*Polish Journal of Veterinary Sciences Vol. 22, No. 2 (2019), 305–312*

DOI 10.24425/pjvs.2019.129221

*Original article*

# Influence of bovine follicular fluid on thawed bovine spermatozoa – assessment by CASA system and flow cytometry

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## Abstract

Aim: The aim of this study was to analyze the effect of bovine follicular fluid on the survival, morphology and kinetic parameters of bovine thawed spermatozoa under laboratory conditions.

Materials and methods: The semen from 5 bulls of proven fertility was incubated in follicular and physiological fluid for 8 hours. During this time assessment using the CASA system was performed. At the beginning and the end of incubation process evaluation by flow cytometry was conducted.

Results: The results of the sperm motility assessment showed a significant decrease in the analyzed parameters both in the follicular and physiological fluid. A significant reduction in all parameters characterizing movement properties in the semen incubated in the follicular fluid was found. In the physiological fluid, a similar trend was demonstrated only for the following properties: VAP, VSL, VCL, ALH, BCF. A significant difference was found for both fluids in: VCL ($p=0.026$), ALH ($p=0.038$) and LIN ($p<0.001$) at the beginning of incubation. The results of the plasma membrane integrity assessment showed a statistically significant increase in the percentage of dying sperm at the 8th hour of the incubation in the follicular fluid. In the case of semen incubation in physiological fluid, a statistically significant decrease in the percentage of live non-damaged cells was found with a simultaneous increase in the subpopulation of undamaged dead cells.

Conclusions: Follicular fluid rapidly accelerates the capacitation process. The results of flow cytometry support the hypothesis concerning the ability of follicular fluid to prolong sperm survival.

**Key words:** bovine follicular fluid, spermatozoa, CASA, flow cytometry

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## Introduction

Follicular fluid is the extracellular fluid, accumulating in the antrum of antral ovarian follicles from the stadium of the 3-rd degree. It is formed by the transsudation of blood plasma within the blood-vesicle barrier and the secretory activity of granulosa cells and follicular thecas (Gosden et al. 1988, Fu et al. 2016, Guerrieiro et al. 2018). Its quantity and composition changes during the development of the follicle. As the follicular fluid creates the environment in which the oocyte and granular cells develop, the substances contained in it may affect the quality of the ova (Gosden et al. 1988, Matoba et al. 2014). The composition of the follicular fluid is similar to the composition of the blood serum from venous vessels (Godsen et al. 1988). However, there are some differences regarding the amount and proportion of the individual components. Significant differences between the fluid originating from dominant and secondary follicles have been demonstrated (Renaville et al. 2008, Bender et al. 2010). Follicular fluid is rich in a variety of substances such as proteins, cytokines, steroid hormones (estrogen, progesterone, pregnenolone, testosterone), protein hormones (FSH, LH), sodium, calcium, potassium, magnesium, fatty acids, growth factors, peptides, amino acids, carbohydrates, anticoagulants, enzymes, reactive oxygen species and many others (Gosden et al. 1988, Bender et al. 2010, Basuino et al. 2016, Fu et al. 2016, Guerrieiro et al. 2018). Such a large number of substances contained in the follicular fluid reflects its multitude of functions: maintaining meiosis blockade, protecting the oocyte against proteolysis and "throwing out" during ovulation, enhancing sperm attraction and motility, triggering the acrosomal reaction and buffering effect and protecting the oocyte from negative effect of the external environment (McNatty 1978, Gosden et al. 1988, Espey et al. 1994, Dell'Aquila et al. 1997, Rodriguez et al. 2001, Wang et al. 2001). Because of its properties it positively influences the cell proliferation, often added to the media used for oocyte maturation, fertilization and embryo production *in vitro* (Alia et al. 2004, Coelho Cruz et al. 2014, Sahteshkumar et al. 2017, Guierriero et al. 2018).

