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METHOD FOR UTILIZATION OF SOLUBILIZED ZONAE PELLUCIDAE IN SPERM ZONA-ADHESION TEST

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Abstract: Glycoproteins of zona pellucida are natural ligands for human spermatozoa and they play crucial role in sperm maturation during fertilization process in vivo. The utilization of zona pellucida ligands on polystyrene surfaces can be used for developing of method for physiological separation of human spermatozoa, based on their zona pellucida adhesion abilities.In this article we propose a simple and effective procedure for utilization of native zona pellucida glycoproteins in the development of sperm-zona adhesion test. The most important checkpoints were to choose the appropriate concentration of HCl for zona solubilisation and to mark down the most convenient conditions for zona glycoproteins immobilization. We report the optimal concentration of the zona ligands, immobilization buffer, support material, adsorption method, incubation temperature and shape of the adhesion area.

This study was conducted with 180 zonae pellucidae from 36 healthy donors. Zonae pellucidae were solubilised in five concentrations of HCl (2.5 mM, 5 mM, 10 mM and 20 mM) and were used in two-fold serial dilutions in three buffers: carbonate buffer (CB), phosphate buffer (PBS) and Tris buffer (TBS). The zona glycoproteins were physically adsorbed in solution or allowed to dry on glass and polystyrene at 4ºC, 25ºC and 37ºC. In addition, two types of dried adhesion area shapes were examined: dot and line.The rate of sperm adhesion was assessed by placing 0.5x106/ml motile spermatozoa from 46 fertile men on the immobilized zona proteins and counting the attached spermatozoa. Sperm-zona adhesion score was calculated as number of attached spermatozoa per mm2. The results from the conducted experiments showed that optimal sperm adhesion is reached by solubilisation of the zonae by 10 mM HCl followed by dilution into CB in concentration 50 µg zonae proteins/ml and immobilizing the solution by air drying 0.1 µl as dots on polystyrene plates at 25ºC.Here we describe a useful method for utilization of zona pellucida glycoproteins that may be applicable both in developing new diagnostic tests for human sperm quality assessment and designing functional sperm selection procedure for IVF in the future.

INTRODUCTION

During the natural process of fertilization the spermatozoa undergo series of consecutive modifications in response to soluble and/or membrane attached molecules from the different sections of female reproductive system. Only functionally matured spermatozoa are able to penetrate the cumulus complex and zona pellucida before meeting the oocyte (Gadella, 2012).

Zona pellucida appears as one of the major selection barriers in front of immature or defective spermatozoa (Kim *et al.,* 2008). It has been demonstrated that the sperm-zona binding potential is related to their fertilisation ability during IVF (Liu *et al.,* 1989; Oehninger *et al.,* 1989). Some authors claim that very common cause of fertilisation failure in couples with unexplained infertility is the defective sperm-zona binding (Liu *et al.,* 2000; Liu *et al.,* 2004; Aitken, 2006).

Impaired sperm-zona interaction is mainly caused by defects in sperm structure and function (Liu *et al.,* 2000). Disturbance in spermatogenesis causes decreased membrane fluidity, followed by disordered assembly of the membrane multimeric zona recognition complex (Reid *et al.,* 2011). Also, high levels of H_2O_2 (Oehninger *et al.,* 1995), genetic mutations in men ZPBP1 gene (Yatsenko *et al.,* 2012) and many other lead to sperm's inability to recognize and adhere to zona pellucida.

Impaired sperm zona-binding cannot be identified by the routine semen analysis. The parameters of the common spermiogram - concentration, motility and morphology (defined by non-strict criteria) are not sufficient indicators of the spermatozoa functional abilities (Liu *et al.,* 1992; Avarez *et al.,* 2003; Cayli *et al.,* 2003; Eliasson, 2010). In this regard additional sperm function tests have been developed including: the assessment of calcium ionophore-induced acrosome reaction rate (Katsuki *et al.,* 2005), the hyaluronan binding assay (HBA) (Ye *et al.,* 2006), the sperm penetration assay (SPA) (Hwang *et al.,* 2013), the zona-induced acrosome reaction (ZIAR) (Franken *et al.,* 2000), magnetic activated cell sorting (MACS) (Gil *et al.,* 2013) etc. The sperm functional tests have been proven as useful in predicting the fertilization ability of the spermatozoa (Pregl *et al.,* 2013).

Different approaches can be applied to assess the sperm functional qualities. For example, HBA is used to evaluate the sperm ability to adhere to hyaluronan, as main composition of the cumulus matrix. The simplified technique involves optimal immobilization of the ligand on flat glass surface allowing easy counting of the adhered spermatozoa. The defined test conditions provide no variations in the results and thus HBA can be applied in routine practice for functional evaluation of spermatozoa according to their hyaluronan binding abilities (Szucs *et al.,* 2015).

