# Effects of long-term liquid commercial semen extender and storage time on the membrane quality of boar semen

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**ABSTRACT**: The objective of this study was to assess the sperm membrane integrity in extended boar semen during storage time using specific spectrum laboratory methods. Boar semen was diluted with the long-term liquid commercial extenders Androhep (A), Androstar (AS), Androstar plus ($AS^+$), LD and M III and was stored up to 96 h. The sperm membrane integrity was evaluated by motility, viable spermatozoa, short hypoosmotic swelling test (sHOST) and by the activity of the enzyme aspartate aminotransferase (AST). Negative changes in the quality of sperm membrane in relation to storage time were observed after 48 h for sHOST, after 72 h for viable spermatozoa and after 72 h for motility. The percentage of viable spermatozoa was decreased by 0.27% each hour. A statistically significant difference between extenders A and LD was observed in sHOST after 72 h and 96 h storage ($P < 0.05$). The AST activity did not show any statistically significant differences in extenders and in storage time. In overall assessment Androhep was the best of the tested extenders, followed by AS, $AS^+$, M III and LD in terms of motility, viable spermatozoa and sHOST. The correlations among laboratory methods were highly significant ($P < 0.001$). In conclusion, the results documented that the sperm membrane integrity was statistically significantly affected by extenders and storage time ($P < 0.001$). Boar semen quality was the best in extender A. sHOST is a very sensitive and relatively simple method for the assessment of sperm membrane integrity in diluted semen.

**Keywords**: boar semen; long-term liquid extender; storage time; membrane integrity

Liquid preservation is still a preferred method of boar semen storage (Weitze, 1991) when diluted semen is stored at 15–20°C for several days before it used for artificial insemination (Johnson et al., 2000). A possibility of using semen stored in liquid condition up to 5 days after collection would greatly improve both production efficiency and economic profit for the AI industry if the present farrowing rate and litter size are maintained (Waterhouse et al., 2004). However, the fertility of liquid semen is gradually lost during extended periods. Several studies have shown the influence of different extenders on longevity (Vyt et al., 2004) and boar sperm quality (Huo et al., 2002; Waterhouse et al., 2004; Ambrogi et al., 2006). We know that short-term extenders are widely used but long-term extenders are interesting because they must preserve not only the sperm cell viability but also the sperm motility for a required period. Therefore, an assessment of integrity of the sperm membrane of diluted semen is important for determining which extender to use and how long the semen can be stored. Only spermatozoa with intact plasma membranes and intact acrosome are able to fertilize an oocyte *in vivo* (Yanagimachi, 1994).

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The quality of insemination dose is one of the factors that participate in successful insemination. The routine assessment of boar semen quality includes the evaluation of sperm concentration, morphology, viability and motility in AI centres. In the present study besides traditional methods (sperm motility and percentage of viable spermatozoa) short hypoosmotic swelling test (sHOST) and the activity of the enzyme aspartate aminotransferase (AST) were applied for an assessment of the sperm membrane integrity. The short hypoosmotic swelling test (sHOST) is a modification of HOST (Pérez-Llano et al., 2001). This test is based on the semi-permeability of the intact cell membrane that allows the sperm to swell under hypoosmotic conditions. HOST enables to evaluate the functional status of the sperm membrane. Pérez-Llano et al. (2001) reported a significant correlation of sHOST with *in vivo* fertility and with farrowing rate. The activity of aspartate aminotransferase (AST) is another interesting marker used to indicate sperm damage. AST is an intracellular enzyme of spermatozoa; an increase in the level of this enzyme in seminal plasma is considered as a determinant of cellular damage (Larson et al., 1996). This enzyme is important for the metabolism and function of spermatozoa (Strzeżek et al., 1981).

The objective of this study was to assess the sperm membrane integrity in extended boar semen during storage time using specific spectrum laboratory methods.

