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INFLUENCE OF SUPPLEMENTING DILUENT OF SEMEN WITH POMEGRANATE JUICE ON ROOSTERS SEMEN QUALITY DURING *IN VITRO* STORAGE

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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 layer 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 24 h, 48 h or 72 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 times (24 h, 48 h or 72 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 72 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.

تأثير إضافة عصير الرمان الى مخفف السائل المنوي في نوعية السائل المنوي للديكة خلال فترة خزنه

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المستخلص

كمحاولة لايجاد طريقة ملائمة لخزن السائل المنوي للديكة، اجريت التجربة الحالية لدراسة تأثير ادخال عصير الرمان في مخفف السائل المنوي في نوعية السائل المنوي المخزون لفترة تصل الى 72 ساعة. استخدم في هذه الدراسة 60 ديك لكهورن ابيض بعمر 40 اسبوع، اذ تم توزيعها عشوائياً على ست معاملات يتكون كل منها من 10 ديكة. وكانت معاملات التجربة كما يلي: المعاملة الاولى : السائل المنوي الطازج، المعاملة الثانية : السائل المنوي المخفف بنسبة 2:1 بمخفف Al – Daraji2 من دون اية اضافة، المعاملات 3 – 6 : السائل المنوي المخفف بنسبة 2:1 بمخفف Al – Daraji2 المضاف اليه عصير الرمان بتراكيز 2 مل، 4 مل، 6 مل و 8 مل / 100 مل من المخفف على التوالي. وتم تقويم عينات السائل المنوي بعد خزنها بدرجة 4 – 6 ^{0}م لمدة 24 ساعة، 48 ساعة أو 72 ساعة بالنسبة للحركة الجماعية والفردية للنطف والنسبة المئوية للنطف الميتة والمشوهة وتشوهات الاكروسومات.

اظهرت النتائج ان اضافة عصير الرمان الى مخففات السائل المنوي للديكة والتي تم خزنها لفترات خزن مختلفة (24 ساعة، 48 ساعة أو 72 ساعة) ادت الى تحسن معنوي ($أ < 0.05$) في حركة النطف وحيويتها وسلامتها وسلامة اكروسوماتها بالمقارنة مع مجموعة المقارنة (المعاملة الاولى). اضافة لذلك، فأن المعاملتين 5 و 6 قد تفوقت على جميع المعاملات الاخرى فيما يتعلق بهذه الصفات، بينما لم تكن هناك فروقات معنوية بين المعاملات 2 و 3 و 4 فيما يخص جميع صفات السائل المنوي المتضمنة في الدراسة الحالية.

يستنتج من الدراسة الحالية، إن ادخال عصير الرمان في مخفف Al – Daraji2 حسّن وبصورة معنوية نوعية السائل المنوي للديكة والمخزون لفترة تصل الى 72 ساعة. إضافة لذلك، فأن التأثير الايجابي لعصير الرمان الملاحظ في هذه الدراسة ربما يكون بسبب تعزيز مقاومة النطف لعملية تأكسد الدهون التي تحدث بصورة طبيعية اثناء فترة خزن السائل المنوي للطيور.

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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 (31). 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 (15).

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 (11). 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 (27). 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 (13).

The current methods of semen storage are only effective for short times of time (up to 12 h) and need to be improved (32). 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 (9). Douard et al. (12) 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 (33).

Evidence suggests the nutritional antioxidants such as Pomegranate juice (PJ) can contribute to the reduction of oxidative stress and atherogenesis (7). Kalpan et al. (16) 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 (19) 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 (20) reported that 1998 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.

Materials and Methods

This study was conducted at the Poultry farm, Department of Animal Resources, College of Agriculture during the period from 1/11/2004 to 1/1/2005 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 (17) during the whole

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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 tow 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)(3) 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. 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 24, 48 and 72 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 (30). Viability was assessed by Fast green stain – Eosin B stain – glutamate extender (4). The proportion of morphologically normal spermatozoa was measured by using a Gentian violet – eosin stain (1). Acrosomal abnormalities were determined according the procedure reported by Al – Daraji (2).

Changes in the motility, viability and morphological integrity of spermatozoa after *in vitro* storage for different times (0 h, 24 h, 48 h or 72 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 (28).

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 times (24 h, 48 h or 72 h) in comparison with T1 group (Figures 1 and 2). T5 and T6 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 times (24 h, 48h or 72 h) compared with fresh semen group (T1) (Figures 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 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 explained by that PJ had very potent antioxidant activity. Presser and Fuhrman (24) 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 (22) 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. (7) 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 (6) 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. (29) demonstrated an effective role for PJ and cold pressed pomegranate seed oil as potential natural food preservatives, therapeutic agent, antioxidant and / or health protective.