Different approach has been applied in the sperm zona-binding studies where whole or half zona with native structure are used as adhesion surface

(Kizilay *et al.,* 2017). It has been proven that sperm-zona binding assays show high predictive value for the outcomes of IVF (Liu *et al.,* 1988; Oehninger *et al.,* 2000). Many studies agreed on high predictive value of the hemizona assay (HZA) for sperm fertilization ability (Gamzu *et al.,* 1994) and the pregnancy outcomes (Arslan *et al.,* 2006). Notwithstanding the high prognostic ability of HZA, the test experiences some practical drawbacks (Franken *et al.,* 1991). Therefore optimization of the sperm zona-binding assessment is required before introducing it into routine practice (Yao *et al.,* 1996).

The aim of this study is to describe a sperm-zona adhesion (SZA) test that evaluates the sperm functional quality towards the zona pellucida. The optimal conditions for the utilization of native zonae pellucidae ligands in the SZA test are reported. The practical qualities of the described SZA test are also described. The evaluated SZA score was compared to the common semen parameters to determine whether SZA test can be used as an independent indicator of sperm functional quality.

MATERIALS AND METHODS

Semen samples

Semen samples were obtained from regular semen analysis (spermiogram) of 46 patients of Women's Health Hospital "Nadezhda"- Sofia after signing written and informed declaration. The participants were classified as normozoospermic or teratozoospermic according to WHO 5th edition.

The fresh ejaculate was received after 2-4 days of sexual abstinence. The samples were allowed to liquefy at 37°C for 30 minutes before they were assessed for physical characteristics – volume and pH. The sperm count, concentration and the motility analysis were performed by using computer assisted sperm analysis (MedeaLAB-CASA software, ver. 5.3). The sperm morphology was determined manually by non-strict criteria.

Zona pellucida isolation

This study was conducted with 180 zonae pellucidae from immature oocytes at germinal vesicle (GV) stage. They were obtained from follicular puncture of 36 healthy donors during IVF procedures in Women's Health Hospital "Nadezhda"- Sofia, between Jan.2018 – Mar. 2018. Written and informed declaration was signed from the participants.

Follicular aspirates were denuded from granulosa cells and placed in plastic vials containing Global for fertilisation medium (LIfeGlobal Group, USA). The intact zonae pellucidae were removed from the oocytes mechanically using a micromanipulator Integra TI (Cooper Surgical, USA).

Zona pellucida preparation

Sixteen of the zonae were used for determination of the solubilisation procedure. Each zona was placed in 1 µl drop of hydrochloric acid (HCl) (2.5

mM HCl, 5 mM HCl, 10 mM HCl or 20 mM HCl). The experiment included 4 repetitions of every HCl concentration variant. The degree of each zona solubilisation for 70 sec has been recorded.

The other 164 zonae were used in the determination of the immobilization conditions. They were acid solubilized in 164 µl of 10 mM HCl separately. The solutions were then combined and neutralized with NaOH to pH 7.

Bradford protein quantification

The protein concentration in the achieved zona solution was determined by Bradford method and measured against human serum albumin (HSA) standard curve at concentrations of 0.01 - 1 mg/ml. Five µl of the sample and the standards were added to 250 µl of Coomassie Brilliant Blue G (Sigma; USA). After 30 minutes incubation at room temperature, the absorbance at 595 nm was measured using Multimode Detector DTX 880 (Beckman Coulter; USA) (Ernst *et al.,* 2010). The zona pellucida protein concentration was presented as µg/ml.

Immobilisation conditions

Two-fold serial dilutions of the solubilized zonae were prepared using carbonate buffer pH 9.6 (CB; Merck, USA), phosphate buffered pH 7.4 (PBS; Merck, USA) and Tris buffered saline pH 8.0 (TBS; Merck, USA). The serial dilutions were air dried at room temperature on polystyrene plates (Corning, USA).

The optimal concentration of the zona-ligands for the sperm zona-adhesion test was determined by the quantity and the distribution of the adhered spermatozoa to the immobilized zona proteins in the different concentrations on a polystyrene surface. The following criteria were used to choose the most suitable coating buffer: (1) the buffer should not alter the binding activity of the dissolved ligands; (2) the immobilized proteins should not dissolve in aqueous solution; (3) the immobilized proteins should not dissolve after sperm adhesion.

The optimal support material, adsorption method, immobilization temperature and shape of the immobilized area were assessed in parallel tests. Zona-ligands were used in 50 µg/ml concentration diluted with CB pH 9.6. The solution was immobilized on two support materials: microscope glass (Merck; USA) and 96-well microtitration polystyrene plates (Corning, USA). 50 µl of the zona-ligands solution were incubated on both support materials in non-vaporizing conditions (with cover) in three repetitions for incubation at 37ºC, 25ºC or 4ºC for 1h. 0.1 µl of the zona-ligands solution was placed either as a drop or a line on both support materials in three repetitions for air drying at 37ºC, 25ºC or 4ºC.

Evaluation of sperm-zona adhesion score

The zona pellucida glycoproteins immobilized areas were calculated using Olympus WHB 10x/20mm eyepiece (Spectra Services, Inc).

