Demonstration of the effect of epidermal growth factor on ram sperm parameters using two fluorescent assays

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ABSTRACT: The goal of this study was to examine the effect of epidermal growth factor (EGF) on sperm viability using two fluorescent techniques and to analyze the obtained results in relation to sperm motility, determined by subjective estimation. Fresh ram semen diluted in a Biladyl commercial extender was cooling stored (at 4 °C in a fridge) for four days in the presence of EGF at doses of 0, 10, 100, 200 or 400 ng/ml. Thereafter, sperm samples were analyzed for progressive motility (Motility test) and membrane integrity using two fluorescent techniques: SYBR-14/PI (Method 1) or PI/DAPI (Method 2). Application of Method 1 did not detect an effect of EGF at any concentration on sperm membrane integrity. A positive effect of EGF (200 ng/ml) on sperm membrane integrity was found using Method 2 of staining, and this result was confirmed by the sperm motility test, which demonstrated an EGF-stimulating effect (200 or 400 ng/ml) on a percentage of progressively moving spermatozoa. Strong positive correlations between Methods 1 and 2 (r = 0.785), Method 1 and Motility (r = 0.803), Method 2 and Motility (r = 0.699), as well as between both techniques taken together and the Motility test (r = 0.853) were found. Regression analysis confirmed that Method 2 was more exact than Method 1, and the results obtained with Method 2 are comparable with those of the Motility test. Dependence of the viability or motility on EGF concentrations (linear regression function) was significant only for Method 2 or the Motility test. The obtained results suggest a stimulating effect of EGF (at higher concentrations) on ram sperm functions (viability/membrane integrity and motility). Furthermore, they indicate substantial differences between two fluorescent techniques in the determination of sperm membrane integrity. Only the data obtained using PI/DAPI were confirmed by a functional Motility test. These findings suggest that the technique chosen for analysis of sperm viability can influence the conclusion concerning the effects of the treatment on sperm function.

Keywords: viability; motility; membrane integrity

Sperm quality and viability are the key factors which determine efficiency of insemination. The integrity of cell membranes is important for sperm viability. Although conventional assays of sperm quality, as sperm concentration and motility, may provide basic information, new techniques to make sperm evaluation more exact and reliable are still required.

The fluorescent technique SYBR-14 in combination with propidium iodide (PI) has been described to assess sperm viability in different species including boars, bulls, rams, rabbits, mice and humans (Garner and Johnson, 1995) as well as in freeze-thawed bovine sperm (Garner et al., 1994; Thomas et al., 1997). The SYBR-14/PI assay distinguishes between populations of living (green color), dead (red color) and dying (yellow, combination of green and red color) sperm cells with damaged membranes. The mechanism by which SYBR-14 stains live sperm more intensely than dead sperm is not completely known yet. However, it is assumed that several biochemical characteristics, like membrane potential, play a role in enhancing fluorescence by a vital dye such as SYBR-14 (Garner and Johnson, 1995).

An alternative probe that can distinguish between viable and non-viable sperm cells is propidium iodide (PI) – impermeant dye, which stains (in red)

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only cells with damaged membranes (Harrison and Vickers, 1990; Pintado et al., 2000). PI has been successfully used to detect non-viable cells in boar and bull spermatozoa (Pintado et al., 2000).

Parameters of activity/motility are important from the viewpoint of their association with sperm quality, i.e., with sperm capacitation, acrosome reaction and *zona pellucida* binding (Robertson et al., 1988). Therefore, a positive correlation between sperm viability and activity/motility may exist. However, there are some reports that many spermatozoa, which stain as vital, are in fact immotile (Hong et al., 1988), or that not all immotile spermatozoa lose their membrane integrity (Bialkowska et al., 2004). This inconsistency requires additional studies to elucidate the relationships between sperm viability and motility parameters.

