An evaluation of damage to bovine spermatozoa during processing, freezing and thawing. I Leakage of glutamic-oxaloactetic transaminase [GOT] from spermatozoa

B. SOMADE*, G. J. KING AND J. W. MACHPERSON

Department of Animal Science, University of Guelph, Guelph, Ontario, Canada, NIG. 2W1

Abstract

The activity of glutamic-oxaloacetic transaminase (GOT) in the seminal plasma of thirty ejaculates after collection; after extension, cooling and equilibration just before freezing; and after freezing and thawing were determined. After correction for the reduction in the activity of GOT caused by the extender and low temperatures the activity of the enzyme was higher (P 0.05) in post thaw semen than in either ejaculated or equilibrated semen just before freezing. However, the pre-freeze leakage of GOT from spermatozoa was similar to the leakage caused by freezing and thawing. The significance of these findings in relation to the use of GOT activitiy in seminal plasma as an index of spermatozoa damage were discussed.

Introduction

Numerous attempts have been made to establish laboratory methods for the evaluation of ejaculated and frozen-thawed semen samples and possibly correlate the results with fertility. Much of the original work on relationships between physical and chemical characteristics of bull semen and the subsequent fertility of the ejaculate has been reviewed (Salisbury and Van-Demark, 1961). It was concluded that no single semen quality measurement or assessment is effective in explaining more than 20 percent of the variation in fertility between bulls or between ejaculates of the same bull.

Clearly, the artificial insemination industry could benefit from a procedure or test that did correlate more highly with fertility. Ideally, this technique would be simple enough for routine performance as a quality control assessment on all ejaculates. The leakage of various enzymes including hyaluronidase (Foulkes and Watson, 1975; Graham and Pace, 1967) acrosin (Shill and Wolff, 1974) and gultamic-oxaloacetic transaminase (GOT) have all been used in an attempt to estimate viability of spermatozoa after freezing. GOT in the fresh ejaculate has been shown to be significantly correlated with percent motility; sperm concentration, percent live spermatozoa and negatively correlated with percent abnormal spermatozoa (Roussel and Stalloup, 1965). The leakage of GOT from spermatozoa has also been shown to result in an increase in the activity of this ensyme in the seminal plasma (Graham and Pace.

* *Present Address: Department of Animal Science, University of Ife, Ile-Ife, Nigeria.*

1967). A positive correlation was also established between fertility and the amount of GOT left in the spermatozoa after freezing (Graham and Pace, 1967).

If the leakage of GOT from spermatozoa is to be used to assess damage to spermatozoa routinely, it is necessary to employ a method for separating spermatozoa from seminal plasma that will cause little damage to the cell as well as determine the activity of the enzyme in seminal plasma quickly. The purpose of the present experiment was to evaluate the reliability of GOT in seminal plasma as an index of spermatozoa damage during processing, freezing and thawing.

Materials and methods

The GOT activity in the seminal plasma of fresh, equilibrated and frozen-thawed semen for six ejaculates from each of five bulls was measured using a commerical reagent set. The ejaculates were processed and frozen as previously described (Somade, 1976).

Spermatozoa were removed from the seminal plasma by discontinuous gradient centrifugation (Brown, Crabo, Graham and Pace, 1971) seminal plasma was carefully aspirated with a disposable pipette. L-aspartic acid and -ket oglutaric acid undergo transamination in the presence of GOT. In this reaction, L-aspartic acid is converted to oxaloacetic acid. The colour developer used in this experiment was a diazonium salt 6 - benzamido - 4 methoxy - n - toluidine diazonium chloride, which reacts selectively with oxaloacetic acid to produce a colour whose intensity reflects the transaminase activity (Babason, Shapiro, Williams and Phillips, 1962). The amount of oxaloacetic acid formed is proportional to the amount of GOT present. A commercial reagent set for the determination of GOT activity was used. The GOT activity was recorded in Trans AC units, one Trans AC unit being defined as the amount of enzyme that will form one micromole of oxaloacetic acid per minute per litre of seminal plasma at 37°C. This activity was then converted to Trans AC units per g of spermatozoa protein.

