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# ATP Content and Sperm Motility of Extended Bovine Semen under Different Storage Conditions

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**Söderquist, L.: ATP content and sperm motility of extended bovine semen under different storage conditions. Acta vet. scand. 1991, 32, 511–518.** – The effects of incubation temperature (+20°C vs +35°C) and media type on the ATP content and motility of spermatozoa were determined in fresh bovine semen in order to develop a method for assaying post-thaw quality. Semen was obtained from 3 bulls at 2 occasions. The spermatozoa were washed using a Ficoll-containing medium before being resuspended in each of 4 different media (**I.** 0.9 % NaCl; **II.** Tris-buffer solution; **III.** seminal plasma; **IV.** seminal plasma + Tris-buffer solution) and incubated for 6 h. The least-squares means for ATP content were higher ($p \leq 0.05$) at +20°C than +35°C for all media except no. I.
By contrast, the least-squares means for sperm motility were higher ($p \leq 0.05$) at +35°C than at +20°C in media II and III. A decrease over time in ATP content and motility at both temperatures was also observed.
The single most important factor responsible for changes in ATP content and sperm motility was the temperature and the medium, respectively.

extender; temperature; bull; spermatozoa; seminal plasma.

## Introduction

To remain motile, spermatozoa require a continuous supply of energy, the primary source of which is provided by the dephosphorylation of adenosine triphosphate (ATP) (*Salisbury et al.* 1978). Hence, one could expect that the production rate of ATP by spermatozoa and their ATP content would be correlated with their motility. Additional energy is presumably also required for cell maintenance. Bull spermatozoa are able to synthesize the ATP needed for motility by metabolising exogenous substrates, either anaerobically, by glycolysis, or aerobically by oxidative phosphorylation coupled to respiration (*Mann* 1964). Since the spermatozoon and the normal fluids surrounding it contain all the enzymes necessary to carry out essential metabolic processes for motility with or without oxygen, spermatozoa are adapted to a variety of conditions. Thus, the metabolic diversity exhibited by spermatozoa makes it possible to control metabolic activity by varying certain environmental factors such as temperature and the kinds of substrate in the environment (*Salisbury et al.* 1978). Early studies (e.g. *Prinzen* 1977, *Folkes & MacDonald* 1979, *Berger* 1983, *Wood et al.* 1986) were mainly focused on the correlations between ATP content, sperm motility and fertility. As a consequence, little is known about the influences of extenders and temperature on sperm motility and ATP content.

To develop a method for assaying post-thaw quality requires that certain physiological characteristics of bull spermatozoa be investigated.

The aims of the present investigation were to determine the effects of temperature and

various types of extenders on the ATP content and motility of spermatozoa in fresh bovine semen.

## Material and methods

*Animals*

Three bulls, A, B and C, of the Swedish Red and White breed, 35, 22 an 24 months old, were used. The bulls were all stabled at the clinic of the Department of Obstetrics and Gynaecology for at least 1 month, and semen was regularly collected before they were entered in the study. Bull A had been used in an A.I. centre for routine semen collection for about 2 years. Bulls B and C were rejected by the A.I. centre for use in artificial insemination because of the higher than average number of times that their frozen semen had to be discarded, owing to low post-thaw motility (< 50 %). The ejaculate and the characteristics of the spermatozoa (volume, concentration, motility and morphology) were all within the normal range for bulls according to the standards at the semen laboratory of the Department of Obstetrics and Gynaecology.

*Procedures*

Semen was collected with an artificial vagina on 2 occasions. Two to 3 ejaculates were collected (over a period of approximately 15–30 min) on each occasion, to ensure that at least 10 ml of semen was available for the experiments. The ejaculates were immediately pooled after collection, and the sperm motility of the native semen was estimated at +35°C by phase contrast microscopy (450 ×). The sperm concentration was determined in a photometer (LINSON, LIC Instruments, Stockholm).