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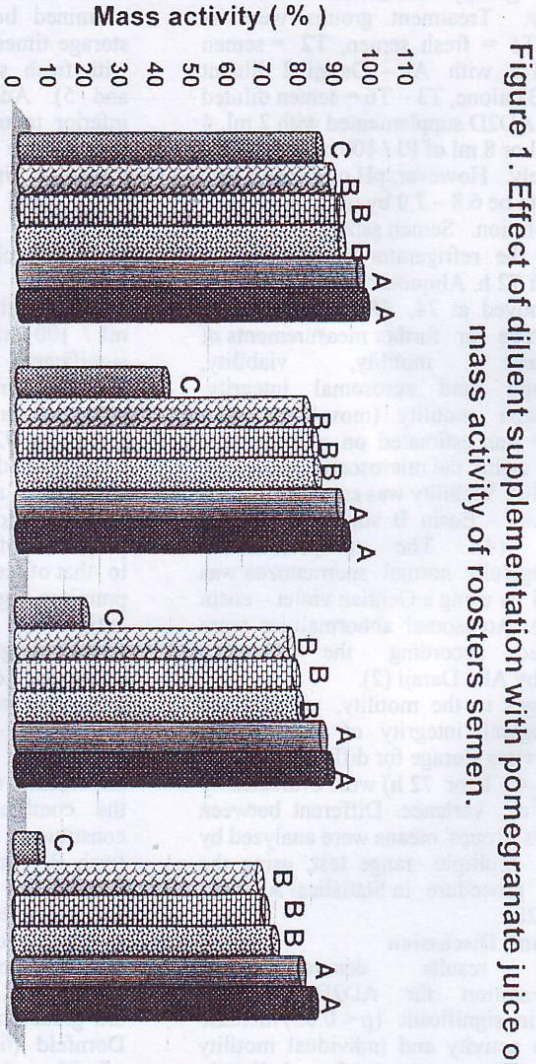

Figure 1. Effect of diluent supplementation with pomegranate juice on mass activity of roosters semen.

T1 = fresh semen, T2 = semen diluted with AD2 D) alone , T3 = semen diluted with AD2 D and supplemented with PJ (2 ml / 100 ml), T4 = semen diluted with AD2 D and supplemented with PJ (4 ml / 100 ml), T5 = semen diluted with AD2 D and supplemented with PJ (6 ml / 100 ml), T6 = semen diluted with AD2 D and supplemented with PJ (8 ml / 100 ml), AD2 D = Al – Daraji 2 diluent.
Each value represented the mean of 12 measures that conducted during 12 consecutive weeks.
Bars with different superscripts differ significantly ($p < 0.05$).

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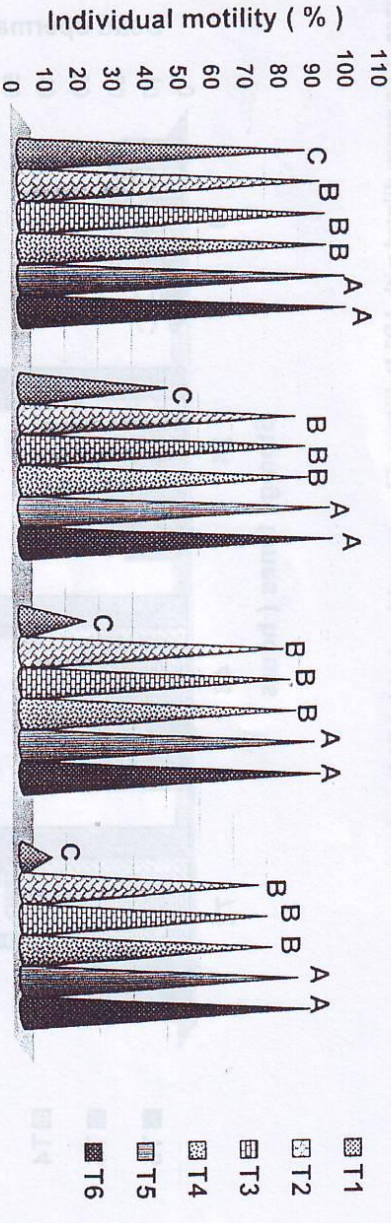
Figure 2. Effect of diluent supplementation with pomegranate juice on individual motility of roosters semen.



