-related apoptosis, was not changed under influence of EGF. Several authors suggest that annexin V-labeled changes in sperm are related to the capacitation and/or acrosome reaction processes rather than to apoptosis (Kurz et al. 2005; Martin et al. 2005). This statement is in concert with our observations, where in the most of spermatozoa the annexin V-positivity was localized in the acrosomal part of the sperm head – the region where the above mentioned processes occur. All together these facts can indirectly indicate that, in our study, the annexin V-labeled spermatozoa does not necessarily mean apoptotic ones. However, to state this with a certainty, a co-localization (annexin V/Yo-Pro-1) study is necessary.

For analysis of plasma membrane integrity we chose fluorescently labeled lectin – PNA. In fixed sperm PNA labels intact acrosomes of sperm cells (Flesch et al. 1998). We used native (unfixed) semen preparations in order to

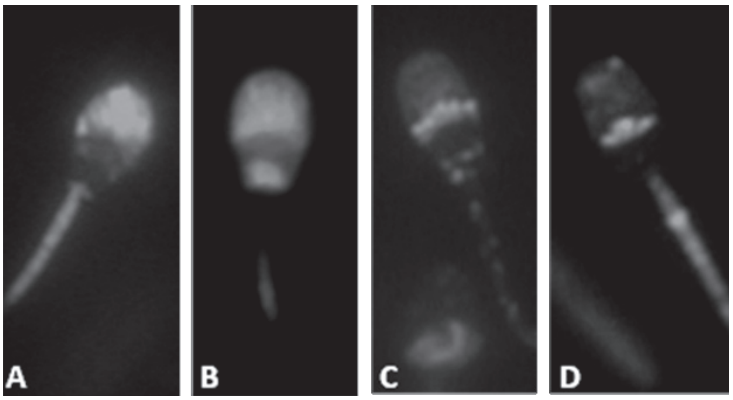

Figure 3. Localization of annexin V-labelled membrane PS externalization in ram sperm. **A**. Acrosomal part of the sperm head and tail. **B**. Both acrosomal and post-acrosomal part. **C**. Equatorial segment. **D**. Post-acrosomal part: head-tail connection and tail.

visualize only spermatozoa with damaged plasma membrane irrespectively to acrosome integrity. About 40% of sperm cells were permeable for PNA, i.e. had compromised plasma membrane in the sperm head region. This value is much higher than percentages of apoptotic, dead or membrane destabilized sperm cells measured in our study. Moreover, the motility of spermatozoa during the storage retained at relatively high level (from 91 to 65%). Therefore, it seems that the loss of integrity in the acrosomal part of the plasma membrane does not affect substantially the motility of spermatozoa. EGF at higher doses lowered the number of sperm with PNA-labeled membrane, i.e. this growth factor can act as a membrane stabilizer.

Because of a wide variability from study to study in correlation between sperm quality and fertilizing capacity, the seeking for the methods of reliable prediction of sperm fertility is still actual. Several attempts to find correlation between male fertility and functional parameters have been reported. In particular, O´Meara et al. (2008) failed to find any positive correlation between the *in vivo* fertility of rams and the *in vitro* determined functional parameters of ram frozen-thawed semen. On the other hand, significant correlation between field fertility and most of sperm viability parameters was found in bull frozen-thawed semen (Januskauskas et al. 2003).

Testing individual male fertility by artificial insemination is expensive and labor intensive procedure. The most adequate method to assess the sperm fertility may be *in vitro* fertilization, since this procedure evaluates the spermatozoa-oocyte interactions occurring during fertilization process, allowing determination of different endpoints in early stages of the embryo development. Garcia-Alvarez et al. (2009) used co-incubation of ram spermatozoa with bovine oocytes with intact *zona pellucida* in the *in vitro* fertilization test, and they documented that heterologous *in vitro* fertilization tests can be useful to predict the *in vivo* fertility of rams. However, similar to O´Meara et al. (2008), they also did not find any correlation between the male fertility and sperm functional parameters (motility, viability, apoptosis) measured in the laboratory tests. The absence of correlation between fertility and functional parameters may be influenced by the fact that in both above cited studies frozen-thawed sperm were analyzed. Therefore, it is not to be excluded that in case of fresh semen correlation values could be different.

In our study, using heterologous system for ram sperm fertility testing (bovine oocytes with intact *zona pellucida)*, about 53–55% of the oocytes were penetrated by ram semen and about 30–33% of them were fertilized, however, despite our expectations no effect of EGF was noted. The effect of EGF on sperm fertility using sperm penetration assay was reported earlier in human (Naz and Kaplan 1993), where EGF at lower (0.1–10 nmol/l) concentrations did not affect the penetration rate and at higher ones (25–100 nmol/l) it even decreased this parameter. For comparison, in our study we used EGF at 200 ng/ml, which is equivalent to 30 nmol/l, and no decrease in sperm fertility was recorded. Analyzing sperm fertility *in vitro,* it should be considered that the quality of bovine oocytes recovered from the slaughterhouse-derived ovaries may vary from bath to bath, which can substantially influence sperm fertility evaluation in the fertilization test. Therefore, the factor of female gamete quality should be taken into account in such studies.

Taken together our observations indicate that EGF, when added at higher doses, was effective in the improving several functional characteristics of ram cooling stored sperm (motility, membrane integrity, apoptosis). However, these EGF effects were not reflected in sperm fertilizing ability *in vitro*.

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