The interrelationship between quality parameters of sperm before and after separation by gradient centrifugation

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ABSTRACT: Significance of a short-time survival test for the prediction of quality parameters of sperm obtained by gradient centrifugation was evaluated from an aspect of their functional resistance. Frozen semen samples from 27 bulls before and after separation were assessed by a 120-min survival test. The evaluation of morphological examination of ejaculates was carried out using the SASMO computer program. Statistically significant interrelationship between sperm quality parameters in the initial insemination doses, the amounts of obtained sperm after separation and their functional and vital resistance in the survival test was documented.

Keywords: bull; sperm; qualitative separation

Male gametes of high quality are necessary to increase the outcomes of assisted reproduction technology. Sperm separation methods based on the quality parameters utilise different movement characteristics and sperm plasma membrane integrity.

Bangham and Hancock (1955) used the effect of separation by sperm filtration through fine glass beads to obtain ejaculates free of dead and abnormal sperm. The filtration process through sephadex columns was tested by various authors (Graham et al., 1976; Landa et al., 1980; Graham and Graham, 1990; Samper et al., 1991; Veznik and Svecova, 1992; Anzar and Graham, 1995). Further procedures were developed with the purpose to yield ejaculate fractions with high proportions of functionally competent sperm. They are applied above all in the process of sperm preparation for assisted reproduction where the swim up method is commonly used; that results in obtaining a higher proportion of motile and intact sperm and is supplemented with the Percoll gradient method. The gradient centrifugation technique that uses Percoll or another matrix is an alternative to semen filtration and is one of the assisted reproduction methods used above all in human medicine. Parrish et al. (1995) applied these protocols for the in vitro fertilisation with bull semen. Somfai et al. (2002) reviewed the results obtained with the swim up and Percoll gradient methods by various authors and compared them. Majority of authors preferred the use of Percoll gradient centrifugation. Brandeis and Manuel (1993) reported that the swim up methods selected preferably motile sperm, while a higher percentage of sperm with intact acrosomes was obtained by Percoll. Parrish et al. (1995) and Somfai et al. (2002) focused on these methods with the aim to prepare bull sperm for *in vitro* fertilisation. The purpose of the present study to evaluate quality parameters concerning cell functions of spermatozoa before and after separation.

MATERIAL AND METHODS

Frozen semen samples from 27 bulls of Czech Pied breed aged two years with fertility level of 52.3–67.3% tested by 56-day non-return rate were

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investigated in the present study. All bulls originated from one AI station.

Qualitative assessment of semen after thawing of the pellets was based on the evaluations during the short-time survival test.

Methods. Besides conventional examination, the ejaculates were tested at initial time (IH) and in the 120-min survival test (2H):

sperm motility was determined by microscopy at 200 to 400× magnification

percentages of live spermatozoa were evaluated by differential counts after supravital staining with primulin

speed of sperm movement was assessed by the propulsivity test according to Baker et al., modified by Hynie (1960)

mitochondrial activity was manifested after the reaction with nitrotetrazolium blue and expressed by an index according to Veznik (1970)

sperm morphology was evaluated by the SASMO programme based on a multiparametric diagnostic method (Veznik et al., 2001)

the response to hypoosmotic conditions was tested by HOS test according to Jeyendran et al. (1984)

Pisum sativum lectin binding to acrosomal membrane was assessed as described by Sirivaidyapong et al. (2000)

Thawed pellets were subjected to the separation process through gradient media so as to obtain functionally and morphologically high quality sperm. Percoll concentrations for bull semen were 90% and 45%. The following components according to Parrish et al. (1995) and Somfai et al. (2002) were used for preparation of 90% Percoll: 10 ml 10× concentrated Sp-TALP medium, 90 ml concentrated Percoll, 29 mg $CaCl_2$, 8 mg $MgCl_2(6H_2O)$, 109 mg $NaHCO_3$.

