The Journal of Animal & Plant Sciences, 25(5): 2015, Page: 1222-1226
ISSN: 1018-7081

EFFECT OF DIFFERENT CONCENTRATIONS OF ASCORBIC ACID ON SEMEN QUALITY AND HATCHABILITY OF INDIGENOUS ASEEL CHICKEN

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ABSTRACT

Aseel, a famous chicken breed, has problems of low fertility that leads to poor hatchability. In commercial breeder setups, the hatchability issues are being addressed by fresh semen artificial insemination (AI). The success of AI mainly depends on semen quality and its life in female reproductive tract. Now a days, different antioxidants are being used to improve the semen quality. This study was designed to optimize the ascorbic acid addition, in semen extender, as an antioxidant. Semen from twenty roosters was collected by abdominal massage. After pooling, the semen was extended in modified ringer's solution (1:3) supplemented with different concentrations of ascorbic acid (0, 0.25, 0.5, 0.75, 1, 1.25 and 1.5% w/v). For longevity testing, *in-vitro* sperm parameters (motility, live ratio and morphology defects) were evaluated, at 0, 6, 12, and 24 h intervals during storage at 4°C. The protection of ascorbic acid against oxidative stress was evident within 6 h storage by semen motility improvement, (69% in 1% ascorbic acid vs 63% in control; $p<0.05$). Similarly, for sperm live ratio, the difference became clear within 6 h (80% for 0.75, 1, 1.25% vs 72% for control; $p<0.05$). After 24 h storage, the improvements, in 1% ascorbic acid samples, became more evident ($p<0.05$) in terms of motility, live sperm and morphological defects. However, the higher concentration of ascorbic acid (1.5%) showed compromised results. This represents the toxic effect of ascorbic acid on higher concentrations. To access our final target, for hatchability improvement, 100 Aseel hens were inseminated with semen. Total hens were divided in two groups and inseminated with best supplemented (1% ascorbic acid) or control (0% ascorbic acid) semen. The supplemented semen enhanced egg hatchability (55% vs 45%; $p>0.05$). These results indicate that the success rate of AI can be improved by using 1% ascorbic acid in poultry semen extender.

Key words: Ascorbic acid, Indigenous Aseel chicken, Semen quality, Hatchability.

INTRODUCTION

Aseel, a historical indigenous chicken breed is reared as a game bird in Pakistan (Ahmad *et al.*, 2014). The breed is famous for its stamina, pugnacity, majestic gate and dogged fighting properties (Panda and Mahapatra, 1989). In rural areas, Aseel is also favored because of their immunity, adaptability to harsh environment and production of organic meat (Mohan *et al.*, 2008). However, this breed is facing the issues of more broodiness, less egg production, small egg size, low fertility and poor hatchability rate (Iqbal *et al.*, 2012). One of the contributing factors in low fertility and hatchability is the poor semen quality from male side.

In commercial poultry systems, artificial insemination (AI) is being used successfully to overcome the fertility issues (Khaezi *et al.*, 2010). For AI, semen is handled in *in-vitro* conditions during which reactive oxygen species (ROS) are produced (Partyka *et al.*, 2012). These ROS has several deleterious effects, including sperm DNA damage, oxidation of polyunsaturated fatty acids (lipid peroxidation), oxidation of amino acids and inactivation of specific enzymes (Aitken *et al.*, 2010; Peris *et al.*, 2007). In chicken sperm, membrane has higher amounts of polyunsaturated fatty acids (Partyka *et al.*, 2012) which make it more vulnerable to lipid peroxidation (LPO). The natural defense system against the ROS damage depends on enzymatic and non-enzymatic antioxidants (Fejercakova *et al.*, 2013). To boost up this defense system, a variety of antioxidants may be added, either through diet or in extenders during semen extension.

Ascorbic acid is the water-soluble antioxidant, found naturally in chicken spermatozoa and seminal plasma (Surai *et al.*, 2001). It is well established that dietary supplementation of ascorbic acid improves quality of poultry semen (Elansary *et al.*, 1999; Khan *et al.*, 2012; McDaniel *et al.*, 1998). Information regarding the direct role of ascorbic acid on semen quality is limited. The current study was designed to evaluate the *in-vitro* effect of ascorbic acid on semen quality of Aseel chicken, during hypothermic storage. The outcome from the present research would provide fundamental awareness about ascorbic acid usage as an antioxidant for the improvement of sperm quality and hatchability.

MATERIALS AND METHODS

Experimental Birds: Aseel roosters (n=20) and hens (n=100), 40-50 weeks aged were selected and caged individually. The standard breeder diet (100g; daily) was offered to each bird. The availability of water was ad libitum through automatic system. The lightening schedule was 16h light and 8h darkness which was maintained by natural sun light and artificial light. The experiment was conducted from September to December 2013 which comprises of 10 weeks.