During ovulation, some part of the follicular fluid gets into the fallopian tube and becomes a part of the environment where the spermatozoa reside until the moment of fertilization (McNutt et al. 1991, Basuino et al. 2016). Therefore, it may affect some of the properties of male gametes, which determine their ability to fertilize. It has been demonstrated that follicular fluid has the potential to induce capacitation and acrosomal reaction, which was not found for the tubal fluid (this fluid did not cause acrosomal reaction - only increased and sustained gamete motility) (McNutt et al. 1991, McNutt et al. 1994). Most likely it is caused by the significantly higher content of bovine serum albumin and glycosaminoglycans in follicular fluid (Lui et al. 1977, Parrish et al. 1988) than in tubal fluid (McNutt et al. 1991). The negative effect of the follicular fluid on the stability of nuclear chromatin in spermatozoa, which is common in gametes after the acrosomal reaction has also been proven (Lymberopoulos et al. 2007).

In the majority of available publications concerning the influence of follicular fluid on spermatozoa, fresh semen was used (McNutt et al. 1991, McNutt et al. 1994), whereas currently fresh semen is very rarely used for artificial insemination. Therefore, the aim of the present study was to investigate the effect of follicular fluid on the survival, movement and morphological features of bull spermatozoa, subjected to the freezing and thawing process, originating from insemination straws used commercially.

## Materials and Methods

### Animals – fluid donors

Follicular fluid was collected from 6 cows, multiparous (5 Polish Holstein-Friesian and 1 Polish Red and White) and with oestrus signs, not intended for insemination at the moment of collection. The donors came from two dairy farms containing 80 and 375 lactating cows, respectively. The age of the animals ranged from 5 to 9 years, the weight from 650 to 750 kg and the milk yield from 4500 to 11,989 kg of milk per lactation. Cows on both farms were kept in a free-range housing system and fed with Total Mixed Ratio (TMR). Animals were milked twice and three times a day on the smaller and larger farm respectively. All cows were gynecologically examinated prior to the collection of follicular fluid using Easi Scan ultrasound (BCF) to exclude the presence of diseases, especially in the reproductive track. The fluid was collected only from healthy animals showing behavioral and clinical oestrus signs (mounting tolerance, swelling of the vulva, enlargement of the clitoris, vaginal discharge of clear mucus, uterine reactivity, lack of corpus luteum on the ovaries). The crucial factor was the presence of a palpable, dominant follicle, with a diameter of 16-24 mm.

### Process for collecting follicular fluid

Aspiration of follicular fluid was carried out using a self-designed set, consisting of a 42.7 cm long metal probe, 10 mm channel diameter, 6 mm outlet diameter, and a 10 ml syringe, 0.9 mm diameter needles, length

40 mm and a non-cut insemination cover. The probe was inserted into the vagina, up to the external opening of the uterine cervix. Through manipulation of the ovary via rectal palpation, the dominant follicle was placed next to the tip of the probe (the only separating element being the vaginal wall). The assistant then introduced the aspiration set into the probe until the follicle was pierced. At this point, the follicle content was aspirated by pulling back the plunger of the syringe. If blood or cervical mucus were aspirated, the sample was excluded from the study.

### Transport and processing of the follicular fluid

Based on literature data, a modified algorithm for the processing and storage of follicular fluid was created (McNutt et al. 1991, McNutt et al. 1994, Alia et al. 2004, Fu et al. 2016, Guerreiro et al. 2018). The collected fluid was placed in a chilled box (4°C) and immediately transported to the laboratory. In every case, the time from the fluid collection to the delivery to the laboratory did not exceed 2 hours. After transport, the fluid was centrifuged at 10,000 x g for 12 minutes at 4°C to eliminate any contaminants. After centrifugation, the supernatant was collected and frozen at -25°C until the experiment started.