The optimal immobilization conditions were determined by comparison of the distribution and the mean number of attached spermatozoa in three repetitions of each semen sample. The fresh semen samples were centrifuged at 600 g for 5 min

in conical plastic tubes. The supernatant was discarded and the pellet was overlaid with 1 ml Global for fertilisation (LIfeGlobal Group, USA) supplemented with 1 mg/ml human serum albumin (HSA; LifeGlobal Group, USA). The tubes were placed inclined at 45º degree for 30 minutes at 37ºC. After incubation 200 µl of the supernatant were pulled and the motile spermatozoa were counted.

Motile spermatozoa were diluted to 0.5x106/ml and 50 µl were placed on the immobilized zona-ligands followed by incubation at 37ºC. After 30 minutes the attached spermatozoa were counted by two separate biologists and the mean result was calculated as the number of attached spermatozoa in the immobilized area [mm^2]. The results are presented as mean ± SD attached spermatozoa per mm^2 [sp/mm^2].

Statistical analysis

The values from the conducted experiments are presented as mean value ± SD. The relation between the common semen parameters and the sperm-zona adhesion score was analyzed by Pearson correlation. Differences in the common semen parameters between the defined groups were investigated using Student's t-test. Statistical analysis was carried out using IBM SPSS Statistics v.21. $p<0.05$ was considered significant.

RESULTS AND DISCUSSION

Preparation of zona pellucida ligands

The solubilization of zona pellucida was compared between variable concentrations of hydrochloric acid: 2.5 mM HCl, 5 mM HCl, 10 mM HCl and 20 mM HCl. The results are presented in Table 1.

Table 1: Comparison of the zona pellucida solubilization degree for 70 sec by various HCl concentrations and sperm adhesion observed on the immobilized zona solutions.

[c] HCl	Solubilization of 1 ZP for 70"	Sperm adhesion observed
2.5 mM	Partial	No
5 mM	Partial	No
10 mM	Full	Yes
20 mM	Full	No

Complete zona pellucida solubilization by low concentrations of HCl (2.5 mM and 5 mM) was achieved for more than 4 hours of exposure (data not shown). All zonae pellucidae placed in 10 mM HCl and 20 mM HCl were fully dissolved in 70 sec (Table 1).

The dissolved zona solutions were immobilized on polystyrene surface using carbonate buffer and the sperm adhesion was observed. No sperm adhesion was observed on the immobilized 2.5 mM HCl and 5 mM HCl solubilized zonae solutions. Although 20 mM HCl dissolves zona pellucida a little faster than 10 mM HCl (data not shown), there was no sperm adhesion on the immobilized 20 mM HCl solubilized zona solution (Table 1).

Rapid solubilization allows direct monitoring of the process and immediate neutralization of the solution so as to avoid further denaturing of the glycoproteins. Despite the immediate neutralization of the solutions, sperm binding was not observed on 20 mM HCl solubilized zonae. High concentration of HCl may alter the sperm-binding residues of the zona pellucida glycoproteins thus affecting the sperm zona-recognition. Zonae were fully dissolved for 70 sec by 10 mM HCl and they preserved their sperm-binding ability (Table 1).

Various approaches to dissolve zona pellucida are described in the literature (Mintz, 1962; Lee *et al.,* 1992; Bastiaan *et al.,* 1999; Franken *et al.,* 2000; Manna *et al.,* 2001; Kolbe *et al.,* 2005; Yamatoya *et al.,* 2011). Successful enzymatic solubilization by trypsin has been applied on murine zonae (Mintz, 1962), but our study group observed no alteration of the human zona three-dimensional structure (data not shown).

It has been shown that acidic solubilization of zonae by Ham's F10 medium pH 2 (Lee *et al.,* 1992) and 10 mM HCl (Bastiaan *et al.,* 1999; Franken *et al.,* 2000) preserved the ability of the zona-ligands to induce an acrosomal response in human spermatozoa. In our study we were looking to obtain a solution of zona pellucida glycoproteins without any additives found in the commercial cell mediums, so we focused on the hydrochloric acid solubilization.

In our experimental setting, we decided to use 10 mM HCl for solubilization of the zonae, because it dissolved the zonae completely for 70 sec and preserved the zona ligands sperm-binding abilities (Table 1). Ligands from 164 zonae pellucidae, solubilized by 10 mM HCl were used in the subsequent experiments.

Immobilization conditions

Six condition variables were considered for optimization in the immobilization procedure: (1) concentration of the zona proteins (two-fold serial dilutions starting at 200 μg/ml); (2) coating buffer (PBS, TBS or CB); (3) immobilization method (adsorption in solution or by air drying); (4) support material (polystyrene or glass); (5) immobilization temperature (4ºC, 37ºC or 25ºC) and (6) shape of the adhesion area (dot or line).

Determination of the ligand concentration

164 zonae were acid solubilized by 10 mM HCl and the solution was neutralized by adding NaOH to pH 7. The protein content in the achieved solution was determined by Bradford method. It has been estimated that the protein concentration in the solution is 325 μg/ml.