To determine if there was a reduction in the activity of the enzyme due to the extender and/or freezing which could have masked leakage of GOT from spermatozoa a short term experiment was performed. The GOT activity in the seminal plasma from eight ejaculates was used. Two ml of seminal plasma from each ejaculate was diluted 1:2 with extender and the GOT activity was measured. The extended seminal plasma was cooled to 5°C over a period of one hour and equilibrated for five hours and theGOT activity measured. The equilibrated seminal plasma was then frozen in liquid nitrogen, thawed and GOT activity measured. The GOT activity of extender alone was also measured.

Data collected was subjected to analysis of variance using the differences between the original values of GOT activity at ejaculation and those recorded prior to freezing as period one and the differences between values obtained just before freezing and post thaw values as period two. Periods were considered fixed effects while bulls and collections were considered random effects.

Results

Rather than a steady increase in the activity of GOT in seminal plasma because of the increase in the number of damaged spermatozoa due to processing and freezing, a slight decrease in activity was observed (Table 1). The GOT activity of the semen extender alone was 58 ± 6 Trans AC units per 1 and the seminal plasma activity was 240 ± 80 units per 1 (Table 2). Thus the addition of the extender to seminal plasma at a ratio of 2:1 after correction for dilution effect would be expected to increase activity. However, a reduction in activity to 215 ± 82 units per 1 was observed. Cooling and equilibration further reduced activity as did freeze-thawing. Based on the data in table 2 correction factors were calculated for equilibrated semen and frozen-thawed semen. The following assumption were made:

Table 1: THE EFFECT OF PROCESSING, FREEZING AND THAWING BOVINE SEMEN ON THE LEAKAGE OF GOT FROM BOVINE SPERMATOZOA (TRANS AC UNITS PER G OF SPERMATOZOA PROTEIN)

Name of bull	Ejaculated semen	Equilibrated semen	Frozen-thawed semen
Magician	1.11	.87	.95
Pure Gold	1.17	.85	1.23
Roycidale	1.20	1.20	1.10
Pursuit	1.20	80	1.25
Astronaut	.82	.85	0.64
Mean sd	1.1 ± .40[a]	0.91 ± .40[a]	1.03 ± .40[a]

[1] *Mean of six ejaculated*

Mean with the same superscripts are not significantly different.

TABLE 2: THE EFFECT OF EXTENDER COOLING AND FREEZE-THAW ON THE ACTIVITY OF GOT IN SEMINAL PLASMA[a]

Treatment	Trans AC units per litre
Extender only	58 ± 6
Seminal plasma	240 ± 80
Seminal plasma plus extender	215 ± 82
Slow cooled to 5 °C and equilibrated	164 ± 56
Seminal plasma plus extender cooled to 5 C equilibrated and frozen	138 ± 66
Seminal plasma plunge frozen	219 ± 82

[a] All values for extended seminal plasma were corrected for dilution factor.

1. It was assumed that the results obtained for the reduction of GOT were valid for bovine semen processed and frozen as described in this project.
2. It was assumed that cooling and equilibration resulted in 23.7 percent inactivation of GOT and freeze-thawing in a further 12.1 percent reduction for a total of 35.8 percent reduction.
3. Finally it was assumed GOT that leaked from spermatozoa during processing and freezing was also reduced in activity. Suppose a, b and c Trans AC units per g of spermatozoa protein were the observed activities of GOT in seminal plasma at the three times of sampling, ejaculated semen (E.S), equilibrated semen (E.Q.S) and frozen-thawed semen (F.T.S.), respectively. The expected Got values for the three times would be:

E.S.	E.Q.S.	F.T.S.
a	a - .237a	a - .358a

while in fact the recorded values were:

a	b	c

The difference would be the contribution by damaged spermatozoa.