### Evaluation of the influence of follicular fluid on bull sperm after thawing

Assessment of the influence of follicular fluid on the survival, morphological and motor properties of spermatozoa was carried out using the semen from 5 bulls of proven fertility, being in a possession of a commercial animal reproductive center (Mazowieckie Centrum Hodowli i Rozrodu Zwierząt). At the first stage of the experiment, the follicular fluid was thawed in a 37°C water bath for 5 minutes to adjust temperatures. Physiological saline (0.9% NaCl) stored in an incubator at 37°C and the follicular fluid (pooled from 4 different cows) were then placed in open tubes in an incubator with an atmosphere containing 5% $CO_2$ at 38.5°C for 30 minutes, to equilibrate both liquids. At the final stage of this process, 12 commercial insemination straws were thawed in a water bath at 37°C for 30 seconds. The semen was then pooled in a 5 ml tube and kept in a water bath used for thawing. 1 ml of the semen was then mixed with 2 ml of the follicular fluid (Experimental group – EG) or 0,9% NaCl (Control Group – CG) in separated tubes. Both tubes were incubated in the atmosphere containing 5% $CO_2$ at 38.5°C for 8 hours. From hour 0 (15 minutes after mixing) to the end of the study, the semen was assessed using the CASA system. The evaluation by flow cytometry was carried out at the beginning and the end of the study (0 and 8th hour).

### CASA (Computer-Assisted Sperm Analysis) system evaluation

Sperm evaluation with the CASA system (computer assisted sperm analysis) was performed using the HTM IVOS ver. 12.2 (Hamilton Thorne Biosciences, Beverly, MA, USA) operating on Animal Motility software (Hamilton Thorne Biosciences, Beverly, MA, USA). Standard settings for semen analysis were used (Niżański et al. 2006, Domosławska et al. 2013, Gotowiecka 2018). The following parameters were evaluated during each assessment: VAP (Average Path Velocity) - mean sperm speed after the approximate movement path (μm/s); VSL (Straight Line Velocity) - average speed of sperms along a straight line determined between the initial and final position of the gamete (μm/s); VCL (Track Speed Velocity) - average sperm speed after the actual path of movement (μm/s); ALH (Amplitude of Lateral Head Displacement) - amplitude of lateral deviations of the head (μm); BCF (Beat Cross Frequency) - frequency of lateral deviations of the head (Hz); STR (Straightness) - sperm motility expressed in VSL/VAP ratio in%; LIN (Linearity) - linearity of the sperm movement expressed in VSL/VCL ratio in %; percentage of motile sperm (MOT, %), percentage of spermatozoa with progressive movement (PMOT, %) (Niżański 2006). Depending on the speed of movement of sperm following populations were classified: (Gotowiecka 2018): rapid movement sperm (RAPID) VAP> 100 μm/s, slow (SLOW) VAP <9 μm/s or VSL <20 μm/s and no showing movement (STATIC).

### Flow cytometry

The FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) was used to assess sperm cells membrane continuity and integrity of the acrosomal membrane. Calculations and analyses were made using CellQuest ver. 3.3 (Becton Dickinson). All non-sperm cellular elements and non-cellular particles were selected and rejected during gating on dot plots obtained (Partyka et al. 2010, Gotowiecka, 2018). Evaluation of the percentage of sperm cells with damaged or normal cell membranes was performed using SYBR-14 fluorescent dyes and propidium iodide (PI) (Live/Dead® Sperm Viability Kit, Invitrogen ™, Eugene, OR, USA). Assessment of the percentage of living and dead sperm with damaged or normal acrosomal membrane was done with lectin PNA isolated from Arachis hypogaea conjugated with Alexa Fluor 488 (InvitrogenTM, Eugene, OR, USA) and propidium iodide (InvitrogenTM, Eugene, OR, USA).