## MATERIAL AND METHODS

Twenty-one ejaculates from 21 fertile hybrid AI boars aged 1 to 3 years were collected using the gloved-hand technique in autumn 2006 and 2007 in the Czech Republic. The gel portion was removed using double gauze. The following parameters were evaluated in fresh native boar semen: semen volume, sperm motility, sperm concentration, morphologically abnormal spermatozoa, percentage of viable spermatozoa, sHOST, activity of the enzyme aspartate aminotransferase (AST) and total number of spermatozoa per ejaculate. The sperm motility was assessed subjectively using phase contrast microscopy with a heating stage (38°C) at 100× magnification. The sperm concentration was determined by a cytometric method using Bürker's chamber. Morphologically abnormal spermatozoa were assessed according to the staining method of Čeřovský (1976) and evaluated microscopically under oil immersion and 1 500× magnification.

The boar semen was diluted according to Čeřovský (1982) in a semen dilution rate of 1 + 4 in Androhep (A), Androstar (AS), Androstar plus ($AS^+$), M III (Minitüb, Germany), LD (Magapor, Spain) and was stored at a temperature of 17°C up to 96 h. Sperm motility, percentage of viable spermatozoa, sHOST and AST activity were evaluated in 24 h, 48 h, 72 h and 96 h after semen dilution.

The percentage of viable spermatozoa was estimated by a supravital staining technique using the eosin-nigrosine stain mixture. One drop from each sample was mixed with 1 drop of 1% eosin Y and 2 drops of 10% nigrosine were added after 30 s. Two hundred spermatozoa per slide were evaluated under a light microscope (1 500×). Viable spermatozoa remain unstained – white, while dead cells are stained red.

The short hypoosmotic swelling test (sHOST) was assessed by the method according to Pérez-Llano et al. (2003) using the eosin-nigrosine staining technique. Sperms are incubated in a 75 mOsm hypoosmotic solution for five minutes. At least 200 spermatozoa were evaluated per slide and the results of sHOST were included in two categories: sHOST positive and sHOST negative. sHOST positive was defined in this study.

The activity of the enzyme aspartate aminotransferase (AST) was measured with a BIOLATEST kit (Lachema, Brno, CR) and with an ENCORE spectrophotometer. A 2 ml sample of native or diluted semen was centrifuged at 1 700 rpm for 10 min and the supernatant was used to assess the AST activity.

Basic statistical characteristics of the results arithmetic means, standard deviations, correlation coefficients and significance ($P$) were calculated by the QC Expert program. Statistical significance was checked by the analysis of variance ANOVA – Scheffe test at significance levels of $P < 0.05$, $P < 0.01$, $P < 0.001$.

## RESULTS

The initial quality of semen used in this study was as follows (mean ± SD): sperm motility 70.24 ± 8.79%; semen volume 281.33 ± 119.39 ml; sperm concentration 331.24 ± 154.71 × $10^3$/$mm^3$; morphologically abnormal spermatozoa 26.55 ± 19.81%; total number of spermatozoa per ejaculate 87.18 ±

Table 1. Percentage of sperm motility in different long-term extenders during storage periods

| Storage periods | Extender | | | | |
|---|---|---|---|---|---|
| | androhep | androstar | androstar plus | LD | M III |
| 24 h | 58[a] | 54[a] | 53[a] | 52[a] | 51[a] |
| 48 h | 56[a] | 50 | 49 | 49[a] | 46 |
| 72 h | 53 | 47 | 46 | 43 | 41 |
| 96 h | 45[b] | 37[c] | 36[b] | 30[c,b] | 31[b] |
| Total | 53[A] | 47 | 46 | 43[C] | 42[D] |

[a,b,c,d]means within the column [a,b]$P < 0.05$; [a,c]$P < 0.01$; [a,d]$P < 0.001$
[A,B,C,D]means within the row [A,B]$P < 0.05$; [A,C]$P < 0.01$; [A,D]$P < 0.001$

46.51 × $10^9$; viable spermatozoa 68.05 ± 9.20%; sHOST 63.57 ± 11.94% and the activity of AST 153.39 ± 133.71 mU/$10^9$ spermatozoa.