The spermatozoa were washed using a Ficoll-containing washing medium prepared according to *Harrison* (1976). The concentration of the Ficoll (Ficoll 400, Pharmacia Fine Chemicals, Uppsala, Sweden) in the washing medium was 7.5 % (w/v). Pooled semen was washed at 2 different temperatures, +20°C and +35°C. Aliquots of semen were carefully layered on top of the Ficoll solution (5 ml) in 4 tubes at +20°C and 4 tubes at +35°C. After loading, the tubes were submitted to a 2-stage centrifugation procedure: after a gentle initial acceleration of 250 g for 5 min the centrifugation proceeded at 1000 g for another 10 min. The different temperatures (+20°C and +35°C) were maintained thoroughout the centrifugation procedure as well as during the rest of the experiment. After centrifugation the supernatants (seminal plasma) were completely removed by careful aspiration. After aspirating the washing medium the spermatozoa, which formed loose pellets at the bottom of the tubes, were resuspended and diluted in 4 ml of each of the following 4 media: **medium I,** NaCl (0.9 % (w/v)); **medium II,** a buffer solution (Tris-fructose-citric acid solution (30.28 g/17.00 g/12.50 g per 1000 ml distilled water; appropriate amounts of antibiotics were also included)); **medium III,** seminal plasma (received from the supernatant layers removed after the washing procedure described earlier) and **medium IV,** a mixture containing equal parts of mediums II and III (described above).

Aliquots were taken from each of the eight tubes to estimate the sperm concentration. The sperm concentration was counted twice in a haemocytometer, according to a method described by *Bane* (1952), and the mean values were used for the final calculation of the ATP contents. The preparations were incubated for 6 h in a water bath at +20°C and +35°C, respectively. Aliquots for determination of ATP content and sperm motility were then taken every half h during a 6 h incubation period. The ATP content was measured using a bioluminescent technique,

and duplicate samples (100 µl each) were pipetted from each tube every half h and placed on ice; 100 µl icecold TCA (Tri Chloratic Acid, 12 %) was then added, and the ingredients were thoroughly mixed. All samples were placed on ice for at least half an h. A 200-µl amount of Tris/EDTA/Triton (100 nM/4 mM/0.2 % (v/v)) was then added and admixed. From the mixture 25 µl was taken and pipetted into a test tube containing 0.8 ml Tris/EDTA/Triton solution and mixed. A 200-µl amount of ATP monitoring reagent (LKB-Sweden AB) was then added, and the ATP content was determined using an LKB Luminometer 1250 001. For internal calibration 10 µl of ATP standard was added and read (*Söderquist & Larsson* 1985). Samples were also taken every half h to estimate sperm motility by phase contrast microscopy (450 ×). Thus 12 observations within each medium and temperature were performed. Altogether 576 observations were analysed.

*Statistical methods*

To estimate the effects of media type and temperature on ATP content and sperm motility the method of least-squares was used as applied in the general linear model (GLM) procedures of the Statistical Analysis System (SAS Institute Inc. 1985).

The model chosen was:

$$Y_{ijklm} = \mu + BC_i + TP_j + M_k + T_l + (BC*TP)_{ij} + (BC*M)_{ik} + (TP*M)_{jk} + (M*T)_{kl} + e_{ijklm}$$

where:

$Y_{ijklm}$ = ijklm'th observation on ATP content and sperm motility

$\mu$ = least square mean

$BC_i$ = fixed effect of i'th bull and collection occasion (i = 1..6)

$TP_j$ = fixed effect of j'th temperature (j = 1..2)

$M_k$ = fixed effect of k'th medium (k = 1..4)

$T_l$ = fixed effect of incubation time (l = 1..12)

$(BC*TP)_{ij}$, $(BC*M)_{ik}$, $(TP*M)_{jk}$, $(M*T)_{kl}$ } the effects of the 2 factor interactions between the fixed effects mentioned above

$e_{ijkl}$ = random residual effect

Due to the irregular interval between 1st and 2nd semen collections it was not possible to separate the effects of bull and collection occasion within bull. Therefore the combined effect of bull and collection occasion was used in the model. To illustrate the effect of incubation time on ATP content and sperm motility within incubation temperature and media a separate analysis of variance within temperature and media was performed (Figs. 1 & 2).

**Results**

The analysis of variance showed that all the independent variables were statistically significant ($p \leq 0.01$). However, the 2 factor interactions, although significant, did not explain much of the variation in the dependent variables. Overall means and standard deviations for ATP content and sperm motility were 34.8 ± 4.8 nmol/spermatozoa × $10^8$ and 58.4 ± 7.6 %, respectively.

Least-squares means of ATP content and sperm motility in each of the different media, at +20°C and +35°C, and their variation over time are shown in Figs 1 & 2.

