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Research Article

The use of Pomegranate Juice for Counteract Lipid Peroxidation that Naturally occurred During Liquid Storage of Roosters' Semen

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ABSTRACT

Materials and Methods: In an attempt to find a suitable *in vitro* storage method for roosters' semen, an experiment was conducted to study the influence of inclusion pomegranate juice (PJ) into semen diluent on semen quality during liquid storage for up to 36 h. A total of 60 White Leghorn roosters, 40 weeks of age, randomly divided into 6 treatment groups (10 males each) were used in this study. Treatment groups were as follows: T1 = fresh semen, T2 = semen diluted 1:2 with Al-Daraji 2 diluent (AD2D) alone, T3 – T6 = semen diluted 1:2 with AD2D supplemented with 2 ml, 4 ml, 6 ml or 8 ml of PJ / 100 ml of diluent, respectively. Semen samples were assessed after *in vitro* storage at 4 – 6°C for 12 h, 24 h or 36 h as regards mass motility, individual motility and percentages of dead spermatozoa, abnormal spermatozoa and acrosomal abnormalities. **Results:** Results revealed that supplementing the diluent of roosters semen with PJ (T3, T4, T5 and T6) and then storing it for different storage periods (12 h, 24 h or 36 h) resulted in significant ($p < 0.05$) improvement in spermatozoa motility, viability, morphology and acrosomal integrity in comparison with the control group (T1). Moreover, T5 and T6 surpassed other treatments with respect to these semen characteristics, while there were no significant differences between T2, T3 and T4 concerning all semen traits included in this study. **Conclusion:** In conclusion, the substitution of AD2D diluent composition with PJ significantly improves the quality of roosters semen that is *in vitro* stored for up to 36 h. Furthermore, the positive effect of PJ observed in this study may be due to enhanced sperm resistance to lipid peroxidation that naturally occurred during *in vitro* storage of avian semen.

Key Words : Pomegranate juice, lipid peroxidation, liquid storage, roosters' semen.

INTRODUCTION

Free radicals are unstable molecules that include the hydrogen atom, nitric oxide and molecular oxygen. These occur naturally in the body as a result of chemical reactions during normal cellular processes. In an attempt to stabilize, they attack other molecules in the body potentially leading to cell damage and triggering the formation of another free radical resulting in a chain reaction.[1] The recent interest in the role of free radicals and other reactive oxygen and nitrogen species in the physiology and pathology of cells and organisms has stimulated interest in the oxidant compounds, protecting vital cellular targets against oxidative attack and contributing to the maintenance of low, steady – state levels of reactive oxygen and nitrogen species.[2]

A characteristic feature of most, if not all, biological membranes is an asymmetrical arrangement of lipids within the bilayer. The lipid composition of plasma membrane of avian spermatozoa is markedly different from that of somatic cells. They have very high levels of phospholipids, sterols, saturated and polyunsaturated fatty acids. Therefore sperm cells are particularly susceptible to the damage induced by excessive reactive oxygen species (ROS) release.[3] This unusual structure of sperm mem-

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brane is responsible for its flexibility and the functional ability of sperm cells. However, spermatozoa lipids are the main substrates for peroxidation, which may provoke severe functional disorder of sperm.[4] Peroxidation of polyunsaturated fatty acids (PUFAs) in sperm cell membranes is an autocatalytic, self – propagating reaction, which can give a rise to cell dysfunction associated with loss of membrane function and integrity.[5]

The current methods of semen storage are only effective for short periods of time (up to 12 h) and need to be improved.[6] One of the conditions necessary to store semen *in vitro* is a cool temperature, generally 2-5˚C. However, the use of low temperatures in combination with a buffered saline medium containing glycolytic substrates and intermediates of the citric acid cycle are not sufficient to ensure prolonged *in vitro* survival of avian spermatozoa.[7,8] found that the phospholipid profiles and content of turkey spermatozoa are severely affected by *in vitro* storage, and the evolution of phospholipids is parallel to the decrease in semen quality. However, the major changes occur during the first hours (1-4 h) of semen storage. This could preferentially originate from the lipid peroxidation and endogenous metabolism of the fatty acids of the membrane phospholipids and induce membrane destabilization.[9]