The results of sperm membrane integrity assessed on the basis of sperm motility, viable spermatozoa, sHOST, AST activity during 24 h, 48 h, 72 h and 96 h storage in different extenders are presented in Tables 1–4. Table 1 shows changes in sperm motility with storage time in different long-term semen extenders. The average motility of spermatozoa in native semen was 70% and the average motility after 24 h was 58% in A, 54% in AS, 53% in AS$^+$, 52% in LD and 51% in M III. A significant decrease in sperm motility was observed after 96 h of preservation in all extenders ($P < 0.05$). The best motility was determined in extender A (53% sperm motility) in comparison with the other extenders in total evaluation of the trial. Significant differences were found between extenders A and LD ($P < 0.01$) and between A and M III ($P < 0.001$).

The percentage of viable spermatozoa also decreased with storage time (Table 2). There were significant differences in the viable spermatozoa percentage between 24 h and 96 h of preservation in A (64 vs. 49%), AS$^+$ (59 vs. 38%), LD (60 vs. 37%), $P < 0.001$, and in AS (59 vs. 42%), $P < 0.01$. A significant decrease in the viable spermatozoa percentage was recorded in M III extender after 72 h ($P < 0.05$). The total mean percentages of viable spermatozoa were 57% in A, 51% in AS, 49% in AS$^+$, 49% in LD and 48% in M III. Statistically significant differences in viable spermatozoa values were found between A and AS$^+$ and between M III and LD ($P < 0.01$). The percentage of viable spermatozoa was estimated to decrease by 0.27% each hour.

Table 3 shows the results of evaluation of the plasma membrane integrity in boar sperm using sHOST. The percentage of incidence of coiled tail spermatozoa reacting positively to the test was de-

Table 2. Percentage of viable spermatozoa in different long-term extenders during storage periods

| Storage periods | Extender | | | | |
|---|---|---|---|---|---|
| | androhep | androstar | androstar plus | LD | M III |
| 24 h | 64[a] | 59[a] | 59[a] | 60[a] | 59[a] |
| 48 h | 59[a] | 54[a] | 52[a] | 53[a] | 51[a] |
| 72 h | 55 | 48 | 47 | 46 | 46[b] |
| 96 h | 49[d,b] | 42[c,b] | 38[d,b] | 37[d,b] | 36[d,c] |
| Total | 57[A] | 51 | 49[C] | 49[C] | 48[C] |

[a,b,c,d]means within the column [a,b]$P < 0.05$; [a,c]$P < 0.01$; [a,d]$P < 0.001$
[A,B,C,D]means within the row [A,B]$P < 0.05$; [A,C]$P < 0.01$; [A,D]$P < 0.001$

Table 3. Percentage of sHOST in different long-term extenders during storage periods

| Storage periods | Extender | | | | |
|---|---|---|---|---|---|
| | androhep | androstar | androstar plus | LD | M III |
| 24 h | $58^{a}$ | $54^{a}$ | $52^{a}$ | $49^{a}$ | $51^{a}$ |
| 48 h | 49 | $43^{c,a}$ | $43^{a,c}$ | $42^{a}$ | $43^{a}$ |
| 72 h | $44^{d,A}$ | $39^{d}$ | $37^{d,a}$ | $34^{c,B}$ | $35^{c}$ |
| 96 h | $37^{d,A}$ | $31^{d,c}$ | $27^{d,b}$ | $26^{d,B}$ | $29^{d,c}$ |
| Total | $47^{A}$ | 42 | $40^{C}$ | $38^{C}$ | $40^{C}$ |

$^{a,b,c,d}$means within the column $^{a,b}P < 0.05$; $^{a,c}P < 0.01$; $^{a,d}P < 0.001$
$^{A,B,C,D}$means within the row $^{A,B}P < 0.05$; $^{A,C}P < 0.01$; $^{A,D}P < 0.001$

creased during storage in AS, AS$^{+}$ ($P < 0.01$) after 48 h while in LD, M III ($P < 0.01$) and A ($P < 0.001$) a decrease was observed after 72 h of preservation. There was a significant difference between extenders A and LD after 72 h and 96 h ($P < 0.05$). The best sperm membrane integrity in the total evaluation was in A (47%) in comparison with AS (42%), AS$^{+}$ (40%), LD (38%) and M III (40%). Statistical differences were observed between extenders A and AS ($P < 0.05$), AS$^{+}$, LD and M III ($P < 0.001$).