The optimal zona-ligand concentration was determined by the quantity of the adhered spermatozoa on the immobilized zona proteins from the serial dilutions. No sperm adhesion was observed below ligand concentration of 10 µg/ml. It is possible that at low concentrations glycoproteins are found over long distances and the required ligand density for sperm adhesion cannot be achieved (**Fig. 1**).

Although the adhesion ligands are denser at the concentration of 200 µg/ml, 53% less adhesion was observed in comparison to 50 µg/ml (**Fig. 1**). The higher density of the zona proteins could interfere with the spatial ligand organization and could obstruct the sperm-ligand recognition (Dwevedi, 2016).

To validate the results that the adhesion of the spermatozoa is specific to the zona proteins, the test was repeated using human serum albumin (HSA). No sperm adhesion was observed on the immobilized HSA at any concentration, also no sperm adhesion was observed on the air dried coating buffer alone (**Fig. 1**).

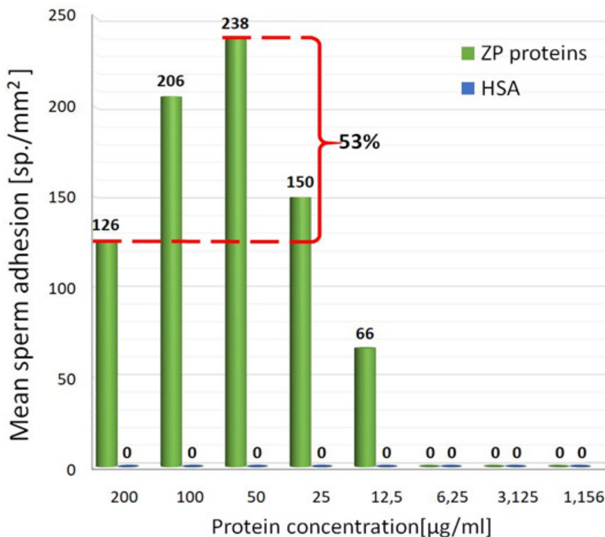

Fig. 1: Mean sperm adhesion per mm2 on two-fold serial dilutions of zona pellucida proteins in range of 1,156 µg/ml to 200 µg/ml.

The distribution of the attached spermatozoa on the adhesion area was also compared between the serial dilutions of zonae ligands. Homogenously adhered spermatozoa were observed on the immobilized protein concentration of 50 µg/ml (**Fig. 2**).

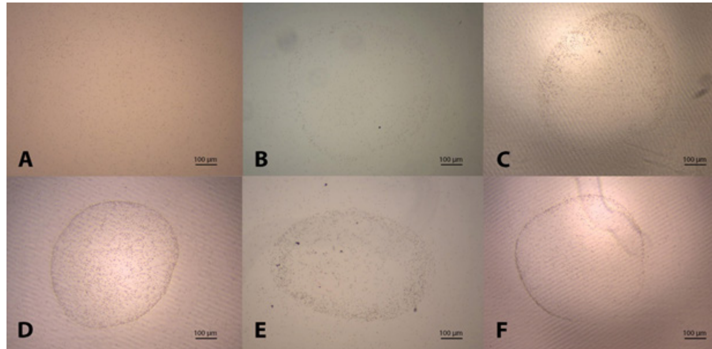

Fig. 2. Sperm adhesion on: **(A)** 6.25 µg/ml ligand; **(B)** 12.5 µg/ml ligand; **(C)** 25 µg/ml; **(D)** 50 µg/ml; **(E)** 100 µg/ml and **(F)** 200 µg/ml.

In this study we decided to describe the zona-ligand quantity as protein concentration in ml whereas other authors present the zona-ligand concentration as number of zonae pellucidae per 1 µl diluent (ZP/ µl). Liu *et al.* (2000) used 2.5 ZP/ µl of acid solubilized zonae for induction of acrosome reaction in 30% of $100x10^3$ spermatozoa in solution. In our experiments the optimal ligand concentration of 50 µg/ml refers to 0.025 ZP/ µl, much less than the used for acrosome reaction induction. The optimal ligand concentration for SZA test is established at 50 µg/ml zona ligands (0.025 ZP/µl).

Optimization of the coating buffer

The optimal immobilization buffer was determined by its effect on the ligands binding activity and the adsorption of the zona proteins to the support material. Results for the stability of the immobilization with carbonate buffer (CB), phosphate buffered saline (PBS) and Tris buffered saline (TBS), regarding polystyrene support material are presented in Table 2.

Table 2 Decision table for immobilization buffer between carbonate buffer (CB), phosphate buffered saline (PBS) and Tris buffered saline (TBS)

Criteria:	CB	PBS	TBS
Does not alter the activity of the dissolved ligands	+	+	-
Ensures stable immobilization in liquid phase	+	-	+
Ensures stable immobilization after sperm adhesion	+	-	-

PBS is a non-toxic, physiological pH cell buffer which is applied in cell culture. It is used for washing and screening cell cultures but is not a suitable buffer for immobilization of zona pellucida ligands. PBS did not retain the ligands on the surface upon application of the spermatozoa and their aqueous medium. Low degree of sperm adhesion was observed and a gradual dissolution of the immobilized proteins (**Fig. 3**).

