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Effects of pomegranate juice as diluent supplementation on sperm quality during liquid storage of chicken semen

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Abstract

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 72 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 1 ml, 3 ml, 5 ml or 7 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 activity, individual motility and percentages of dead spermatozoa, abnormal spermatozoa and acrosomal abnormalities. Results revealed that supplementing the diluent of roosters semen with PJ (T3, T4, T5 and T6) and then store 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 control group (T1). Moreover, T5 and T6 surpasses 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. In conclusion, the substitution of AD2D diluent composition with PJ significantly improves quality of roosters semen that *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, semen quality**,** liquid storage, chicken semen

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1. Introduction

Free radicals are unstable molecules that include the hydrogen atom, nitric oxide and molecular oxygen. These naturally occur 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 (Sies, 1997). 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 oxidants compounds, protecting vital cellular targets against oxidative attack and contributing to the maintenance of low, steady – state levels of reactive oxygen and nitrogen species (Janaszewska and Bartosz, 2002). 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 (Culver, 2001). This unusual structure of sperm membrane is responsible for its flexibility and the functional ability of sperm cells. However, spermatozoa lipids are the main substrates for peroxidation, what may provoke severe functional disorder of sperm (Sanocka and Kurpisza, 2004). 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 (Ford, 2004).

The current methods of serum storage are only effective for short periods of time (up to 12 h) and need to be improved (Thurston, 1995). 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 (Blesbois et al., 1999). Douard et al. (2000) found that phospholipids profile and content of turkey spermatozoa are severely affected by *in vitro* storage, and the evolution of phospholipids is parallel to decrease in semen quality. However, the major changes occurred 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 (Wishart, 2004).

Evidence suggests the nutritional antioxidants such as Pomegranate juice (PJ) can contribute to the reduction of oxidative stress and atherogenesis (Aviram et al., 2000). Kalpan et al. (2001) 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 in the PJ had a significant anti oxidative stress and antiatherosclerotic activity. Lansky et al (2002) 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, Lee and Watson (1998) reported that medical monograph recommends 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 probable role of PJ in counteracting the detrimental effects of lipid peroxidation that naturally occurred during *in vitro* storage of roosters' semen.

2. 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 % CP and 2850 ME / kg. Semen was routinely collected from all roosters twice a week by abdominal massage (Lake and Stewart, 1978) 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)(Al-Daraji, 2004) alone, T3 – T6 = semen diluted with 1:2 AD2D supplemented with 1 ml, 3 ml, 5 ml or 7 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 24 h, 48 h, or 72 h. Aliquots of semen samples were removed at 12, 24 and 36 h after *in vitro* 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 (1976). Viability was assessed by Fast green stain – Eosin B stain – glutamate extender (Al-Daraji et al., 2002). The proportion of morphologically normal spermatozoa was measured by using a Gentian violet – eosin stain (Al-Daraji, 1998). Acrosomal abnormalities were determined according the procedure reported by Al-Daraji (Al-Daraji, 2001). 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. Different between treatments groups' means were analyzed by Duncan's multiple range test, using the ANOVA procedure in Statistical Analysis System (SAS, 1996).

3. Results and Discussion

The results denoted that supplementation the AD2D with PJ resulted in significant ($p < 0.05$) increase in mass activity and individual motility when semen samples 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. Resulted also revealed that the inclusion of PJ into diluent resulted in significant ($p < 0.05$) decrease in percentages of dead spermatozoa, abnormal spermatozoa and

acrosomal abnormalities when semen samples were examined before storage or after certain storage periods (12 h, 24 h 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. It was obvious from the data of this experiment that the addition of PJ especially at the concentrations of 6 and 8 ml / 100 ml of diluent 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 36 h.These positive results may be explained by that PJ had very potent antioxidant activity. Presser and Fuhrman (2000) 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 (BHA) (Longtin, 2003) concluded that antioxidant capacity of PJ is dependent not only vitamin C content of juice but also antioxidant – rich tannins and flavonoids compounds.

However, he suggested that antioxidant capacity of PJ is a function of the combined action of a number of constituents. Aviram et al. (2000) reported that fresh PJ contains 85 % water, 10 % total sugars, and 1.5 % pectin, ascorbic acid, and polyphenolic flavonoids. Furthermore, in PJ, fructose and glucose are present in similar quantities, calcium is 50 % of its ash content, and the principal amino acids are glutamic and aspartic acid. Aviram and Dornfeld (2001) 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, Schubert et al. (1999) demonstrated an effective role for PJ and cold pressed pomegranate seed oil as potential natural food preservatives, therapeutic agent, antioxidant and / or health protective. Besides, 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.

