Antioxidant Effects of Aloe vera as Semen Additive in Cryopreservation of Cattle Bull Semen

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ABSTRACT

The present study was done at the Frozen Semen Laboratory, U.T. of Jammu & Kashmir, Hukkal, Jammu and Division of V.G.O., F.V. Sc & A.H., SKUAST- J, R.S. Pura, during the period between December 2017 and May 2018. This investigation was carried out as a pioneer study with the objective to study the effect of natural antioxidant Aloe vera as a semen additive on cryopreservation of semen. Semen samples (n=10) from mature cattle bull stationed at Frozen semen laboratory, U.T. of Jammu & Kashmir, Hukkal, Jammu, were used to evaluate the effect of Aloe vera additive at post-dilution and at post-thaw stage. The semen sample was extended with Tris-Egg-Yolk-Citric-acid-Fructose-Glycerol (TEYCAFG) extender and were split into two groups: Group 1: TEYCAFG without any additive/ control and group 2: TEYCAFG + Aloe vera (5µl/ml). Progressive motility, live spermatozoa, acrosomal integrity, sperm abnormality, hypo-osmotic swelling test (HOST) was evaluated at both post-dilution and post-thaw stage, whereas, oxidative stress tests viz. malondialdehyde (MDA), catalase (CAT) and superoxide dismutase (SOD) were evaluated at only post-thawed stage. Group 2 i.e. Aloe vera group, showed significant (p<0.05) increased progressive motility, live spermatozoa, acrosomal integrity and HOST positive spermatozoa, while significant (p<0.05) decreased sperm abnormalities in post-thawed semen. In oxidative stress evaluation the MDA level was significantly (p<0.05) decreased, whereas, CAT level significantly (p<0.05) increased in group 2 in comparison to control group. It was concluded that addition of Aloe vera (5µl/ml) as semen additive improves semen quality and minimize oxidative stress to the spermatozoa during cryopreservation of semen.

Keywords
Semen, Cattle bull, Aloe vera, Oxidative stress

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Introduction

Artificial insemination is assisted reproductive technology that has made possible the effective use of best breeding bulls, thus greatly improving the genetic potential of the breeding herds (Januskauskas and Zilinskas, 2002). Cryopreservation technique has allowed specific opportunities for the cryopreservation of semen and widespread dissemination of precious genetic resources through sperm banks that
collaborate in breed improvement programme by means of artificial insemination (Shaikh, 2019; Holt, 1999).

During cooling and freezing process, sperm cells are susceptible for harmful effect from unbalanced osmotic pressure, protein denaturation, cellular acidosis, energy loss, membrane breakdown, crystallization of cell body, destabilization of the cytoskeleton and formation of free radicals or reactive oxygen species (Haris et al., 2020; Urgur et al., 2019).

All radicals and non-radicals from oxygen are considered reactive oxygen species (ROS), which has high electron reactivity and instability. The ROS can react with a great number of compounds, acting as donors or receivers of the electron (Agarwal et al., 2005), and they are considered the main promoters of damage to living organisms (Bernard and Krause, 2007). Oxidative stress occurs when there is an imbalance between the production of ROS and the protective action of the antioxidant system, responsible for its neutralization and removal (Pinto et al., 2019; Jedrzejowska et al., 2013).

To avoid oxidative stress, the cells have a defense system compound by antioxidants, which are divided into enzymatic and non-enzymatic substances with low molecular weight (Halliwell and Gutteridge, 1999). However, during semen cryopreservation, antioxidant concentrations are reduced during the dilution stage, which results in the imbalance and consequently in cellular and oxidative stress (Pinto et al., 2019; Bilodeau et al., 2000).

The plasma membrane of sperm cells had supplementary content of unsaturated fatty acids and their cytoplasmic components are deficient in oxidants. Therefore, sperm cells are highly susceptible to lipid peroxidation (LPO) in presence of ROS, leading to impaired function activity (Shaikh, 2019; Shaikh et al., 2016, Hu et al., 2010, Hu et al., 2009).