Isotonic solution of 45 % Percoll was obtained by dilution of 25 ml of 90% Percoll and 25 ml of Sp-TALP medium.

Sperm quality index (SQIG – sperm quality index of groups according to Veznik et al., 2006) calculated by a summation of scores obtained for respective characteristics of ejaculates from individual animals, was used for the assessment of relationships between semen quality parameters and sperm counts in fractions obtained by separation. The following parameters were compared:

sperm motility

percentage of live sperm

index of mitochondrial activity

percentage of abnormal sperm

Twenty-seven ejaculates and sperm fractions recovered from Percoll separation were analysed by dynamic tests; thawed pellets were subjected to the 120-min survival test, and in the sperm fractions, the initial values and values after 3 and 6 h incubations were determined.

Besides an analysis of the investigated group as a whole, the samples were assigned into two groups on the basis of the recovery of sperm fractions rela-

Table 1. Sperm analysis after thawing of pellets from 27 bulls with an average concentration of 45 970 spermatozoa/mm^3. Mean values of evaluated criteria after thawing of pellets and in the 120-min survival test

	Motile sperm (%)	Live sperm (%)	Speed of sperm movement (μm/s)	ER index	Abnormal sperm (%)	it	Primary defects (%)	Secondary defects (%)	Acrosome integrity			
									A	B	C	D
Initial value												
Mean	57.4	64.8	65.6	85.7	34.6	1.1	9.6	23.9	91.1	83.2	7.9	8.9
SD	7.5	6.5	7.9	21.3	8.3	0.04	4.2	6.2	2.7	4.6	3.5	2.7
Min	40	47.25	44.9	39	21	1.02	3.5	14.5	81	71.5	3	5
Max	67.5	75.25	84.26	131	47	1.18	18	39	95	90	17	18.5
In the 120-min survival test												
Mean	41.02	58.7	52.6	73.9	44.9	1.13	10.3	33.6	87.3	74.7	12.6	12.7
SD	11.1	8.3	15.3	24.6	8.7	0.05	4.1	6.2	2.4	6.5	5.6	2.5
Min	12.5	38.25	24.6	36	28.5	1.03	4	24	79.5	58.5	3.5	9
Max	57.5	70	69.2	124	61.5	1.23	18.5	46.5	91	85	27.5	20

A = *Pisum sativum* bound sperm; B = strong binding; C = weak binding; D = no binding; it = teratospermia index

tive to the sperm count in the original pellet with the aim to highlight differences in quality of sperm from respective ejaculates. Fractions reaching values (1) less than 19% and (2) more than 35% were assigned to the two groups, respectively. Of the 27 analysed animals, nine and six individuals were included in these two groups, respectively.

Statistical analyses were performed with the use of Stat-Plus programme (Matouskova et al., 1992).

RESULTS

Sperm analysis of pellets from 27 AI bulls gave evidence of a good quality of insemination doses. The detected initial values generally ranged within conventional limits (Table 1). Except three bulls, motility of sperm was maintained within these limits after the 120-min test, even though it decreased by 41.0% in comparison with the initial value. Sperm motility decreased below 30% in three bulls after

Table 2. List of ejaculates aligned according to the sperm quality index of groups (SQIG)