Evidence suggests that nutritional antioxidants such as Pomegranate juice (PJ) can contribute to the reduction of oxidative stress and atherogenesis.[10,11] reported that PJ supplementation to mice with advanced atherosclerosis reduced their cell oxidative stress, their cells cholesterol flux, and even attenuated the development of atherosclerosis. Moreover, tannin – fraction of PJ had significant anti oxidative stress and antiatherosclerotic activity.[12] noticed that both the juice and the oil of pomegranate contain numerous and diverse bioflavonoids, which have been shown to be both potently antioxidant and inhibitory of one or both of the enzymes cyclooxygenase and lipooxygenase. However,[13] recommended the use of pomegranates in the treatment of AIDS disease owing to their antioxidant properties and botanical uniqueness. Therefore the present study was designed to examine the role of PJ in counteracting the detrimental effects of lipid peroxidation that naturally occur during *in vitro* storage of roosters' semen.

MATERIALS AND METHODS

This study was conducted to determine whether the addition of PJ to semen diluent could improve quality of roosters' semen when *in vitro* stored for up to 72 h. Sixty males (White Leghorn, 40 wk of age) divided in 6 treatment groups of 10 males were used for experimentation. They were raised in floor pens and fed a commercial diet containing 16.5% crude protein and 2850 metabolizable energy / kg. Semen was routinely collected from all roosters twice a week by abdominal massage[14] during the whole experimental period which lasted 12 weeks (40 – 52 weeks of age). After each collection, pools of semen (each pool from five males in each treatment group, therefore there were two pools for each treatment group) were transferred to the laboratory. Treatment groups were as follows: T1 = fresh semen, T2 = semen diluted 1:2 with Al-Daraji 2 diluent (AD2D)[15] alone, T3 – T6 = semen diluted with 1:2 AD2D supplemented with 2 ml, 4 ml, 6 ml or 8 ml of PJ / 100 ml of diluent, respectively. However, pH of diluents was adjusted to be 6.8 – 7.0 by using phosphate buffer solution. Semen samples were then stored at the refrigerator (4-6˚C) for 12 h, 24 h, or 36 h. Aliquots of semen samples were removed at 12, 24 or 36 h storage for further measurements of spermatozoa motility, viability, morphology and acrosomal integrity. Spermatozoa motility (movement in a forward) was estimated on a percentage basis by using the microscopic method of Sexton.[16] Viability was assessed by Fast green stain – Eosin B stain – glutamate extender.[17] The proportion of morphologically normal spermatozoa was measured by using a Gentian violet – eosin stain.[18] Acrosomal abnormalities were determined according the procedure reported by.[19]

Changes in the motility, viability and morphological integrity of spermatozoa after *in vitro* storage for different periods (0 h, 12 h, 24 h or 36 h) were evaluated by analysis of variance. Difference between treatments groups' means were analyzed by Duncan's multiple range test, using the ANOVA procedure in Statistical Analysis System.[20]

RESULTS AND DISCUSSION

The results denoted that supplementation of the AD2D with PJ resulted in a significant ($p < 0.05$) increase in mass motility and individual motility when semen samples were evaluated directly after collection or after different storage periods (12 h, 24 h or 36 h) in comparison with T1 group (Tables 1 and 2). Treatments 5 and 6 surpass other treatments of PJ (T3 and T4) with respect to these two traits. However, there were no significant differences between T2, T3 and T4 concerning these two characteristics.

Our results also revealed that the inclusion of PJ into diluent resulted in significant ($p < 0.05$) decreases in the per-

Table 1: Effect of AD2E supplementation with pomegranate juice on mass motility (Mean ± SE) of roosters' semen *in vitro* stored for certain storage periods.

