ADDITIVES EFFECT OF ALPHA-TOCOPHEROL, PENTOXIFYLLINE AND TAURINE ON POST THAW SEMEN IN POITOU DONKEYS

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ABSTRACT

The study was conducted with the objective to evaluate the post thaw semen qualities of Poitou donkey by addition of α-tocopherol, Pentoxifylline and Taurine in semen extender. Semen was divided in four different treatments having final concentration of 150 x 10^6 sperm/ml with use of cryoprotectant (5% DMF) containing without supplement (control) and following supplements with concentration: á-tocopherol (1 mM), Pentoxifylline (3.5 mM) and Taurine (25 mM), all stored in liquid nitrogen for 24 hrs. All samples after thawing process were maintained at 37°C, were analyzed and performed. Spermatozoa motility, live sperm count, host reactive, acrosomal integrity and DNA integrity were recorded and performed for each additive and also for an untreated control. In between the overall treatments, Pentoxifylline (T2) and Taurine (T3) revealed the higher (P<0.01) post thaw sperm motility (53.78±0.87 and 52.28±0.90%) compared to α-tocopherol-T1 (50.15±0.80%) and control (39.65±0.52%) while, T2 and T3 differed non-significantly. Between the treatments, T2 and T3 revealed the higher (P<0.01) post thawed live sperm count (60.62±0.82and 59.46±0.84%) compared to T1 (57.12±0.77%) and control (45.43±0.56%).In between the treatments, T2 and T3 revealed the higher (P<0.01) values of post thaw host reactive sperms (43.28±0.77 and 42.18±0.84%) compared to T1 (40.21±0.72%) and control (30.15±0.40%). Whereas, T2 and T3 differed non-significantly. Between the treatments, T2 and T3 revealed the higher (P<0.01) post thaw Acrosomal integrity (87.03±0.52and 87.03±0.45%) compared to T1 (84.62±0.34%) and control (80.34±0.24%) while T2 and T3 differed non-significantly. In between the treatments (T2 and T3) revealed the higher (P<0.01) DNA integrity (88.28±0.37 and 87.50±0.28%) compared to both treatment T1 (84.75±0.26%) and control (81.03±0.37%) while T2 and T3 differed non-significantly. In above study Pentoxifylline was found to improve sperm motility, live sperm count, host reactive, acrosomal integrity and DNA integrity and may also enhance the quality of frozen equine semen by increasing the fertility rate probably.

Key words: Poitou donkey, additives, semen extender, cryoprotectant, post thaw

Introduction

Poitou breed of donkey originating in France in a region called as Poitou. It is one of the largest donkey breeds and is selected for size so that it can be used for the production of large working mules.The Poitou donkey is instantly recognizable for a number of unusual characteristics, shaggy coat that hangs in long cords when ungroomed because of the long, soft hair. The coat is always dark brown or black and having a white underbelly, nose and rings around its eyes (Brayer and Fall, 1995; www.scz.org). The temperament of the Poitou donkey is described as “friendly, affectionate and docile”. Poitou donkeys are used to breed large mules and also for agricultural work, driving and riding. The equine breeders prefer to get their mares crossed with superior donkey stallions for mule’s production, but availability of superior jacks is limited in the field. The indigenous donkeys are incompatible for natural covering of the mares due to their short build. This problem can be overcome by artificial insemination with fresh and frozen semen. Artificial insemination (AI) is possible on the farm or with transported fresh, cooled semen. Jacks are easy to train for collection although this technique is not currently used on a wide spread basis. Donkey semen is very concentrated and in general has good fertility. It can be handled similar to that of the stallion and skim milk extenders seem to be useful to keep sperm alive during transport in artificial insemination programs (Singhui, 1990).