TBS is used in biochemical tests to dilute proteins, but is also not suitable for immobilizing zona pellucida ligands in our experimental setting. The attached spermatozoa pull the immobilized layer until it peeled, thus preventing the evaluation of the adhered spermatozoa (**Fig. 3**).

CB is widely used in other assays for protein immobilization like the immunosorbent assays. By using CB for immobilization of the zona ligands stable immobilization was achieved and the counting of the adhered spermatozoa in the following experiments was facilitated (**Fig. 3**). The optimal immobilization buffer for SZA test was the carbonate buffer.

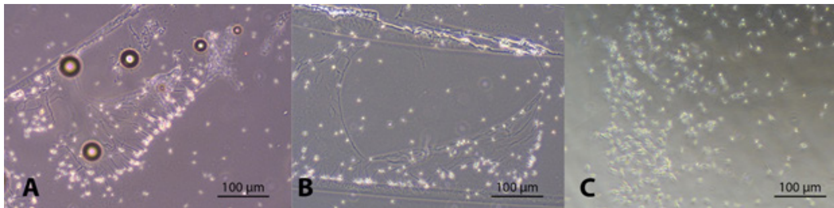

Fig. 3. Sperm adhesion to zona pellucida ligands, diluted in:
(A) PBS; **(B)** TBS and **(C)** CB.

Choice of the immobilization method

The quantity of the attached spermatozoa to 50 µg/ml zona-ligands was considered in determination of the immobilization method. Two types of physical immobilization were compared: ligand adsorption in solution and ligands adsorbed by air drying. Results are presented in Fig. 4.

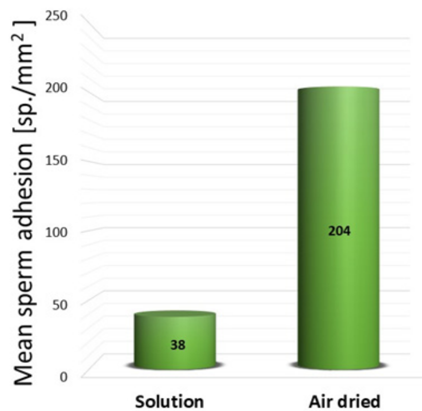

Fig. 4 Mean sperm adhesion per mm2 on zona-ligands immobilized in solution and air-dried.

The average sperm adhesion on the immobilized by air drying zona-ligands was significantly higher (204 sp/mm² ± 5), whereas weak sperm adhesion was observed on ligands immobilized in solution (38 sperm/mm² ± 5) (Fig. 4). The higher number of adhered spermatozoa on the air dried ligands indicates for better ligand immobilization than the immobilization in solution. Similarly the dry adsorption method is applied in other sperm binding tests such as the spermatozoa–hyaluronan binding assay (Esterhuizen *et al.,* 2015). In our experimental setting the optimal zona-ligand immobilization method for the SZA was the dry adsorption.

Choice of the support material

The zona-ligands were immobilized on glass and on polystyrene support materials and the mean quantity of the adhered spermatozoa in 1 mm² was compared. The results are presented in Fig. 5.

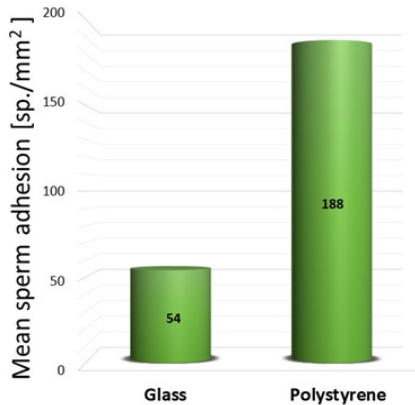

Fig. 5 Mean sperm adhesion per mm² on glass and polystyrene support material.

The average sperm attachment on the glass surface was 54 sp/mm² ± 5, whereas on the polystyrene plate was 188 sp/mm² ± 5 (**Fig. 5**). The higher number of adhered spermatozoa on the polystyrene plate indicates for better ligand immobilization.

The visual appearance of the adsorbed ligands also differed between the two support materials. The glass immobilization resulted in unevenly scattered crystals of the ligands, whereas continuous layer is observed on the polystyrene plate (**Fig. 6**).

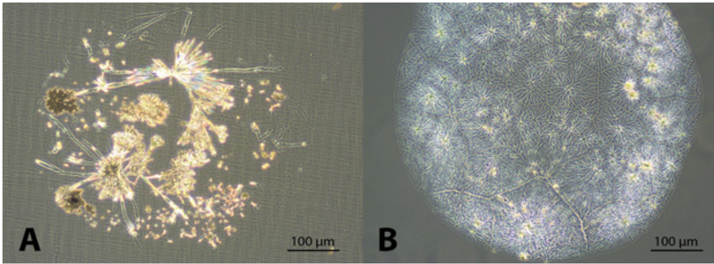

Fig. 6 Zona ligands immobilization on: **(A)** glass and **(B)** polystyrene.