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

Treatments	Storage periods (hours)			
	0	12	24	36
T1	83.6 ± 1.3^{c}	41 ± 1.1^{c}	18.9 ± 1.8^{c}	6.9 ± 1.3^{c}
T2	87.1 ± 2.0^{b}	80.6 ± 1.6^{b}	76.7 ± 1.3^{b}	68.5 ± 1.0^{b}
T3	89.6 ± 1.1^{b}	82.9 ± 1.3^{b}	78.1 ± 1.6^{b}	70.1 ± 1.1^{b}
T4	89.9 ± 2.3^{b}	83.8 ± 1.5^{b}	79.3 ± 1.4^{b}	72.9 ± 2.1^{b}
T5	95.2 ± 1.2^{a}	90.1 ± 1.5^{a}	85.8 ± 1.2^{a}	80.3 ± 1.9^{a}
T6	96.9 ± 1.7^{a}	91.9 ± 1.4^{a}	87.9 ± 1.6^{a}	83.9 ± 1.2^{a}

T1 = fresh semen, T2 = semen diluted with AD2D) alone, T3 = semen diluted with AD2D and supplemented with PJ (1 ml / 100 ml), T4 = semen diluted with AD2D and supplemented with PJ (3 ml / 100 ml), T5 = semen diluted with AD2D and supplemented with PJ (5 ml / 100 ml), T6 = semen diluted with AD2D and supplemented with PJ (7 ml / 100 ml), AD2D = Al-Daraji 2 diluent.
Each value represented the mean of 12 measures that conducted during 12 consecutive weeks.
Values in a column with different superscripts differ significantly ($P < 0.05$).

Table.2. Effect of diluent 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	$86.6 + 3.3^{c}$	$41.3 + 3.3^{c}$	$20.9 + 3.3^{c}$	$5.9 + 1.0^{c}$
T2	$91.9 + 5.7^{b}$	$84.9 + 5.5^{b}$	$78.7 + 3.8^{b}$	$66.2 + 4.3^{b}$
T3	$91.3 + 3.6^{b}$	$85.0 + 2.9^{b}$	$79.1 + 5.0^{b}$	$66.8 + 3.9^{b}$
T4	$93.8 + 2.7^{b}$	$87.9 + 3.6^{b}$	$81.3 + 4.2^{b}$	$69.3 + 2.6^{b}$
T5	$98.0 + 3.9^{a}$	$92.6 + 2.7^{a}$	$89.1 + 3.3^{a}$	$82.3 + 4.0^{a}$
T6	$99.1 + 4.4^{a}$	$95.8 + 3.0^{a}$	$93.9 + 5.6^{a}$	$85.7 + 2.9^{a}$

T1 = fresh semen, T2 = semen diluted with AD2D) alone, T3 = semen diluted with AD2D and supplemented with PJ (1 ml / 100 ml), T4 = semen diluted with AD2D and supplemented with PJ (3 ml / 100 ml), T5 = semen diluted with AD2D and supplemented with PJ (5 ml / 100 ml), T6 = semen diluted with AD2D and supplemented with PJ (7 ml / 100 ml), AD2D = Al-Daraji 2 diluent.
Each value represented the mean of 12 measures that conducted during 12 consecutive weeks.
Values in a column with different superscripts differ significantly ($P < 0.05$).

Table.3 Effect of diluent 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	25.6 + 2.2 [a]	62.3 + 3.0 [a]	87.6 + 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	17.1 + 2.0 [b]	30.2 + 1.3 [b]	43.2 + 1.3 [b]	52.0 + 1.7 [b]
T4	14.3 + 1.3 [b]	28.0 + 2.2 [b]	41.0 + 2.3 [b]	51.9 + 2.3 [b]
T5	7.1 + 2.0 [c]	15.7 + 1.6 [c]	24.8 + 1.6 [c]	34.0 + 1.7 [c]
T6	5.0 + 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 AD2D) alone, T3 = semen diluted with AD2D and supplemented with PJ (1 ml / 100 ml), T4 = semen diluted with AD2D and supplemented with PJ (3 ml / 100 ml), T5 = semen diluted with AD2D and supplemented with PJ (5 ml / 100 ml), T6 = semen diluted with AD2D and supplemented with PJ (7 ml / 100 ml), AD2D = Al-Daraji 2 diluent.
Each value represented the mean of 12 measures that conducted during 12 consecutive weeks.
Values in a column with different superscripts differ significantly ($P < 0.05$).