E.S.	E.Q.S.	F.T.S.
0	b-[a-0.237a]	c-[a-0.358a]

GOT from spermatozoa was reduced in activity comparable to that in plasma (Assumption 3). The corrected contribution from spermatozoa would be:

E.S.	E.Q.S.	F.T.S.
0	b-(a-0.237a)-(b-(a-0.237a)0.237a	c-(a-0.358a)-(c-(a-0.358a) 0.358a

Therefore, the total GOT (original amount in seminal plasma plus amount released by damaged spermatozoa) will be

E.S. = 0
E.Q.S. = a + [b-[a-0.237a]-[b-[a-0.237a]0.237
= .418a + .763b
F.T.S. = a+[c-[a-0.358a]-[c[a-0.358a]0.358
= 578a + .642c

The above factors for equilibrated semen and frozen-thawed semen were used to correct all the original GOT data before an analysis of variance was carried out.

The corrected values for GOT activity in seminal plasma are presented in table 3. The leakage of GOT into the seminal plasma from spermatozoa became evident. Activities of GOT was 1.10, 1.15 and 1.29 Trans AC units per g of spermatozoa protein for ejaculated, equilibrated and frozen-thawed semen respectively. The GOT activity in

TABLE 3: THE EFFECT OF PROCESSING, FREEZING AND THAWING BOVINE SEMEN ON THE LEAKAGE OF GOT FROM BOVINE SPERMATOZOA (TRANS AC UNITS PER G OF SPERMATOZOA PROTEIN)

Name of bull	Ejaculated semen	Equilibrated	Frozen thawed semen
Magician	1.12	1.13	1.23
Pure Gold	1'17	1.14	1.47
Roycidale	1.20	1.42	1.40
Pursuit	1.20	1.11	1.49
Astronaut	.80	.98	.90
Meam sd	1.10 ± .4[a]	1.15 ± .4[a]	1.29 ± .4[b]

[1] *Mean of six collections*

Mean with same superscript are not significantly different.

TABLE 4: THE LOSS OF GOT FROM BOVINE SPERMATOZOA DURING COOLING AND EQUILIBRATION FROM 37°C to 5°C (Period I) AND DURING FREEZING AND THAWING (PERIOD II)

	Trans AC units/g spermatozoa protein
Period 1	.05
Period 2	.14

14

.05

frozen-thawed semen was significantly different from those of ejaculated and equilibrated semen. There was no variation in GOT loss among bulls, collections or periods (Table 4).

Discussion

The CIBA foundation symposium on cellular injury (1964) concluded that when cells are subjected to cold shock there is damage to the limiting plasma membrane and cell contents then leak into the surrounding

medium on thawing. The measurement of the concentration of one or a combination of substances that leaked into the medium has been suggested as a means of accessing the degree of damage to the cells. (De Reuck and Knight, 1964). A convenient marker substance for bovine spermatozoa was found to be GOT because correlations between sperm cell concentration and enzyme in the seminal plasma indicated that GOT was present primarily in spermatozoa and that leakage from cells into the medium was proportional to cell damage (Brown **et al.,** 1971). The original data on extended and frozen bovine semen in this study did not support this hypothesis (Table 1). However, after the equilibrated and frozen-thawed GOT levels were corrected for the reduction in the activity of GOT caused by extension, cooling and freezing the increased leakage of GOT from spermatozoa during cooling and freezing became apparent (Table 3). It would appear that the stress induced by the combination of the extender and the reduction in the activity of GOT at 5°C was substantial enough to mask the expected increase in the activity of GOT in seminal plasma. The mean GOT activity in seminal plasma was greater after freezing and thawing than in the seminal plasma of ejaculated or equilibrated semen ($P<0.05$). However when periods were fixed effects there was no difference in the activity of GOT betwen periods one and two, bulls or collections.

