BIOLOGY OF REPRODUCTION 3, 140–146 (1970)

The Release of Glutamic Oxaloacetic Transaminase from Bovine Spermatozoa as a Test Method of Assessing Semen Quality and Fertility[1]

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Received January 29, 1970

Bovine semen was analyzed to establish the source of glutamic oxaloacetic transaminase (GOT) and to determine the feasibility of using GOT release from the cell for determining semen quality. Experiments comparing the concentration of GOT in extracellular media after spermatozoa had been subjected to minimal and maximal stress and another measuring the amount of GOT in semen before and after a bilateral corpus epididymectomy led to the conclusion that GOT is primarily associated with the spermatozoa. The correlation between the releasable amount of GOT and sperm concentration was $r = .80$ ($p \leqslant .01$). From a second group of experiments it was concluded that GOT release from the sperm cell was influenced by centrifuging at various gravitational forces and by freezing with different concentrations of glycerol or ethylene glycol. Freezing spermatozoa after different equilibration periods at 5° or thawing at different temperatures did not significantly alter GOT release. Another experiment involving 98 samples of semen from 35 Holstein bulls showed a significant correlation ($r = .21$, $p \leqslant .05$) between GOT/10^9 spermatozoa and fertility. A significant correlation ($r = .25$, $p \leqslant .05$) was also found between fertility and the amount of enzyme left in the sperm cell after normal freezing, indicating that the amount of enzyme left in the cell is important for fertility.

None of the methods previously employed to predict the fertilizing capacity has been highly successful. Motility is the most widely used technique today. The advent of frozen semen increased the nature of the problem because sperm motility is present after thawing but has resulted in either limited or no fertility in boar, turkey, and stallion semen.

Sherman (1959) showed that some cells had motility but were nonetheless injured or weakened after freezing and thawing. Mann (1964) stated that cytochrome *c* and hyaluronidase were released by the spermatozoa when mechanical damage was inflicted upon the cell. The CIBA foundation symposium on cellular injury (1964) concluded that as cells were injured, membranes were inactivated or destroyed and cellular material was lost. Graham and Pace (1967) found that glutamic oxaloacetic transaminase (GOT) and lactate dehydrogenase (LDH) increased in the extracellular media upon plunging spermatozoa into liquid nitrogen. Plunging the cells into liquid nitrogen destroyed all motility and apparently damaged cell membranes to cause an increase in the concentration of enzyme in the plasma. Flipse (1960) and Roussel and Stallcup (1965) found that GOT was highly correlated with bovine spermatozoa and not with accessory gland fluids.

Research was conducted to test the hypothesis that cellular stress results in intracellular substances being released under differ-

[1] Scientific Journal Series Paper No. 7148, Minnesota Agricultural Experiment Station.

ent conditions and to correlate the amount of release of enzyme with the fertilizing capacity of bovine spermatozoa. Mann (1964) showed a detrimental effect on spermatozoa by centrifugation. Different concentrations of glycerol afforded different degrees of protection to the cell for motility and fertility (Erickson, Graham, and Frederick, 1954; Miller and VanDemark, 1954; Cragle *et al.*, 1955; and Saroff and Mixner, 1955). The rate of thawing frozen semen had little effect on motility and fertility (Hafs and Elliott, 1954; and Pickett, Hartig, and Bean, 1962). Sullivan and Mixner's review (1963) showed only small effects on motility and fertility by varying the length of time the semen was equilibrated with glycerol.

These stress effects were used in an attempt to establish their relationship to GOT loss. GOT was chosen as the indicator because of its stability. Pace (1968) concluded that little change in GOT activity was found when the enzyme diluted with egg yolk buffer was frozen at various rates, thawed at various rates, or when the samples were repeatedly frozen and thawed.

It should be strongly emphasized that GOT is used only as an indicator as to what may be occurring to the cell and should not be mistaken for the enzyme necessary for fertility.

MATERIALS AND METHODS

GOT analysis. To establish the amount of enzyme in the seminal plasma at any given period, sperm cells were removed from extracellular medium by filtering through glass fibers and cellulose powder (Graham and Pace, 1967).

Before semen was stressed, one sample was filtered and identified as "pre-treatment release." This was the base line control. Another sample was plunged into liquid nitrogen and was identified as "maximum releasable GOT." Semen frozen at a slower rate (5°/min) was referred to as "normal freezing."

Frozen samples were thawed in 5° water one-half hour before analysis and then were filtered to remove the sperm cells.