Epidermal growth factor (EGF) is one of the important cytokines with a role in male fertility (Ahmad and Naz, 1993). The elimination of circulating plasma EGF was shown to suppress spermatogenesis and decrease sperm content and motility in adult mouse males (Liu et al., 1994). Administration of exogenous EGF was also reported to markedly repair the testicular injuries caused by testicular torsion (Uguralp et al., 2004), cryptorchidism (Kurokawa et al., 2005) and streptozotocin-induced diabetes (Noguchi et al., 1990) in rodents. Furthermore, EGF administration has been shown to be useful for the treatment of male infertility in rats with induced varicoceles (Cheng et al., 2006). EGF was found to stimulate capacitation and acrosomal exocytosis in human, mouse and bovine sperm through the activation of tyrosine kinase (Furuya et al., 1993a,b; Naz and Kaplan, 1993) or protein kinase C (Lax et al., 1994). These kinases are activated by EGF through specific receptors in human, mouse, rabbit, bull and boar sperm (Naz and Ahmad, 1992; Damjanov et al., 1993; Naz and Minhas, 1995; Oliva-Hernandez and Perez-Gutierrez, 2008).

The published data show that EGF effects on the functions of ejaculated sperm are dependent on EGF concentration and animal species. In humans, EGF may have a negative, rather than positive role in sperm function (Naz and Kaplan, 1993). It has been shown that at lower (0.1–10nM) concentrations EGF did not affect sperm penetrating ability, whilst at higher (> 25nM) concentrations EGF decreased penetration rates, acrosome reaction and sperm motility parameters. On the other hand, in boar sperm, EGF at 10 and 100 ng/ml affected neither acrosome status nor membrane integrity or motility either in intact or acrosome reaction-induced sperm, but significantly improved the parameters of sperm movement (Oliva-Hernandez and Perez-Gutierrez, 2008).

Therefore, EGF can be potentially used to improve the characteristics of fresh and stored semen used in farm animal breeding and assisted reproduction. Nevertheless, the effect of EGF on sperm membrane integrity using the techniques mentioned above has not been yet been studied.

The aim of this study was to evaluate the effect of EGF given as an additive to ram semen extender to preserve cooling-stored spermatozoa. Sperm membrane integrity was examined using two known fluorescent techniques. The SYBR-14/PI (Method 1) is based on direct counting of live sperm cells stained by green fluorescent dye (SYBR-14), whereas dead or dying cells are stained by the red fluorescent dye – PI. Sperm were analyzed under a fluorescent microscope in a wet preparation following spermatozoa immobilization in 1% glutaraldehyde. The second technique – PI/DAPI (Method 2) is based on the labelling of dead cells by red fluorescence (PI). Sperm was analyzed in a smear-dried preparation; the sperm viability was calculated as the PI-sperm ratio to the total sperm counts (DAPI). The obtained results were compared *vice versa* and with sperm progressive movement (the motility test).

MATERIAL AND METHODS

Sperm collection and incubation

Semen was collected from six rams of the Lacaune breed with proven health and reproductive status. After measuring volume and density the sperm was diluted (1 : 3) in a Biladyl extender (pH 6.3, osmolarity 311 mOsmol/kg; Minitub, Nitra, Slovakia) and transported to the laboratory. Individual ejaculates were mixed up to obtain heterospermic samples, which were divided into five tubes (4 ml per tube). Epidermal growth factor, isolated from mouse submaxilliary gland (EGF, Sigma-Aldrich, Germany) was added to the relevant tubes at doses of 0 (control), 10, 100, 200 or 400 ng/ml. Sperm samples diluted in the Biladyl extender with or without EGF were stored in open plastic tubes (4 ml per tube) at cooling conditions (4 °C) for four days. Afterwards, the sperm samples were washed by centrifugation at 600 g for 10 min and consequent washing in

HBS medium (Hepes-buffered saline added with 1% BSA, pH 7.4) and subjected to the motility test or stained using the two fluorescent techniques described below.