*ATP content*

As can be seen from Table 1 the least-squares means for the ATP content in media II, III and IV were higher at +20°C than at +35°C (Fig. 1). In medium I the ATP content at +20°C and that measured at +35°C were about the same. A statistically significant ($p \leq 0.05$–$0.001$) decrease over time in ATP

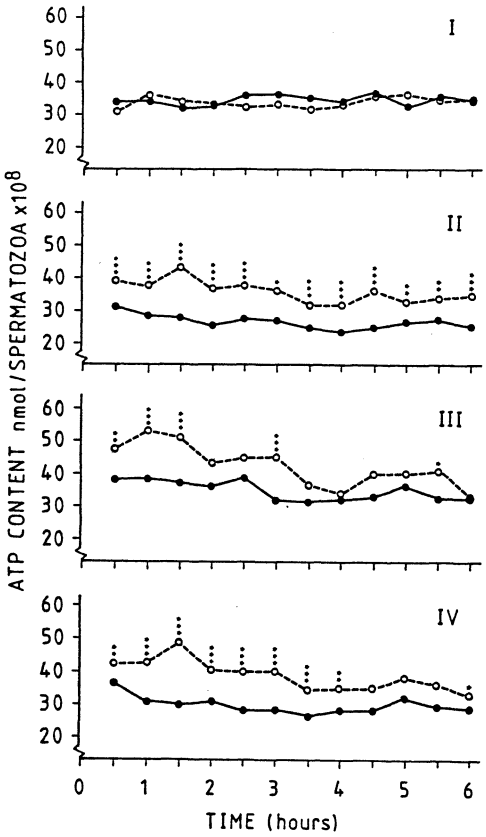


Figure 1. Variation in least-squares means of the **ATP content** (nmol/spermatozoa x $10^8$) in each of the test media during a 6-h incubation at either +20°C (○ - - - ○) or + 35°C (● - - - ●). Medium **I** is 0.9 % NaCl, medium **II**Tris-buffer solution, medium **III** seminal plasma and medium **IV** seminal plasma + Tris-buffer solution. Statistically significant differences between temperatures on each sampling occasion are indicated in the figure. The following levels of significance are used: ns = not significant; * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$.

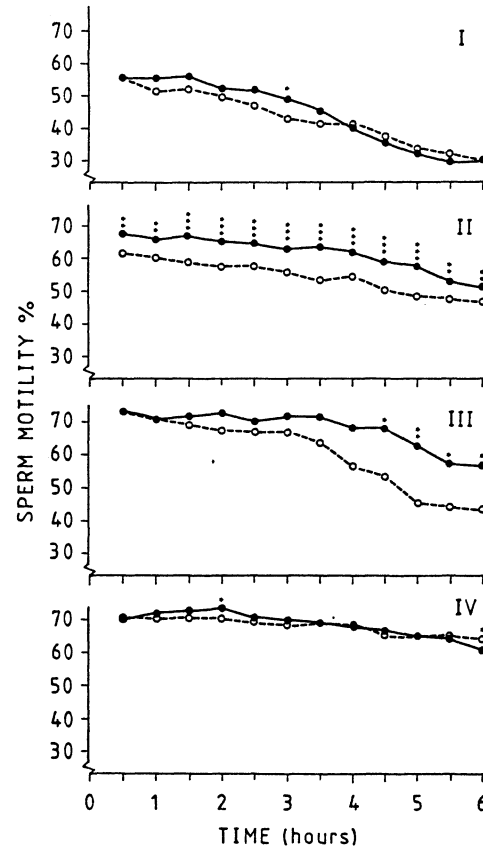


Figure 2. Variation in least-squares means of the **sperm motility** (%) in each of the test media during a 6-h incubation at either +20°C (○ - - - ○) or + 35°C (● - - - ●). Medium **I** is 0.9 % NaCl, medium **II**Tris-buffer solution, medium **III** seminal plasma and medium **IV** seminal plasma + Tris-buffer solution. Statistically significant differences between temperatures on each sampling occasion are indicated in the figure.

content occurred at both incubation temperatures in media II, III and IV. In **medium I** the ATP content fluctuated somewhat, but remained quite constant over time at both temperatures. In medium I no statistically significant difference in ATP content was found between spermatozoa kept at +20°C and spermatozoa kept at +35°C. In **media II, III** and **IV** (Fig. 1) consistent temperature-related differences in the ATP content were