GOT analysis was conducted using the Technicon Auto Analyzer methodology test N-25. GOT reacts with substrate to produce oxaloacetic acid which then reacts with a diazonium salt, 6-benzamido-4-methoxy-*m*-tolidine diazonium chloride to produce color.

The enzyme was measured in international units (IU) of enzyme/ml. This value was then converted to IU/10^9 spermatozoa.

Statistical analysis. Analysis of variance was used to determine significant differences among treatments. Tukey's procedure as described by Steel and Torrie (1960) was used in making comparisons among treatment means at the 5% level. The computed value was then centered around the treatment means on bar graphs. Treatment means were considered to be significantly different if no overlapping occurred. Simple correlation coefficients were determined to test the interrelationship between the amount of GOT released and fertility.

Sources of semen. To distinguish cellular GOT from seminal plasma GOT, semen from each of 33 Holstein bulls was diluted one part semen to one part Tris–Tricine buffer (5.18 g Tricine, 0.227 g TRIS per 100 ml H_2O; pH 6.8; 325 milliosmols/liter). An aliquot of the diluted semen was filtered immediately, and the filtrate was placed into liquid nitrogen; the remainder of the semen was plunged directly into liquid nitrogen and stored until analyzed.

To determine the amount of seminal plasma GOT, semen from a 2-year-old Holstein bull was collected and placed in liquid nitrogen and then a bilateral corpus epididymectomy was performed. Approximately three-fourths of an inch of the corpus epididymis was removed. The proximal and distal ends of the remaining epididymis were ligated with catgut. Ejaculates were taken from the bull until the tract was free of spermatozoa and then at varying intervals to 380 days after the operation. All samples were analyzed for GOT.

To determine if stress due to centrifugation resulted in the release of GOT, semen from three bulls was extended one part semen to five parts Minn GO buffer (Rajamannan, Graham, and Smith, 1964). The extended semen was placed in four centrifuge tubes and centrifuged at 1,470; 3,900; 12,000, and 21,500*g* for 10 min. The samples were then filtered, and the extracellular media was placed in liquid nitrogen until analyzed.

To determine if the concentration of cryophylactic agents affects the release of GOT, semen from three bulls was extended one part of semen to twenty parts of Minn GO buffer containing glycerol or ethylene glycol at final concentrations of 0, 1, 4, 7, 10, or 15% v/v. The extended semen was allowed to equilibrate 10 hr with the extender at 5° after which pretreatment and maximal release samples were obtained along with a sample which had been normally frozen in hermetically sealed ampules (0.8 ml per ampule).

To determine if the rate of freezing affects the release of GOT, semen from 21 Holstein bulls was placed into Tris–Tricine buffer with 20% egg yolk and 7% glycerol (final concentration) at the rate of one part semen to two parts buffer. The buffered semen was equilibrated 4 hr with the extender at 5° after which pretreatment and maximal release samples were obtained along with samples which had been normally frozen in nitrogen vapors.

To determine if the length of equilibration time affects the release of GOT, semen from three bulls was extended 1 part of semen to 20 parts of Minn GO buffer with a final concentration of 7% glycerol. Pretreatment and maximal release values were immediately obtained. The remaining semen was frozen normally at 3, 5, 8, 13, or 23 hr after collection.

To determine if thawing conditions affect the GOT release, semen was extended and glycerol added as above, ampuled, and then pretreatment and maximal release values were immediately obtained. After a 10-hr equilibration at 5° the remaining semen was normally frozen. At the time of GOT analysis a sample of normally frozen semen from each bull was thawed at each of the following temperatures; 5, 37, and 50° waterbaths and at 22° room air.

To determine the correlation between GOT and fertility, 98 samples of semen were obtained from 35 Holstein bulls in service which had an average fertility of 71% with a range from 60–77% fertility on a 60- to 90-day nonreturn. The samples were diluted with Minn GO buffer and cooled at 5° at approximately ½°/minute. Additional buffer containing glycerol was then added to the semen over a half-hour period resulting in a final glycerol concentration of 7%. The final sperm cell concentration was 36×10^6/ml. After the final dilution, semen was placed into 1.0-ml ampules (0.8 ml/ampule), sealed and allowed to equilibrate with the extender for 6 hr. At the end of the equilibration period semen from each collection was used to obtain pretreatment and maximal release values along with samples which had been normally frozen. The GOT values from each bull's semen were then correlated to the bull's average fertility. Fertility was not obtained on each collection of semen since the number of breeding units would have been too low to obtain an accurate estimate of fertility. Therefore, it was assumed that the bull's average fertility was a more accurate estimate of fertility than obtaining an estimate with only a few first services.