Evaluation of sperm progressive movement (Motility test)

Sperm samples were removed from the fridge and, following centrifugation and washing in HBS, incubated at 39 °C. Sperm motility (progressive movement) in the samples was estimated subjectively on the basis of a percentage ratio of progressively (straight-line forward movement) moving spermatozoa assessed in several view fields of a light microscope at 200× magnification. Total sperm motility in the sample was calculated as an average value from the motility values measured after 15 min, 1, 3 and 5 h of incubation at 39 °C.

Analysis of sperm viability

Method 1: SYBR-14/PI. The SYBR-14/PI (Method 1) is based on the direct counting of live sperm cells stained by a green fluorescent dye – SYBR-14. Dead or dying cells are stained by the red fluorescent dye propidium iodide. Sperm was analyzed under a fluorescent microscope in a wet preparation after spermatozoa immobilization in 1% glutaraldehyde solution.

The samples of sperm suspension following incubation in a fridge were centrifuged at 600 g for 10 min and the sperm pellets were resuspended in fresh HBS medium to give the original volume. The sperm samples were diluted (1 : 10) using fresh HBS medium (pH 7.4) and aliquots of 500 µl per each group were made. SYBR-14/PI staining was performed using a LIVE/DEAD Sperm Viability Kit (Molecular Probes, Lucerne, Switzerland) according to the manual. Briefly, 1 µl of the SYBR-14 dye stock solution (1mM in DMSO) was added to 49 µl of the HBS medium to make 50 µl of the working solution at a concentration of 0.02mM. Then, 2.5 µl of the working solution were added to each 500 µl sample of diluted semen, resulting in a final SYBR-14 concentration of 100nM. The sperm samples were incubated at 37 °C for 15 min; afterwards 2.5 µl of PI was added to each sample (final PI concentration – 12µM) and the samples were additionally stained for 10 min.

Following staining the spermatozoa (in 3 µl of sperm sample) were immobilized using a 1% glutaraldehyde solution (1 µl), placed onto microslides, covered with Vectashield mounting medium (4 µl; H-1000, Vector Laboratories Inc., Burlingame, CA, USA) and covered by a coverslip. Sperm samples were analyzed under the Leica fluorescent microscope (Leica Microsystems, Wetzlar, Germany) equipped with specific wave-length filters for FITC and TRITC channels using the PlanApo dry objective at 40× magnification. Sperm images were acquired by the DFC 480 camera and processed using the IM500 Leica software.

When stained with SYBR-14 and excited at 488 nm, the living sperm exhibited bright green fluorescence. Non-motile or apparently dead sperm showed a faint fluorescent signal with lower intensity compared to living sperm. Additional PI staining made dead sperm cells to fluoresce bright-red, but some sperm (apparently moribund) showed both green and red fluorescence. Since fluorescence intensity may be slightly increased over time [1], sperm images were made within a few minutes. Total sperm number was counted from merged images (Figure 1.). Sperm viability was calculated as the ratio of SYBR-14-stained cells to total sperm number.

Method 2: PI/DAPI. PI/DAPI staining (Method 2) is based on the labelling of dead cells by red fluorescence (PI) and analysis of a smear-dried preparation; sperm viability was calculated as the PI-sperm to total sperm count ratio, which are labelled by a blue fluorescent dye (DAPI; Figure 2).

Following incubation in a fridge, the samples of sperm suspension were centrifuged at 600 g for 10 min and the sperm pellets were resuspended in fresh HBS medium to the original volume. PI stock solution (0.5 mg/ml) was added to each tube up to a final concentration 5 µg/ml and the suspension was incubated for 15 min at 37 °C. Following incubations in PI, 3 µl of the sperm suspension were mixed with 1 µl of 1% glutaraldehyde solution and smeared over the microslide. Excessive liquid was removed by a short drying step (2 min) in air and the sample was covered with Vectashield anti-fade medium containing DAPI fluorochrome (H-1200, Vector Laboratories Inc.) and covered by a coverslip. The sperm samples were analyzed under the Leica fluorescent microscope (Leica Microsystems) equipped with specific wave-length filters for TRITC or DAPI channels using a PlanApo dry objective at 40× magnification. Sperm images were acquired using the DFC 480 camera and processed

using IM 500 Leica software. Total sperm number was determined from DAPI-stained cell counts; PI-stained cells were subtracted from total cell counts and sperm viability was calculated as a ratio of PI-unstained cells to the total sperm number.