Cryopreservation causes extensive chemical and physical damage to sperm membranes of all mammalian species of either lethal or sub lethal nature (Watson, 2000), which are attributed to alterations in the transition from the lipid phase, increase in LPO of the membrane induced by reactive oxygen species (ROS) and mechanical stress on cell membranes due to osmotic stress and temperature changes (Ortega *et al*., 2009; Camera *et al*., 2011). Oxidative stress at the level of tissues, organs or organelles is derived from the imbalance between the production and elimination of reactive oxygen species in stallion sperm (Ball *et al*., 2001) studies show that during the freezing-thawing process an ove reproduction of reactive oxygen species (ROS) takes place and there is a reduced activity of enzymes involved in the antioxidant defense (Alvarez and Story, 1982) which results in damages to the plasma membrane.

The beneficial effects of antioxidants in maintaining cell structure and function of sperm after thawing indirectly proved the hypothesis that oxidative stress is installed during cryopreservation (Agarwal *et al*., 2003). Although semen contains antioxidants, which balance the lipid peroxidation and prevent excessive peroxide formation (Griveau *et al.,* 1995). However, the endogenous antioxidative capacity of semen may be insufficient during prolonged storage. The aim of study was to examine the effect of different additives α-tocopherol, pentoxifylline and taurine on post thawed semen quality of Poitou donkey.

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Matterials and Methods

All the chemicals used in research work were purchased from Sigma Chemical (Sigma-Aldrich, St. Louis, MO).

Semen collection and processing

Semen was collected from four Poitou donkeys (eight ejaculates per donkey) by following the routine procedure with an artificial vagina using (Colorado style). Immediately after collection, the gel fraction was separated by filtering with sterile gauze and gel free ejaculated semen was diluted with primary extender in ratio (1:1) and further divided into 4 equal aliquots. The aliquots of extended semen were centrifuged in 50 ml centrifuge tube (1800 rpm for 3 min) at 10^0C to get clear sperm pellet. Each sperm pellet was extended with secondary semen extender (Modified Glucose-EDTA-lactose) containing 5% DMFas a cryoprotectant alone (Control) or supplemented with either of the semen additives i.e. alpha-tocopherol (1 mM), pentoxifylline (3.5 mM) or Taurine (25 mM) to get final concentration of 150 x 10^6 sperm/ml. After that semen was packed in 0.5 ml capacity French medium straws filled with extended semen from each treatment by automatic straw filling and sealing machine. Then straws were cryopreserved in liquid nitrogen. After 24 hrs of storage of frozen semen the semen straws were thawed at 37°C for 30 sec. in water bath and carried out for post thaw semen quality evaluation.

Sperm evaluation

After the thawing, semen was maintained at 37°C for different parameters of sperm cells activity were measured such as sperm motility, viability, hyposmotic swelling, acrosomal integrity and DNA integrity test.

Evaluation of post thaw sperm motility and viability of Poitou donkey

Evaluation of post thaw sperm motility of cryopreserved semen samples were analyzed by a Computer Assisted Semen Analyzer (CASA) (HTB CEROS II, Version 1.3, Hamilton Thorne Research and Beverly, MA, USA) equipped with a thermo stage (Mini Therm®, Hamilton Thorne Inc. Beverly, MA, USA). Briefly 4 µl of each aliquots of semen sample from one control and three treatment groups (50 µl of semen sample dissolved in 1 ml of sodium citrate diluting fluid to make a 1:20 dilution) was loaded in disposable chambers with a 20 µm chamber depth (Leja® Standard Count 8 Chamber Slide, 20 µm, Leja® Products B.V., Netherlands, Fig. 9). The HTB CEROS II system was set to measure 30 frames per field at a frame rate of 60 Hz. A minimum of seven fields and 500 spermatozoa were measured for each sample. Viability of the frozen semen samples were counted by using eosin-nigrosin stained smears of semen sample and analyzed under microscope at 100X oil immersion (Nikon Instech Co. Ltd., Kanagawa, Japan). To prepare smear, one drop of semen was placed on clean, grease free, pre-warmed glass slide and mixed with one drop of eosin-nigrosin stain using blunt fine glass rod. After a minute, a thin smear from the mixture was prepared on glass slide and air dried. Total three smears were prepared from each sample and a total of 200 sperm were counted from each smear for live sperm percentage.