Register of bulls	Initial value					In the 120-min survival test					Mean SQIG	Succession of bulls
	motile sperm (%)	live sperm (%)	ER index	abnormal sperm (%)	SQIG	motile sperm (%)	live sperm (%)	ER index	abnormal sperm (%)	SQIG		
PR 562	57.5	71.75	104	24.5	**18**	50	70	113	33.5	**11**	**14.5**	1
REN 381	65	67.5	131	32.5	**22**	37.5	65	114	36.5	**23**	**22.5**	2
RST 96	57.5	70.5	110	33	**25**	50	66.25	102	42	**20**	**22.5**	3
SAS 28	65	70.25	103	30	**21**	42.5	67.25	79	42.5	**28**	**24.5**	4
RDA 184	65	69.75	87	45.5	**39**	57.5	66.75	124	46.5	**19**	**29**	5
ME 95	57.5	65.75	116	30	**27**	42.5	62.25	86	43	**31**	**29**	6
REN 385	57.5	66.75	87	22.5	**28**	42.5	65.5	43	28.5	**32**	**30**	7
PR 572	57.5	63.25	95	23.5	**32**	52.5	59.75	79	35.5	**28**	**30**	8
REN 384	65	69.25	83	21.6	**24**	45	59.25	57	31.5	**36**	**30**	9
MOR 10	67.5	69.75	79	21	**24**	50	56.25	47	31	**40**	**32**	10
FAN 174	65	75.25	70	35	**37**	47.5	70	64	45	**30**	**33.5**	11
LM 333	50	35	120	25.5	**29**	35	56.25	103	43	**39**	**34**	12
MOR 13	65	71.75	76	42	**39**	45	68	100	56	**31**	**35**	13
LM 319	60	69.5	80	34.5	**39**	40	56.5	81	42	**40**	**39.5**	14
REN 367	57.5	66	85	45.5	**48**	55	61.75	85	48	**32**	**40**	15
HB 259	65	65.25	75	27	**39**	35	57.5	72	37.5	**43**	**41**	16
POL 3	52.5	56.75	114	39.5	**46**	42.5	56.25	95	46	**41**	**43.5**	17
ULK 453	57.5	62.5	93	44.5	**49**	55	60	77	51.5	**38**	**43.5**	18
BJ 102	62.5	69.75	82	39	**37**	40	60	68	61.5	**52**	**44.5**	19
MKM 212	62.5	64.75	53	35	**53**	47.5	61.75	72	50.5	**40**	**46.5**	20
HB 273	60	64	81	45	**53**	42.5	58.75	56	52.5	**54**	**53.5**	21
MOR 157	45	47.25	92	31	**52**	32.5	45.75	56	45	**57**	**54.5**	22
HG 116	45	65.75	60	46	**65**	40	57.75	36	52	**59**	**62**	23
BJR 245	40	54.25	39	30.5	**65**	15	38.25	38	43	**64**	**64.5**	24
FAN 170	45	57.25	66	39.5	**65**	20	52.5	43	52	**65**	**65**	25
ZB 7	55	61	54	47	**69**	32.5	43.75	62	60	**66**	**67.5**	26
A-HG 64	47.5	51	79	43	**65**	12.5	40.75	43	57.5	**73**	**69**	27

2H; however, the percentage of live sperm did not fall below this limit. Membrane integrity, which was demonstrated by supravital staining for live and dead sperm, was declined by 9.7% in average versus initial value. Membrane integrity decreased less than by 8.3% was maintained in 62.9% ejaculates after 2H 37.1% of ejaculates showed a decrease in live sperm percentage by 11.8% to 29.5% (about 18% in average).

Morphological analysis of spermatozoa stained according to Hancock at the initial time point showed a total of 34.6% abnormal sperm; among these, 9.6% were developmental defects. After the survival test, the percentage of total defects increased to 44.9% due to an increase of acquired defects to 33.6% in average.

Mitochondrial activity evaluated by the ER index showed low values that varied between respective ejaculates (average index values: IH 85.7, 2H 73.9).

After the calculation of the sperm quality index (SQIG), the distribution of individual bulls was obtained and presented in Table 2.

Thawed pellet spermatozoa were separated using density gradient media to obtain sperm of good function and morphology. The results of quality assessment of sperm fractions are shown in Table 3.

An extremely high variability in percentages of sperm obtained after separation of thawed pellets was noted. The average sperm recovery (± SD) from thawed pellets after separation was 26.6 ± 16.9% of original sperm count. The linear regression of SQIG to sperm counts with the correlation coefficient –0.5753 was significant.

