International Journal of Biological, Life and Agricultural Sciences
ISSN: 2415-6612
Vol:6, No:10, 2012

Adding Olive Oil into Diluents for Improving Semen Quality and Storage Ability of Roosters' Semen during Liquid Storage

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***Abstract*—**The aim of this study was to investigate the effects of supplementing the diluent of roosters' semen with different levels of olive oil on motility, viability, morphology and acrosome integrity of chicken spermatozoa after *in vitro* storage for up to 72 h. Semen was collected from 60 White Layer males (62 wk of age) kept in separated floor pens and randomly divided into six treatment groups (10 males in each group). Experimental groups were as follows: T1 :fresh semen, T2 : semen extended 1:1 with Al – Daraji 2 diluent (AD2D) alone, T3 – T6 :semen samples extended 1:1 with AD2D supplemented with 2 ml, 4 ml, 6 ml or 8 ml of olive oil / 100 ml of diluent, respectively. Semen samples were then stored at 5 °C for 24 h, 48 h or 72 h. There was a clear influence of diluent supplementation with olive oil on the spermatozoa motility profile; olive oil groups (T3, T4, T5 and T6) recorded the highest scores of mass activity and individual motility during all storage periods compared to T1 and T2 groups. In addition, the inclusion of olive oil into semen diluent (T3, T4, T5 and T6) gave significantly higher percentages of viable spermatozoa, normal morphologically spermatozoa and intact acrosomes irrespective of storage period. These results clearly show that supplementation the diluent of roosters' semen with olive oil can improve semen quality when semen samples *in vitro* stored at 5 °C for up to 72 h.

***Keywords*—**Olive oil, diluent, liquid storage, semen quality of roosters.

I. Introduction

OXIDATIVE stress, defined as an overproduction of free radicals, or a diminution in antioxidant defense mechanisms, determines cellular damage with functional alterations of the involved tissue. Free radicals can attack any biochemical component of the cell, but lipids are a major target. Lipid peroxidation of cell membranes and plasma lipoproteins represents a primary event in the establishment of oxidative stress [1]. Susceptibility of low density lipoprotein (LDL) to oxidative modifications depends on its fatty acid composition and cellular and extra cellular antioxidants, which serve to trap reactive oxygen species and to inhibit the chain reaction of free radicals [2].

The lipid composition of chicken semen is an important determinant of its quality and fertilizing capacity [3]. Chicken spermatozoa are characterized by comparatively high levels of 20:4 *n*-6 and 22:4 *n*-6 fatty acids within their phospholipids [4]. As a result of this high proportion of polyunsaturated fatty acids (PUFAs) chicken semen is susceptible to lipid peroxidation [5], which could lead to sperm deterioration during storage [6]. Wishart [7] reported that as a likely result of high proportions of PUFAs, avian spermatozoa showed a significant susceptibility to lipid peroxidation, which was associated with loss of viability, motility and fertilizing ability of spermatozoa *in vitro*. Kelso et al. [8] found that the concentration of spermatozoa and the proportion of live cells in the semen samples from the older birds were significantly lower than that observed for the younger age group. However, the metabolic activity of spermatozoa from the older donors was significantly reduced, being only 25 % of that for the young birds. There was a marked decrease with age (approximately ten – fold) in the activity of antioxidant enzyme glutathione peroxidase of spermatozoa [8].

Antioxidant nutrients are important for limiting damaging oxidative reactions in cells, which may predispose to the development of major clinical conditions such as oxidative stress disorders. There is great interest in the possibility that the antioxidant potential of plant – derived phenolic compounds, such as flavonoids, may reduce the risk of developing these conditions [9].

Several epidemiological studies suggest that olive oil significantly contributes to the well known effects of the Mediterranean diet in lowering the incidence of degenerative pathologies, including coronary heart disease, cancer and other oxidative stress disturbances [10]. In this respect, converging evidence indicates that the protective effects of olive oil could be ascribed not only to its high oleic acid content but also to the antioxidant properties of its polyphenols, absent in seed oil [11]. D'Angelo et al. [12] demonstrated that olive oil effectively counteracts the cytotoxic effects of reactive oxygen species in various cellular systems.

To our knowledge, however, no data are available in the literature concerning the antioxidant activity of olive oil as regards counteracting the deleterious effects of lipid peroxidation that naturally occurred during *in vitro* storage of roosters' semen. Therefore, the present experiment was designed to determine the probable role of olive oil antioxidants in suppress the detrimental effects of lipid

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peroxidation by adding different levels of olive oil to the semen diluent that held *in vitro* at 5 °C for certain storage periods (0h, 24 h, 48 h or 72 h).