Semen Collectionp: Semen was milked down by abdominal massage technique twice weekly (Riaz *et al.,* 2004). During the whole research same time, same person and same place were used for semen collection to avoid the stress on males. Ejaculates containing any contamination of urine, feces or blood were rejected. The ejaculates with more than 60% motility were pooled for further experiment.

Semen processing and storage: The modified ringer's solution was used as base medium for extension and storage of semen. The composition of modified ringer's solution was; sodium chloride 0.68, potassium chloride 0.173, calcium chloride 0.064, magnesium sulphate 0.025 and sodium bicarbonate 0.24; % w/v in distill water (Tabatabaei, 2012). To nullify the male effect, after collection and evaluation the individual semen samples were pooled. To maintain sperm cell concentration in countable range semen was further diluted in 1:3 ratios in the modified ringer's solution supplemented with different concentration of ascorbic acid (0.0, 0.25, 0.5, 0.75, 1.0, 1.25 and 1.5, % w/v). Semen was refrigerated at 4^{o}C and evaluated at 0, 6, 12 and 24 hours of incubation for motility, live sperm and morphological abnormalities. The whole experiment was repeated for five times.

Semen Evaluation: For motility estimation, a drop of extended semen was placed on clean, pre warmed glass slide (37^{o}C). After applying cover slip, motility was assessed under 10x objective of light microscope. At least three fields were observed before final decision.

The eosin nigrosin staining technique was used to estimate live spermatozoa. One drop of semen and stain was placed on pre wormed (37^{0}C) glass slide and gently mixed them. A thin smear was prepared on another glass slide and observes under 40x objective of light microscope. The spermatozoa containing unstained head was counted as live while stained head of spermatozoa counted as dead (Lukaszewicz et al. 2008). At least 300 sperms were counted before final decision.

The eosin nigrosin stained slide was used for the estimation of morphological defects under 100x oil emersion lens of light microscope. The morphological defects which were observed included swollen head, coiled tail and bent tail (Lukaszewicz et al. 2008). At least 300 sperms were counted before final decision.

Artificial Insemination: As per commercial farm practices, pooled semen from 20 roosters was extended in 1:1 ratio to maintain proper sperm concentration for AI. For *in-vivo* evaluation, only best concentration of ascorbic acid supplementation (1%) was compared with control group (modified ringer's solution only). Aseel hens (n=100) were equally divided into two groups and inseminated with either treatment or control group. AI was performed once weekly for three weeks. AI was performed by everting hen's vaginal orifice by applying pressure on left side of abdomen (Quinn and burrows 1936). About 0.25 ml of diluted semen was injected within 15 min of collection with the help of dropper. Egg collection was started after one week of insemination. Eggs were collected daily and labeled. The 100 egg were collected for each group. After that eggs were shifted to hatchery and incubated in hatcher with standard conditions. For the estimation of fertility, candling was performed at day 14 of incubation.

Statistical Analysis: Repeated measure analysis of variance (ANOVA) under completely randomized design was performed for seven concentrations of ascorbic acid and four time points (7x4 factorial) taking time as repeated measure. Data were presented as Mean±SEM. The significant differences in means were compared by using Duncan's Multiple Range (DMR) test. Fertility and hatchability rate was compared using Chi square analysis. All analysis were performed using statistical software SAS (Version 9.1 SAS Inst., Inc., Car, NC, USA).

RESULTS

The pooled semen sample was divided into seven aliquots to treat with different concentrations of ascorbic acid. For all evaluated sperm parameters, no differences were observed between ascorbic acid concentrations and control at 0 h of incubation.

Motility was higher ($p<0.05$) in 1% ascorbic acid than all other treatments at 6 and 12h of incubation while at 24 h of incubation 0.75%, 1% and 1.25% ascorbic acid contain higher ($p<0.05$) motility than other treatments (Table 1).

The live sperm was higher ($p<0.05$) in 0.75%, 1% and 1.25% ascorbic acid at 6h of incubation. At 12 and 24h of incubation 1% ascorbic acid contain higher ($p<0.05$) proportion of live sperms (Table 2).

At 6 and 12h of incubation morphological defects were non-significant ($p>0.05$) among all treatments while at 24 h of incubation 1% ascorbic acid contain lesser ($p<0.05$) morphological defects than other treatments (Table 3).

The overall means of sperm motility, live sperm and morphological defects evaluated at different time

intervals (0, 6, 12 and 24 h) during 24 h storage at 4 °C was better ($p<0.05$) in 1% ascorbic Acid.

Similarly the fertility was better (55%) for the group that was inseminated with semen extended in modified ringer's solution supplemented with 1% ascorbic acid and as compare to control group (45%) ($p>0.05$).