Table 1a. Movement parameters assessed by CASA system of sperm cells during incubation in the follicular (FF) and physiological (NaCl) fluid (part 2).

| Movement parameter / Medium\ hour of incubation | MOT (%) | PMOT (%) | RAPID (%) | SLOW (%) | STATIC (%) |
|---|---|---|---|---|---|
| **FF 0 h** | **29.2** ± 10.38[a1] | **26.4** ± 9.15[a2] | **28** ± 10.12[a3] | **6.6** ± 3,71 | **64** ± 13.73[a4] |
| **FF 2 h** | **32** ± 7.21 | **29.8** ± 6.91 | **31** ± 7.18 | **5** ± 4,74 | **62.8** ± 11.12 |
| **FF 4 h** | **25.8** ± 8.61 | **22.2** ± 8.90 | **23.2** ± 8.90 | **2.8** ± 0,84 | **71.4** ± 9.42 |
| **FF 6 h** | **22** ± 5.79 | **12.8** ± 9.04 | **13.6** ± 8.29 | **6.4** ± 2,70 | **71.6** ± 3.91 |
| **FF 8 h** | **7.2** ± 7.33[b1] | **1.8** ± 2.39[b2] | **1.8** ± 2.39[b3] | **8.8** ± 3,56 | **84.2** ± 11.05[b4] |
| **NaCl 0 h** | **31.8** ± 8.87[c1] | **28.8** ± 8.64[c2] | **29.8** ± 9.31[c3] | **6** ± 1.87 | **62** ± 8.75[c4] |
| **NaCl 2 h** | **29.6** ± 5.86 | **26.4** ± 5.73 | **27.2** ± 5.76 | **6** ± 3.39 | **64.2** ± 6.06 |
| **NaCl 4 h** | **18.4** ± 4.72 | **12.4** ± 5.94 | **13** ± 5.92 | **10** ± 6.75 | **71.8** ± 7.95 |
| **NaCl 6 h** | **9.8** ± 5.63 | **3.4** ± 2.88 | **3.6** ± 3.29 | **13.8** ± 7.33 | **76.6** ± 9.94 |
| **NaCl 8 h** | **1.6** ± 1.52[d1] | **0.4** ± 0.55[d2] | **0.6** ± 0.55[d3] | **7.8** ± 5.72 | **90.6** ± 6.73[d4] |

Statistically important differences were found for following comparisons: $a^{1-4}$ vs $b^{1-4}$ ($p<0.05$); $c^{1-4}$ vs $d^{1-4}$ ($p<0.05$)

Table 1b. Movement parameters assessed by CASA system of sperm cells during incubation in the follicular (FF) and physiological (NaCl) fluid (part 1).