Evaluation of hyposmotic swelling test

Total three slides were prepared from each sample and a total of 200 sperm were counted from each sample, in at least 5 different fields of view and the percentage of sperm that reacted with different swelling patterns of tail were observed under 40x objective.

Evaluation of acrosomal integrity and DNA integrity

Acrosomal integrity was evaluated by using the Giemsa staining technique following staining procedure. Prepared smears were examined under oil immersion (100X). Total 200 sperms were counted from each sample and sperms with intact acrosome were calculated. The DNA quality of a semen sample was evaluated by conducting DNA integrity test using Nuclear dye Acridine orange (AO) following procedure by acridine orange staining according to Liu and Baker (1992).

Statistical analysis

Data were collected and statistically analyzed. Analysis included mean values, standard error and analysis of variance (ANOVA) using F-test. The data were analyzed using conventional statistical procedures as described by Snedecor and Cochran (1994).

Results and Discussion

Sperm motility and viability status

The mean values of post thaw sperm motility in the different treatments (T1, T2 and T3) for individual Poitou donkeys varied from 44.37±0.90 to 53.87±1.07, 48.87±0.70 to 57.50±1.20 and 46.75±1.60 to 55.87±0.97%, respectively (Table 1) and in untreated control (C) samples the individual post thaw sperm motility varied from 35.75±0.70 to 41.75±0.50%. The overall post thaw sperm motility in treatments (T1, T2 and T3) were found significantly higher ($P<0.01$) compared to control. Between the overall treatments, T2 and T3 revealed the higher ($P<0.01$) post thaw sperm motility (53.78±0.87 and 52.28±0.90%) compared to T1(50.15±0.80%) and control (39.65±0.52%) while T2 and T3 differed non-significantly. The mean values of post thaw live sperm count in the different treatments (T1, T2 and T3) for individual Poitou donkeys varied from 51.37±0.98 to 60.25±1.02, 55.87±0.71 to 64.25±0.66 and 53.62±1.61 to 62.25±0.87%, respectively (Table 2) and in untreated control (C) samples the individual post thaw sperm motility varied from 41.62±0.70 to 47.62±0.56%. The overall post thaw live sperm count in treatments (T1, T2 and T3) were found significantly higher ($P<0.01$) compared to control. Between the treatments, T2 and T3 revealed the higher ($P<0.01$) post thawed sperm motility (60.62±0.82 and 59.46±0.84%) compared to T1 (57.12±0.77%) and control (45.43±0.56%). Jang *et al.* (2006) studied in eosin Y and nigrosin staining (ENS), effect of taurine on sperm characteristics for in vitro storage of boar semen. The live sperm (%) for 6 hrs. incubation were significantly higher in 25 mM (76.0±0.6 %) and 50 mM taurine groups (78.0±0.7), respectively. The present study showed higher values for live sperm above 80 % in taurine (25 mM) at 2 hrs. of incubation period. The value for live sperm count were found higher in pentoxifylline than taurine and alpha-tocopherol in post thaw semen of Poitou donkey semen. Stephens *et al*. (2013) studied the effect of post thaw addition of pentoxifylline and taurine to semen and found increased in sperm motility and longevity in equine frozen semen. Similar to these previous reports the present study observed increase in sperm motility and longevity

of post thaw semen.

Hypo-osmotic swelling test (HOST)