II. Materials and Methods

Sixty White Leghorn males (52 wk of age), divided into 6 treatment groups of 10 males were maintained in separated floor pens and used as semen donors in this study. Males were fed *ad libitum* a diet containing 16.5 % protein and 2850 kcal / kg metabolizable energy. Semen samples were routinely collected twice a week from all males by abdominal massage method of Burrows and Quinn [13] during the whole experimental period which lasted 10 weeks (62 – 72 weeks of age). Care was taken to minimize contamination of the semen by feces or urates. After each collection, 12 pools of semen (each pool from five males in each treatment group) of approximately 3 ml each were immediately transferred to the laboratory. Treatment groups were as follows: T1 : fresh semen, T2 : semen diluted 1:1 with Al-Daraji 2 diluent (AD2D) alone and T3 – T6 : semen samples diluted 1:1 with AD2D supplemented with 2 ml, 4 ml, 6 ml or 8 ml of olive oil (Olive pomice oil, Rafael Salgado, 28529 Rivas, Madrid, Spain) / 100 ml of diluent, respectively. AD2D was prepared as described by Al- Daraji [14] which consisted of 0.64 g potassium citrate, 8.67 g sodium glutamate, 4.3 g sodium acetate, 0.34 g magnesium chloride, 12.7 g potassium diphosphate, 0.65 g potassium mono phosphate, 4 mg vit A, 16 mg vit C, 8 mg vit.E, 1.95 g TES and 5 g fructose.These components were dissolved in 1 liter of distilled water. However, the pH of diluents was adjusted to be 6.8 – 7.1 and the osmotic pressure 360 – 400 mOsm / kg H2O by using phosphate buffer solution. Semen samples were then stored at 5 °C for 24h, 48 h or 72 h, Aliquots of semen samples were removed at 0 h, 24 h, 48 h or 72 h after *in vitro* storage for further assessment of spermatozoa motility, viability and integrity. Spermatozoa motility (mass activity and individual motility) was estimated on a percentage basis by using the microscopic method [15]. Viability was evaluated by using fast green stain – Eosin B stain – glutamate extender [16]. The proportion of morphologically abnormal spermatozoa was measured by utilizing a Gention violet – Eosin stain [17]. Acrosomal abnormalities were determined according the procedure reported by Al-Daraji [18].

Changes in the motility, viability and morphology of spermatozoa after *in vitro* storage for certain periods (0, 24 h, 48 h, or 72 h) were evaluated by analysis of variance. Differences between treatment groups' means were analyzed by Duncan's multiple range test, using the ANOVA procedure in Statistical Analysis System [19].

