



RESEARCH ARTICLE

Effect of Diluent Supplementation with Different Levels of Orange Juice on Semen Quality during Liquid Storage of Roosters' Semen

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ABSTRACT

The effect of adding orange juice to the extender of roosters' semen on mass activity, individual motility, dead and abnormal spermatozoa and acrosomal abnormalities were studied. A total of 60 White layer cocks, 32 weeks of age, randomly divided into 6 groups of 10 cocks each were used in this experiment. The treatment groups were; T1 - the control (fresh semen) ; T2 - the semen extended 1 : 1 with Al-Daraji 2 extender (AD2E) alone , whereas T3 , T4 , T5 and T6 represented semen samples extended with AD2E extender and supplemented with 1 , 4 , 7 or 10 ml of orange juice / 100 ml extender. Results revealed that after 0, 24, 48 or 72 h *in vitro* storage, the supplementation of roosters semen extender with 7 and 10 ml orange juice / 100 ml of extender (T5, T6) caused significant ($P < 0.05$) increases in mass activity and individual motility of spermatozoa and significant decreases ($P < 0.05$) in percentages of dead spermatozoa, abnormal spermatozoa and acrosomal abnormalities compared with control group. However, T2, T3 and T4 groups showed significant improvements in all of these parameters in comparison with control group. Moreover, it was also noticed that there no significant differences ($p > 0.05$) between T5 and T6 and among T2, T3 and T4 regarding all semen characteristics included in this study. In conclusion the supplementation of orange juice into semen extender plays an important role in protecting spermatozoa against the harmful effects of lipid peroxidation during *in vitro* storage of roosters' semen for up to 72 hours.

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INTRODUCTION

An important reason for the decrease in motility and viability during the storage of semen is the formation of lipid peroxides from oxygen radicals (17). The sperm plasma membranes contain a high amount of unsaturated fatty acids. Therefore, it is particularly susceptible to peroxidative damage, the lipid peroxidation destroys the structure of the lipid matrix in the membranes of spermatozoa, and it is associated with a loss of motility and membrane integrity (29).

System that satisfy the metabolic requirement for oxygenation of avian spermatozoa during storage have improved the maintenance of fertilizing ability , but have led to consideration that the limiting factor of sperm maintenance may now be the deleterious effects of oxygen free-radicals and resultant lipid peroxidation. This has been identified as significant and problematic for both

chicken and turkey spermatozoa, which having a high proportion of polyunsaturated fatty acids (PUFAs), are therefore considered to be likely to be particularly susceptible to lipid peroxidation (31).

Indeed, even at low temperatures, spermatozoa of both chickens and turkey were suffering from lipid peroxide during liquid storage (12). However, a correlation between semen quality and fertilizing ability and lipid peroxides following storage has yet to be made.

Blesbois et al. (8) pointed out that in chicken peroxides are already present at the time of ejaculation, in equal concentration between spermatozoa and seminal plasma (2 to 4×10^{-2} nM Malonaldehyde/ 10^9 spermatozoa). However, sperms are subject to oxygen toxicity resulting from lipid peroxidation, which can result in membrane damage, reduced motility and lower fertility (11).

Semen contains appreciable amounts of antioxidants that balance lipid peroxidation and prevent excessive

peroxide formation (21). However, the endogenous antioxidative capacity of semen may be insufficient during storage or dilution (23). *In vitro* studies suggested that the addition of some antioxidants to diluted semen could improve the motility and survival of spermatozoa (25; 26).

Recently, natural foods and food-derived antioxidants such as vitamin C and phenolic phytochemicals have received growing attention, because they are known to function as chemopreventive agents against oxidative damage (18). Gardener et al. (16) studying the relative contributions of vitamin C, carotenoids and phenolics to the antioxidant potential of fruit juices, including orange, grapefruit, pink grapefruit, apple, pineapple and vegetable-juices and found that both vitamin concentrate-ions and total phenolic contents strongly correlated with antioxidant capacity. Antioxidant capacities of different juices varied markedly, orange juice being 5-7 folds more active than the vegetable juice and had the highest antioxidant power.