The Aloe vera plant has been known and used for centuries for its health, beauty, medicinal and skin care properties. 2000 years ago, the Greek scientists regarded Aloe vera as the universal panacea. The Egyptians called Aloe “the plant of immortality”. There are over 300 Aloe species, which have been used in cultures like: Greece, Egypt, India, Mexico, Japan and China (Marshall, 1990). Studies on Aloe vera have reported for its anti-diabetic, anti-cancer, antibiotic, hypotensive, hepatoprotective and blood purifying properties (Fakhirldin and Sodani, 2014; Kosif et al., 2008; Tiwari and Rao, 2002).

To best of our knowledge, no study has been done for investigation of the role of addition of Aloe vera in semen of cross-bred bull. In continuation to our previous reported findings in cryopreservation of cross-bred cattle bull semen (Singh et al., 2020) the present study was designed with the objective to evaluate the oxidative stress to the spermatozoa during cryopreservation of semen and the effects of Aloe vera as semen additive.

Materials and Methods

The present study was undertaken with the objective to determine anti-oxidant effect of Aloe vera during cryopreservation of cattle bull semen. Semen samples (n=10) were collected twice a week from mature cattle bull randomly by artificial vagina method. The bulls were kept at Frozen semen laboratory, U.T. of Jammu & Kashmir, Hukkal, Jammu, where collection of the semen, initial evaluation, extension and freezing of the semen took place, while the post-thawed semen samples were evaluated at the Division of Veterinary Gynaecology and Obstetrics
and Division of Pharmacology and Toxicology, F.V.Sc & A.H., SKUAST- J, R.S.Pura. The present study was undertaken during the period between December 2017 and May 2018. The fresh semen samples were evaluated for volume, mass activity (Tomar et al., 1966), spermatozoa concentration using Accucell photometer (IMV Technologies, France) and progressive motility (Salisbury et al., 1978). The semen samples qualifying the minimum initial standards were further processed. The qualified samples were extended with tris egg yolk citric acid fructose glycerol (TEYCAFG) kept at 37°C. The semen samples were extended in such a way to yield approximately 40 million spermatozoa/ml (Anel et al., 2003). The extended semen samples were split into two parts. The first part was considered as group 1: TEYCAFG without any additive/ control and group 2: TEYCAFG + Aloe vera (5µl/ml). The pH was adjusted within the range 7.2 to 7.4. The semen samples were filled in 0.5 ml capacity, polyvinyl straw (IMV, France). The equilibration was done at 4° C for 4 hours and freezing of the straws was done as per standard protocol of the lab using programmable biofreezer (Digit Cool-530, IMV Technologies, France) with freezing rate 4°C to -10°C @ 5°C, -10°C to -100°C @ 40°C, -100°C to -140°C @ 20°C, after attainment of -140°C temperature straws were plunged in liquid nitrogen and stored in liquid nitrogen container pending future evaluation. Thawing was done at 37° C for 30 seconds for evaluation of post-thawed samples.

The processed semen samples (n=10) were evaluated at two stages post-dilution and post-thaw stage. At post-dilution stage the per cent progressive motility, live spermatozoa & acrosomal integrity as per Salisbury et al., (1978), sperm abnormalities (Kumar, 1993) and hypo-osmotic swelling test (Jeyendran et al.,1984), whereas, at post-thaw stage in addition to above parameters done at post-dilution stage it was also evaluated for oxidative stress test viz. Malondialdehyde (MDA) as described by Shafiq-ur-Rehman (1984); Catalase as per Aebi (1983) and Superoxide dismutase (SOD) as described by Marklund and Marklund (1974). The results were analysed statistically using Analysis of Variance (ANOVA) (Snedecor and Cochran, 1989).

Results and Discussion

In the present investigation EY-tris (egg yolk-tris-citric acid-fructose-glycerol) was used as control dilutor, EY-tris plus Aloe vera (5µl/ml) were used as experimental groups.

Progressive motility

The per cent progressive motility (Mean ± SE) post-dilution and post-thawing in control group were 67.0 ± 1.52 and 48.0 ± 2.49; whereas in Aloe vera added group were 69.0 ± 3.14 and 55.0 ± 2.24, respectively (Table 1). Perusal of the table (1) it was observed that the progressive motility (%) differed non significantly between post-diluted control and Aloe vera added group, whereas, in post-thaw a significant (p<0.05) difference in progressive motility was observed between control and Aloe vera group.