Table 1. Least-squares means (ls-means)* for **sperm motility** and **ATP content** in different media incubated at +20°C and +35°C, respectively.

| Medium | Incubation temperature +20°C | +35°C |
|---|---|---|
| | Sperm motility % | |
| I | 42.4[1a] | 44.1[1a] |
| II | 54.4[1b] | 61.5[2b] |
| III | 60.0[1c] | 67.9[2c] |
| IV | 68.2[1d] | 68.5[1c] |
| | ATP content nmol/sperm-atozoa x $10^8$ | |
| I | 33.7[1a] | 34.6[1a] |
| II | 36.0[1b] | 26.8[2b] |
| III | 42.7[1c] | 35.3[2a] |
| IV | 39.0[1d] | 30.0[2c] |

Means in a **row** followed by the same **numeral** are *not* significantly different ($p \leq 0.05$).
Means in a **column** followed by the same **letter** are *not* significantly different ($p \leq 0.05$).
* Standard error of ls-means for sperm motility = 0.6 (equally for all ls-means).
* Standard error of ls-means for ATP content = 0.9 (equally for all ls-means).

found. The decrease in ATP content in **medium III** at +20°C was especially pronounced ($p \leq 0.001$) during the last half of the incubation period.

*Sperm motility*

The least-squares means for sperm motility (Table 1) were higher at +35°C than at +20°C in media II and III, whereas no significant difference was found in media I and IV. Ranked on the basis of their least-squares means for sperm motility, medium I was lowest and medium IV was highest at both +20°C and +35°C. A statistically significant ($p \leq 0.05$–$0.001$) decrease over time in sperm motility occurred at both temperatures in all media investigated (Fig. 2), but was especially pronounced ($p \leq 0.001$) in medium I. In addition, the lowest values of sperm motility at both the start and end of the inbubation period were recorded in **medium I.** The total decrease in sperm motility in medium I at +20°C was similar to that observed at +35°C, and there was no statistically significant temperature-related difference in this parameter. The decrease was more pronounced during the last half of the incubation period. In **medium II** the temperature-related difference in sperm motility was already statistically significant ($p \leq 0.01$) at the beginning of the incubation period. This difference in sperm motility, which was mainly caused by 1 bull (C), remained significant throughout the incubation period. In **medium III** the drop in sperm motility was larger during the 2nd half of the incubation period than during the 1st half, at both temperatures (Fig. 2). Still, the decrease was more pronounced at +20°C, mainly because there were very conspicuous drops in sperm motility at +20°C in some of the replicates for bulls B and C. In **medium IV** no consistent temperature-related differences in sperm motility were found during the incubation (Fig. 2) and the pattern of change in sperm motility over time at +20°C was similar to that observed at +35°C.

## Discussion

Normally, the concentration of spermatozoa in freshly ejaculated bull semen is high. To avoid autotoxication due to the accumulation of toxic metabolic products and the considerable acidity developed if the semen is stored as such, considerable extension is necessary. By mixing spermatozoa with different kinds of extenders all the ingredients necessary for sustaining and protecting the spermatozoa can be obtained. Furthermore a reduction in temperature has been the chief means of retarding chemical reactions

and prolonging sperm life, since the metabolic rate of spermatozoa tends to be proportional to the absolute temperature (*Salisbury et al.* 1978). Under anaerobic conditions spermatozoa must rely on an extracellular source of energy to protect their intracellular reserves and cell components. The content of ATP and with it the motility of ejaculated spermatozoa therefore both depend on the maintenance of normal metabolism of glycosable material (*Mann* 1964).

According to earlier studies washing of ejaculated bovine spermatozoa in Ficoll, does not harm them (*Harrison* 1976). By using this technique the spermatozoa can be separated from seminal plasma, allowing factors affecting motility and viability in bull spermatozoa to be studied (*Baas et al.* 1983). In the present study the spermatozoa washed in this way were resuspended in 4 different media. The least squared means for sperm motility were higher at +35°C than at +20°C in media II and III, while the ATP content was higher at +20°C than at +35°C in media II, III and IV. *Hammerstedt & Hay* (1980) reported that after incubating spermatozoa in a buffer solution containing glucose their ATP content was higher at room temperature than at body temperature, whereas their motility was higher at body temperature than at room temperature. These results support the trends found in the present study. The least squared means for the ATP content were also similar to the means presented by *Hammerstedt & Hay* (1980).