RESULTS AND DISCUSSION

The pretreatment mean release from 33 samples of bull semen diluted 1:1 with Tris–Tricine buffer was 127 IU GOT/10^9 spermatozoa. The maximal mean release was 307 IU GOT/10^9 spermatozoa. The difference between these means was highly significant ($p \leqslant .01$). The correlation between the maximal amount of GOT released and sperm cell concentration was $r = .80$. Hence most of the GOT came from the spermatozoa.

The data from an epididymectomized bull confirmed that the GOT was associated with the cell. Prior to surgery the semen contained 800 IU GOT/ml. After epididymectomy, seminal plasma GOT activity declined rapidly (Table 1) for 12 days and then stabilized at approximately 20 IU GOT/ml. At day 12, sperm could no longer be found in the ejaculated fluid. Therefore, GOT was mainly associated with the sperm cell and amounts found in seminal plasma arose mainly as the result of leakage from the sperm cell. This agrees with the work of Flipse (1960), Roussel and Stallcup (1965) and Graham and Pace (1967).

Factors Affecting the Release of GOT

Centrifugation. Figure 1 shows that increasing gravitational force on sperm cells caused an increase in GOT in the extracellular media above that of pretreatment release. The amount of GOT released by centrifugation at 12,000 or 21,500 *g* was significantly different from that of the pretreatment release

TABLE 1

QUANTITATIVE ANALYSES OF GOT/ML OF EJACULATED FLUID BEFORE AND AFTER A BILATERAL CORPUS EPIDIDYMECTOMY OF A BULL

Days before and after	IU GOT/ml
7 (before)	800
9 (after)	180
10 (after)	76
12 (after)[a]	17
16 (after)[a]	27
104 (after)[a]	22
178 (after)[a]	23
237 (after)[a]	15
380 (after)[a]	19

[a] Seminal plasma free of spermatozoa.

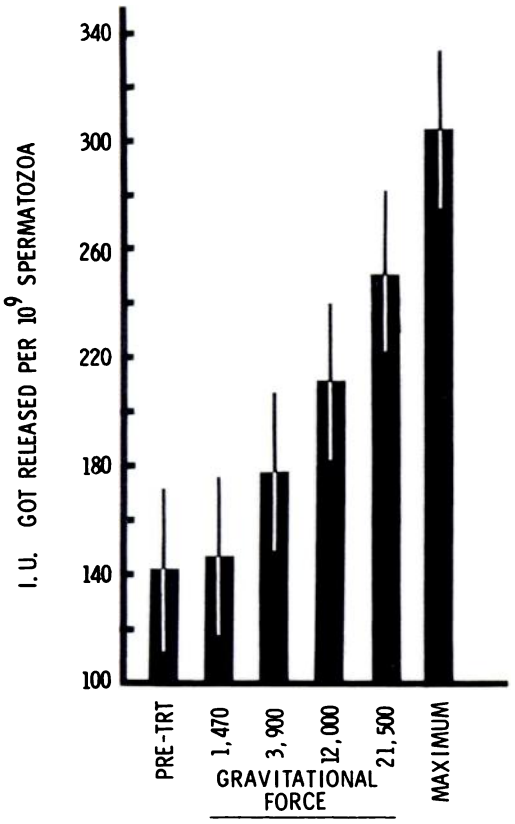

FIG. 1. The effect of gravitational force on the release of GOT from bovine spermatozoa into the extracellular media as compared to controls (pretreatment and maximum releasable GOT). The Tukey's test values shown by narrow bars indicate significance if no overlapping occurs.

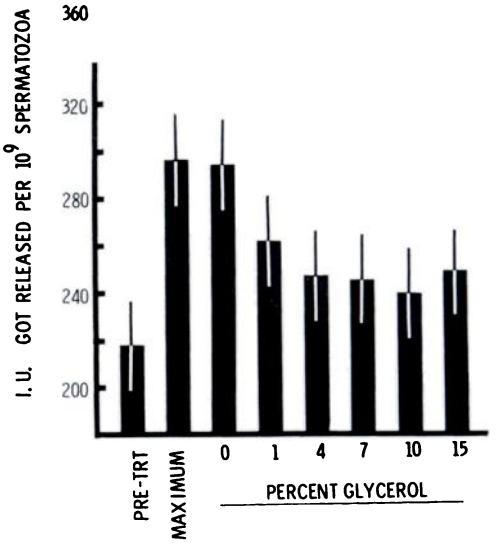

FIG. 2. The effect of normal freezing in different concentrations of glycerol on the release of GOT from bovine spermatozoa into the extracellular media as compared to controls (pretreatment and maximum releasable GOT). The Tukey's test values shown by narrow bars indicate significance if no overlapping occurs.