Treatments	Storage periods (hours)			
	0	12	24	36
T1	82.1 ± 4.0 [c]	38.0 ± 3.6 [c]	17.3 ± 2.0 [c]	1.1 ± 0.9 [c]
T2	87.2 ± 3.6 [b]	88.6 ± 4.0 [b]	74.6 ± 1.7 [b]	62.0 ± 4.3 [b]
T3	88.1 ± 1.7 [b]	84.3 ± 3.0 [b]	75.2 ± 3.9 [b]	63.2 ± 3.9 [b]
T4	89.0 ± 3.3 [b]	84.9 ± 2.2 [b]	76.0 ± 6.0 [b]	64.3 ± 2.6 [b]
T5	92.3 ± 1.6 [a]	90.3 ± 1.8 [a]	86.3 ± 3.6 [a]	78.6 ± 1.3 [a]
T6	94.2 ± 5.3 [a]	92.1 ± 3.3 [a]	89.9 ± 5.5 [a]	79.9 ± 5.0 [a]

T1 = Fresh semen, T2 = semen diluted with AD2E alone, and T3 – T6 = semen diluted with AD2E and supplemented with 2, 4, 6 and 8 ml of pomegranate juice / 100 ml of extender, respectively : * Each value represented the mean of 24 consecutive measurements that conducted during 12 consecutive weeks on the basis of 2 measurements each week : [a, b, c] Values in a column with different superscripts differ significantly (P < 0.05).

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

Treatments	Storage periods (hours)			
	0	12	24	36
T1	83.6 ± 3.3 [c]	39.3 ± 3.3 [c]	18.9 ± 3.3 [c]	1.9 ± 1.0 [c]
T2	88.9 ± 5.7 [b]	81.9 ± 5.5 [b]	75.7 ± 3.8 [b]	63.2 ± 4.3 [b]
T3	89.3 ± 3.6 [b]	83.0 ± 2.9 [b]	77.1 ± 5.0 [b]	64.8 ± 3.9 [b]
T4	89.8 + 2.7 [b]	84.9 ± 3.6 [b]	78.3 ± 4.2 [b]	66.3 ± 2.6 [b]
T5	94.8 ± 3.9 [a]	90.6 ± 2.7 [a]	87.1 ± 3.3 [a]	80.3 ± 4.0 [a]
T6	95.7 ± 4.4 [a]	92.8 ± 3.0 [a]	89.7 ± 5.6 [a]	82.7 ± 2.9 [a]

T1 = Fresh semen, T2 = semen diluted with AD2E alone, and T3 – T6 = semen diluted with AD2E and supplemented with 2, 4, 6 and 8 ml of pomegranate juice / 100 ml of extender, respectively : * Each value represented the mean of 24 consecutive measurements that conducted during 12 consecutive weeks on the basis of 2 measurements each week : [a, b, c] Values in a column with different superscripts differ significantly (P < 0.05).

centages of dead spermatozoa, abnormal spermatozoa and acrosomal abnormalities when semen samples were examined before storage or after certain storage periods (12 h, 24h or 36 h)

compared with fresh semen group (T1) (Tables 3, 4 and 5). Additionally, T5 and T6 were inferior to other PJ treatments as regards these three characteristics, while there were no significant differences between T2, T3 and T4 in relation to these three features.

The addition of PJ (especially at the concentrations of 6 and 8 ml / 100 ml of diluents) to the semen diluent resulted in significant improvement in quality of roosters' semen either directly after semen collection or when semen *in vitro* stored for up to 72 h. These positive results may be due the PJ having very potent antioxidant activity. Presser and Fuhrman[21] demonstrated that antioxidant properties of PJ were significantly superior to that of red wine and approaching that of premium green tea and the synthetic antioxidant, butyrated hydroxyanisole