Medium I (0.9 % NaCl) differed from the other media in that it contained no available extracellular source of energy and provided no buffering capacity. These differences are probably the major reasons why there was a great decrease in sperm motility over time and why the extent of the decrease was not affected by temperature. However, there was no significant decrease over time in the ATP content of spermatozoa in medium I, suggesting that the majority of the spermatozoa were still alive although their motility had decreased significantly over time. *Shapiro et al.* (1985) reported that a low intracellular pH suppressed motility and respiration either by inhibiting mitochnodrial respiration or by inhibiting axoneme motion, and as a result little ATP was utilized.

In contrast to medium I, medium II (Tris-buffer solution) contained, an energy source (fructose). Moreover, the latter was also a more suitable medium for maintaining sperm viability due to its high buffering capacity. This was reflected in the consistently higher level of sperm motility and the smaller decrease in motility in medium II compared with medium I. The highest levels of sperm motility were observed in media III and IV where seminal plasma was present. Earlier studies (*Baas et al.* 1983) reported that seminal plasma contains factors that help to sustain the motility of spermatozoa as well as factors that speed up the rate at which spermatozoa lose their activity. The latter effect might explain why there was a clear reduction in sperm motility during the 2nd half of the incubation period (in medium III), which was especially pronounced at +20°C. The statistically significant difference in motility between spermatozoa at +20°C and spermatozoa at +35°C during the 2nd half of the incubation was mainly caused by the drop in sperm motility at +20°C in bulls B and C. Furthermore the marked and rather early drop in the motility sperm from bulls B and C (in some cases) at +20°C suggests that spermatozoa from these bulls not only had low post-thaw motility but also were more sensitive to incubation in pure seminal plasma at +20°C. A statistically significant and more evident decrease in ATP content was also observed at +20°C in medium III, reflecting the well known fact

that +20°C is not the best temperature for storing undiluted bull semen. On the other hand, the detrimental effect on sperm motility was much less evident when the incubation was carried out in medium IV, which was a mixture of seminal plasma and Tris-buffer. In this case the motility of spermatozoa from all 3 bulls was maintained at a very high level throughout the incubation at both +20°C and +35°C. Only a minor decrease over time in sperm motility and ATP content was found. Thus, medium IV, seemed to be most suitable for the spermatozoa.

The fact that ATP content was lower at +35°C that at +20°C in media II, III and IV could have been due to a more rapid breakdown of ATP at +35°C, which would be required to maintain the higher level of sperm motility associated with the more rapid metabolism at the higher temperature. During incubation a significant decrease over time in ATP content occurred at both +20°C and +35°C (except in medium I) which might reflect a gradual reduction in mitochondrial activity during incubation; as a result ATP might have been consumed faster than it was produced. In conclusion, the present study showed that the ATP content and sperm motility are affected by temperature, and medium type and change during the course of incubation. The most important single factor responsible for changes in the ATP content and sperm motility was the temperature and the medium, respectively.

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**Sammanfattning**

*ATP innehåll och spermiemotilitet i spädd sperma under olika förvaringsförhållanden.*

Effekten av inkuberingstemperatur (+20°C och +35°C) och mediatyp på ATP-innehållet och spermiemotiliteten undersöktes i färsk tjursperma i syfte att utveckla en metod för bedömning av spermakvaliteten efter upptining. Sperma från 3 tjurar samlad vid 2 olika tillfällen användes. Spermierna tvättades med hjälp av centrifugering genom en Ficoll-lösning innan de späddes med 4 olika medier (**I.** 0.9 % NaCl; **II.** Tris-buffert; **III.** seminalplasma; **IV.** seminalplasma + Tris-buffert) och inkuberades under 6 t. Least-squares means för ATP var högre ($p \leq 0.05$) vid +20°C jämfört med vid +35°C, i alla medier utom i medium I. Däremot var least-squares means för spermiemotiliteten högre ($p \leq 0.05$) vid +35°C jämfört med vid +20°C i medium II och III. En nedgång över tiden i både ATP-innehållet och spermiemotiliteten ägde rum under inkuberingen vid båda temperaturerna. Den viktigaste enskilda orsaken till förändringar i ATP-innehållet och spermiemotiliteten var temperaturen respektive mediet.

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