The adsorption of the ligands occurred passively through hydrophobic interactions between the amino acid side chains and the support material, which are influenced by the dilution buffer, the time and the temperature of the immobilization conditions (Datta *et al.,* 2013). In our study the ligand adsorption on glass was not sufficient for the optimal protein immobilization required for the following test. The adhered zona-ligands should be equally distributed in the test area in order to calculate the precise density of adhered spermatozoa. The polystyrene provided even distribution of the ligands and it was selected as optimal support material in the immobilization of zona pellucida proteins for SZA.

Determination of the immobilization temperature

Protein adsorption at three incubation temperatures (4°C, 25°C and 37°) was compared. Results, referring to the incubation of air-dry zona-ligands on polystyrene are discussed. When immobilization was performed at 4°C and 37°C, the spermatozoa adhered to the ligands, but the immobilized protein layers were unstable and they detached in the aqueous medium. Immobilization at 25°C resulted in stable protein layer during and after sperm adhesion (**Fig. 7**). The optimal immobilization temperature of the proteins depends on the type of the support material and on the interactions between the protein and the surface of the support material (Datta *et al.,* 2013). Immobilization at 4°C and 37° also resulted in very weak sperm adhesion to the ligands, whether immobilized in solution on polystyrene or on glass surface. Therefore the zona-ligands were air-dried on polystyrene plates at 25°C for the SZA test.

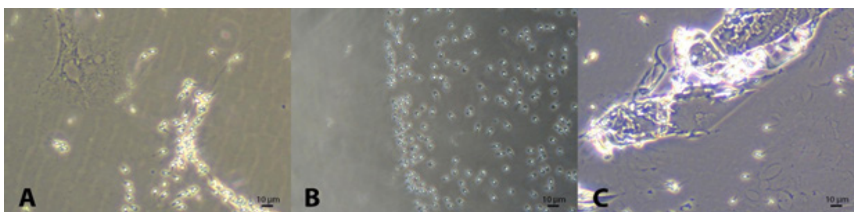

Fig. 7 Sperm adhesion on zona pellucida ligands immobilized at **(A)** 4°C; **(B)** 25°C and **(C)** 37°C.

Determination of the adhesion area shape

The following immobilization area shapes were tested: whole surface (as the adsorption was carried in solution), dot and line (when air drying immobilization was applied). The distribution of the adhered spermatozoa and the convenience in calculating their number were considered when determining of the immobilization area shape.

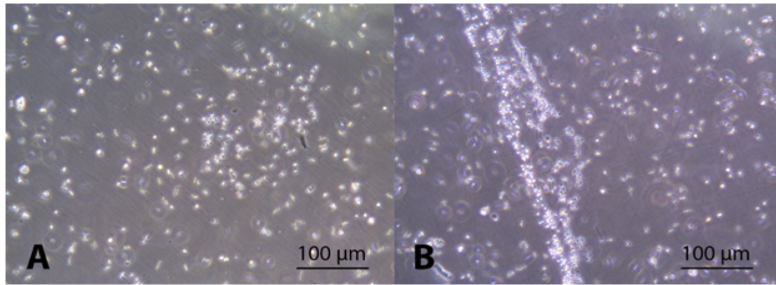

Fig. 8 Sperm adhesion on zona pellucida ligands immobilized: **(A)** on the whole well surface; **(B)** as a line.

Zona pellucida ligand immobilization on the entire area of the bottom of a 50 µl well resulted in sperm adhesion at random areas. Clusters of attached spermatozoa were observed consequently the sperm adhesion density calculation would be controversial (**Fig. 8**).Immobilization of 0.1 µl zona-ligands solution in lines resulted in variable lengths and widths of the adhesion area. Also the exact area cannot be calculated due to the dried irregular shape of the immobilized area (**Fig. 8**). When the zona-ligands were adsorbed as 0.1µl drop the ligand layer dried as a circle (**Fig. 9**). The diameter of the circle can be measured using eyepiece micrometer and the area of the immobilization circle in mm^2 can be calculated. The sperm adhesion density calculated by the number of adhered spermatozoa per 1 mm^2 (sp/mm^2) was established as SZA score (**Fig. 9**).

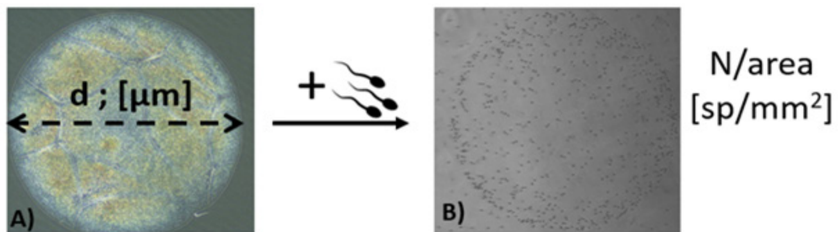

Fig. 9 SZA score evaluation: **(A)** the diameter of the immobilized area was measured; **(B)** the attached spermatozoa were counted and the sperm zona-adhesion score were calculated.