Statistics

In each experimental group at least six view fields were examined, so that at least 200 sperm cells were counted per experiment. Experiments were performed in five replications. In total, more than 1000 cells were analyzed per group.

The obtained results were processed using one-way analysis of variance (ANOVA), linear and non-linear regression and correlation methods. For all the methods and EGF concentrations the arithmetic means were calculated. Comparisons of arithmetic means between groups with different EGF concentrations for all methods were performed using one-way ANOVA with fixed effects using the model equation:

$$y_{ij} = m + a_i + e_{ij}$$

where:
y_{ij} = j obtained observation in i group
m = a general mean,
a_i = the fixed effects of i group
e_{ij} = a random error of observation distributed as N (0, s^2)

The elementary contrast between mean values was evaluated by a Bonferroni test. For evaluation of the precision of the used methods, Pearson's correlation, Spearman's rank correlation and F-tests were computed for the EGF concentrations, with the comparison of residual mean squares MS_e from one-way ANOVA for the methods used. For the determination of dependence of the viability (Methods 1 and 2) and progressive movement (Motility test) from EGF concentrations linear and non-linear (power function) regression methods with computation of coefficients of determination (R^2) were used. Since percentage values have been used in this study, in order to eliminate any abnormality of the distribution and/or non-homogeneity of the data, all the values were subjected to the arcsin transformation (y = arcsin$\sqrt{\%}$). Statistical analysis was performed using the Statistix analytical software (Version 8.0) (Anonymous, 2001).

RESULTS

Using Method 1 of viability staining, we observed three patterns of sperm staining. Live spermatozoa with intact membranes were labelled only with SYBR-14 dye and their heads fluoresced green (L); dead spermatozoa, were permeable for PI and their heads fluoresced red (D), and moribund (dying) spermatozoa were permeable for both stains and fluoresced yellow (M) on merged images (Figure 1). The spermatozoa with both red- and yellow-colour heads were counted as dead. The majority of the spermatozoa accumulated stain(s) in the head, whilst flagella were either unstained or stained only with PI. SYBR-14 staining of sperm cells appeared to be more intense than PI staining; however, no visible differences in morphology between live and dead spermatozoa were noted.

Using Method 2, spermatozoa with damaged plasma membranes were labelled with PI. Only sperm cells with PI-stained heads (either in the anterior or posterior sperm region), and which turned

Table 1. Effect of EGF on the viability (measured by two methods) and motility of ram sperm. Values are mean ± standard error of mean, after arcsin transformation

Methods	EGF dose added (ng/ml medium)				
	0	10	100	200	400
1. SYBR-14/PI					
Viability (%)	63.40 ± 1.82	62.42 ± 1.15	64.41 ± 1.19	65.94 ± 2.14	66.41 ± 1.42
2. PI/DAPI					
Viability (%)	72.96 ± 1.71	72.77 ± 0.87	77.17 ± 0.52	78.43* ± 0.70	75.93 ± 1.33
Motility test					
Motility (%)	46.25 ± 1.67	49.22 ± 0.53	50.18 ± 0.25	51.17* ± 0.83	52.76**± 1.00

Significant differences: *$P_1 \leq 0.05$, **$P_1 \leq 0.01$, compared to control (0 ng/ml EGF)

