 Effect of supplementation of glutamic acid and aspartic acid in egg yolk tris and egg yolk-citrate-glucose diluents on cryopreservation of Murrah buffalo bull spermatozoa

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Abstract: Present study was designed to assess the effect of supplementation of glutamic acid and aspartic acid in egg yolk tris and egg yolk-citrate-glucose diluents on spermatozoa quality of Murrah buffalo bull after equilibration and thawing. Semen was collected from four Murrah buffalo bulls biweekly from each bull with the help of artificial vagina (AV) using standard protocol. A total of thirty two ejaculates were collected from four bulls. The fresh ejaculates were pooled and divided into eight equal parts. The two basic diluents used for extending semen were Egg yolk Tris (EYT) and Egg yolk-citrate glucose (EYCG). Glutamic acid (4 mM) and aspartic acid (0.40 mM) were supplemented in different combinations. Diluted semen was equilibrated and cryopreserved. The progressive motility, live and dead spermatozoa and acrosomal damage was assessed after equilibration and thawing. Post thaw acrosomal damage was significantly higher \((P<0.05)\) in EYCG than EYT. Supplementation of aspartic acid to EYT and EYCG significantly \((P<0.05)\) decreased the post thaw acrosomal damage as compared to control. Supplementation of glutamic acid to EYT and EYCG significantly \((P<0.05)\) improved all the studied spermatozoa qualities. The addition of both aspartic and glutamic acid together did not produce any beneficial effect on spermatozoa quality. It can be concluded that tris buffer was a better substitute to citrate buffer and glutamic acid alone may be supplemented to semen extender to improve the spermatozoa quality after equilibration and thawing.

Key Words: Tris buffer, citrate buffer, glutamic acid, aspartic acid, post thaw spermatozoa quality, Murrah buffalo bull

Introduction

Buffalo contribute significantly to the agricultural economy of many developing countries including India. One of the major bottlenecks in optimizing their productivity is the inherent low reproductive efficiency. Semen cryopreservation has become an important tool in assisted reproduction but sub-lethal damage to spermatozoa during freeze–thaw procedures results in poor post-thaw motility and functional characteristic changes in cryopreserved spermatozoa. Reduced fertility of frozen–thawed spermatozoa is mainly due to the changes in the plasma membrane integrity and low antioxidant status that makes them susceptible to oxidative stress and peroxidative damage (Aitken et al. 1998; Chatterjee and Gagnon 2001). It has been demonstrated that there is a decline in motility, viability and forward progression of spermatozoa in frozen–thawed semen in the female reproductive tract which causes a reduction in fertility (Salamon and Maxwell 2000).

Dilution of semen in a suitable buffer is one of the important factors affecting sperm survival during cryopreservation (Rasul et al. 2000). Several studies have tested citrate, Tris or citric acid as buffers for deep-freezing of buffalo spermatozoa (Matharoo and Singh 1980; Chinnavaiya and Ganguli 1980; Ahmad et al. 1986; Dhami and Kodagali 1990; Singh et al. 1991; Dhami et al. 1994; Siddique et al. 2006; Yániz and Santolaria 2012) and reported that tris alone was better buffer than citrate however, combination of tris and citrate in equal proportions provided maximum protection to spermatozoa during cryopreservation. Glutamic acid is quantitatively first major amino acid whereas, aspartic acid is the fourth major amino acid of the seminal plasma (Bustamante and Setchell 2000) of buffalo bull. Glutamic acid has been reported to be effectively used as an additive in the extender of buffalo bull semen (Topraggaleh et al. 2014; Dawra et al. 2015). The increase in aspartic acid level in seminal plasma levels either by dietary supplementation (Talevi et al. 2013) or by direct addition to semen extender (Macchia et al. 2010) improved the spermatozoa quality parameters. In view of the importance of glutamic acid and aspartic acid in spermatozoa protection during cryopreservation the present study was undertaken to assess the effect of addition of glutamic acid and aspartic acid in different...
materials and methods

The experiment was conducted at the district dairy demonstration farm of the College of Veterinary Sciences and Animal Husbandry, Uttar Pradesh Pandit Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalaya Evan Go Anusanndhan Sansthan, Mathura, Uttar Pradesh, India which is located in the semi-arid region of the country at longitude 78°E and latitude 27°N and at an altitude of 176 m above mean sea level.

