

Effect of IGF-I on spermatozoa membrane protein profile and correlation between seminal plasma IGF-I and antioxidant enzymes in buffalo semen

S SELVARAJU¹, T SIVA SUBRAMANI², B S RAGHAVENDRA³ and J P RAVINDRA⁴

National Institute of Animal Nutrition and Physiology, Bengaluru, Karnataka 560 030 India

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ABSTRACT

The present study assessed the effect of insulin like growth factor-I (IGF-I) on spermatozoa membrane protein profile and relationship between IGF-I and antioxidant levels in buffalo (*Bubalus bubalis*) semen. Immediately after semen collection (n = 8), the seminal plasma and spermatozoa cells were separated by centrifugation (5000 g/10 min) and the seminal plasma IGF-I and antioxidant enzymes estimations were carried out. The spermatozoa were diluted (20 million cells/ml) in a tris-egg yolk extender, IGF-I (100 ng/ml of the semen) was added and incubated at 37°C for 2 h. Membrane proteins were extracted with 0.1% Triton X 100 and protein profile was carried out by denaturing gel electrophoresis. No change in membrane protein profile was observed in spermatozoa treated with IGF-I as compared to control. The mean buffalo seminal plasma IGF-I, superoxide dismutase (SOD) and glutathione peroxidase (GPx) concentrations were 139.07±23.61 ng/ml (range: 51.45 to 267.43), 0.38±0.03 Unit/mg of protein (range: 0.16 to 0.74) and was 168.15±35.54 IU/mg of protein (range: 77 to 401), respectively while the total protein concentration (G %) was 2.89±0.20 (range: 0.88 to 5.27). IGF-I had no significant, correlation with SOD (r = -0.17) and GPx (r = -0.38). The correlation between SOD and GPx was significant and positive (r = 0.77). The present study revealed that IGF-I had no correlation with SOD and GPx levels and its addition did not influence spermatozoa membrane protein profile in a spermatozoa incubated for 2 h.

Key words: Buffalo, GPx, IGF-I, SOD, Spermatozoa membrane protein

IGF-I has been suggested to be one of the important factors for the germ cell development, maturation and motility of the spermatozoa (Vickers *et al.* 1996). IGF-I has been reported to play a role in maintaining spermatozoa motility and viability in bulls (Henricks *et al.* 1998, Gancarczyk *et al.* 2006) and buffalo bulls (Selvaraju *et al.* 2008) possibly through its antioxidant effect. The semen cryopreservation process and reactive oxygen species cause alterations in some protein molecules that influence the metabolism and the fertility characteristics of the ejaculate (Killian *et al.* 1993). Since antioxidants protect spermatozoa membrane proteins, addition of IGF-I may protect spermatozoa protein profile. Perusal of literature revealed that no information is available on the influence of IGF-I on buffalo spermatozoa membrane protein profile. Also correlation among seminal plasma IGF-I, SOD and GPx has not been reported in detail. Hence the objectives of the present study were to assess the effect of

IGF-I on spermatozoa membrane protein profile and correlation among seminal plasma IGF-I, SOD and GPx levels in buffaloes.

MATERIALS AND METHODS

Ejaculates were collected from Surti buffalo bulls (n = 8) using artificial vagina. Four ejaculates from each bull were collected. The ejaculates with a concentration of more than 500 million spermatozoa per ml and more than 60 per cent progressive forward motility were used for the experiment. Fresh semen (0.5 ml) was centrifuged at 5 600 g for 10 min at 4°C. The seminal plasma was separated and stored at -20°C until further analysis. The samples were analysed and mean values for each parameters for a particular bull were used for analysis.

Experiment I: Effect of IGF-I on membrane protein profile: The spermatozoa diluted (20 million cells/ml) in tris-egg yolk extender were added with physiological concentration of IGF-I (100 ng/ml of the semen) and incubated at 37°C for 2 h. After incubation, the spermatozoa were washed 3 times 560 g for 5 min at 4°C) with 1 mL of tris-calcium chloride buffer (TC buffer, 40 mM Tris, 2 mM

Present address: ¹Scientist (SS) (e-mail: selvarajuars@rediffmail.com), ^{2,3}Research Fellows (e-mail: dnasiva@gmail.com, raghu0077_2002@yahoo.co.in), ⁴Principal Scientist (e-mail: jpravindra@yahoo.com), Animal Physiology Division.