The objective of this study was to investigate the effect of adding different levels of natural orange juice to semen extender on quality of roosters' semen after liquid storage for up to 72 hours

MATERIALS AND METHODS

This experiment was carried out to investigate the effect of diluent supplementation with orange juice on semen quality of roosters during liquid storage. A total of 60 White Layer cocks, 32 weeks of age, randomly divided into 6 groups (10 cocks each) and housed in separate terrestrial pens were used in this study. Cocks fed a commercial ration (16% protein and 2850 kcal metabolic energy / kg of diet) *ad libitum*. The semen was collected from all roosters manually by dorsal – abdominal massage (19), on a week basis, for 8 consecutive weeks (32-40 weeks of age). Semen samples for each treatment pen were divided into 3 test tubes of 1 ml each to provide 3 replicates pooled samples per each treatment. In order to maximize, the quality and quantity of collected semen, collection was always performed under the same conditions (environment, time, persons, and massage method). Only clean ejaculates were used for treatments and evaluating. The experimental groups were as follows : T1 = fresh semen (control group) ; T2 = semen extended 1 : 1 with Al-Daraji 2 extender (AD2E) (4) alone ; T3 = semen extended with AD2E and supplemented with 1 ml of orange juice / 100 ml of extender ; T4 = semen extended with AD2E and supplemented with 4 ml of orange juice / 100 ml of extender ; T5 = semen extended with AD2E and supplemented with 7 ml of orange juice / 100 ml of extender and T6 = semen extended with AD2E and supplemented with 10 ml orange juice / 100 ml of extender . However, the levels of orange juice involved in the AD2E were choose on the basis of results of the preliminary experiment that conducted before the initiation of present study (unpublished data). The pH of extenders was adjusted to be 6.8 – 7.2 by using phosphate buffer solutions. Semen samples were then stored at the refrigerator temperature (4-6°C) for certain storage periods (24, 48 and 72 h). Orange juice were extracted

from fresh orange and then the juice filtrated by filter paper before it supplemented to the extenders.

An aliquot of semen from each treatment group was evaluated directly after collection and then after *in vitro* storage for 24, 48 and 72 h for mass activity, individual motility, percentages of dead spermatozoa, abnormal spermatozoa and acrosomal abnormalities. Spermatozoa motility (movement in a forward) was estimated on a percentage basis by using the microscopic method of Sexton (28). The measurement of dead spermatozoa was achieved by using a Fast green stain – Eosin B stain – glutamate extender (5). Percentage of abnormal spermatozoa was determined by using a Gentian Violet – Eosin stain (1). As an alternative to evaluate acrosomal abnormalities in birds, staining procedure for fixed samples have been developed to distinguish which spermatozoa have retained or lost the acrosome (2). Data were statistically analysis using the general linear model for analysis of variance of SAS (27). Test of significance for the difference between means of different levels within each classification was done by Duncan's multiple range tests (27).

RESULTS

The results obtained from this experiment confirm that the supplementation of the AD2E extender with 7 or 10 ml orange juice / 100 ml of extender (T5, T6) improved mass activity, individual motility, percentage of live and normal spermatozoa and intact acrosomes in comparison with control (T1), T2, T3 and T4 groups (Tables 1, 2, 3, 4 and 5) when semen samples evaluated directly after collecting or after *in vitro* storage for 24, 48 and 72 h. Also T2, T3 and T4 surpasses control group with respect to these characteristics. Furthermore, there were no significant differences ($P > 0.05$) between T2, T3 and T4, and between T5 and T6 concerning these spermatozoa traits.

Table 1: Effect of AD2E supplementation with orange juice on mass activity (Mean \pm SE) of roosters' semen *in vitro* stored for certain storage periods.

Treatments	Storage periods (hours)			
	0	24	48	72
T1	83.1 \pm 4.0 ^c	39.0 \pm 3.6 ^c	18.3 \pm 2.0 ^c	2.1 \pm 0.9 ^c
T2	88.2 \pm 3.6 ^b	81.6 \pm 4.0 ^b	75.6 \pm 1.7 ^b	63.0 \pm 4.3 ^b
T3	89.1 \pm 1.7 ^b	83.3 \pm 3.0 ^b	77.2 \pm 3.9 ^b	64.2 \pm 3.9 ^b
T4	90.0 \pm 3.3 ^b	84.9 \pm 2.2 ^b	78.0 \pm 6.0 ^b	65.3 \pm 2.6 ^b
T5	95.3 \pm 1.6 ^a	90.3 \pm 1.8 ^a	87.3 \pm 3.6 ^a	79.6 \pm 1.3 ^a
T6	96.2 \pm 5.3 ^a	93.1 \pm 3.3 ^a	90.9 \pm 5.5 ^a	80.9 \pm 5.0 ^a

T1 = Fresh semen, T2 = semen diluted with AD2E alone, and T3 – T6 = semen diluted with AD2E and supplemented with 1, 4, 7 and 10 ml of orange juice / 100 ml of extender, respectively; * Each value represented the mean of 8 consecutive measurements; ** Values in a column with different superscripts differ significantly ($P < 0.05$).

