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SHORT COMMUNICATION

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Influence of γ-glutamyltransferase and alkaline phosphatase activity on *in vitro* fertilisation of bovine frozen/thawed semen

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ABSTRACT

The aim of this work was to evaluate whether the residual amount of γ-glutamyl-transferase (GGT) and alkaline phosphatase (ALP) in bovine sperm after freezing/thawing is correlated with fertility parameters, including blastocyst rates after *in vitro* fertilisation (IVF). The enzyme activities were determined in both spermatozoa and supernatant after centrifugation. While ALP was only correlated with sperm viability, GGT activity was correlated with sperm motility ($r_s = .4$; $p < .05$) both in sperm and supernatant. Interestingly, GGT activity was also correlated with cleavage ($r_s = .5$; $p < .05$ and .8; $p < .01$, for sperm and supernatant respectively) and blastocyst ($r_s = .6$ and .9, for sperm and supernatant respectively; $p < .01$) rates obtained after IVF. These results suggest that GGT could play an important role in the protection of sperm against oxidative stress and could be considered a reliable marker to assess frozen/thawed sperm quality in bovine.

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Enzyme determination; fertility test; *in vitro* fertilisation

Introduction

The great variability in fertility among bulls strongly affects the efficiency of Artificial Insemination and *in vitro* embryo production. Laboratory tests, routinely performed in a Semen Collection Center, do not enable the determination a priori of fertility. A more accurate estimation of semen fertility is currently achieved by increasing the number of tests performed in the laboratory. The identification of male fertility markers is, therefore, fundamental to improve the selection of males for breeding.

In cattle, fertility-associated proteins such as osteopontin (Cancel et al. 1997) and lipocalin-type prostaglandin (PG) D synthase (Gerena et al. 1998) have been identified. It has been reported that lactic dehydrogenase may represent an accurate marker for semen quality in stallions (Pesch et al. 2006). Furthermore, older studies showed that alkaline phosphatase is suitable for differentiation of oligo- and azoospermia in stallions (Turner & McDonnell 2003).

In standard *in vitro* fertilisation (IVF) procedure frozen-thawed sperm is commonly used but it is known that freezing may determine several types of sperm damages including enzyme leakage. Pace and Graham (1970) demonstrated that the residual amount of glutamic oxaloacetic transaminase (GOT) after freezing was correlated with *in vivo* bull fertility. To our knowledge, no information are available about the influence of residual concentrations of gamma glutamyl transferase (GGT) and alkaline phosphatase (ALP) after freezing/thawing and bovine semen fertility.

Therefore, the aim of this work was to investigate whether the concentration of GGT and ALP in frozen/thawed bovine sperm is correlated to various fertility parameters, including the *in vitro* fertilising capability, evaluated in terms of cleavage and blastocyst rates after IVF.

Materials and methods

The study was carried out on frozen/thawed semen from eight adult fertile bulls (three ejaculates/bull) housed at the Semen Collection Center "CO.FA" (Cremona, Italy). After thawing, the total and progressive motility were evaluated by using CASA. In addition to this, sperm were fixed and stained with Trypan blue-Giemsa as reported by Boccia et al. (2007). Sperm cells were microscopically evaluated at

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40 × magnification (Nikon Diaphot 300), to assess the incidence of viable cells with intact acrosome.

Furthermore, IVF was carried out on abattoir-derived cumulus-oocyte-complexes that were *in vitro* matured, fertilised and cultured according to our standard procedure (Sattar et al. 2011). In particular, frozen-thawed sperm from each ejaculate were separated by Percoll gradients and used at the concentration of 1×10^6 sperm/mL to inseminate on average 225 oocytes, over 4 replicates. Cleavage and blastocyst rates were recorded on day 7 (day 0 = IVF).

Frozen semen straws were thawed in a water bath at 37 °C for 30 s and centrifuged at 1000*g* for 10 min to separate sperm cells from the supernatant. Sperm cells were lysed in CHAPS lysis buffer (150 nM KCl, 50 mM HEPES, 0.1% CHAPS, protease inhibitor, pH 7.4 (protease inhibitor kit, Thermo Scientific) and centrifuged using 1.5 mL microcentrifuge tubes (USA Scientific). The activity of GGT and ALP was measured in supernatant and in spermatozoa with a kinetic procedure using Spinreact Reagents (Spinreact, SA, St. Esteve de Bas Girona, Spain) and the absorbance read with a spectrophotometer (Thermo Scientific, BIOMED 6, Waltham, MA) (Pero et al. 2010). In order to eliminate the differences in sperm concentration among samples, the values were corrected for the dilution factor used, to give the final concentration of 5×10^7 sperm/straw for each sample. The concentration of enzyme activity was expressed in U/L.

Results and discussion

As depicted in Table 1, differences in enzyme concentration between spermatozoa and supernatant were analysed by the Mann–Whitney Rank Sum Test. Correlations between different parameters were analysed by the Spearman rank correlation coefficient (r_s). The average cleavage and blastocyst rates (over 4 replicates) per each ejaculate were correlated with the corresponding enzyme levels per straw.

All fertility parameters showed a certain degree of variation but the greatest variation was recorded for blastocyst rate (ranging from 3.3% to 50.9%). As already demonstrated by Roussel and Stallcup (1966) a high variability in enzymatic activity was also recorded among straws and the concentrations of GGT and ALP in the supernatant were higher ($p < .001$ and $p < .05$, respectively) than in sperm cells (Table 1).