No significant differences were found in the results when the AST activity was used to evaluate the integrity of sperm membrane in different long-term extenders and periods of storage (Table 4). The total mean value of the AST activity was 64.0, 62.8, 61.6, 53.6, 55.5 mU/$10^{9}$ spermatozoa in A, AS, AS$^{+}$, LD and M III, respectively. However, an increase in the AST activity was observed after 72 h of storage in all extenders. The effect of extender and storage time is statistically significant in motility, viable spermatozoa and in sHOST ($P < 0.001$).

Table 5 demonstrates correlations among the methods for testing the membrane integrity. All correlations among the methods were statistically significant ($P < 0.001$) but there was a negative correlation among motility, viable spermatozoa, sHOST and AST activity. We noted that a leakage of this enzyme into the external environment decreased the quality of sperm. The strongest correlation was calculated between viable spermatozoa and both motility ($r = 0.85$, $P < 0.001$) and sHOST ($r = 0.81$, $P < 0.001$).

## DISCUSSION

The present study was focused on an assessment of the sperm membrane integrity in extended boar

Table 4. Activity of AST (mU/$10^{9}$ spermatozoa) in supernatants of boar spermatozoa in different long-term extenders during storage periods

| Storage periods | Extender | | | | |
|---|---|---|---|---|---|
| | androhep | androstar | androstar plus | LD | M III |
| 24 h | 54.9 (32.4) | 47.7 (28.4) | 52.9 (38.2) | 45.9 (25.8) | 43.9 (21.2) |
| 48 h | 62.4 (38.1) | 57.7 (33.0) | 55.6 (36.6) | 52.7 (33.8) | 55.8 (40.8) |
| 72 h | 71.3 (42.1) | 72.2 (52.5) | 63.3 (43.2) | 58.3 (37.5) | 59.5 (40.0) |
| 96 h | 67.5 (28.7) | 73.6 (54.0) | 74.5 (46.6) | 57.4 (33.6) | 62.8 (48.1) |
| Total | 64.0 (36.5) | 62.8 (45.6) | 61.6 (42.4) | 53.6 (33.5) | 55.5 (39.7) |

results are presented as mean values (SD)
differences within the column and within in the row are not significant $P > 0.05$

Table 5. Correlation coefficients among different methods for testing the quality of boar sperm

| | Sperm motility | Viable spermatozoa | sHOST | AST |
|---|---|---|---|---|
| Sperm motility | – | 0.85[d] | 0.73[d] | –0.53[d] |
| Viable spermatozoa | 0.85[d] | – | 0.81[d] | –0.56[d] |
| sHOST | 0.73[d] | 0.81[d] | – | –0.43[d] |
| AST | –0.53[d] | –0.56[d] | –0.43[d] | – |

[d]interaction is statistically significant ($P < 0.001$)

semen during storage time using specific spectrum laboratory methods. The initial quality of semen showed a higher percentage of morphologically abnormal spermatozoa (up to 25% is tolerated for A.I. in the Czech Republic). It could be caused by variation of temperature in the summer season. Several studies have shown that elevated ambient temperatures, heat stress and/or hot weather have an adverse effect on sperm production (McNitt et al., 1970; Colenbrander et al., 1993; Frydrychová et al., 2007; Wolf et al., 2009) and sperm morphology in boars (Larsson et al., 1984; Malmgren, 1989). On the other hand, it could also be an influence of the hereditary background (Čeřovský, 2005).