In the present study in control group the per cent progressive motility of diluted semen (Table 1) was 67.0 ±1.52 which was in nearly similar to the findings of Pathak et al., 1990; 65.30 ± 1.20 per cent in diluted semen of cattle bull. Though the higher per cent progressive motility in the diluted semen was reported by Rao et al., 1996; 76.90 ± 0.87 and Gupta et al., 1990; 73.23 ± 0.38. In the present study per cent post thaw motility in control group (Table 1) was 48.0 ± 2.49 which was less than reported by Kishore (2009), 80.74; Pawshe et al., (2016), 61.7 ±
1628; Uysal et al., (2007), 55.5 ± 2.5 and Ulfin and Raina (2003), 55.34 ± 1.02, while it was higher than reported by Hu et al., (2010), 36.88 ± 1.53 and Li et al., (2016), 14.7 ± 1.4. The main reason for the decrease in the per cent post thaw progressive motility might be due to cryopreservation damage, ROS production and damage caused due to formation of ice crystal formation in mitochondria and Axomemes during cryopreservation that impairs sperm motility.

**Live spermatozoa**

The per cent live spermatozoa (Mean ± SE) post-dilution and post-thawing in control group were 74.4 ± 1.82 and 50.8 ± 1.17; whereas in Aloe vera group were 76.2 ± 2 and 59.0 ± 1.65, respectively (Table 1). Perusal of the table (1) it was observed that the live spermatozoa (%) differed non significantly between post-diluted control and Aloe vera group, whereas, in post-thaw a significant (p<0.05) difference in live spermatozoa was observed between control and Aloe vera group.

In the present study in the control group values of per cent live spermatozoa in diluted semen (Table 1) was 74.4 ± 1.82, which was in agreement with the findings of Keshava (1996), 74.28; Abdel-khalek et al., (2008), 73.0 ± 1.3 and Ulfin and Raina (2003), 72.44 ± 0.51. In the present study the values of per cent live spermatozoa of post thaw semen of control group (Table 1) was 50.8 ± 1.17 which was less than reported by Bhalde et al., (1991), Rao et al., (2017) and Abdel-khalek et al., (2008) who reported 57.64 ± 0.78, 56.24 ± 0.01 and 66-73 per cent, respectively. Decreasing propotion of live spermatozoa in post thaw semen may be due to cryo damage caused to formation of ice crystals in extra and intracellular environment, increasing solute concentration (Mazur, 1984) and sperm susceptibility for freezing and thawing temperature, ROS production and lipid peroxidation (Bucak et al., 2008)

**Acrosomal integrity**

The per cent acrosomal integrity (Mean ± SE) post-dilution and post-thawing in control group were 79.7 ± 1.52 and 53.9 ± 0.94; whereas in Aloe vera group were 81.2 ± 1.34 and 67.6 ± 1.43, respectively (Table 1). Perusal of the table (1) it was observed that the acrosomal integrity (%) differed non significantly between post-diluted control and Aloe vera group, whereas, in post-thaw a significant (p<0.05) difference in acrosomal integrity was observed between control and Aloe vera group.

The evaluation of acrosomal integrity is important for determination of semen quality as enzyme necessary for fertilization present in the acrosomal cap. In the present study the values of per cent acrosomal integrity of diluted semen of control group (Table 1) was 79.7 ± 1.52, which was in agreement with the finding of Lone et al., (2017), 80.17 ± 3.26, whereas it was less than the finding of Rao et al., (2017), 85.77 ± 0.01. In the present study the values of per cent acrosomal integrity of post thaw semen of control group (Table 1) was 53.9 ± 0.94 which was in agreement with the finding of Sandeep et al., (2015) and Paudel et al., (2010) which were reported as 36.25 ± 0.91 and 31.5 ± 1.3, respectively.

**Sperm abnormalities**

The per cent sperm abnormalities (Mean ± SE) post-dilution and post-thawing in control group were 7.2 ± 0.63 and 16.8 ± 0.61; whereas in Aloe vera group were 7.9 ± 0.97 and 14.2 ± 0.96, respectively (Table 1). Perusal of the table (1) it was observed that the sperm abnormalities (%) differed non
significantly between post-diluted control and Aloe vera group, whereas, in post-thaw a significant (p<0.05) difference in sperm abnormalities was observed between control and Aloe vera group.