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

Treatments	Storage periods (hours)			
	0	12	24	36
T1	24.6 ± 2.2 [a]	61.3 ± 3.0 [a]	86.6 ± 1.3 [a]	99.0 ± 5.8 [a]
T2	17.0 ± 1.9 [b]	31.1 ± 2.8 [b]	44.9 ± 2.0 [b]	54.6 ± 2.9 [b]
T3	16.1 ± 2.0 [b]	29.2 ± 1.3 [b]	42.2 ± 1.3 [b]	51.0 ± 1.7 [b]
T4	14.3 ± 1.3 [b]	28.0 ± 2.2 [b]	41.0 ± 2.3 [b]	52.9 ± 2.3 [b]
T5	6.1 ± 2.0 [c]	14.7 ± 1.6 [c]	24.8 ± 1.6 [c]	35.0 ± 1.7 [c]
T6	4.0 ± 1.8 [c]	12.2 ± 2.0 [c]	21.3 ± 2.2 [c]	31.8 ± 2.2 [c]

T1 = Fresh semen, T2 = semen diluted with AD2E alone, and T3 – T6 = semen diluted with AD2E and supplemented with 2, 4, 6 and 8 ml of pomegranate juice / 100 ml of extender, respectively : * Each value represented the mean of 24 consecutive measurements that conducted during 12 consecutive weeks on the basis of 2 measurements each week : [a, b, c] Values in a column with different superscripts differ significantly (P < 0.05).

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

Treatments	Storage periods (hours)			
	0	12	24	36
T1	24.6 ± 2.6 [a]	61.9 ± 3.7 [a]	91.2 ± 6.7 [a]	100.0 ± 0.0 [a]
T2	15.0 ± 1.7 [b]	30.0 ± 2.9 [b]	51.3 ± 3.9 [b]	64.2 ± 5.1 [b]
T3	13.3 ± 2.0 [b]	27.7 ± 1.3 [b]	49.9 ± 2.2 [b]	61.3 ± 3.9 [b]
T4	10.9 ± 1.0 [b]	27.0 ± 2.9 [b]	48.0 ± 1.7 [b]	61.9 ± 2.8 [b]
T5	5.1 ± 2.9 [c]	14.0 ± 1.7 [c]	31.3 ± 2.6 [c]	42.8 ± 4.0 [c]
T6	4.3± 0.8 [c]	11.8 ± 3.3 [c]	28.8 ± 1.9 [c]	40.3 ± 5.1 [c]

T1 = Fresh semen, T2 = semen diluted with AD2E alone, and T3 – T6 = semen diluted with AD2E and supplemented with 2, 4, 6 and 8 ml of pomegranate juice / 100 ml of extender, respectively : * Each value represented the mean of 24 consecutive measurements that conducted during 12 consecutive weeks on the basis of 2 measurements each week : [a, b, c] Values in a column with different superscripts differ significantly (P < 0.05).

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

Treatments	Storage periods (hours)			
	0	12	24	36
T1	23.0 ± 2.7 [a]	71.8 ± 3.5 [a]	91.2 ± 2.9 [a]	99.9 ± 6.5 [a]
T2	18.6 ± 1.3 b	36.0 ± 2.2 [b]	49.8 ± 3.9 [b]	61.3 ± 5.8 [b]
T3	16.1 ± 2.0 [b]	34.6 ± 4.3 [b]	46.3 ± 1.7 [b]	60.0 ± 3.6 [b]
T4	15.7 ± 3.9 [b]	34.9 ± 1.8 [b]	47.0 ± 4.0 [b]	59.1 ± 2.7 [b]
T5	6.0 ± 4.4 [c]	13.1 ± 1.4 [c]	27.7 ± 3.9 [c]	38.3 ± 4.8 [c]
T6	3.3 ± 1.3 [c]	14.9 ± 3.0 [c]	24.9 ± 2.6 [c]	36.9 ± 3.0 [c]

T1 = Fresh semen, T2 = semen diluted with AD2E alone, and T3 – T6 = semen diluted with AD2E and supplemented with 2, 4, 6 and 8 ml of pomegranate juice / 100 ml of extender, respectively : * Each value represented the mean of 24 consecutive measurements that conducted during 12 consecutive weeks on the basis of 2 measurements each week : [a, b, c] Values in a column with different superscripts differ significantly (P < 0.05).