| Movement parameter / Medium/ hour of incubation | VAP (µm/s) | VSL (µm/s) | VCL (µm/s) | ALH (µm/s) | BCF (Hz) | STR (%) | LIN (%) |
|---|---|---|---|---|---|---|---|
| **FF 0 h** | **124.2** ± 13.38[a1] | **115.1** ± 11.62[a2] | **175.4** ± 20.53[a3] | **5.98** ± 0.98[a4] | **32.34** ± 5.45[a5] | **92** ± 1.41[a6] | **67.6** ± 1.14[a7] |
| **FF 2 h** | **116.46** ± 13.12 | **108.18** ± 11.61 | **166.1** ± 21.03 | **5.8** ± 0.74 | **27.74** ± 6.47 | **92.6** ± 1.14 | **68** ± 4.06 |
| **FF 4h** | **85.56** ± 17.80 | **78.38** ± 18.12 | **121.76** ± 22.12 | **4.82** ± 0.57 | **25.18** ± 3.48 | **90.6** ± 2.70 | **65.6** ± 5.03 |
| **FF 6 h** | **58.96** ± 12.75 | **51.3** ± 16.09 | **86.44** ± 11.07 | **3.72** ± 0.35 | **21.96** ± 4.45 | **85.6** ± 8.08 | **60** ± 11.31 |
| **FF 8 h** | **45.54** ± 2.24[b1] | **38.2** ± 4.36[b2] | **76.24** ± 5.61[b3] | **3.54** ± 1.44[b4] | **20.34** ± 6.79[b5] | **81.8** ± 5.89[b6] | **50.6** ± 5.27[b7] |
| **NaCl 0 h** | **135.86** ± 11.26[c1] | **124.64** ± 11.11[c2] | **212.48** ± 22.56[c3] | **7.64** ± 1.13[c4] | **33.02** ± 4.72[c5] | **90.8** ± 2.68 | **59.2** ± 3.35 |
| **NaCl 2 h** | **114.82** ± 19.71 | **105.64** ± 17.85 | **170** ± 34.53 | **6.3** ± 1.32 | **27.44** ± 1.96 | **91** ± 2.00 | **63** ± 3.94 |
| **NaCl 4 h** | **71** ± 16.19 | **64.6** ± 16.89 | **105.1** ± 17.55 | **4.4** ± 0.29 | **26.98** ± 6.27 | **88.8** ± 3.49 | **61** ± 6.00 |
| **NaCl 6 h** | **47.34** ± 4.77 | **39.68** ± 5.22 | **75.62** ± 12.18 | **4.42** ± 1.36 | **20.16** ± 8.56 | **82** ± 7.00 | **53.2** ± 8.35 |
| **NaCl 8 h** | **51.8** ± 7.43[d1] | **43.06** ± 8.52[d2] | **82.3** ± 18.07[d3] | **3.36** ± 2.11[d4] | **13.28** ± 6.15[d5] | **81.6** ± 8.02 | **55.8** ± 6.06 |

Statistically important differences were found for following comparisons: $a^{1-7}$ vs $b^{1-7}$ ($p<0.05$); $c^{1-5}$ vs $d^{1-5}$ ($p<0.05$); $a^{3-4,7}$ vs $c^{3-4,7}$ ($p<0.05$)

## Statistical analysis

The analysis was performed using the PQStat software for Windows 1.6.2 by PQStat Software. Only the results obtained at the beginning (0 h) and at the end of semen incubation (8 h) were evaluated due to small differences at individual stages of the study (tests were conducted every 2 hours). Student's t-test for dependent trials was used to assess the differences between the start of the experiment (0 h) and after its end (8 h) for groups incubated in the follicular fluid (PP) and physiological fluid (NaCl) respectively. To assess the differences between the percentage of sperm at the same time of the experiment (separately for 0 h and 8 h) for PP and NaCl, Student's t-test for independent samples was used.

The study was carried out after obtaining the approval of the II Local Ethics Committee of18/03/2015.

Table 2. Plasma membranes integrity of sperm cells during incubation in the follicular (FF) and physiological (NaCl) fluid.

| Spermatozoa (%) / Medium/ hour of incubation | Dead (%) | Dying (%) | Live (%) |
|---|---|---|---|
| FF 0 h | **52.50** ± 9.10 | **15.32** ± 2.61[a] | **32.18** ± 10.34 |
| FF 8 h | **52.36** ± 6.59 | **19.80** ± 2.30[b] | **27.84** ± 7.52 |
| NaCl 0 h | **47.59** ± 8.82[c] | **14.51** ± 0.81 | **37.90** ± 9.55[e] |
| NaCl 8 h | **60.40** ± 7.96[d] | **14.50** ± 2.41[g] | **25.10** ± 6.69[f] |

Statistically important differences were found for following comparisons: a vs b (p=0.005); c vs d (p=0.049); e vs f (p=0.027) and b vs g (p=0.007)

Table 3. Acrosomal membranes integrity of sperm cells during incubation in the follicular (FF) and physiological (NaCl) fluid.