In the present study the values of per cent sperm abnormalities in diluted semen of control group (Table 1) was 7.2 ± 0.63, which was in agreement with the finding of Rao et al., (2017), 7.28 ± 0.02 in crossbred bull, whereas, it was higher than as reported by Mittal et al., (2014), 4.91 ± 0.14 in bhadawari bull, while it was lower than as reported by Gupta et al., (1990), 9.14 ± 0.09 in cattle bull. In the present study per cent post thaw sperm abnormalities in control group (Table 1) was 16.8 ± 0.61 which was in agreement with the finding of Rao et al., (1999), range of 15.66 ± 0.39 to 16.75 ± 0.37 in Jersey bulls, while it was higher than as reported by Mittal et al., (2014), 7.75 ± 0.17 in Bhadawari bull; Sariozkan et al., (2009a), 14.93 ± 1.07 in cattle bull and Sariozkan et al., (2009b), 15.0 ± 1.1 in cattle bull, whereas lower than the finding of Gupta et al., (1990) and Rao et al., (2017) who reported as 17.63 ± 1.99 and 18.36 ± 0.04 in crossbred bull, respectively.

Hypo-osmotic swelling test (HOST)

The hypo-osmotic swelling test positive spermatozoa per cent (Mean ± SE) post-dilution and post-thawing in control group were 71.0 ± 1.10 and 45.4 ± 0.93; whereas in Aloe vera group were 72.5 ± 1.89 and 55.1 ± 0.99, respectively (Table 1). Perusal of the table (1) it was observed that the hypo-osmotic swelling test positive spermatozoa (%) differed non significantly between post-diluted control and Aloe vera group, whereas, in post-thaw a significant (p<0.05) difference in hypo-osmotic swelling test positive spermatozoa was observed between control and Aloe vera group.

In the present study the per cent hypo-osmotic swelling test (HOST) positive spermatozoa of diluted semen in control group (Table 1) was 71.0 ± 1.10 which was in agreement with the findings of Rao et al., (2017) and Lone et al., (2017) who reported as 72.54 ± 0.01 in crossbred bull and 70.91 ± 5.92 in buffalo bull, however lower values of per cent hypo-osmotic swelling test (HOST) of diluted semen was observed by Kumar et al., (2018) who reported as 62.05 ± 0.80 in pure Jersey bulls and 62.11 ± 0.89 in crossbred Jersey bulls. In the present study the values of per cent hypo-osmotic swelling test (HOST) of post thaw semen of control group (Table 1) was 45.4 ± 0.93, which was agreement with the finding of Kumar et al., (2018), 47.27 ± 1.05 in pure Jersey bulls; 45.94 ± 1.33 crossbred Jersey bulls and Sariozkan et al., (2009a) who reported as 43.70 ± 1.96 in cattle bull, while lower values were observed by Paudel et al., (2010), Rao et al., (2017) and Taraphdar (1999) who reported as 39.6 ± 1.3 in crossbred bulls, 38.73 ± 0.01 in crossbred bull and 36.52 in cattle bull, respectively. Whereas, higher finding was observed by Lone et al., (2017), 51.30 ± 4.43 in buffalo bull and Sohail et al., (2015), 49.97 ± 3.62 in sahiwal cattle bull. The structural changes produced in the post thaw sperm cells membrane are primarily linked to altered abilities for energy sourcing which later on influence both cellular metabolism and other sperm functions (Dziekonska et al., 2009; Gillan et al., 2004).

Malondialdehyde (MDA)

The MDA levels (Mean ± SE) post-thaw semen samples in control group and Aloe vera group were 3.49 ± 0.19 and 1.71 ± 0.04, respectively (Table 2). Perusal of the table (2) it was observed that MDA levels in post-thaw semen samples differed significantly (p<0.05) between control and Aloe vera group.
In the present study MDA level (nmol H$_2$O$_2$ produced/ $10^8$ spermatozoa) in post thaw semen of control group (Table 2) was 3.49 ± 0.19 which was lower than the value observed by Paudel et al. (2010), 712.1 ± 49.1 nmol MDA level/10$^9$ spermatozoa in crossbred bull and Lone et al., (2017), 496.02 ± 39.28 nmol MDA level/10$^9$ spermatozoa in buffalo bull. The finding of other scientists could not be compared with our finding due to disparity in method of evaluation and units of measurement.