T1 = fresh semen, T2 = semen diluted with AD2 D alone, T3 = semen diluted with AD2 D and supplemented with PJ (2 ml / 100 ml), T4 = semen diluted with AD2 D and supplemented with PJ (4 ml / 100 ml), T5 = semen diluted with AD2 D and supplemented with PJ (6 ml / 100 ml), T6 = semen diluted with AD2 D and supplemented with PJ (8 ml / 100 ml), AD2 D = Al – Daraji 2 diluent.
Each value represented the mean of 12 measures that conducted during 12 consecutive weeks.
Bars with different superscripts differ significantly ($p < 0.05$).

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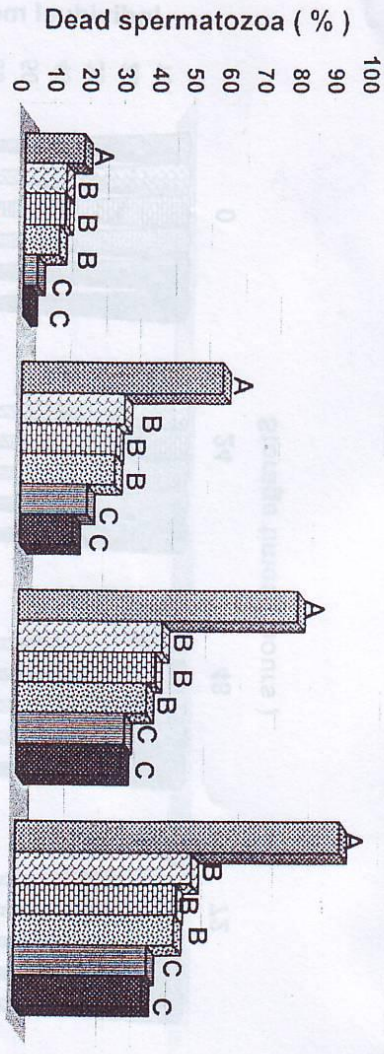
Figure 3. Effect of diluent supplementetion with pomegranate juice on dead spermatozoa of roosters semen.



T1 = fresh semen, T2 = semen diluted with AD2 D diluent, T3 = semen diluted with AD2 D and supplemented with PJ (2 ml / 100 ml), T4 = semen diluted with AD2 D and supplemented with PJ (4 ml / 100 ml), T5 = semen diluted with AD2 D and supplemented with PJ (6 ml / 100 ml), T6 = semen diluted with AD2 D and supplemented with PJ (8 ml / 100 ml), AD2 D = Al – Daraji 2 diluent.
Each value represented the value of 12 measures that conducted during 12 consecutive weeks.
Bars with different superscripts differ significantly ($p < 0.05$).

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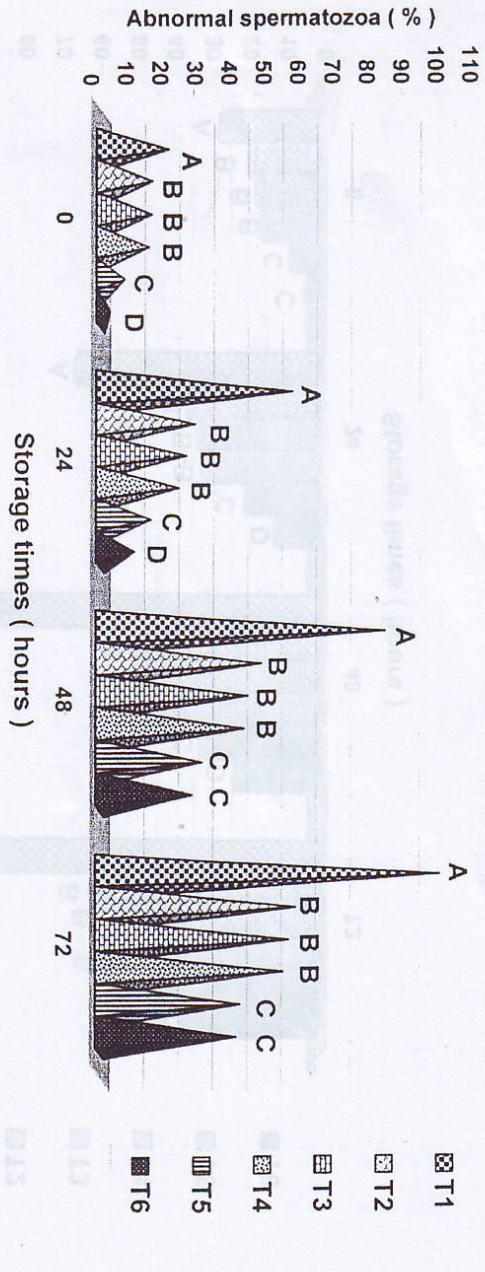

Figure 4. Effect of diluent supplementation with pomegranate juice on abnormal spermatozoa of roosters semen.