CaCl₂, 1 mM PMSF, 0.01% sodium azide, pH 7.3). The spermatozoa cells were re-suspended with 1 mL of TC buffer with 0.1% Triton X-100 and incubated for 1 h at 4°C. During incubation, the samples were vortexed once in 10 min. At the end of incubation, samples were centrifuged at 5600 g for 30 min to pellet cellular debris. The supernatant containing spermatozoa membrane protein was dialysed using dialysis membrane (10 Kda cut off). The proteins were precipitated with 9 volumes of cold ethanol and vortexed once in 15 min for min at 4°C. The precipitated proteins were then recovered by centrifugation at 560 × g for 10 min. After 3 subsequent ethanol washes, the precipitates were solubilised in distilled water. Denaturing SDS-PAGE was carried out (4% stacking gel and 15% separating gel acrylamide) and stained with silver staining.

Experiment 2: Correlation between seminal plasma IGF-I, SOD and GPx

IGF-I concentration: Seminal plasma IGF-I concentration was assessed by immuno-radiometric assay kit according to the manufacturer's protocol. The assay was validated for parallelism by using different dilutions of a seminal plasma sample and also by adding different concentrations of standard given in the kit. As per the information given by the manufacturer, the assay had a sensitivity of 2 ng/ml. All samples were assayed at the same time and the intra assay coefficient of variation was 3.26%.

Total protein: Bicinchoninic acid (BCA) method was used for the quantitative estimation of total protein using the protein estimation kit.

Superoxide dismutase: SOD activity in the seminal plasma was determined as per Nishikimi *et al.* (1972). Seminal plasma (50 µl) was added to 2.6 ml of phosphate buffer (0.017 M, 8.3 pH at 20°C), 0.1 ml of phenazine methosulphate (0.093 mM) and 0.1 ml of nitro blue tetrazolium (1.5 mM). The reaction was started by adding 0.1 ml of NADH (2.34 mM) and the solutions were mixed well. The increase in absorbance at 560 nm was taken for 2 min at 30 sec intervals using double beam UV spectrophotometer. Unit of SOD was defined as activity of enzyme required for suppressing the increase in absorbance by 50%. SOD is expressed as Unit/mg of seminal plasma protein.

Glutathione peroxidase: GPx activity in the seminal plasma was determined as per Hafeman *et al.* (1974). Briefly, the enzyme assay mixture consisted of 1 ml GSH (2.0 mM), 1.0 ml sodium phosphate buffer (0.40 M, pH 7.0 also containing 0.4 mM EDTA), 0.5 ml sodium azide (0.01 M), 0.2 ml seminal plasma and distilled water to make up the volume to 4.0 ml. The enzyme assay tubes were incubated at 37°C for 5 min. To this, 1.0 ml of 1.25 mM H₂O₂ (prewarmed to 37°C) was added. After 3 min interval 1 ml of incubation mixture was removed and add with 4 ml of meta phosphoric acid precipitation solution [Metaphosphoric acid

solution: 1.67 g glacial metaphosphoric acid (a mixture of HPO₃ and NaPO₃), 0.2 g EDTA and 30 g NaCl to 100 ml of double distilled water]. To the 2 ml of the above filtrate, 2 ml of 0.4 M disodium hydrogen phosphate and 1 ml of 5, 5-dithiobis -2-nitrobenzoic acid [40 mg 5,5-dithiobis (2-nitrobenzoic acid) in 100 ml of 1% trisodium citrate] was added. After mixing thoroughly, the absorbance was recorded at 412nm within 2 min. A blank (0.15 M KCl substituted for enzyme source) was carried through the incubation simultaneously with the samples since nonenzymatic GSH oxidation by H₂O₂ occurred during incubation. An enzyme unit of activity was defined as a decrease in the log [GSH] of 0.001 per min, after the decrease in log [GSH] per minute of the nonenzymatic reaction was subtracted.