The mean values of post thaw HOST reactive sperms in the different treatments (T1, T2 and T3) for individual Poitou donkey varied from36.50±1.34to43.87±0.65, 39.25±0.94 to 47.75±1.32 and 37.62±1.73 to 46.87±1.16%, respectively (Table 3) and in untreated control (C) samples the individual post thawed HOST reactive sperms values varied from 28.50±0.59 to31.75±0.59%. The overall post thaw HOST reactive sperms in treatments (T1, T2 and T3) were found significantly higher (P<0.01) compared to control. Between the treatments, T2 and T3 revealed the higher (P<0.01) values of post thaw HOST reactive sperms (43.28±0.77 and 42.18±0.84%) compared to T1 (40.21±0.72) and control (30.15±0.40%). Whereas, T2 and T3 differed non-significantly. Hyposmotic swelling test (HOST) seems to be more appropriate for predicting the fertilizing capacity of frozen-thawed semen, because membrane damage appears to be a more important limiting factor (Colenbrander *et al*., 2003). Correa and Zavos (1994); Neild *et al*. (1999); Samardzija *et al.* (2008) and Mansour (2009) found positive correlation between the percentage of motile spermatozoa and HOST positive sperm. Similarly, the present study also showed both motility and HOST significantly increased for pentoxifylline than alpha-tocopherol and taurine in post thaw stage of Poitou donkey.

Acrosomal integrity in post thaw semen

The mean values of post thaw acrosomal integrity in the different treatments (T1, T2 and T3) for individual Poitou donkeys varied from 83.5 ± 0.49 to 85.87 ± 0.95, 85.75 ± 0.80 to 89.87±1.32 and 85.62 ±1.24 to 88.37± 0.80%, respectively (Table 4) and in untreated control (C) samples the individual post thaw acrosomal integrity varied from 79.62 ± 0.49 to 80.75± 0.45 %. The overall post thaw acrosomal integrity in treatments (T1, T2 and T3) were found significantly higher (P<0.01) compared to control. Between the treatments, T2 and T3 revealed the higher (P<0.01) post thaw acrosomal integrity (87.03 ± 0.52 and 87.03 ± 0.45%) compared to T1 (84.62 ±0.34%) and control (80.34 ± 0.24%) while T2 and T3 differed non-significantly. Bhakat *et al*. (2011) reported that the addition of pentoxifylline and alpha-tocopherol improved the acrosome integrity and their findings supported the present study. Silva *et al.* (2009) studied the effect of pentoxifylline (3.5 mM) with Tris-egg yolk diluents and glycerol (5%) in equine semen after thawing and at 2 hrs. of incubation period such treatment resulted in significant rise (P<0.05) in acrosome integrity similarly, present study showed improved intact acrosomal integrity of sperm in post thaw semen of Poitou donkeys.

DNA integrity in post thaw semen

The mean values of post thaw DNA integrity in the different treatments (T1, T2 and T3) for individual Poitou donkeys varied from 84.12±0.51 to 85.87±0.58, 87.12±0.76 to 89.25±0.80 and 86.87±0.55 to 88.12±0.81%, respectively (Table 5) and in untreated control (C) samples the individual DNA integrity varied from 79.25±0.81 to 82.37±0.46%. The overall post thaw DNA integrity in treatments (T1, T2 and T3) were found significantly higher (P<0.01) compared to control. Between the treatments (T2 and T3) revealed the higher (P<0.01) DNA integrity (%) compared to both treatment (T1) and control while T2 and T3

Table 1: Post thaw motility (Mean± SE) of Poitou donkeys

Poitou donkeys					
Treatment	M-28	M-29	M-30	M-31	Overall motility (%)
T1	53.87±1.07^{c}	44.37±0.90^{b}	49.62±1.00^{b}	52.75±0.90^{b}	50.15±0.80^{BY}
T2	57.50±1.20^{b}	48.87±0.70^{c}	51.50±2.16^{c}	57.25±0.60^{d}	53.78±0.87^{CY}
T3	55.87±0.97^{c}	46.75±1.60^{c}	53.62±1.90^{c}	52.87±1.10^{c}	52.28±0.90^{CX}
Control	41.37±0.40^{aD}	35.75±0.70^{a}	39.75±0.70^{a}	41.75±0.5^{a}	39.65±0.52^{AX}

Note: Values with different lowercase superscripts differ significantly (P<0.05) and uppercase superscripts (ABCD) differ significantly (P<0.0 1) within column