Table.4 Effect of diluent 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	16.0 + 1.7 [b]	31.0 + 2.9 [b]	52.3 + 3.9 [b]	65.2 + 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	13.9 + 1.0 [b]	28.0 + 2.9 [b]	49.0 + 1.7 [b]	62.9 + 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	5.3 + 0.8 [c]	12.8 + 3.3 [c]	29.8 + 1.9 [c]	41.3 + 5.1 [c]

T1 = fresh semen, T2 = semen diluted with AD2D) alone, T3 = semen diluted with AD2D and supplemented with PJ (1 ml / 100 ml), T4 = semen diluted with AD2D and supplemented with PJ (3 ml / 100 ml), T5 = semen diluted with AD2D and supplemented with PJ (5 ml / 100 ml), T6 = semen diluted with AD2D and supplemented with PJ (7 ml / 100 ml), AD2D = Al-Daraji 2 diluent.
Each value represented the mean of 12 measures that conducted during 12 consecutive weeks.
Values in a column with different superscripts differ significantly ($P < 0.05$).

Table.5 Effect of diluent 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	24	48	72
T1	25.0 + 2.7 [a]	73.8 + 3.5 [a]	93.2 + 2.9 [a]	99.7 + 6.5 [a]
T2	19.6 + 1.3 [b]	37.0 + 2.2 [b]	50.8 + 3.9 [b]	62.3 + 5.8 [b]
T3	18.1 + 2.0 [b]	36.6 + 4.3 [b]	48.3 + 1.7 [b]	62.0 + 3.6 [b]
T4	16.7 + 3.9 [b]	35.9 + 1.8 [b]	47.0 + 4.0 [b]	60.1 + 2.7 [b]
T5	8.0 + 4.4 [c]	15.1 + 1.4 [c]	29.7 + 3.9 [c]	40.3 + 4.8 [c]
T6	4.3 + 1.3 [c]	15.9 + 3.0 [c]	25.9 + 2.6 [c]	37.9 + 3.0 [c]

T1 = fresh semen, T2 = semen diluted with AD2D) alone, T3 = semen diluted with AD2D and supplemented with PJ (1 ml / 100 ml), T4 = semen diluted with AD2D and supplemented with PJ (3 ml / 100 ml), T5 = semen diluted with AD2D and supplemented with PJ (5 ml / 100 ml), T6 = semen diluted with AD2D and supplemented with PJ (7 ml / 100 ml), AD2D = Al-Daraji 2 diluent.
Each value represented the mean of 12 measures that conducted during 12 consecutive weeks.
Values in a column with different superscripts differ significantly ($P < 0.05$).

These phytochemicals may act as antioxidants, include enzymes, act as pro or antioestrogens, or modulate bacterial populations in the body or media (Lampe, 1999). 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 (Arao et al., 2004). Record et al. (2001) 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. Halvorsen et al. (2002) found that analyses of fruits demonstrated that pomegranate contained very high concentrations of antioxidants, i.e., 11.33 mmol/100g. 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 (Cao et al., 1998). These phenolic compounds have already been implicated in the protection by pomegranate consumption against diseases and disorders that associated with oxidative stresses. On the other hand, Douard et al. (2000) noticed that motility tests (missal motility and the proportion of motile spermatozoa), viability and the proportion of morphologically normal living spermatozoa 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 (Douard et al., 2000).

However, 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 liquid – crystalline phase that affects the physical and biochemical properties of spermatozoa (Parks and Lynch, 1992). Lipids of spermatozoa are involved in mechanisms of cell resistance to cold shock, aerobic peroxidation and are believed to be metabolized activity (Sanocka and Kurpisz, 2004). Wishart (2005) concluded that as a likely result of 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. Baumber et al. (2000) found that the decrease in sperm motility associated with ROS occurs in combination with significant decrease in viability, acrosomal integrity, and mitochondrial membrane potential and significant increase in lipid peroxidation. Long and Kramer (2003) 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 (2005) 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. Vitamin C boosts sexual appetite and increases men's semen volume. 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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