**Catalase**

The catalase levels (Mean ± SE) post-thaw semen samples in control group and Aloe vera added group were 1.18 ± 0.36 and 1.41 ± 0.48, respectively (Table 2). Perusal of the table (2) it was observed that catalase levels in post-thaw semen samples differed significantly (p<0.05) between control and Aloe vera group.

In the present study catalase activity (µmol H$_2$O$_2$ decomosed/ min/ $10^8$ spermatozoa) in post thaw semen of control group (Table 2) was 1.18 ± 0.31 which was higher than finding of Zhao et al., (2015), 1.16 ± 0.82 (U/ml) in Qinchuan bulls, whereas lower than the finding of Sharma et al., (2016) who reported as 23.36 ± 0.25 in rainy season, 24.25 ± 0.30 winter season and 24.22 ± 0.56 in summer season in buffalo bull. There was reduction in the catalase activity with the level of freezing.

CAT activity reduced at post thaw stage when compared to pre freeze stage (Lone et al., 2017). CAT is an enzymatic antioxidant found in all living organisms exposed to oxygen, which decomposes harmful peroxides and converts them into water and oxygen (Chelikani et al., 2004).

**Superoxide dismutase (SOD)**

The SOD levels (Mean ± SE) post-thaw semen samples in control group and Aloe vera added group were 0.95 ± 0.06 and 1.26 ± 0.06, respectively (Table 2). Perusal of the table (2) it was observed that SOD levels in post-thaw semen samples differed significantly (p<0.05) between control and Aloe vera group.

The level of Superoxide dismutase (Unit/ $10^8$ spermatozoa) in present study in post thaw semen of control group (Table 2) was 0.95 ± 0.06 which was lower than the finding of Nair et al., (2006) who reported as 3.50 ± 0.19 in cattle and 1.98 ± 0.09 in buffalo bulls, however it was higher than the finding of Lone et al., (2017) who reported as 0.16 ± 0.03 (units/mg protein) in buffalo bulls. Superoxide is a free radical which is converted to oxygen and hydrogen peroxide by the dismutation action of antioxidant enzyme superoxide dismutase (Tariq et al., 2015).

**Effects of Aloe vera**

In the present study in Aloe vera added group the per cent progressive motility, live spermatozoa, acrosomal integrity, sperm abnormalities and hypo-osmotic swelling test (HOST) positive spermatozoa of diluted semen and post-thawed semen were recorded as 69.0 ± 3.14 and 55.0 ± 2.24; 76.2 ± 2 and 59.0 ± 1.65; 81.2 ± 1.34 and 67.6 ± 1.43; 7.9 ± 0.97 and 14.2 ± 0.96; 72.5 ± 1.89 and 55.1 ± 0.99, respectively (Table 1).

In attempt to study the oxidative stress MDA, Catalase and SOD levels were studied. In Aloe vera added group the (Mean ± SE) of MDA, Catalase and SOD levels of post-thawed samples were recorded as 1.71 ± 0.04, 1.41 ± 0.48 and 1.26 ± 0.06, respectively (Table 2).
Table 1 The effect of *Aloe vera* on different physio-morphological characteristics at post-dilution and post-thaw semen of cross-bred bulls

| Parameter (%)          | Stage               | Control            | *Aloe vera* (5µl/ml) |
|------------------------|---------------------|--------------------|----------------------|
| **Progressive motility** | Post-dilution      | 67.0 ± 1.52<sup>a</sup> | 69.0 ± 3.14<sup>a</sup> |
|                        | Post-thaw           | 48.0 ± 2.49<sup>a</sup> | 55.0 ± 2.24<sup>b</sup> |
| **Live spermatozoa**   | Post-dilution      | 74.4 ± 1.82<sup>a</sup> | 76.2 ± 2<sup>a</sup>     |
|                        | Post-thaw           | 50.8 ± 1.17<sup>a</sup> | 59.0 ± 1.65<sup>b</sup>  |
| **Acrosomal integrity**| Post-dilution      | 79.7 ± 1.52<sup>a</sup> | 81.2 ± 1.34<sup>a</sup>  |
|                        | Post-thaw           | 53.9 ± 0.94<sup>a</sup> | 67.6 ± 1.43<sup>b</sup>  |
| **Sperm abnormalities**| Post-dilution      | 7.2 ± 0.63<sup>a</sup>  | 7.9 ± 0.97<sup>a</sup>   |
|                        | Post-thaw           | 16.8 ± 0.61<sup>a</sup> | 14.2 ± 0.96<sup>b</sup>  |
| **HOST**               | Post-dilution      | 71.0 ± 1.10<sup>a</sup> | 72.5 ± 1.89<sup>a</sup>  |
|                        | Post-thaw           | 45.4 ± 0.93<sup>a</sup> | 55.1 ± 0.99<sup>b</sup>  |