Table 2: Live sperm count (Mean±SE) in post thaw semen of Poitou donkeys

Poitou donkeys					
Treatment	M-28	M-29	M-30	M-31	Overall live (%)
T1	60.25±1.02^{b}	51.37±0.98^{b}	56.62±0.66^{b}	60.25±0.98^{b}	57.12±0.77^{BY}
T2	64.12±1.17^{c}	55.87±0.71^{c}	58.25±1.89^{bc}	64.25±0.66^{c}	60.62±0.82^{CY}
T3	62.25±0.87^{bc}	53.62±1.61^{bc}	60.75±1.52^{c}	61.25±1.04^{bc}	59.46±0.84^{CY}
Control	47.25±1.03^{a}	41.62±0.70^{a}	45.25±0.64^{a}	47.62±0.56^{a}	45.43±0.56^{AY}

Note: Values with different lowercase superscripts differ significantly (P<0.05) and uppercase superscripts (ABCD) differ significantly (P<0.01) within column

Table 3: Hypo osmotic swelling test (HOST) (Mean±SE) in post thaw semen of Poitou donkey

Poitou donkeys					
Treatment	M-28	M-29	M-30	M-31	Overall HOST (%)
T1	43.87±0.65^{b}	36.50±1.34^{b}	37.75±1.17^{b}	42.75±0.80^{bc}	40.21±0.72^{BY}
T2	47.75±1.32b^{c}	39.25±0.94^{b}	39.87±0.91^{b}	46.25±0.82^{c}	43.28±0.77^{CY}
T3	46.87±1.16^{c}	37.62±1.73^{b}	41.12±1.26^{b}	43.12±1.05^{bc}	42.18±0.84^{CY}
Control	31.75±0.59^{a}	28.50±0.59^{a}	28.87±0.51^{a}	31.50±0.86^{a}	30.15±0.40^{AY}

Table 4: Acrosomal integrity (Mean±SE) in post thaw semen Poitou donkeys

Poitou donkeys					
Treatment	M-28	M-29	M-30	M-31	Overall Acrosome integrity (%)
T1	85.87±0.95^{b}	83.5±0.49^{b}	84.37±0.56^{b}	84.75±0.72^{b}	84.62±00.34^{BX}
T2	89.87±1.32^{c}	85.75±1.08^{b}	86.75±0.98^{c}	85.75±0.80^{b}	87.03±0.52^{CX}
T3	88.37±0.80^{c}	85.62±1.24^{b}	88.25±0.72^{c}	85.87±0.62^{b}	87.03±0.45^{CY}
Control	80.62±0.59^{a}	79.62±0.49^{a}	80.37±0.41^{a}	80.75±0.45^{a}	80.34±0.24^{AX}

Note: Values with different lowercase superscripts differ significantly (P<0.01) and uppercase (ABCD) differ significantly (P<0.01) within column

Table: 5. DNA integrity (Mean±SE) in post thaw semen of Poitou donkey

Poitou donkeys					
Treatment	M-28	M-29	M-30	M-31	Overall Acrosome integrity (%)
T1	84.50± 0.60	84.12± 0.51	84.50± 0.45	85.87± 0.58	84.75± 0.26^{BY}
T2	88.50± 1.00	87.12± 0.76	88.25± 0.44	89.25± 0.80	88.28± 0.37^{CY}
T3	87.75± 0.69	86.87± 0.55	87.25± 0.26	88.12± 0.81	87.50± 0.28^{CY}
Control	81.25± 0.52	79.25± 0.81	81.25± 0.52	82.37± 0.46	81.03± 0.34^{AX}

Values with different uppercase superscripts (ABCD) differ significantly (P<0.01) within column

differed non-significantly. Pentoxifylline (PTX) is a methylxanthine phosphodiesterase inhibitor which reduces superoxide anions responsible for DNA apoptosis when used at a concentration of 3.6 mM (Maxwell *et al.,* 2002). Present study showed that pentoxifylline at concentration of 3.5 mM improved intact DNA sperm in post thaw semen of Poitou donkeys.

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