The wholesome effects in semen quality that accompanied with enrichment of semen extender with orange juice may be account for it excellent source of vitamin C (22, 24), which act as water – soluble antioxidant to scavenge aqueous peroxel radicals before these destructive substance have a chance to damage the lipids (34). On the other hand, the major finding in recent

years was the possibility of vitamin E in recycling from its oxidized (radical) form by means of vitamin C. However, vitamin C works synergistically with vitamin E (fat – soluble antioxidant) and the antioxidant enzyme glutathione peroxidase, performing their protective effects against lipid peroxidation and preserving cell membrane integrity (9, 12). Furthermore, vitamin C can participate with vitamin E to protect molecules such as DNA from oxidative damage (33).

Many studies confirm our conclusions; Surai et al. (32) reported that vitamin C as a water-soluble antioxidant was found in avian seminal plasma and it was negatively correlated with accumulation of reactive

Table 2: Effect of AD2E supplementation with orange juice on individual motility (Mean \pm SE) of roosters' semen *in vitro* stored for certain storage periods.

Treatments	Storage periods (hours)			
	0	24	48	72
T1	84.6 \pm 3.3 ^c	40.3 \pm 3.3 ^c	19.9 \pm 3.3 ^c	4.9 \pm 1.0 ^c
T2	89.9 \pm 5.7 ^b	82.9 \pm 5.5 ^b	76.7 \pm 3.8 ^b	64.2 \pm 4.3 ^b
T3	90.3 \pm 3.6 ^b	84.0 \pm 2.9 ^b	78.1 \pm 5.0 ^b	65.8 \pm 3.9 ^b
T4	91.8 \pm 2.7 ^b	85.9 \pm 3.6 ^b	79.3 \pm 4.2 ^b	67.3 \pm 2.6 ^b
T5	96.0 \pm 3.9 ^a	91.6 \pm 2.7 ^a	88.1 \pm 3.3 ^a	81.3 \pm 4.0 ^a
T6	98.1 \pm 4.4 ^a	93.8 \pm 3.0 ^a	91.9 \pm 5.6 ^a	83.7 \pm 2.9 ^a

T1 = Fresh semen, T2 = semen diluted with AD2E alone, and T3 – T6 = semen diluted with AD2E and supplemented with 1, 4, 7 and 10 ml of orange juice / 100 ml of extender, respectively; * Each value represented the mean of 8 consecutive measurements; ** Values in a column with different superscripts differ significantly (P < 0.05).

Table 3: Effect of AD2E supplementation with orange juice on percentage of dead spermatozoa (Mean \pm SE) of roosters' semen *in vitro* stored for certain storage periods.

Treatments	Storage periods (hours)			
	0	24	48	72
T1	23.6 \pm 2.2 ^a	60.3 \pm 3.0 ^a	85.6 \pm 1.3 ^a	98.0 \pm 5.8 ^a
T2	16.0 \pm 1.9 ^b	30.1 \pm 2.8 ^b	43.9 \pm 2.0 ^b	53.6 \pm 2.9 ^b
T3	15.1 \pm 2.0 ^b	28.2 \pm 1.3 ^b	41.2 \pm 1.3 ^b	50.0 \pm 1.7 ^b
T4	13.3 \pm 1.3 ^b	27.0 \pm 2.2 ^b	40.0 \pm 2.3 ^b	50.9 \pm 2.3 ^b
T5	5.1 \pm 2.0 ^c	13.7 \pm 1.6 ^c	23.8 \pm 1.6 ^c	33.0 \pm 1.7 ^c
T6	3.0 \pm 1.8 ^c	11.2 \pm 2.0 ^c	20.3 \pm 2.2 ^c	30.8 \pm 2.2 ^c

T1 = Fresh semen, T2 = semen diluted with AD2E alone, and T3 – T6 = semen diluted with AD2E and supplemented with 1, 4, 7 and 10 ml of orange juice / 100 ml of extender, respectively; * Each value represented the mean of 8 consecutive measurements; ** Values in a column with different superscripts differ significantly (P < 0.05).

Table 4: Effect of AD2E supplementation with orange juice on percentages of abnormal spermatozoa (Mean \pm SE) of roosters' semen *in vitro* stored for certain storage periods.