| Spermatozoa (%) / Medium/ hour of incubation | Dead intact (%) | Dead damaged (%) | Live intact (%) | Live damaged (%) |
|---|---|---|---|---|
| FF 0 h | **23.80** ± 2.80[a] | **40.36** ± 9.71[c] | **31.36** ± 10.14 | **4.48** ± 1.99[k] |
| FF 8 h | **15.02** ± 5.06[b] | **48.61** ± 9.33[d] | **27.76** ± 6.67 | **8.60** ± 2.80[i] |
| NaCl 0 h | **27.69** ± 4.44[m] | **26.88** ± 7.76[g] | **40.86** ± 5.75[e] | **4.57** ± 2.15[l] |
| NaCl 8 h | **38.92** ± 8.01[c] | **26.46** ± 6.96[h] | **29.95** ± 4.23[f] | **4.68** ± 1.84[j] |

Statistically important differences were found for following comparisons: a vs b (p=0.020); c vs d (p=0.011); e vs f (p=0.031), c vs g (p=0.041); b vs c (p<0.001); d vs h (p=0.003); i vs j (p=0.031); c+k vs d+i (p=0.002); e+l vs f+j (p=0.030); m+g vs c+h (p=0.030); d+i vs h+j (p=0.001).

## Results

The results obtained during sperm motility assessment at the beginning and end of the incubation period showed a significant decrease in the parameters characterizing the movement of gametes (MOT, PMOT, RAPID, STATIC), both in the follicular and physiological fluid (Table 1a). During the evaluation of the sperm movement properties, a significant reduction in all parameters examined in the semen incubated in the follicular fluid was found (VAP, VSL, VCL, ALH, BCF, STR, LIN) (Table 1b). However, in the physiological fluid, a similar trend was demonstrated only for the following properties: VAP, VSL, VCL, ALH, BCF (Table 1b). Comparing the results of all the above-mentioned parameters obtained during incubation in both fluids, a significant decrease was observed for: VCL (p=0.026), ALH (p=0.038) and increase for LIN (p<0.001) at the beginning of the incubation (Table 1b). For the other properties, no differences were found at both the 0 or 8$^{th}$ hour of incubations. The results of the plasma membrane integrity assessment showed a statistically significant increase in the percentage of dying sperm at the 8th hour of the study during incubation of sperm in the follicular fluid (Table 2). However, no differences was found in the range of dead and viable cells. For the semen incubated in the physiological fluid, a significant decrease in the percentage of viable sperm and increase of dead ones was noted (Table 2). When comparing the average percentage of particular groups of spermatozoa in both fluids at the following readings during the experiment, a higher proportion of dying gametes in the semen incubated in the follicular fluid at the 8th hour of the experiment was noted (Table 2). Analysis of the integrity of the acrosomal membrane and the survival of sperm in the flow cytometer allowed 4 subpopulations of cells to be obtained: live with intact acrosome, live with damaged acrosome, dead with intact acrosome and dead with damaged acrosome. Comparison of the percentages of individual subpopulations in the semen incubated in the follicular fluid showed significant differences within the population of the damaged and undamaged dead sperm (Table 3). The group of dead cells with disturbed integrity of the acrosomal membrane increased by almost the same percentage points, as the percentage point by which the group of dead gametes decreased (8.25% vs. 8.78%). In the case of semen incubation in physiological fluid, a statistically significant decrease in the percentage of live non-damaged cells was found (a decrease of 10.91%, p=0.031) in a simultaneous increase in the subpopulation of undamaged dead cells (an increase of 11.23%, no statistical significance) (Table 3). When comparing the effects of both fluids, a statistically significant difference was found for the subpopulation of dead cells already damaged at the

beginning of the study. The sum of damaged gametes did not differ between the two fluids at this stage of incubation (Table 3).

## Discussion

The influence of follicular fluid on the morphological and motility parameters of bull spermatozoa is extremely important in determinating whether such an atypical environment can have a negative effect on the spermatozoa. Research conducted by McNutt et al. (1991) showed that the addition of follicular fluid positively influenced the process of capacitation and hyperactivation in fresh semen. It also caused a decrease in the percentage of motile spermatozoa, a decrease in motility parameters: track speed velocity (VCL) and amplitude of lateral head displacement (ALH) and an increase in the linearity of the sperm movement expressed in the VSL/VCL ratio (LIN) (McNutt et al. 1991). However, this research was carried out using fresh semen, which nowadays is rarely used for the artificial insemination in cattle. In addition, spermatozoa morphology and motility in fresh semen are much better than in frozen/thawed semen (Gotowiecka 2018).