(BHA). Longtin[22] concluded that the antioxidant capacity of PJ is dependent not only vitamin C content of juice, but also other antioxidant – rich tannins and flavonoids compounds. However, he suggested that the antioxidant capacity of PJ is a function of the combined action of a number of constituents.[7] reported that fresh PJ contains 85% water, 10% total sugars, and 1.5% pectin, ascorbic acid, and polyphenolic flavonoids. Furthermore, fructose and glucose are present in similar quantities in PJ. Calcium is 50% of its ash content, and the principal amino acids are glutamic and aspartic acid.[23] showed that the consumption of PJ exhibited powerful antioxidant effects and identified polyphenols as the active compounds responsible for the effects of PJ against LDL oxidation and oxidative stress. Moreover,[24] demonstrated an effective role for PJ and cold pressed pomegranate seed oil as potential natural food preservatives, therapeutic agent, antioxidant and / or health protective agents.

Pomegranate is also a rich source of other food factors, including vitamins, minerals, sugars, and non – nutritive phytochemicals which may exhibit biological activity in a number of ways. These phytochemicals may act as antioxidants, act as antioestrogens, or modulate bacterial populations in the body or media.[25] The antioxidant activity of pomegranate is often assumed to be of greatest importance in combating a number of degenerative diseases, as free radical – related damage has been implicated in causing many of these conditions.[26,27] observed that following consumption of PJ for 2 weeks, plasma concentrations of ascorbic acids, α – and β – carotene, retinol and tocopherol were all significantly increased.[28] reported that pomegranate fruit contained very high concentrations of antioxidants (11.33 mmol / 100 g). Other fruits with high antioxidant content included grape, orange, plum, pineapple, lemon, date, kiwi, clementine and grapefruit which contained between 0.83 and 1.43 mmol antioxidants per 100 g. Flavonoids and other phenolic compounds appear to be some of the other antioxidants that contribute to the high antioxidant capacity measured in pomegranate; which have antioxidant activities that are several times stronger than those of vitamins E and C.[29] These phenolic compounds have already been implicated in the protection by pomegranate consumption against diseases and disorders that are associated with oxidative stresses.

In contrast,[8] noticed that motility tests (missal motility and the proportion of motile spermatozoa), viability and the proportion of morphologically normal living sper-

matozoa were severely decreased when semen *in vitro* stored at 4°C for up to 48 h. These changes in semen quality and the failure of *in vitro* storage may be explained by membrane phospholipids lysis followed by endogenous metabolism or by a complex combination of lysis, metabolism, and lipid peroxidation.[8] Lipids appear to be involved in the success of *in vitro* semen storage. In the case of cooling without freezing, the membrane lipid moieties are in a liquid – crystalline phase that affects the physical and biochemical properties of spermatozoa.[30] Lipids of spermatozoa are involved in mechanisms of cell resistance to cold shock, aerobic peroxidation and are believed to be metabolized activity.[4] Wishart[31] concluded that as a likely result of the high proportion of PUFAs, avian spermatozoa showed a significant susceptibility to lipid peroxidation, which was associated with loss of viability and fertilizing ability of spermatozoa.[8] found that the decrease in sperm motility associated with ROS occurs in combination with significant decrease in viability, acrosomal integrity, mitochondrial membrane potential and significant increases in lipid peroxidation. Long and Kramer[32] pointed out that lipid peroxidation is a significant factor affecting the fertility of stored turkey sperm and that methods to prevent or reduce lipid peroxidation remain to be elucidated. Moreover, Roger[33] reported that PJ is also packed with vitamins A, C and E, all of which boost sexual libido in men and women. Vitamin A increases testosterone and estrogen levels, while a lack of vitamin E in the diet could mean a lower sex drive and reduced fertility.[34] Vitamin C boosts sexual appetite and increases men's semen volume.

CONCLUSION

In conclusion, substitution of semen diluent with PJ was found to significantly improved storage ability of roosters' semen, which was assessed for motility, viability and morphology. In addition, with respect to lipid peroxidation that naturally occurred during *in vitro* storage of avian semen, our finding confirm the protective effects of PJ against lipid peroxidation during liquid storage of roosters' semen for up to 36 h.

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