Treatments	Storage periods (hours)			
	0	24	48	72
T1	23.6 \pm 2.6 ^a	60.9 \pm 3.7 ^a	90.2 \pm 6.7 ^a	100.0 \pm 0.0 ^a
T2	14.0 \pm 1.7 ^b	29.0 \pm 2.9 ^b	50.3 \pm 3.9 ^b	63.2 \pm 5.1 ^b
T3	12.3 \pm 2.0 ^b	26.7 \pm 1.3 ^b	48.9 \pm 2.2 ^b	60.3 \pm 3.9 ^b
T4	11.9 \pm 1.0 ^b	26.0 \pm 2.9 ^b	47.0 \pm 1.7 ^b	60.9 \pm 2.8 ^b
T5	4.1 \pm 2.9 ^c	13.0 \pm 1.7 ^c	30.3 \pm 2.6 ^c	41.8 \pm 4.0 ^c
T6	3.3 \pm 0.8 ^c	10.8 \pm 3.3 ^c	27.8 \pm 1.9 ^c	39.3 \pm 5.1 ^c

T1 = Fresh semen, T2 = semen diluted with AD2E alone, and T3 – T6 = semen diluted with AD2E and supplemented with 1, 4, 7 and 10 ml of orange juice / 100 ml of extender, respectively; *Each value represented the mean of 8 consecutive measurements; ** Values in a column with different superscripts differ significantly (P < 0.05).

Table 5: Effect of extender supplementation with orange juice on percentage of acrosomal abnormalities (Mean \pm SE) of roosters' semen *in vitro* stored for certain storage periods.

Treatments	Storage periods (hours)			
	0	24	48	72
T1	22.0 \pm 2.7 ^a	70.8 \pm 3.5 ^a	90.2 \pm 2.9 ^a	99.7 \pm 6.5 ^a
T2	17.6 \pm 1.3 ^b	35.0 \pm 2.2 ^b	48.8 \pm 3.9 ^b	60.3 \pm 5.8 ^b
T3	15.1 \pm 2.0 ^b	33.6 \pm 4.3 ^b	45.3 \pm 1.7 ^b	59.0 \pm 3.6 ^b
T4	14.7 \pm 3.9 ^b	33.9 \pm 1.8 ^b	45.0 \pm 4.0 ^b	58.1 \pm 2.7 ^b
T5	5.0 \pm 4.4 ^c	12.1 \pm 1.4 ^c	26.7 \pm 3.9 ^c	37.3 \pm 4.8 ^c
T6	2.3 \pm 1.3 ^c	13.9 \pm 3.0 ^c	23.9 \pm 2.6 ^c	35.9 \pm 3.0 ^c

T1 = Fresh semen, T2 = semen diluted with AD2E alone, and T3 – T6 = semen diluted with AD2E and supplemented with 1, 4, 7 and 10 ml of orange juice / 100 ml of extender, respectively; *Each value represented the mean of 8 consecutive measurements; ** Values in a column with different superscripts differ significantly (P < 0.05).

oxygen species and positively correlated with the percentage of spermatozoa displaying normal morphology. Moreover, vitamin C is an important for the spermatozoa since it has been shown to restore the physiological constitution of PUFA in cell membrane under certain conditions (20). Andrzej et al. (6) found that the lack or low levels of ascorbic acid in semen highly correlated with the damage to male germ cells. These results are in agreement with Al-Daraji (3) and El-Nasry et al. (14), who found that the supplementation of some antioxidants (vitamins A, C or E) to the semen extenders resulted in significant improvement in motility, survivability, morphology and fertilizing ability of roosters semen after *in vitro* storage for different periods. Other works reported that addition of ascorbic acid to sperm preparation medium did provide sperm with complete protection against H₂O₂ – induced DNA damage and generation of H₂O₂ – induced radicals oxygen species (ROS) and was also significantly reduced after treatment with ascorbic acid (10, 13). The antioxidant capacity of orange juice was not only vitamin C content of juice but may also arise from some of the phenolic compounds (30). The major phenolic compound in orange juice is ferulic acid (7), it accounts especially good at neutralizing the free radicals known as superoxide, hydroxyl radical and nitric oxide and it acts synergistically with other antioxidant giving them extra potency and greatly reduces free radical damage to the external and internal membranes of cells (36). These informations give us an indicator, that all improvements in semen quality when treated with orange juice belong to another agent (Ferulic acid) found in juice with vitamin C and they act together to protect spermatozoa from lipid peroxidation during storage. Our conclusion confirms Zheng and Zhang (35) which reported that ferulic acid was beneficial to sperm viability and motility in both fertile and infertile individuals, and that reduction of lipid peroxidative damage to sperm membranes and increase of intracellular cAMP and cGMP may be involved in these benefits, and it is possible that ferulic acid may be used for cure of asthenozoosperm infertility.

Conclusion

The present results lead to conclude that orange juice components especially vitamin C and ferulic acid produce

good repression against lipid peroxidation during liquid storage of roosters semen. Thus, the addition of orange juice to semen extender was suitable agent for preserving semen quality when, semen stored at the refrigerator for up to 72 hours.

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