Assessment of motility parameters using the CASA system

The results obtained during the comparison of the sperm motility at the beginning and end of the incubation period showed a significant decrease in parameters characterizing the movement of gametes (MOT, PMOT, RAPID, STATIC), both in the follicular and physiological fluid (Table 1a). During the evaluation of the sperm movement properties, a significant decrease was observed within all the tested parameters in the semen incubated in the follicular fluid (VAP, VSL, VCL, ALH, BCF, STR, LIN), whereas in the physiological fluid, a similar trend was demonstrated only for the following properties: VAP, VSL, VCL, ALH, BCF (Table 1b). The decrease in sperm motility parameters during the incubation process in various media has been demonstrated by many authors (McNutt et al. 1991, McNutt et al. 1994, Yao et al. 2000). Most probably it is due to depletion of energy reserves and its insufficient supplementation in the fluids used during the test. It should be noted that the influence of the follicular fluid on sperm motility may depend on the species. In human the addition of follicular fluid to semen samples showed a positive effect (in comparison to the control group) on the gamete's motility parameters (Yao and al. 2000, Jeon et al. 2010). However, similar experiments using bull sperm and follicular fluid, returned very variable results (McNutt et al. 1991, McNutt et al. 1994), showing a relationship between motion parameters, and the concentration of follicular fluid. During our study the comparison of influence of both fluids on motile parameters obtained from the CASA system did not show statistically significant differences in parameters: MOT, PMOT, RAPID, SLOW and STATIC, both at the beginning and at the end of incubation in both fluids (Table 1a). However, it should be noted that at the 8$^{th}$ hour of incubation, better results were obtained for the follicular than the physiological fluid (i.e., MOT 7.2% vs 1.6%, PMOT 1.8%. Moreover, a significant difference was found for both fluids in: VCL ($p=0.026$), ALH ($p=0.038$) and LIN ($p<0.001$) at the beginning of incubation, whereas no difference was found at both the 0 and 8 hour incubation for the rest of the investigated parameters. These results correspond with the those obtained by McNutt et al. (1994) during the 4-hour incubation of fresh semen in follicular fluid. It seems that a high concentration of follicular fluid leads to acceleration of the capacitation process, because all the described changes in the VCL, ALH and LIN parameters are typical for the progression of capacitation in bull sperm (McNutt et al. 1994). In addition, it can be concluded that higher concentrations of the follicular fluid accelerates this process. Similarly to McNutt et al. (1994), hyperactivation of gametes has not been demonstrated (decrease of VCL, ALH and increase of LIN) (Kathiravan et al. 2011), which may have a beneficial effect on their survival and fertilization ability, because of slower energy usage (Gotowiecka 2018). This hypothesis is supported by a study of McNutt et al. (1991), where the higher fertilization rate of oocytes was obtained for sperm incubated for 4 hours with 60% addition of follicular fluid in comparison to the control group.

### Evaluation of sperm cell membrane continuity (SYBR-14 / PI)

During the incubation of sperm in the follicular fluid a significant increase of dying spermatozoa at the 8$^{th}$ hour was found. However, although no difference was found between the compared groups of dead and living cells (Table 2). Whereas in semen incubated in the physiological fluid, a significant decrease in the percentage of live and an increase in dead spermatozoa was noted (Table 2). These results suggest the ability of the follicular fluid to maintain sperm viability. Comparing the results obtained during the SYBR test at the subsequent study stages, a significantly higher proportion of dying gametes in the semen incubated in the follicular fluid at the 8th hour of the experiment was demonstrated. However, a clear tendency was observed for a smaller decrease in the percentage of living sperm stored in the follicular fluid and a greater increase in the percentage of dead spermatozoa remaining in the physiological fluid. Interestingly, no increase in the percent-

age of dead spermatozoa in the semen incubated in the follicular fluid was noted, in contrast to the physiological fluid (an increase of 12.81%) (Table 2).