The experiment was carried out during the month of February and March when the environmental temperature was under thermoneutral zone to avoid any seasonal variation in seminal attributes. Semen was collected from four Murrah buffalo bulls (age; 4-6 years) biweekly two times from each bull with the help of artificial vagina (AV) on dummy animal between 8:00 to 9:00 hours using standard protocol. A total of thirty two ejaculates from four bulls. The ejaculates were pooled and divided into eight equal parts to study the effect of diluents and additives.

The two basic diluents used for extending semen were Egg yolk Tris (EYT) and Egg yolk-citrate glucose (EYCG). The diluent EYT contained 2.65 g Tris (hydroxy methylaminomethane), 0.625 g glucose and 0.125 g cystiene hydrochloride whereas EYCG contained 1.40 g sodium citrate, 1.2 g glucose, 0.9 g glycine and 0.125 g cystiene hydrochloride. The pH of both the diluents was adjusted to 6.8 and egg yolk was added to final concentration of 20%, and the total volume was made up to 100 ml.

Glutamic acid and aspartic acid were supplemented in different combinations viz. EYT (without additives, control), T1 (EYT + Glutamic acid, 4.0 mM), T2 (EYT + Aspartic acid, 0.40 mM), T3 (EYT + Glutamic acid + Aspartic acid), T4 (EYCG, without additives), T5 (EYCG + Aspartic acid), T6 (EYCG + Glutamic acid) and T7 (EYCG + Glutamic acid + Aspartic acid). Diluted semen was equilibrated and cryopreserved. In the investigation the concentration of additive was so adjusted that their level in diluted semen remained the same as found in ejaculated semen (Mann and Mann 1981) so that their physiological role can be maintained.

Three percent glycerol was added to extended semen at room temperature and incubated for two hours at 15°C. After two hours equal volume of pre-cooled (10-15°C) 11% glycerol was added three times at the interval of fifteen minutes to achieve a spermatozoa concentration of 80-100 million/ml and glycerol concentration of 7%. The final volume of extended semen was equilibrated for 90 min at 5°C in cold handling cabinet. After equilibration, semen was aspirated into French mini straws, sealed with polyvinyl alcohol powder and cooled horizontally from 5°C to -140°C in liquid nitrogen vapour for 10 minute duration and plunged into liquid nitrogen for storage.

At two stages viz. after equilibration and after 12 hours of cryopreservation (thawed samples) progressive motility, percent live and percent acrosomal damage of spermatozoa was assessed.

The progressive motility was determined by placing 5μl of diluted semen on a warmed glass slide (37°C) and allowed to spread uniformly under the cover slip. The motility was recorded using 200× magnifications with a phase contrast microscope equipped with a thermostatically controlled warm stage. Percent progressive motility (0-100%) was observed at five representative fields of the glass slide. The average of the five scores was recorded. The live sperm percent was evaluated by eosin-nigrosin staining technique (Hancock, 1951). Giemsa stain technique was used for staining acrosome (Hancock 1951) and acrosomal damage was classified according to Watson and Martin (1972).

statistical analysis

Data of spermatozoa quality attributes of different treatments were presented as means ± SE and analyzed by one way analysis of variance (ANOVA) followed by posthoc Tukey’s test (SAS 9.4). Differences in means were considered significant at P < 0.05.