T1 = fresh semen, T2 = semen diluted with AD2 D alone, T3 = semen diluted with AD2 D and supplemented with PJ (2 ml / 100 ml), T4 = semen diluted with AD2 D and supplemented with PJ (4 ml / 100 ml), T5= semen diluted with AD2 D and supplemented with PJ (6 ml / 100 ml), T6= semen diluted with AD2 D and supplemented with PJ (8 ml / 100 ml), AD2 D = Al – Daraji 2 diluent.
Each value represented the mean of 12 measures that conducted during 12 consecutive weeks.
Bars with different superscripts differ significantly ($p < 0.05$).

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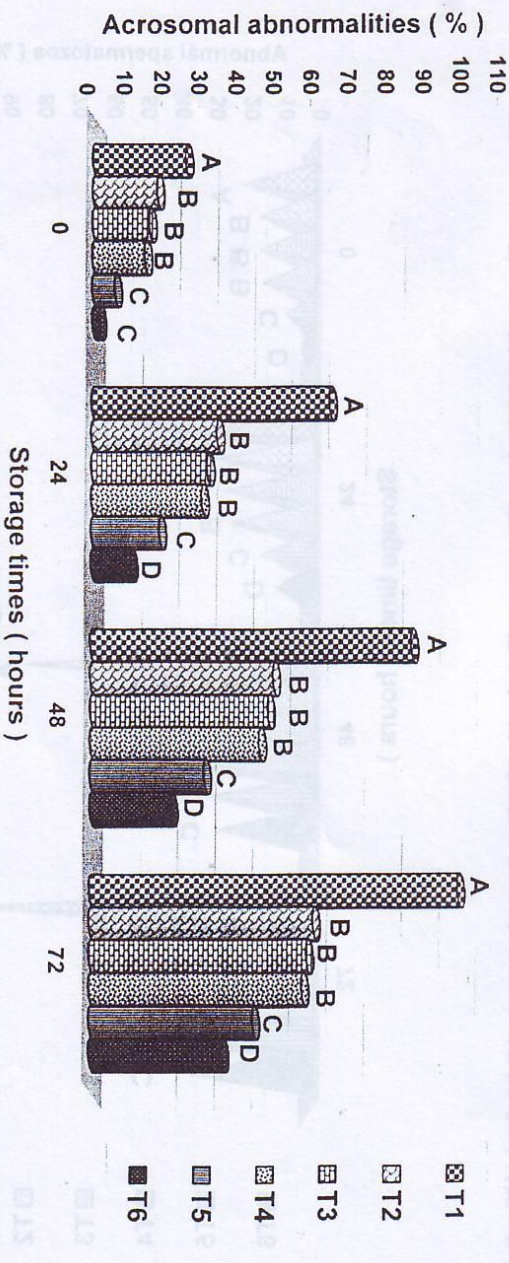
Figure 5. Effect of diluent supplementation with pomegranate juice on acrosomal abnormalities of roosters semen.



T1 = fresh semen, T2 = semen diluted with AD 2 D alone, T3 = semen diluted with AD2 D and supplemented with PJ (2 ml / 100 ml), T4 = semen diluted with AD2 D and supplemented with PJ (4 ml / 100 ml), T5= semen diluted with AD2 D and supplemented with PJ (6ml / 100 ml), T6 = semen diluted with AD2 D and supplemented with PJ (8 ml / 100 ml). AD2 D = Al – Daraji 2 diluent.
Each value represented the mean of 12 measures that conducted during 12 consecutive weeks.
Bars with different superscripts differ significantly ($p < 0.05$).

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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. These phytochemicals may act as antioxidants, include enzymes, act as pro – or antioestrogens, or modulate bacterial populations in the body or media (18). 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 (5). Record et al. (25) 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. (14) 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 (10). 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. (12) 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 (12). 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 (23). Lipids of spermatozoa are involved in mechanisms of cell resistance to cold shock, aerobic peroxidation and are believed to be metabolized activity (27). Wishart (34) 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. (8) 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 (21) 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 (26) 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 72 h.

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