value ($p \leqslant .05$). The amount of GOT released by centrifugation at 21,500 *g* was not significantly different from that of the maximal release value. This indicated that high gravitational forces cause extensive release of GOT by the spermatozoa. This agrees with Mann (1964) whose results showed that centrifugation increased the amount of another enzyme (cytochrome *c*) in the seminal plasma. In addition, results indicate that so-called normal values for GOT in semen will be in error if centrifugation at high gravitational forces is used to separate sperm from seminal plasma. Mann (1964) also agreed by stating that seminal plasma protein results obtained through the use of centrifugation at high speeds should be looked at with suspicion as to actual values. Even though the GOT value at 1470 *g* was about the same as the pretreatment value, centrifugation at this low speed did not remove all of the sperm cells; therefore, filtering was still necessary.

Cryophylactic agents. Figure 2 shows the amount of GOT found in the extracellular media after the sperm cells were frozen normally in different concentrations of glycerol. Pretreatment and maximal values were given as reference points to indicate the relative amount of release that had taken place. At 4, 7, 10, and 15% glycerol the loss of GOT was significantly less ($p \leqslant .05$) than in samples which contained a lesser concentration of glycerol. The use of ethylene glycol in place of glycerol showed similar results.

Approximately 7% glycerol has proved to be the optimal concentration for motility and

fertility as shown by Erickson *et al.* (1954); Miller and VanDemark (1954); Cragle *et al.* (1955), and Saroff and Mixner (1955). Saacke and Almquist (1962) have found that sperm frozen without glycerol showed loss or damage of the cell membranes of the midpiece while sperm frozen in 10% glycerol had membranes intact as viewed by electron microscopy. This could indicate that the increased GOT losses found may be related to damage.

Rate of freezing. Extracellular media containing spermatozoa from 21 Holstein bulls contained 331 IU/10^9 spermatozoa upon measuring pretreatment release, 423 IU after maximal release, and 381 IU after normal freezing in the nitrogen vapors. Both of the frozen samples were significantly higher ($p \leqslant .01$) than the pretreatment release samples. The difference in GOT release between maximal release and normal freezing was also significant ($p \leqslant .01$).

Equilibration time. Figure 3 depicts the amount of GOT released during normal freezing after allowing the semen to equilibrate for 3, 5, 8, 13, and 23 hr. There was a trend showing increased release of GOT from the sperm cells as the equilibration time increased but the increase was not great enough to show statistically significant differences ($p \leqslant .05$). This agrees with the general findings of other investigators using different methods of assessment. Using motility as the criterion for semen quality Sullivan and Mixner (1963) found little difference in the postthaw motility of semen equilibrated from 1 to 24 hr in glycerol. Graham, Erickson, and Bayley (1957) found only small differences in fertility of semen equilibrated for 4, 8, or 12 hr.

Thawing conditions. Figure 4 shows that thawing samples in 22° air, 0, 5, or 37° water change very little the amount of GOT released into the extracellular medium. The differences were not statistically significant. It was concluded that thaw rate has little effect on GOT release. Beisang *et al.* (1969) in a more extensive study on thaw rates of bovine semen found a similar pattern showing no significant differences ($p \leqslant .05$) in GOT release when the thaw rate varied from ice water temperatures to 600°/min in a microwave oven. Hafs and Elliott (1954) and Pickett *et al.* (1962) using fertility and motility as methods of assessment found no differences in semen thawed at temperatures from 5 to 40° indicating little difference in the amount of damage taking place during thawing.

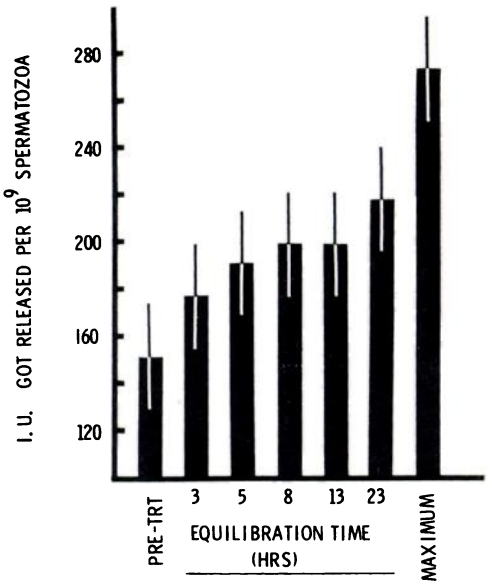

FIG. 3. The effect of equilibration time at 5° on the release of GOT from bovine spermatozoa into extracellular media containing 7% glycerol after normal freezing as compared to controls (pretreatment and maximum releasable GOT). The Tukey's values shown by narrow bars indicate significance if no overlapping occurs.