Sperm motility is an indication of the active metabolism and integrity of membranes (Johnson et al., 2000). A significant decrease in sperm motility was observed after 72 h for all extenders. Ambrogi et al. (2006) also obtained similar results when the low sperm motility after 72 h storage would significantly influence the fertilizing ability of sperm. Kommisrud et al. (2002) found significant differences in motility after 78 h and 102 h. The effects of extenders on sperm motility are evident from total means where significant differences were recorded in the motility between extenders A and LD and between A and M III.

A significant decrease in the percentage of viable spermatozoa was also observed after 72 h in each extender but in M III extender the percentage of viable spermatozoa was already decreased after 48 h. Differences in the percentage of viable spermatozoa between extender A and AS$^+$ and between M III and LD are evident from total means. The percentage of viable spermatozoa was estimated to decrease by 0.27% each hour. The viability of spermatozoa was always higher than the motility irrespective of the extender and storage time. It would be due to the fact that some spermatozoa are living but they are immobile.

The sHOST score for fresh semen was 63.57% in our study whereas Pérez-Llano et al. (2001) reported 78.3%. The sHOST percentage decreased during storage after 48 h in AS, AS$^+$ while in LD, M III and A its decrease occurred after 72 h of preservation. There was a significant difference between extenders A and LD after 72 h and 96 h. Extender A provided the greater plasma membrane resistance to adverse external conditions. The percentage of spermatozoa responding by swelling was lower than the percentage of motility and living spermatozoa. These relations were in agreement with Vazquez et al. (1997) and Pérez-Llano et al. (2001). The difference may be due to the fact that some spermatozoa with membrane damage remain motile (Zou et al., 2000) or the membranes of some spermatozoa are inactivated when they come into contact with the hypoosmotic solution. sHOST evaluates the functional integrity of plasma membrane. Functional heterogeneity was demonstrated by flow cytometry of a sperm population that included spermatozoa with reduced survival capacity despite their motility (Morrell, 1991). Our data showed that sHOST indicates the sperm population with membrane damage in diluted boar semen earlier than sperm motility, percentage of viable spermatozoa and AST activity. This test may be a useful complement to the standard evaluation of boar semen and for determination of preservation capability.

The results of the AST activity used to evaluate the sperm membrane in different long-term extenders and periods of storage did not indicate any significant differences. However, an increase in the AST activity was observed after 72 h storage in all extenders. On the contrary, Azawi et al. (1990) and Pandey et al. (2001) reported significant differences during preservation in different extenders.

In this study, the influence of extender and storage time on motility, percentage of viable spermatozoa and sHOST was statistically significant.

Kommisrud et al. (2002) also reported that the storage time significantly influenced motility and acrosome integrity.

All correlations among the methods of evaluation of the sperm membrane integrity were statistically significant. A negative correlation was only found among motility, viable spermatozoa, sHOST and AST activity. We noted that a leakage of this enzyme into the external environment decreased the sperm quality. The strongest correlation was calculated between viable spermatozoa and motility ($r = 0.85$, $P < 0.001$) and sHOST ($r = 0.81$, $P < 0.001$). A significant correlation between sHOST and motility was observed because motility depends partly on the membrane transport and partly on biochemical activities. Nur et al. (2005) and Lin et al. (1998) also recorded a significant correlation between sHOST and motility. The high correlation among motility, percentage of viable spermatozoa and sHOST was also reported by Věžník et al. (2003) but on the other hand, Serniene et al. (2005) did not find any significant correlation between motility and viable spermatozoa.

In conclusion, the results documented that the sperm membrane integrity was affected by extenders and storage time statistically significantly ($P < 0.001$). Changes in the quality of sperm membrane in relation to storage time were determined after 48h for sHOST, after 72 h for the percentage of viable spermatozoa and after 72 h for motility. In overall assessment Androhep was the best of the tested extenders, followed by AS, $AS^+$, M III and LD in terms of motility, viable spermatozoa and sHOST. The correlations among the methods of evaluation of the sperm membrane quality were highly significant ($P < 0.001$). sHOST is a very sensitive method for assessment of the sperm membrane integrity because it detects damage to the sperm membrane earlier than the other methods used.

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