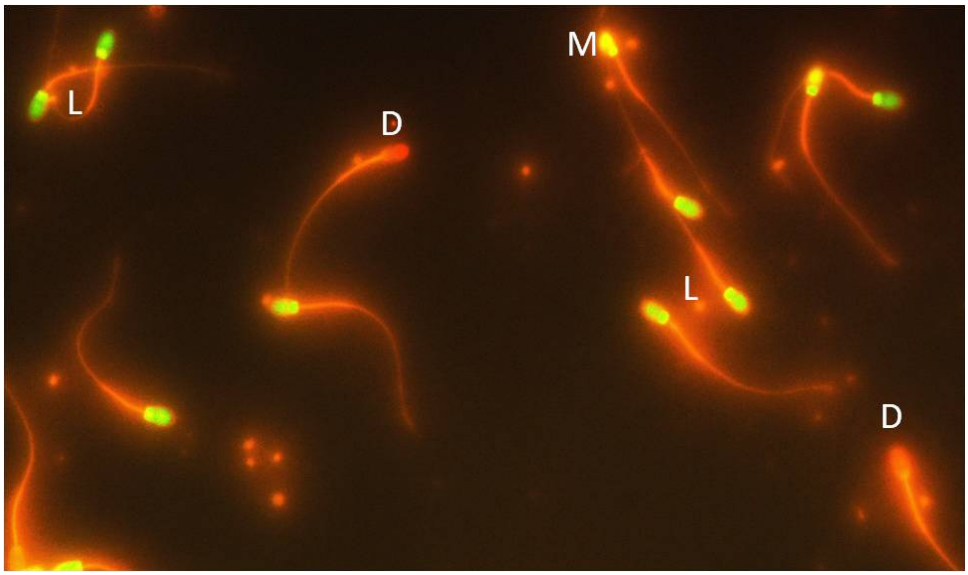

Figure 1. Fluorescent staining of ram spermatozoa with SYBR-14/PI (Method 1). Three patterns of staining are seen on a merged image: green-fluoresced sperm heads (L) denote live spermatozoa with intact membranes, red-fluorescent sperm heads (D) indicate dead spermatozoa, yellow-fluorescent sperm heads (M) represent moribund (dying) spermatozoa (magnification 400×)

pink on a merged image (D), were considered as dead (Figure 2).

A comparison of two methods shows that the SYBR-14/PI method revealed a significantly lower percentage of viable sperm cells (63.4%) than did the PI/DAPI method, where 73% of cells where shown to be viable (Table 1). Using Method 1, no significant differences between the groups receiving EGF at different doses were observed. However, Method 2 revealed that EGF at a dose of 200 ng/ml promoted sperm viability ($P_I < 0.05$). Progressive motility of these sperm samples was in the range of 46–53%, depending on EGF doses added. EGF given at doses of 200 or 400 ng/ml significantly improved sperm motility, which was confirmed on the arcsin$\sqrt{\%}$ transformed data (Table 1). EGF