The majority of authors focused on the analysis of gamete motility parameters, the possibilities to increase migration and the induction of capacitation and acrosomal reaction (McNutt et al. 1991, McNutt et al. 1994, Yao et al. 2000, Jeon et al. 2010, Mondal et al. 2017). Only McNutt et al. (1991) analyzed the viability of sperms, focusing on acrosomal reaction, and obtained significantly higher percentages of live gametes by incubation with follicular fluid. Up to date, there is no information in the available literature about the viability assessment of spermatozoa incubated in follicular fluid, which makes the presented results novel and original.

### Assessment of integrity of the acrosomal membrane

A comparison of the percentages of individual cell populations in the semen incubated in the follicular fluid showed differences for the spermatozoa with damaged and undamaged acrosome (Table 3). The group of dead cells with impaired integrity of the acrosomal membrane increased by almost the same percentage points, by which the group of dead gametes decreased (8.25% vs. 8.78%). A similar tendency was observed in the population of live cells (3.6% - a decrease for live intact, and a 4.12% increase in live damaged, with, however, no statistical significance) (Table 3). This indicates the possibility of acrosomal membrane damage by the follicular fluid in both live and dead cells. It is also important to notice that the difference between the total population of damaged cells at the beginning and at the end of the incubation was statistically significant ($p=0.002$). This confirms the ability of the follicular fluid to interfere with the integrity of the acrosomal membrane. In the case of an 8-hour incubation of semen in physiological saline, a statistically significant decrease in the percentage of live non-damaged cells was found (a decrease of 10.91%, $p=0.031$) in favor of the undamaged dead cell population (an increase of 11.23%, no statistical significance) (Table 3). This indicates a stronger negative effect of the physiological fluid than the follicular fluid on sperm survival. However, no significant increase was observed in the number of damaged cell population, which seems to indicate the absence of a negative influence of physiological fluid on the integrity of the acrosomal membrane. The comparison of the effect of both fluids indicates a significantly higher percentage of damaged dead cells in the semen incubated in the follicular fluid in relation to the control group at the beginning of the study. The population of damaged gametes (live+dead) did not differ between the two fluids at this stage of incubation (Table 3). Research conducted by McNutt et al. (1991, 1994) and Yao et al. (2000) indicated rapid changes caused by the addition of follicular fluid. It should also be noted that the interval time between mixing the semen with the appropriate medium and assessment in the flow cytometer could induce negative changes at the beginning of the incubation period. It can be assumed that it was a sufficient period to induce changes affecting an increased percentage of dead cells with a damaged acrosome membrane at the beginning of incubation in the follicular fluid. Of course, such differences may be due to some imperfections in the laboratory procedures used. This issue requires further research.

Analysis of the final incubation stage showed a significantly higher percentage of cells damaged in follicular fluid than in the control group. An important difference was also found between the percentages of cell populations with damaged acrosome integrity. Interestingly, a significantly higher percentage of the dead undamaged cell population in the physiological fluid was demonstrated. No difference in the percentage of dead cells in the semen incubated in the follicular fluid was demonstrated. Similarly, an increase in the percentage of dead gametes in the 8th hour of storage in the physiological fluid was found (Table 3). These results support the earlier conclusions concerning the ability of follicular fluid to prolong sperm survival compared to the control group. They also correspond to the results of other authors (McNutt et al. 1991, McNutt et al. 1994, Yao et al. 2000, Lymberopulos et al. 2007), who noted a significant effect of the follicular fluid on the changes occurring within the acrosome.

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