The decrease of percentages of live and motile sperm in thawed pellets during the survival test significantly correlated with the sperm recovery in the obtained fractions (correlation coefficients: –0.5198; –0.4545, respectively; $P = 0.01$).

Table 3. Sperm analysis of thawed pellets after separation by gradient centrifugation

	Motile sperm (%)	Live sperm (%)	Speed of sperm movement (μm/s)	ER index	Abnormal sperm (%)	it	Primary defects (%)	Secondary defects (%)	Acrosome integrity			
									A	B	C	D
Initial value												
Mean	79.7	81	74.2	209.8	26.6	1.05	4.1	21.4	92	82	10	8
SD	8.1	10	12.8	27.4	5.9	0.03	1.9	4.2	4	15	13	3
min	63.1	51	50.4	146	17	1	1.5	13.5	84	42	11	2
max	89.9	93	100.5	268	42	1.11	10.5	29.5	98	97	42	17
In the 120-min survival test												
Mean	74.9	77	74.6	209.4	33.8	1.03	4.2	28.2	90	68	21	10
SD	16.2	11	13.3	37.3	7.3	0.03	1.9	4.7	5	15	12	5
min	33.7	41	47	79	21.5	1	1	20	77	34	8	2
max	91.3	90	104.2	262	54	1.11	10	39.5	99	90	51	23
In the 180-min survival test												
Mean	73.9	75	74.3	191.1	41.2	1.05	4.2	34.1	86	62	24	14
SD	11.8	12	13.5	32.1	9.7	0.04	1.9	6.4	6	15	11	6
min	36.4	38	46.9	102.5	25	1	1.5	21	72	30	9	4
max	87.4	86	103.6	240	67.5	1.14	10.5	51	96	86	51	28

A = *Pisum sativum* bound sperm; B = strong binding; C = weak binding; D = no binding; it = teratospermia index

Table 4. Characteristics of separated spermatozoa after the 3 h (3H) and 6 h (6H) survival test

	Motile spermatozoa (%)		Speed of sperm movement		Live spermatozoa (%)		ER index		Abnormal spermatozoa (%)	
	3H	6H	3H	6H	3H	6H	3H	6H	3H	6H
Bulls with fractions reaching values higher than 35% (n = 6)										
Mean	82.1	79.8	72.6	74.1	83,0	80.2	223.2	206.3	31.2	37.6
SD	4.3	2.9	18.4	17.9	5.6	4.5	21.5	28.5	4.1	6.7
Bulls with fractions reaching values less than 19% (n = 9)										
Mean	71.9	70.8	69.01	68.8	73.5	72.1	212.3	187.1	35.7	44.9
SD	15.1	7.3	10.8	11.8	10.7	11.4	27.1	27.7	5.7	8.6

Categorization of samples according to sperm recovery as minimum and maximum values to the average 26.6%, documented significant differences in sperm motility, percentages of live and abnormal sperm in these two categories.

A significant correlation between the results of the survival test and sperm recovery after separation was documented for thawed pellets in the present study (Spearman correlation coefficient 0.638). Comparison of the calculated SQIG values with sperm recovery after separation also showed a significant correlation; that documented positive correlation between ejaculate quality and sperm recovery after separation.

Table 4 shows a lower functional resistance of sperm in small versus large volumes. Even though the differences in the investigated criteria were non-significant, a tendency to a lower potential to survive was evident.

DISCUSSION

Analysis of 27 deep-frozen ejaculates from AI bulls with the use of methods tested by various authors and standard evaluation of sperm documented that the level of the initial parameters after thawing was generally good (Rasul et al., 2001; Januskauskas et al., 2003; Martinez et al., 2006).

The survival test indicated differences in qualities between respective ejaculates, and consistent findings by various authors showed its importance (Kozumplík and Sosnova, 1985 and other authors). A higher resistance of the structures and functions of organelles in contrast to fluid cytoplasmic surface membranes was documented by a significant increase of secondary structural changes in sperm during this test in contrast to the decrease of binding capacity of *Pisum sativum* to sperm acrosome, which was non-significant. Similar findings were obtained when dog ejaculates were examined (Veznik et al., 2003). Due to a limited time of the survival test (120 min), no marked individual differences in structural changes of acrosomes were found in sperm from thawed pellets; that was indicated by relatively low SD values.