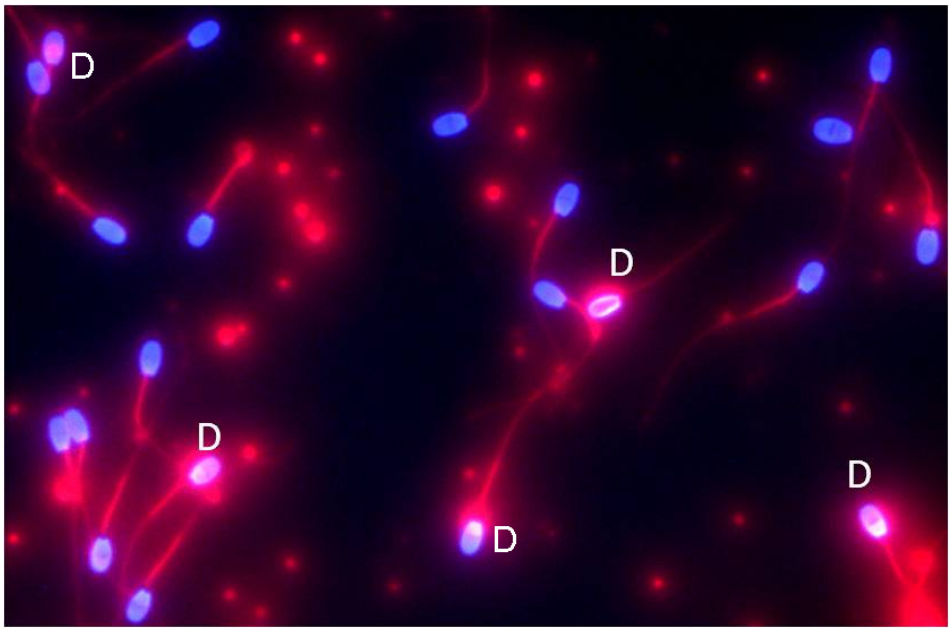

Figure 2. Fluorescent staining of ram spermatozoa using PI/DAPI (Method 2). Sperm cells with pink-colored heads (PI-stained) on a merged image are dead (D) spermatozoa (magnification 400×)

Table 2. Correlations between arithmetic means of traits for EGF concentrations and *F*-test for comparison of residual mean squares MS_e between staining methods and the motility test

Compared methods	Pearson (r)	Spearman (r_s)	*F*-test
Method 1 × Motility	0.8032	0.9000*	3.0489**
Method 2 × Motility	0.6993	0.7000	1.5064
Method 1 × Method 2	0.7854	0.6000	2.0246*
Viability# × Motility	0.8534	0.9000*	–

Significant differences: $*P_I \leq 0.05$, $**P_I \leq 0.01$
= mean of two methods

at concentrations of 10 or 100 ng/ml influenced neither sperm viability nor motility.

High positive correlations (Pearson's correlation) were observed between values obtained using Methods 1 and 2, as well as between values obtained using these two methods for viability staining and motility (Table 2). The highest and the significant values for Spearman's correlation for EGF doses were observed between Method 1 and Motility, as well as between viability (Method 1 plus Method 2) and Motility. *F*-test values confirm that the Method 2 had a similar residual mean square as the Motility, but Method 1 had the larger residual mean square compared to Motility or to Method 2. This suggests that Method 2 is more accurate than Method 1 for viability staining.

The dependence of the viability/motility on EGF concentrations (linear regression function) was significant only for Method 2 (b = 0.0085*) or highly significant for the Motility test (b = 0.0129**), but not for Method 1 (b = 0.0088). The determination coefficients for both viability methods were low (0.1068 and 0.1268, resp.). However, for the Motility test the linear regression was represented by approximately 38% of the total variability (R^2= 0.3798; Table 3). Therefore, these regressions were analyzed by nonlinear regression methods, such as polynomial regression (quadratic or cubic polynomials; data not shown) and by power function ($y = ax^b$; Figure 3). From the comparison of determination coefficients between linear functions of quadratic and cubic polynomial regression and power function (Figure 3) it can be concluded that dependence of the viability on EGF concentrations was significant only for the Method 2 (R^2 = 0.2722*) and the Motility test (R^2 = 0.4745*), but not for Method 1 (by power function, R^2 = 0.0961. The results of the regression analysis confirmed that Method 2 was more accurate than Method 1, and that the results using Method 2 are comparable with those of the Motility test.