The GGT activity in both cells and supernatant was significantly correlated with total motility ($r_s = .45$, $p < .05$ and $r_s = .41$, $p < .05$; respectively), cleavage ($r_s = .47$, $p < .05$ and $r_s = .80$, $p < .0001$; respectively) and blastocyst ($r_s = .60$, $p < .01$ and $r_s = .87$, $p < .0001$; respectively) rates. The ALP activity both in sperm cells ($r_s = .43$; $p < .05$) and supernatant ($r_s = .51$; $p < .05$) was only correlated with sperm viability. Since the high variability and the limited number of samples may affect the power of statistical analysis, in Table 2 data are presented in a descriptive manner to show the relationship between enzyme activity and *in vitro* fertility for each bull.

The high concentrations of GGT and ALP found in both sperm cells and supernatant confirm that the enzymes play a role in sperm function and suggest that their activity may be also critical to guarantee frozen/thawed sperm quality. It is possible to speculate that GGT plays an important role in the protection of spermatozoa from oxidative stress because of the positive correlation found in this study between GGT activity and sperm motility. These results are in agreement with previous studies carried out in stallion (Pesch et al. 2006; Dogan et al. 2009). It was also reported a positive correlation among GGT and cleavage and blastocyst rates after IVF, supporting the hypothesis that this enzyme could be considered a fertility marker for frozen/thawed bovine semen. Moreover, it is

Table 1. Enzymatic activities (U/L) in semen samples.

	Sperm cells			Supernatant		
	Mean ± SE	Min	Max	Mean ± SE	Min	Max
GGT	575.2 ± 50.1^{A}	80.0	1032.1	2884.7 ± 262.8^{B}	727.6	5229.8
ALP	1739.6 ± 177.0^{a}	639.8	3959.1	2779.4 ± 380.7^{b}	714.1	8732.7

^{a,b}Values with different superscripts are significantly different; $p < .05$.
^{A,B}Values with different superscripts are significantly different; $p < .001$.

Table 2. Percentages of motility, viability, cleavage, blastocysts and GGT and ALP activities in sperm cells (SC) and surnatant (S) in bovine frozen-thawed semen samples (means of the 3 ejaculates per 8 bull).

Bulls	Motility Mean ± SE	Viability Mean ± SE	Cleavage Mean ± SE	Blastocysts Mean ± SE	GGT (SC) Mean ± SE	ALP (SC) Mean ± SE	GGT (S) Mean ± SE	ALP (S) Mean ± SE
1	46.3 ± 2.7	70.1 ± 3.5	67.0 ± 4.2	20.8 ± 6.7	34.5 ± 8.4	136.6 ± 45.5	165.9 ± 32.9	232.0 ± 50.3
2	37.0 ± 3.8	81.2 ± 2.3	76.7 ± 2.9	31.0 ± 2.8	27.4 ± 1.8	76.3 ± 10.0	109.6 ± 10.5	121.9 ± 33.4
3	43.3 ± 4.5	74.2 ± 9.0	72.8 ± 5.8	25.4 ± 4.1	34.7 ± 5.3	118.6 ± 12.6	106.7 ± 14.7	250.6 ± 76.9
4	47.3 ± 3.2	78.1 ± 3.1	70.7 ± 5.7	29.0 ± 7.5	25.2 ± 8.7	128.9 ± 12.1	103.5 ± 5.3	94.6 ± 23.6
5	36.7 ± 3.4	75.3 ± 3.8	71.2 ± 7.3	18.2 ± 7.4	20.2 ± 3.6	81.6 ± 14.8	68.0 ± 4.1	165.9 ± 38.3
6	36.3 ± 3.8	59.7 ± 5.6	71.1 ± 1.0	24.5 ± 1.3	19.1 ± 2.5	56.1 ± 14.9	75.5 ± 22.8	44.5 ± 8.3
7	42.3 ± 3.7	76.3 ± 1.7	68.7 ± 2.0	22.2 ± 6.1	28.2 ± 8.0	77.1 ± 27.8	128.4 ± 37.1	240.8 ± 91.7
8	38.3 ± 5.2	68.2 ± 3.8	72.5 ± 3.4	25.9 ± 0.2	28.0 ± 6.8	51.9 ± 7.7	93.7 ± 19.8	73.0 ± 25.0

known that GGT is involved in the transfer of amino acids across the cellular membrane and in glutathione metabolism by transferring the glutamyl moiety to a variety of acceptor molecules including water, certain L-amino acids, and peptides, leaving the cysteine product to preserve intracellular homeostasis of oxidative stress (Alvarez & Storey 1989). It has been suggested that GGT also play an important role in the transfer of the glutathione residues to reactive groups on the sperm surface (Agrawal & Vanha-Perttula 1988). Therefore, the beneficial effect observed on sperm may be due to GGT protective activity against oxidative stress. However, it would be worth investigating in future studies on the relation between GGT activities and oxidative stress parameters.

A relation between ALP and different fertility parameters has been reported in fresh semen in other species (Turner & McDonnell 2003). On the contrary, our results showed that ALP activity in bovine frozen/thawed semen is only correlated with sperm viability, whereas no relationship was found with cleavage and blastocyst rates after IVF. This suggests that in cattle this enzyme should not be considered a suitable tool to predict frozen-thawed sperm fertility.

Conclusions

In conclusion, it was demonstrated that the residual GGT activity after freezing is correlated with the *in vitro* fertilising ability of bovine sperm. Indeed, measurement of GGT in frozen-thawed bovine sperm is a reliable method to predict the embryo output after IVF. Therefore, because of the availability of fast and simple tests for enzyme assay, measurement of GGT could represent a useful tool for a fast determination of frozen/thawed sperm quality, before enrolling bulls in IVF programmes.

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Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing this article.

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