General overview of the method

The results from this study were combined in utilization method of zona-proteins obtained from solubilized native zonae. The following conditions were found most suitable for the direct assessment of sperm zona-adhesion ability:

1. Solubilisation of the intact zonae by 10 mM HCl and neutralization by NaOH to pH 7.
2. Protein quantification of the dissolved zonae by Bradford method.
3. Dilution of the obtained zona-ligand solution to 50µg/ml with CB (pH 9.6).
4. Position 0.1 µl of the diluted zona-ligand solution as drop on polystyrene plate.
5. Incubation at 25°C air until the drop is fully dried.
6. Measuring the diameter of the circle and calculating its area in mm^2.
7. Incubation of 0.5x106/ml motile spermatozoa on the immobilized area at 37°C for 30 minutes.
8. Counting the attached spermatozoa and calculation of the adhered spermatozoa per 1 mm^2.

Slightly different approach in assessing the sperm binding abilities is applied in the commercially available HBA (Ye *et al.,* 2006). In HBA the sperm adhesion ligand is chemically modified hyaluronan, whereas native zonae are used in SZA. The hyaluronan in HBA is also evenly coated in circular area. Yet the size of the immobilized area and the support material differ. Nevertheless the approach for assessment of the sperm functionality is similar and is depicted as reliable in many studies (Huszar *et al.,* 2003; Roudebush et al., 2004; Szucs *et al.,* 2015).

Sperm zona-adhesion score and conventional semen parameters

The sperm zona-adhesion (SZA) score of 46 patients was evaluated by the protocol described above. Their parameters from the common semen analysis were compared in relation to their SZA score. The mean SZA score in the studied group was also evaluated and it was estimated at 155 ± 99 sp/mm^2.

As expected no correlation was recorded between the semen parameters of the common spermiogram and the SZA score. Other sperm functional tests also failed to relate the common semen parameters with the spermatozoa functional abilities (Francavilla *et al.,* 1994; Nijs *et al.,* 2010; Esterhuizen *et al.,* 2015). It is known that the routine semen analysis does not provide information on the sperm functionality (Guzick *et al.,* 2001) and cannot predict their fertilizing ability (Jeulin et al., 1988; Mercan et al., 1998). Although some authors report that zona-bound spermatozoa express good morphology (Bastiaan *et al.,* 2003) our result show no relation between the SZA score and the morphology parameter from the common semen analysis.

To further examine the relation of the SZA score and the common sperm characteristics, the semen parameters between two groups were compared. The patients were divided according to their individual SZA score in regard to the estimated mean sperm zona-adhesion score (155 ± 99 sp/mm^2). The group with SZA score <155 included 29 patients and the SZA score >155 group - 17 (Table 3).

Table 3 Common spermiogram parameters (Mean ± SD) and significance of their differences of the patients in SZA score <155 group and the SZA score > 155 group.

Sperm characteristic	SZA Score < 155, n = 29	SZA Score > 155, n= 17	Sig.
Days of abstinence	3 ± 1	3 ± 1	0,58
Volume, ml	2.8 ± 1.38	3.4 ± 1.5	0,17
pH	8 ± 0.29	8 ± 0.33	0,58
Concentration, $x10^6$/ml	127 ± 63.65	98.5 ± 28.8	0,89
Total count, $x10^6$	334.47 ± 193.2	330.8 ± 182.8	0,95
Rapidly progressive (A), %	28 ± 6.2	30 ± 7	0,29
Slowly progressive (B), %	27.1 ± 7.46	26.7 ± 7.19	0,84
Motile spermatozoa (A+B), %	55.1 ± 10.8	56.8 ± 9.27	0,59
Morphology (non-strict criteria), %	12.1 ± 3.36	10.59 ± 3.3	0,97
SZA Score [sp/mm^2]	93 ± 35	263 ± 80	0.001*

The mean SZA score differed significantly (P = 0.001) although both groups (SZA score <155 and SZA Score >155) displayed similar sperm parameters. Also, the conducted t-test showed no significant differences between the two groups (Table 3). The same result has been achieved when the sperm zona-binding ability was evaluated by the hemizona assay. The comparison between the common sperm parameters of patients with HZI >30 and HZI <30 showed no relation to sperm zona-binding ability (Mercan, 1998).

Therefore in these experimental conditions, the SZA score may be considered as a sperm functional quality indicator independent of the conventional semen analysis parameters – concentration, motility and morphology.

Significance of the sperm zona-adhesion (SZA) test

The established sperm zona-adhesion (SZA) test holds many practical qualities. In comparison to other zona-binding tests - hemizona assay (HZA), zona-binding assay (ZBA) it can be introduced as routine assessment of sperm functionality after further examination of its reliability for sperm fertilization potential.