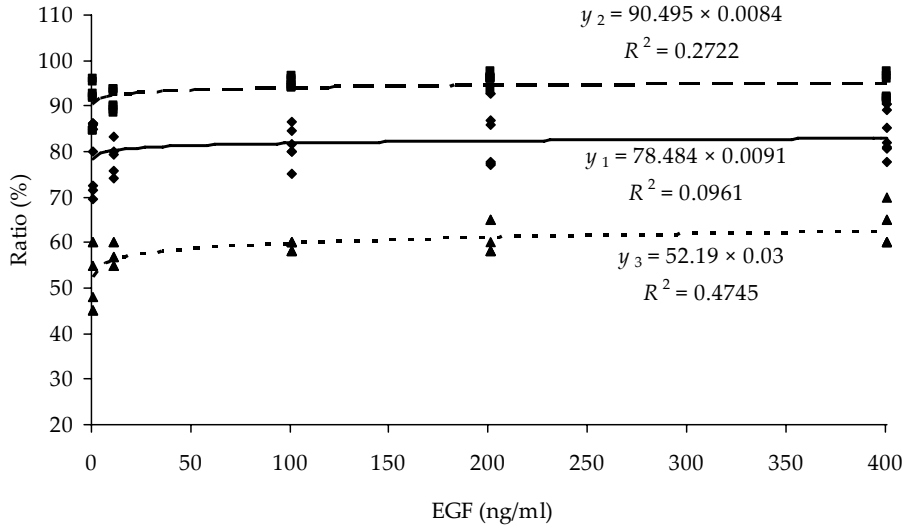

Figure 3. Power regression function of sperm viability/motility in relation to EGF concentrations

y_1 = viability by Method 1, y_2 = viability by Method 2, y_3 = Motility test

Table 3. Linear regression functions ($y = a + bx$) of viability and motility for EGF concentrations (x)

Parameters (y)	a	$\pm s_a$	b	$\pm s_b$	R^2
1. SYBR-14/PI					
Viability	63.2875**	± 0.9833	0.0088	± 0.0047	0.1068
2. PI/DAPI					
Viability	74.2408**	± 0.7989	0.0085*	± 0.0039	0.1268*
Motility test					
Arcsin motility	48.0894**	± 0.6374	0.0129**	± 0.0031	0.3798**

Significant differences: *$P_I \leq 0.05$, **$P_I \leq 0.01$

DISCUSSION

Sperm membrane integrity is an important parameter for sperm quality evaluation, because intact plasma membrane is an essential requirement for sperm cell survival. A number of methods exist to distinguish between spermatozoa that are viable or non-viable, but it still remains unclear, which of these methods is more reliable and should be used for practical applications. For example, membrane integrity of bovine sperm, according to Jankovicova et al. (2008), can be reliably evaluated using either Hoechst 33258/FITC-PSA or Congo red/Giemsa double staining techniques. In our experiments, we have tested and compared two techniques based on the ability/inability of fluorescent dyes to enter intact cell membranes, which enables examination of the membrane integrity of ram spermatozoa stored in a sperm extender with or without EGF. These two fluorescent techniques have been verified by a motility test.

Using the SYBR-14/PI technique, a significantly lower number of sperm cells was identified as viable (63%) in comparison to the PI/DAPI technique (73%). However, indexes of viability determined by both techniques (given as the mean of two staining techniques) almost positively correlated (r = 0.8534) with the index of sperm motility. These viability techniques also positively correlated with each other (r = 0.7854).

Regression analysis confirmed that the PI/DAPI method is more accurate than the SYBR-14/PI method in determining the effect of certain EGF concentrations on sperm parameters and the results obtained with the PI/DAPI better match the Motility data. The difference between the two techniques in assessing sperm membrane integrity may be explained by different approaches to sample preparation. Using the SYBR-14/PI assay, fluorescent analysis was performed in a wet sample, whereas for the PI/DAPI assay, smeared-dried sperm samples (covered with DAPI-contained Vectashield) were analyzed. Therefore, it is possible that the analysis of wet samples (Method 1) overestimated, whilst the procedure of smearing and drying (Method 2) underestimated, the ratio of PI-labelled cells. Patterns of the effects of EGF analyzed by power function regression show the coincidence in trends (parallelism between graphs characterizing two staining methods and the motility test), which indicates the consistency and homogeneity of the data in the tested groups.

Motility is important parameter in the estimation of sperm hyperactivation, which is considered to be an integral part of sperm capacitation, acrosome reaction and binding to the *zona pellucida* (Robertson et al., 1988). In our study a strong positive correlation (r = 0.8534) between sperm motility and viability/membrane integrity has been found. The test of sperm movement showed that about 46–53% (depending on EGF dose) of all spermatozoa in our samples after four days of cooling storage were motile. This parameter is different from the percentage of viable spermatozoa determined either by the SYBR-14/PI (63–66%) or PI/DAPI (73–78%) staining techniques. These observations suggest that the loss of sperm motility does not necessary mean a loss of membrane integrity due to membrane damage, i.e., spermatozoa can maintain their membranes intact even after becoming immotile. Similarly, Hong et al. (1988) have found that many human spermatozoa, fluorescently stained as vital, were in fact immotile. These facts are in agreement with the report of Bialkowska et al. (2004), who showed in the bivalve mollusk Zebra Mussel, that not all immotile spermatozoa lose their membrane integrity. Therefore, the SYBR-14/PI assay has been shown to be effective in testing the viability of sperm from several mammalian species (Garner and Johnson, 1995), but has not