From the above results it was concluded that GOT is released from the cell in proportion to the amount of damage occurring. More extensive work is needed with gravitational force, cryophylactic agents, and equilibration time to find out which would be the best combined conditions for the sperm cell.

Effect of GOT Release on Fertility

Table 2 shows the release of GOT from the sperm cells of 98 samples of semen from 35

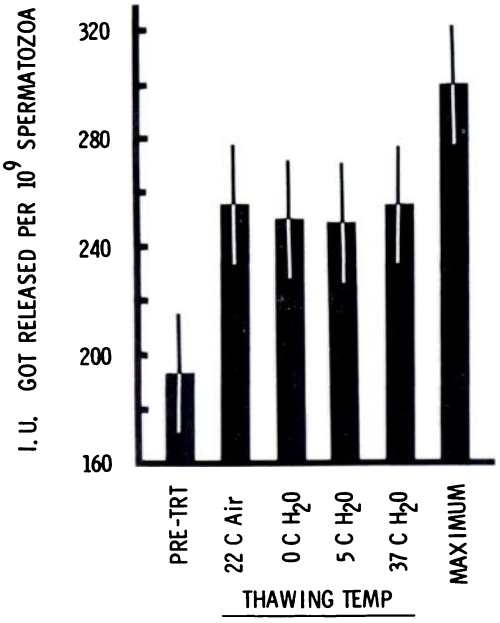

FIG. 4. The effect of thawing temperatures on the release of GOT from frozen bovine spermatozoa into the extracellular media containing 7% glycerol as compared to controls (pretreatment and maximum releasable GOT). The Tukey's test values shown by narrow bars indicate significance if no overlapping occurs.

TABLE 2

MEAN AND STANDARD DEVIATIONS OF GLUTAMIC OXALOACETIC TRANSAMINASE (GOT) IN THE EXTRACELLULAR MEDIA OF 98 SAMPLES OF EXTENDED SEMEN FROM 35 HOLSTEIN BULLS AFTER VARIOUS TREATMENTS AND THEIR CORRELATION TO FERTILITY[a]

Treatment	Mean IU GOT/10^9 spermatozoa	SD	Correlation with fertility
Pretreatment release[b]	232	±67	.09
Maximal release[c]	403	±80	.21*
Normal freezing[d]	310	±71	.11
Maximal release–normal freezing[e]	93	±43	.25

[a] The mean fertility of the 35 bulls was 71% with a range from 60 to 77%.

[b] Enzyme released before stress treatment was started.

[c] Release of enzyme by exerting the maximal stress.

[d] Release of enzyme due to all manipulations through the freezing and thawing process.

[e] The amount of releasable GOT left in the cell after normal freezing.

* Significant ($p \leqslant .05$).

Holstein bulls after different treatments. A correlation between the total amount of GOT released from the cell and fertility ($r = .21$) was significant ($p \leqslant .05$). This would indicate that the sperm cells containing higher amounts of GOT have a higher fertilizing capacity.

Another correlation was determined between the amount of GOT left in the cell after normal freezing (maximum release—normally frozen) and fertility. A significant correlation ($r = 0.25$, $p \leqslant .05$) was found. The GOT left in the cell might give an indication as to what is happening to other enzymes which are in or on the cell that may be important to fertility.

A correlation between the percentage of GOT lost from the sperm cell before freezing (pretreatment release/maximum release) and fertility was established. The correlation was —0.26 which was highly significant ($p \leqslant .01$). This shows that the amount of prefreeze release is important to fertility.

These correlations are not sufficiently high to be used in predicting the fertility of a bull. These correlations are, however, as high as those between motility and fertility as given by Salisbury and VanDemark (1961). It is suspected that there is a difference between ejaculates in their fertilizing capacity and are now attempting to study this (unpublished observation). If this is established, a more meaningful correlation with fertility may be obtained.

It can be concluded that (1) most of the GOT is found in or on the sperm cell initially and not in the seminal plasma; (2) the measurement of cellular injury can be monitored using GOT release; (3) the higher the concentration of GOT/cell the higher the

fertility; and (4) the amount of enzyme left in the cell after freezing is important to fertility.

ACKNOWLEDGMENTS

Gratitude is expressed to the North Central Breeders Research Council for their financial support and help in obtaining the data for this research.

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