The described protocol for utilization of solubilized zonae pellucidae in sperm zona-adhesion test includes 8 consecutive steps and do not require sterile conditions. The materials used in the method are wide available and cost-effective. One polystyrene plate of 96 wells is enough for 32 semen samples in 3 repetitions, whereas in HZA a single zona is used in only one test with no repetitions (Burkman *et al.,* 1988; Franken *et al.,* 1991). The routine HZA assessment would require daily supply of zonae which imposes practical difficulties that are avoided in SZA. With the obtained zona-ligands solution many tests could be performed, because small volume of (0.1 µl) in low concentration (50 µg/ml = 0.025 ZP/ml) is used.

The immobilization of the zona-ligands for the SZA test require approximately 4-5 hours. Apart from the Bradford protein quantification, the procedure for utilization of the zona-ligands includes incubation for approximate 1 hour in the zona-solution air-drying step. Once the zona-ligands are immobilized the SZA score can be evaluated in 1-2 hours after the semen is received.

The SZA test plates can be stored until 2 month at room temperature at dry conditions. In contrast in HZA fresh or thawed zonae are used. The different storage conditions and the freeze-thawing procedures affect the sperm-binding affinity of the zona-ligands and would result in variable results (Yao *et al.,* 1996).

Small amount of the whole semen sample is used for the evaluation of the SZA score by the described method. The rest of the sperm probe can be subjected to other tests or to be prepared for ART procedure. SZA test is also suitable for oligozoospermic patients because only $25x10^3$ motile spermatozoa are needed for performing the SZA test.

Sperm-binding variation between the individual zonae was avoided by using a mixture of dissolved zonae pellucidae from different women. Sperm adhesion to intact or half zona is affected by the zona diameter (Burkman *et al.,* 1988), surface appearance (Familiari *et al.,* 1988) and the thickness of the zona matrix (Franken *et al.,* 1991). Therefore the results of sperm single-zona binding tests would vary and the accurate evaluation of the zona-adhesion is restricted. In hemizona assay (HZA), samples from fertile donors with normal semen parameters are used as internal controls for each test (Oehninger *et al.,* 1989). However there is variability in the donor semen quality that confuses the in vitro assay interpretations (Franken *et al.,* 1991). Hence, other sperm functional tests imply uniform sperm ligands. For instance, HBA scores the sperm ability to bind to chemically modified hyaluronan so that the results for single semen sample are repeatable and independent of donor semen varieties (Roudebush *et al.,* 2004).

The SZA score assessment avoids varieties in the individual semen sample. The sperm zona-adhesion ability is calculated in defined conditions with no need of fertile control samples. Furthermore the set conditions allow comparing SZA score between every subject tested whereas the other zona-binding tests have controversy in comparing their results due to their evaluation on variable control semen samples (Franken *et al.,* 1991).

Other sperm binding tests deliver their results as percentage of bound to the total spermatozoa (HBA) or as index by comparing the number of the bound spermatozoa between the subject and a donor semen (HZI). The results from the described SZA test are presented as adhered spermatozoa per mm2 [sp/mm2] avoiding possible lowering the SZA score if there are more spermatozoa with zona-binding potential than number of immobilized ligands.

Flat surface was used in the described SZA test and the counting of the attached spermatozoa was eased. Ligand immobilization on flat surface has been also applied in HBA and it has proven to be efficient (Huszar *et al.,* 2003;

Roudebush *et al.,* 2004; Szucs *et al.,* 2015). As well as the eased counting of the adhered spermatozoa, the flat surface ligand immobilization allows single spermatozoa separation of the test surface using a micromanipulator.

This approach has been applied in methods for functional sperm selection for ICSI. Most of them are based on the sperm ability to bind immobilized hyaluronan molecules (Parmegiani *et al.,* 2009; Worrilow *et al.,* 2009; Witt *et al.,* 2016). Similarly, the utilization of zona-ligands for SZA test may be applied in the functional sperm selection according to their zona-adhesion abilities. In studies using intact zonae or hemizona the efficacy of the zona selection has been demonstrated. They show that using zona-bound spermatozoa for ICSI results in high quality embryos (Braga *et al.,* 2009) and high pregnancy rates (Jin *et al.,* 2016). The described SZA test provides sperm zona-adhesion ability assessment that could be useful in the assisted reproduction practice as additional semen analysis parameter or as method for functional sperm selection.

CONCLUSIONS

In this study the optimal conditions for utilization of solubilized zonae pellucidae in sperm-zona adhesion test were established. The described SZA test is applicable in the diagnostic assessment of sperm function. This approach allows physical separation of sperm according to their ability for recognition and adhesion to zona pellucida ligands could be used for development of spermatozoa functional selection methods in ART.

AUTHOR CONTRIBUTIONS

T.C. and R.G. conceived the experiment; R.G., M.G., I.I., I.R., M.V. and K.N. conducted the experiment; D.P., T.C. and R.G. analyzed the results. D.P. and R.G. wrote the main manuscript text and prepared tables and figures. T.C. and G.S. edited the manuscript and made its final revision. All authors critically reviewed and approved the final version of the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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