been reliable in non-mammalian (bivalve mollusk) sperm (Bialkowska et al., 2004).

Previously, SYBR14-/PI staining of fresh ram spermatozoa has been described by Garner and Johnson (1995). They reported that 49.2% of sperm stained with SYBR-14, whilst 45.2% were labelled with PI. In our study, the percentage of SYBR-14 stained sperm cells was higher (63–66%) than in the above mentioned study. This difference may be explained by different incubation conditions (type of semen extender and temperature) and analysis (fluorescent microscopy versus flow cytometry) used in our study and the study of the mentioned authors. Some variations could be due to sperm origin. For example, these authors noted some variability in ram sperm viability among males. Since our primary goal was to compare the methods and to examine the effects of EGF but not of individual male variability on ram sperm functions, we avoided any potential influence of individual rams by mixing the sperm doses from several rams to obtain a heterospermic semen population.

EGF has been shown to have a role in both male and female reproduction (Ahmad and Naz, 1993). From the published data regarding the effects of EGF on functions of ejaculated sperm it is obvious that these effects are dependent on concentration and animal species. Thus, in humans, EGF may exert a negative rather than a positive effect on sperm function (Naz and Kaplan, 1993). In particular, EGF at lower (0.1–10nM) concentrations, was shown to not affect sperm penetrating ability as assessed by the sperm penetration test, whilst EGF at higher (> 25nM) concentrations decreased penetration rates, acrosome reaction and sperm motility parameters (Naz and Kaplan, 1993).

At the same time, Furuya et al. (1993a) reported that EGF (at 100 ng/ml) stimulated human sperm capacitation. These two teams used different concentrations of EGF. In pigs, EGF, given at 10 or 100 ng/ml, did not affect acrosome status, membrane integrity or motility of acrosome reaction-induced sperm. However, it significantly improved (at 100 ng/ml) the quality of movement of capacitated spermatozoa (Oliva-Hernandez and Perez-Gutierrez, 2008). EGF (100 ng/ml) has also been reported to stimulate mouse sperm capacitation (Furuya et al., 1993b) and induced acrosomal exocytosis in bovine sperm (Lax et al., 1994).

In our study, EGF given at concentrations less than 200 ng/ml (30nM) did not affect sperm viability or motility. Higher doses of EGF (200 ng/ml or higher) caused an improvement in sperm viability, as assessed by the PI/DAPI assay and Motility test. This is the first demonstration of a stimulating effect of EGF on ram sperm functions. This positive EGF effect was observed under conditions of long-term cooling storage of sperm, routinely used prior to artificial insemination of sheep. Although we did not compare EGF effects under different sperm storage conditions, it cannot be excluded that not only stimulatory, but also protective effects of EGF on ram sperm may occur. Our observations suggest an involvement of EGF in the physiological control of sperm function in rams, as well as its potential usefulness for the improvement of sheep insemination and breeding programs.

Taken together, our observations demonstrate a beneficial and protective effect (preservation of membrane integrity and motility) of EGF on ram sperm parameters. The obtained results indicate substantial differences between the two fluorescent techniques employed for the determination of ram sperm membrane integrity and suggest a higher reliability of the PI/DAPI in comparison to the SYBR-14-/PI assay. However, this conclusion should not be generalized for sperm from all mammalian species. Finally, the presented data suggest that the technique chosen for analysis of sperm viability can affect the conclusion concerning the effects of the treatment on sperm function.

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