Values are given as mean ± SE of 10 animals unless and otherwise stated
Values bearing different superscripts in a row differ significantly (p<0.05)

Table 2 The effect of *Aloe vera* on malondialdehyde (MDA), catalase (CAT) and superoxide dismutase (SOD) activity in post-thawed semen of cross-bred bulls

| Oxidative stress test       | Control            | *Aloe vera* (5µl/ml) |
|-----------------------------|--------------------|----------------------|
| MDA (nmol/10<sup>8</sup> Spermatozoa) | 3.49 ± 0.19<sup>a</sup> | 1.71 ± 0.04<sup>b</sup> |
| CAT (µmol/10<sup>8</sup> Spermatozoa)  | 1.18 ± 0.36<sup>a</sup> | 1.41 ± 0.48<sup>b</sup> |
| SOD (U/ 10<sup>8</sup> Spermatozoa)   | 0.95 ± 0.06<sup>a</sup> | 1.26 ± 0.06<sup>b</sup> |

Values are given as mean ± SE of 10 animals unless and otherwise stated
Values bearing different superscripts in a row differ significantly (p<0.05)

It was observed that in *Aloe vera* added group the per cent progressive motility, live spermatozoa, acrosomal integrity, sperm abnormalities and hypo-osmotic swelling test (HOST) positive spermatozoa of diluted semen differed non significantly between post-diluted control and *Aloe vera* added group, whereas, in post-thaw a significant (p<0.05) difference in per cent progressive motility, live spermatozoa, acrosomal integrity, sperm abnormalities and hypo-osmotic swelling test (HOST) positive spermatozoa was observed between control and *Aloe vera* group (Table 1). In the present study the in post-thawed sample the MDA, Catalase and SOD levels differed significantly (p<0.05) between control and *Aloe vera* group (Table 2).

*Aloe vera* is a pharmaceutical plant which can be useful for curing various diseases and improving body’s physiology. It can be used as a natural antioxidant with high potential of reducing fats oxidation and oxidative stresses (Vinson *et al.*, 2005). Though multiple uses of *Aloe vera* as antioxidant, therapeutic agents etc. has been reported in humans and animals, but its use as semen additive and that to as an antioxidant is completely missing.

Fakhrildin and Sodani (2014) worked on human sperm and observed a beneficial role of antioxidative phenolic compound in *Aloe vera* on human semen and its effects on lipid peroxidation in their study 5µl of *Aloe vera* extract was mixed with 1 ml of each semen sample and then the sperm parameter
including concentration, motility, morphology, agglutination, viability and HOS test were recorded.

This was the only report of Aloe vera where it was used as semen additive. It formed the basis of our curiosity to use Aloe vera in our experiment and to evaluate its antioxidant property and it was observed that though the Aloe vera added diluted semen didn’t showed any improvement in the semen parameters, but the significant (p<0.05) improvement was recorded in semen parameter i.e. per cent progressive motility, live spermatozoa, acrosomal integrity, sperm abnormalities, hypo-osmotic swelling test (HOST) positive spermatozoa as well as MDA, Catalase, SOD levels.

In the absence of the reports of Aloe vera use as semen additive our readings could not be compared and discussed, but its beneficial effect as semen additive in post-thawed semen is noticeable and further studies should be done with other possible concentrations and complete semen analysis.

In conclusion, it was the pioneer study in which due to antioxidant property of Aloe vera it was tried as a semen additive. Due to its beneficial effects in the cryopreserved semen it may be used for the cryopreservation of the semen. The parameters of semen quality improved in the Aloe vera added cryopreserved semen in comparison of control group. The oxidative stress to the semen is also minimized after addition of the Aloe vera. The in vitro fertility assessed by hypo-osmotic swelling test also indicates for the better quality and fertility in Aloe vera added cryopreserved semen, however, the larger number of the semen samples along with other concentrations of Aloe vera coupled with field fertility trials should be done in the cattle population for its final validation.

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