Gradient centrifugation of ejaculates was used to obtain good-quality sperm fractions. Sperm motility in the obtained fractions reached almost 80% (79.7%) and likewise membrane integrity that gives evidence of live spermatozoa gave comparable results (81.0%). These findings are in accordance with Brandeis and Manuel (1993), Parrish et al. (1995) and Somfai et al. (2002). The index of mitochondrial activity showed marked alterations as it reached an average value of 209.8 ± 27.4 in contrast to initial values obtained by analysis of pellets after thawing. The conventional value of the index (180) was not obtained in two fractions only. The percentage of abnormal spermatozoa was in average 26.6 ± 5.9 %. Developmental defects were found in 4.1 ± 1.9% of sperm in average, while acquired defects were detected in 21.4 ± 4.2%.

The above mentioned differences of sperm counts in fractions obtained from respective ejaculates were associated with a quality of thawed pellets. Minimum and maximum recovery in fractions was 2.7% (in bull HG 64) and 73.9% (in bull MOR 10), respectively. The relationship between sperm quality parameters and the recovery after the separation process is evident from the values of quality parameters for thawed pellets from bulls HG 64 and MOR 10 as follows:

sperm motility – initial value (%) 47.5, 67.5; sperm motility after 120-min survival test 12.5, 50.0

live spermatozoa – initial value (%) 51.0, 69.7; live spermatozoa after 120-min survival test 40.7, 56.2
abnormal spermatozoa – initial value (%) 43.0, 21.0; abnormal spermatozoa after 120-min survival test 57.5, 31.0

Differences in quality were manifested particularly by lower percentages of live spermatozoa and a lower morphological resistance of sperm surface structures, confirmed by a higher increase in abnormal spermatozoa.

Average percentages of live sperm were 64.8%, 68.4% and 60.8% in all thawed pellets, in the group of ejaculates with sperm recovery of more than 35% and in the group with recovery less than 19%, respectively. The percentage of live sperm in fractions at the initial time point was 83.8% and 72.7% in groups with a higher and lower recovery, respectively. During the 6 h incubation, the percentage of live spermatozoa decreased to 80.2% in the group with a higher recovery and to 72.1% in the group with a lower recovery. A significant effect of separation on increased percentage of live spermatozoa was evident in both groups; however, the level of sperm survival was lower in the group with lower sperm counts. A decreased ability to survive was also confirmed by increased percentages of abnormal sperm. The average percentage of abnormal sperm at the beginning was 34.6%. The average percentages of abnormal sperm in the groups of ejaculates were 28.3% and 41.1% in group with a higher and lower recovery, respectively. The separation process resulted in a decrease of abnormal sperm to 24.6% and 28.7%, respectively. The percentage of abnormal sperm increased to 37.6% and 44.9% within 6 h, respectively. Increased percentages of abnormal sperm could result from increased numbers of sperm with disturbed membrane structures and hence lower morphological resistance.

A relatively low index of mitochondrial activity (average index values: IH 85.7, 2H 73.9) indicates that not all spermatozoa that showed motility and membrane integrity after thawing were fully functional. That was documented by comparison of sperm motility during the survival test and mitochondrial activity index (i. ER); the agreement of these parameters was documented by a significant regression function ($P = 0.05$) when interrelationships between motility and ER index were evaluated after 2H.

It follows that the examination of sperm function in ejaculates brings important information for the prediction of their ability to survive. This information is not only important for the assessment of sperm recovery after the separation based on their quality, but above all it can indicate their vital resistance. The levels of functional parameters of separated spermatozoa were always good, but persistence of their functions was different depending on the quality of respective ejaculate as a whole.

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