results and discussion

The effect of different treatments on spermatozoa quality after equilibration and post thaw was presented in table 1. After equilibration and thawing progressive motility and live spermatozoa were similar in two basic diluents viz. EYT and EYCG. However, post thaw acrosomal damage was significantly higher (P<0.05) in EYCG than EYT. The results of present study indicated that tris buffer was a better substitute to citrate buffer if they are used alone as semen extender for Murrah buffalo bull semen. In previous studies (Singhet et al. 1975; Matharoo and Singh 1980; Tuli et al. 1981; Siddique et al. 2006; Yániz and Santolaria 2012) the tris buffer was reported to be more effective buffalo semen diluent as compared to citrate buffer. However, Siddique et al. (2006) reported that tris and citrate buffers in equal proportions as diluents were more effective for cryopreservation of buffalo bull semen.

The supplementation of aspartic acid to EYT diluents did not improve the progressive motility and percent live spermatozoa after equilibration and thawing. However, supplementation of aspartic acid decreased significantly (P<0.05) the post thaw acrosomal damage as compared to control. The supplementation of aspartic acid to EYCG significantly (P<0.05) improved the post thaw progressive motility and acrosomal integrity. Supplementation of glutamic acid to EYT and EYCG significantly (P<0.05) improved all the studied spermatozoa qualities. The addition of both aspartic and glutamic acid did not produce any beneficial effect on spermatozoa quality. The glutamic acid is
secreted in highest quantities (4.352 ± 0.257 µM/ml) whereas aspartic acid is also the fourth major free amino acid (0.369 ± 0.025 µM/ml) of the seminal plasma (Al-Hakim et al. 1970) and therefore these amino acids definitely play a significant role in physiological functions and quality of the spermatozoa. In present experiment these amino acids were added in the semen extender in different combinations to maintain their normal physiological levels and there after the spermatozoa quality was assessed after equilibration and thawing. The result showed that supplementation of aspartic acid was not as effective as glutamic acid in improving the spermatozoa quality after equilibration and thawing. The additive effect of aspartic acid along with glutamic acid was also not observed. In previous studies addition of D-aspartate to human semen improved the spermatozoa quality parameters as compared to control (Talevi et al. 2013). Oral administration of DL-aspartic acid improved semen kinematic parameters in rabbit by increasing the seminal plasma contents of D-aspartate (Macchia et al. 2010). From the previous studies it was confirmed that increase in aspartate levels in seminal plasma improved the spermatozoa quality which can be extrapolated for our study. Glutamic acid is formed in substantial amount within seminiferous tubules (Bustamante and Setchell, 2000) and constitutes 90% of total amino acids of rete-testes fluid (Hinton, 1990). Glutamic acid has been reported to be used as an additive in several species such as Angora goat (Bucak et al. 2009) ram (Mehr and Noori 2013), donkey (Dorado et al. 2014), rabbit (Saryözkan et al. 2014) and bull (Tuncer et al. 2011, Topraggaleh et al. 2014) spermatozoa against freeze-thawing damage by virtue of its antioxidative and other unknown properties. Although the mechanism of action of glutamic acid as a cryoprotectant is still not clear but many authors have reported that glutamine possess the antioxidative capacity (Bucak et al. 2009; Farshad and Hosseini 2013; Topraggaleh et al. 2014) which may be attributed to its cryoprotective nature. Supplementation of glutamine to tris-egg yolk based extender increased post thaw motility pattern and plasma membrane integrity of spermatozoa as compared to control in buffalo bull semen (Topraggaleh et al. 2014). The result of present study corroborated with the cryoprotective effects of supplementation of glutamic acid alone or in combination with other additives in semen extender in different species on post thaw motility, acrosomal integrity and percent live spermatozoa.

### Conclusions

From the results of present study, it can be concluded that egg yolk tris buffer was a better substitute to egg yolk-citrate glucose buffer if they are used alone as semen extender for Murrah buffalo bull semen and glutamic acid alone may be supplemented to semen extender to improve the spermatozoa quality after equilibration and thawing in comparison to aspartic acid or combination of aspartic acid and glutamic acid.

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