Table 1. Effect of ascorbic acid on semen motility (%) during 24 h storage at 4°C in indigenous chicken Aseel semen (n=5)

Ascorbic Acid %	Semen motility (%) during incubation			
	0 h	**6 h**	**12 h**	**24 h**
0.00	72±1.2[a]	63± 1.2[b]	52±1.2[bc]	23±1.2[b]
0.25	73±1.2 [a]	62±1.2 [b]	49±2.4[c]	23±1.2[b]
0.50	73±1.2 [a]	63±1.2[b]	50±1.0[bc]	25±1.0[b]
0.75	71±1.0[a]	63±1.2[b]	53±1.2[b]	32±1.2[a]
1.00	74±1.0[a]	69±1.0[a]	64±1.0[a]	35±1.0[a]
1.25	71±1.0 [a]	63±1.2[b]	53±1.2[b]	32±1.2[a]
1.50	73±1.2 [a]	62±1.2[b]	50±1.0[bc]	25±1.0[b]

[a-b] denote difference in columns($p<0.05$)

Table 2. Effect of ascorbic acid on live sperm ratio during 24 h storage at 4°C in indigenous chicken Aseel semen (n=5)

Ascorbic Acid %	Live sperms (%) during incubation			
	0 h	6 h	12 h	24 h
0.00	82±1.2[a]	72±1.2[b]	62±1.2[cd]	38±1.2[c]
0.25	83±1.2[a]	72±1.2[b]	61±1.0[d]	33±1.2[d]
0.50	82±1.2[a]	73±3.0[b]	65±1.0[c]	40±0.4[c]
0.75	83±1.2[a]	80±2.0[a]	70±1.0[b]	60±1.0[b]
1.00	82±1.2[a]	80±1.2[a]	72±2.0[a]	67±1.0[a]
1.25	83±1.0[a]	80±1.0[a]	70±1.0[b]	60±1.0[b]
1.50	82±1.2[a]	70±1.0[c]	65±1.0[c]	40±1.0[c]

[a-d] denote difference in columns($p<0.05$)

Table 3. Effect of ascorbic acid on sperm morphological defects (%) during 24 h storage at 4°C in indigenous chicken Aseel semen (n=5).

Ascorbic Acid %	Morphological defects (%) during incubation			
	0h	6h	12h	24h
0.00	8.0±0.1[a]	14.8±0.4[a]	18.8±0.4[a]	48±1.2[d]
0.25	8.1±0.1[a]	11.2±0.4[a]	13.2±0.4[a]	43±1.2[cd]
0.50	8.0±0.1[a]	11.2±0.8[a]	15.4±0.2[a]	37±1.2[cd]
0.75	8.0±0.1[a]	9.6±0.4[a]	13.2±0.4[a]	32±1.2[bc]
1.00	7.9±0.1[a]	9.2±0.2[a]	12.8±0.4[a]	26±1.0[a]
1.25	7.9±1.2[a]	9.2±0.4[a]	13.2±0.4[a]	32±1.2[bc]
1.50	7.9±0.1[a]	12.0±0.3[a]	15.0±0.2[a]	37±1.2[cd]

[a-d] denote difference in columns($p<0.05$)

DISCUSSION

The present experiment revealed that 1% ascorbic acid provide better resistance against the ROS damage during liquid storage in terms of sperm motility, live sperm, morphological defects and for fertility. Similar results were documented by (Tabatabaei, 2012) who observed that sperm motility, viability and

morphological defects were better when chicken semen was treated with 1% ascorbic acid. In contrast of our results, ascorbic acid fails to improve sperm motility, viability and membrane integrity in turkey semen (Donoghue and Donoghue, 1997) which may be due to the specie difference.

The positive effect of ascorbic acid can be attributed to the fact that it is very efficient antioxidant and scavenges ROS which are noxious to the sperm. Ascorbic acid is a dominant antioxidant when peroxyl radicals are located in the aqueous phase (Donoghue and Donoghue, 1997). In rabbit seminal plasma, the concentration of Ascorbic acid was found ten times higher as compared to serum and in pheasants it was realized that it contributes about 65% of the total antioxidant capacity of seminal plasma (Nowaczewski and Kontecka, 2005; Yousef *et al.*, 2003). Ascorbic acid is also required for male hormones production like testosterone, which is essential for the reproductive performance (Sönmez *et al.*, 2005).

Though, the numerical values of fertility are improved after supplementation of 1% ascorbic acid, but it could not prove it statistically ($p>0.05$). The reason might be the less number of inseminations, because only 50 pure bred Aseel hen were available and based on poor laying performance 100 eggs from each group were used for hatchability analysis. The results need to be retested by applying on big flock size. It is speculated that ascorbic acid as antioxidant improve the storage conditions for sperm in specialized sperm storage tubules of hens by reducing lipid peroxidation. The development of the resistance against the lipid peroxidation with antioxidants like ascorbic acid has the practical significance for the improvement of the refrigerated storage of the chicken semen and fertility.

Conclusion: It is concluded that in vitro semen quality and hatchability of Indigenous Aseel chicken could be improved by supplementation with 1% ascorbic acid.

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