These results are contradictory to previously published data in which there was a trend showing an increased release of GOT from spermatozoa as equilibration time increased. The increase was not enough to show statistically significant difference in GOT release between frozen-thawed semen samples and equilibrated samples (Pace and Graham, 1970). These authors showed that GOT released from spermatozoa of samples from 35 bulls during normal freezing was significantly correlated to fertility ($r = .21$). For the same experiment, a significant correlation ($Y = .25$, $P>0.05$) was found between the amount of GOT left in the spermatozoa after normal freezing and thawing and fertility. There was a reduction in the activity of GOT obtained, from canine kidney as hydrostatic pressure applied to the enzyme in a medium of 10% glycerol by volume in plasma increased. The effect of pressure on the enzyme increased as the temperature of the plasma was decreased to -20°C (Carter, Graham, Lillehel and Blackshear, 1971).

These experiments suggest a reduction in the activity of GOT in plasma due to low temperatures which agrees with some of the results presented here (Table 2).

When bovine semen is extended and frozen as in this experiment, GOT leakage from spermatozoa cannot be used to estimate the degree of damage to spermatozoa because of the compounding effect of the extender and low temperatures which reduce the activity of the enzyme. An extender with a different composition may have a different effect on GOT in seminal plasma. It may still be possible to use GOT levels to monitor spermatozoa damage if correction factors can be established for different extenders and temperatures. Furthermore, these results indicate that the conditions adequate for the preservation of the integrity of a reasonable number of spermatozoa during processing and freezing are not necessarily optimal for the preservation of GOT in seminal plasma. The reduction in the activity of GOT when seminal plasma was

plunged into liquid nitrogen was smaller than any of the other treatments (Table 2). It would appear that the best way to preserve the activity of GOT is to plung freeze the extended semen in liquid nitrogen after extension at near body temperature.

References

Babson, A. L., Shapiro, P. O., Williams, P. A. R. and Phillips, G. E. 1962. The use of diazonium salt for the determination of glutamic oxaloacetic transaminase in serum. *Clin Chim. Acta., 7:* 199-202.

Brown, K. I., Crabo, G. B., Graham, E. F. and Pace, M. M. 1971. Some factors affecting loss of intracellular enzymes from spermatozoa. *Cryobiology. 8:* 220-224.

Cater, K. E., Graham, E. F., Lillehel, R. C. and Blackshear, P. L. 1971. The effect of high hydrostatic pressure and low temperature on lactic dehydrogenase and glutamic oxaloactetic transaminase. *Cryobiology, 8:* 524-528.

DeReuck, A. V. S. and Knight, J. 1964. C I B A Foundation Symposium on Cellular Injury. P. 314. CIBA oundation, London, England.

Foulkes, J. A. and Watson, P. A. 1975. Hyaluronidase activity in seminal plasma as a method of assessing bull sperm integrity. *J. Reprod. Fert. 43:* 349-353.

Graham, E. F. and Pace, M. M. 1967. **Some biochemical changes in spermatozoa due to freezing.** *Cryobiology 4:*75-84.

Pace, M. M. and Graham, E. F. 1970. The release of glutamic oxaloacetic transaminase from bovine spermatozoa as a test method of assessing semen quality and fertility. *Bio. Reprod. 3:* 140-146.

Roussel, J. D. and Stallcup, O. T. 1965. Parallelism between semen characteristics and glutamic oxaloacetic transaminase, and glutamic pyruvic transaminase activities. *J. Dairy Sci. 48:* 1684-1687.

Salisbury, G. W. and Van-Demark, K. N. L. 1961. Physiology of reproduction and artificial insemination of cattle. Freeman and Co., San Francisco.

Schill, W. B. and Wolff, H. H. 1974. Ultrastructure of human sperm acrosome and determination of acrosin activity under conditions of semen preservation *Int. J. Fert. 19:* 217-221

Somade, B. 1976. An evaluation of enzyme leakage and ultrastructural damage to bovine spermatozoa during processing in a commercial laboratory. Ph.D. Thesis. University of Guelph.