Statistical analysis: Statistical analysis was performed by using the SPSS software. Pearson's correlation coefficients were determined to provide a measure of linear association between the IGF-I, SOD and GPx results. All values are expressed as means ±SE. A probability of P<0.05 was set as significance level.

RESULTS AND DISCUSSION

Since the proteins present on the spermatozoa membrane take part during zona binding and penetration, the role of IGF-I on maintaining spermatozoa membrane protein was examined in the present study. Analysis of spermatozoa membrane protein revealed 23 proteins of various molecular weight ranging from 19 kDa to 137 kDa (Fig. 1). Soluble and structural proteins have an important role in the spermatozoal metabolism and bull's fertility (Killian *et al.* 1993). The semen preservation process causes alterations in

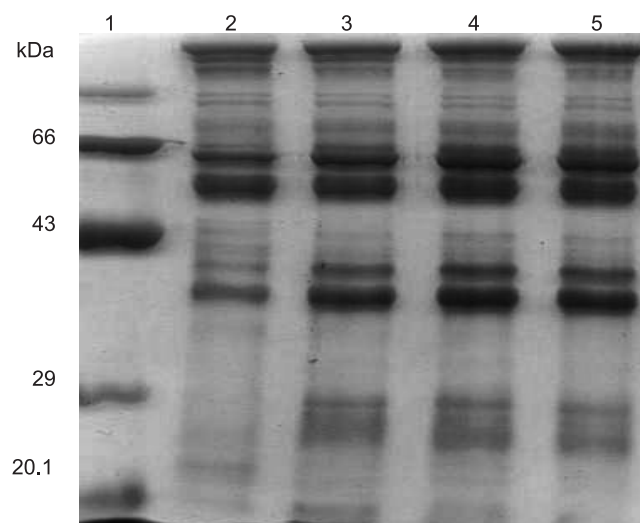


Fig. 1. Assessment of the effect of IGF-I on spermatozoa membrane protein profile in frozen thawed buffalo spermatozoa. (Lane 1: marker, lane 2: frozen thawed sperm, lane 3: spermatozoa incubated without IGF-I for 2 h at 37 °C, lanes 4,5: spermatozoa incubated with IGF-I for 2 h at 37 °C).

some protein molecules that may influence the fertility characteristics of the ejaculate (fertilizing capacity of the spermatozoa from particular ejaculate). Since IGF-I decreases lipid peroxidation levels, the present study was aimed to assess the effect of IGF-I on membrane protein profile. However no difference in spermatozoa membrane protein profile was observed between the control and IGF-I groups might be due to the short incubation period applied for the

spermatozoa. Further 2D-electrophoresis of spermatozoa membrane proteins could confirm the effect of IGF-I on spermatozoa membrane protein profile.

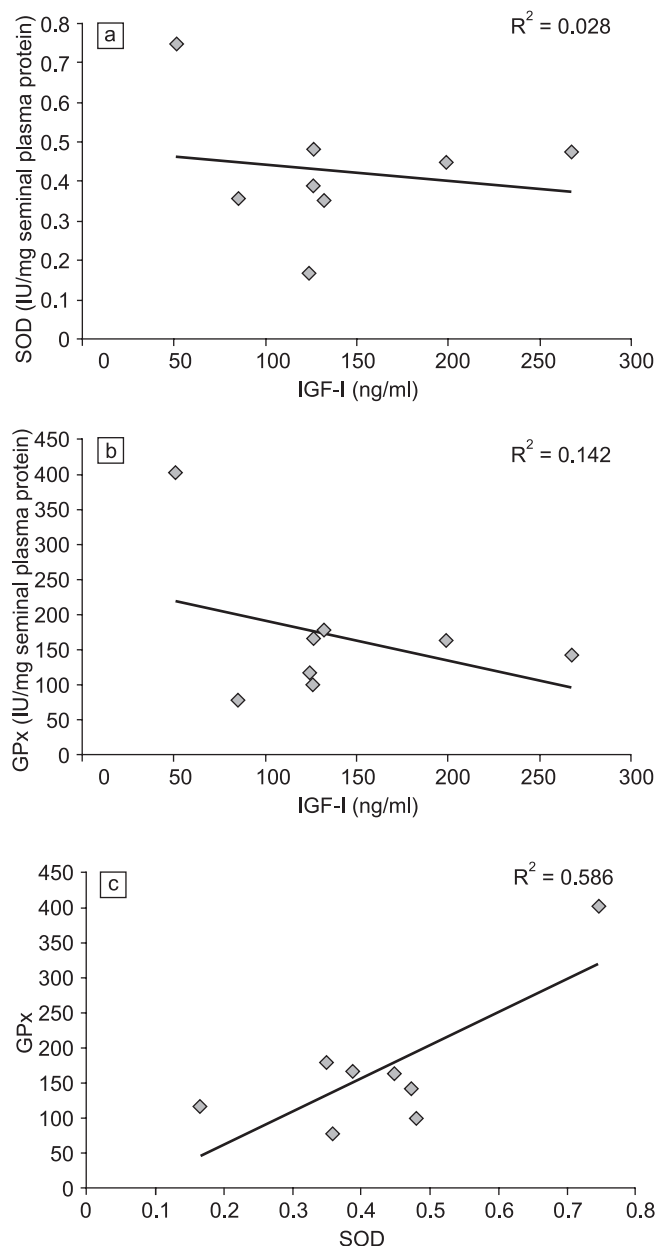
The present study documented the relationship among the seminal plasma IGF-I, SOD and GPx system (Fig. 2a,b,c). The mean buffalo seminal plasma IGF-I concentration (ng/ml) was 139.07 ± 23.61 (range: 51.45 to 267.43). The mean buffalo seminal plasma SOD concentration (Unit/mg of protein) was 0.38 ± 0.03 (range: 0.16 to 0.74). The mean buffalo seminal plasma GPx concentration (IU/mg of protein) was 168.15 ± 35.54 (range: 77 to 401). The mean buffalo seminal plasma total protein concentration (G %) was 2.89 ± 0.20 (range: 0.88 to 5.27). In the present study, lower SOD (0.38 ± 0.03 vs 0.87 ± 0.03 U/mg of protein) but higher GPx (168.15 ± 35.54 vs 29.53 ± 0.11 IU/mg of protein) activity was observed than the earlier report (Nair *et al.* 2006). The variations in the antioxidant levels might be due to different breeds. Further the antioxidant enzymes concentrations in the seminal plasma depend on the levels of stress and the quality of the ejaculate. Diet also influences the antioxidant enzyme levels in the body. Antioxidant enzymes had positive effects on maintaining the motility (Maxwell and Stojanov 1996) and with the concentration of motile spermatozoa (Kobayashi *et al.* 1991).

Since IGF-I act as an antioxidant, correlation between the IGF-I and other seminal plasma antioxidant enzymes was reported in the present study. IGF-I had no significant ($P > 0.05$) correlation with SOD ($r = -0.17$) and GPx ($r = -0.38$). However, IGF-I had positive and small correlation ($P > 0.05$) with total protein ($r = 0.25$). Total protein had negative and moderate correlation with SOD ($r = -0.60$) and GPx ($r = -0.53$). Correlation between SOD and GPx was significantly ($P < 0.05$) positive and moderate ($r = 0.77$). The IGF-I had no correlation with SOD and GPx suggesting that IGF-I secretion might be influenced by independent factors. A positive correlation between SOD and GPx suggested that both are influenced by common factors like copper and zinc intake, oxidative stress etc. IGF-I is synthesized in the Sertoli cells and Leydig cells under the control of their respective gonadotropins, FSH and LH (Lejeune *et al.* 1996). Various factors especially, plan of nutrition, genetic, etc. affecting the gonadotropins secretion and testicular growth may influence IGF-I levels in the seminal plasma.

The present study revealed that IGF-I had no correlation with SOD and GPx levels and its addition did not influence spermatozoa membrane protein profile in a spermatozoa incubated for 2 h.

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Figs 2a–2c. **2a.** Correlation between seminal plasma insulin like growth factor-I (IGF-I) and superoxide dismutase (SOD). **2b.** Correlation between seminal plasma insulin like growth factor-I (IGF-I) and glutathione peroxidase (GPx). **2c.** Correlation between seminal plasma superoxide dismutase (SOD) and glutathione peroxidase (GPx).

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