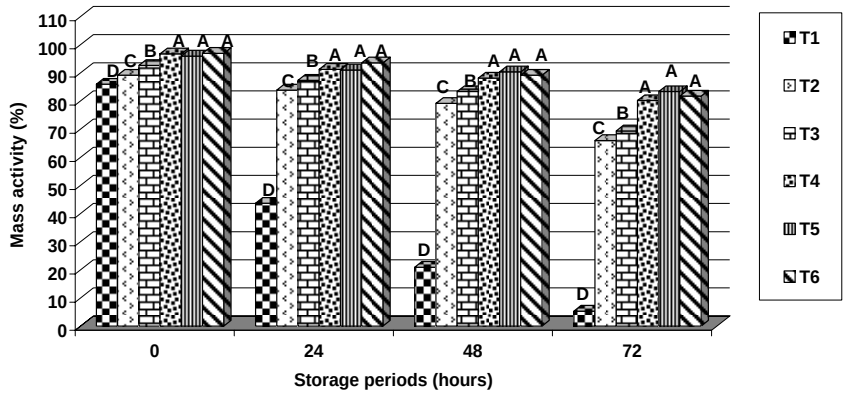
III. Results and Discussions

The effects of semen dilution and holding on mass activity and individual motility of spermatozoa are shown in figures 1 and 2. Spermatozoa motility consistently increased ($p < 0.05$) in semen diluent supplemented with olive oil (T3, T4, T5 and T6) as compared to T1 and T2 groups, regardless of semen storage periods. However, there were no significant differences between T4, T5 and T6 groups in relation to these two traits. The positive influences in spermatozoa motility that obtained with the inclusion of olive oil into semen diluent may be accounts for its very high content of antioxidant components [20]. Giovannini et al [21] pointed out that some biophenols, such as those contained in olive oil, may counteract the reactive oxygen metabolite – mediated cellular damage and related disorders by improving *in vivo* antioxidant defenses. Previous studies of possible mechanisms of olive oil phenol action indicated that these compounds are able to scavenge free radicals and to break peroxidative chain reactions. However, these materials can prevent lipid peroxidation by metal chelation [22]. Eating Mediterranean diet rich in olive oil is correlated with a decreased risk of coronary heart disease. It is thought that the natural antioxidants found in abundance in such a diet may be responsible for its benefit to heart [23]. Masella et al. [24] found that olive oil antioxidants had the following effects: 1- completely prevented the oxidation of LDL; 2- counteracted the time – dependent variations in intracellular redox balance, inhibiting the production of O_2^- and H_2O_2 and the decrease in glutathione content; 3- restored glutathione reductase and peroxidase activities; and 4- restored the mRNA expression of γ − glutamylcisteine synthetase, glutathione reductase, and glutathione peroxidase to control values. Rawashdah [25] noticed that compared with initial values, olive oil diet significantly reduced concentrations of serum total cholesterol and LDL and increased serum triglycerides level. Aydin et al. [26] found that olive oil prevented conjugated linoleic acid – induced mineral exchange between yolk and albumen, presumably by reducing the yolk saturated fatty acids, which are believed to disrupt the vitelline membrane during cold storage. Besides, feeding olive oil completely prevented conjugated linoleic acid – induced embryonic mortality in fertile eggs. This way probably due to restoration of the levels of 16:0, 18:0 and 18:1 (*n* – 9) in the egg yolk. Scaccini et al. [27] reported that the maximal protection observed after the olive oil diet may be explained by the presence of other unidentified antioxidants in addition to vitamin E, derived from oil intake. Therefore, the optimal balance between the content of unsaturated fatty acids and natural antioxidants in dietary oils appears to be of major importance.

The characteristics of the semen samples diluted with AD2D and supplemented with different levels of olive oil, in terms of dead spermatozoa, abnormal spermatozoa and acrosomal abnormalities are given in Fig. 3, 4 and 5. When evaluated 0 h, 24 h, 48 h or 72 h after initiation of *in vitro* storage, olive oil (T3, T4, T5 and T6) sperm viability, normality and acrosomal integrity indices were higher ($p < 0.05$) than for control groups (T1 and T2). However, for all storage periods, no differences were observed between T4 and T5 for any of these three variables evaluated (Fig. 3, 4 and 5). Furthermore, T6 (8 ml olive oil / 100 ml of diluent) recorded the best results as concerns spermatozoa viability and integrity

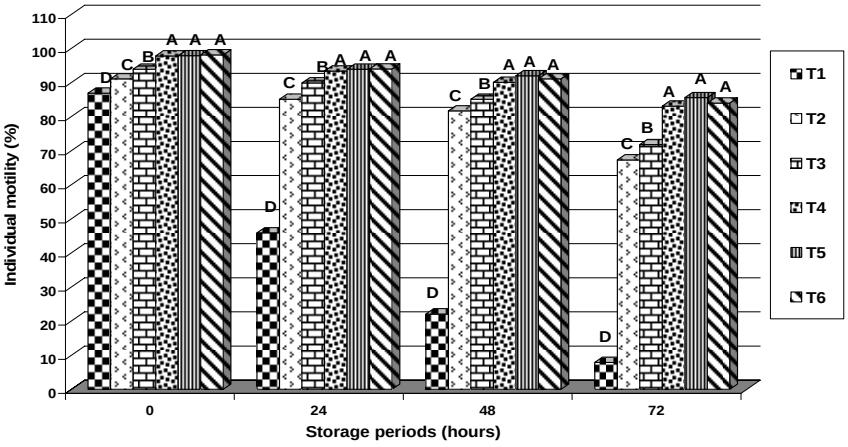
and acrosomal integrity as compared with other treatments included in this study. These finding are in agreement with the results of Al-Daraji [28] who found that the supplementation of some antioxidants (vitamins A, C or E) to the semen diluent resulted in a significant improvement in spermatozoa integrity, viability, fertilizing ability and acrosomal integrity when roosters' semen stored for 24 h at 4 °C. It is speculated that the improvement in spermatozoa viability and integrity are a result of antioxidants suppressing or limiting the damaging effects of lipid peroxidation *in vitro*. Possibly the improvement to these sperm parameters are at the level of the membrane, as lipid – and water – and – lipid – soluble antioxidants maintained viability, membrane integrity, and motility of turkey sperm after 48 h *in vitro* storage but the water – soluble antioxidant tested did not [29].Because of their lipid solubility, olive oil can permeate plasma membrane of spermatozoa and suppress free radical damage. However, Donoghue and Donoghue [29] concluded that the antioxidant activity in seminal plasma and sperms is not enough to prevent lipid peroxide damage after extension and *in vitro* storage, and those supplemental antioxidants could improve semen shelf life.

Enhancement of the antioxidant capacity of semen by inclusion olive oil into semen diluent could present a major opportunity for improving storage ability of roosters' semen. The beneficial consequences of an effective protection of olive oil against lipid peroxidation are likely to result from two related mechanisms: (1) Defense against peroxidative damage is essential to maintain the structural integrity of the spermatozoa and (2) Minimization of lipid peroxidation will prevent any reduction in the concentrations of the functionally important C_{20-22} polyunsaturated fatty acids of the sperm phospholipids.



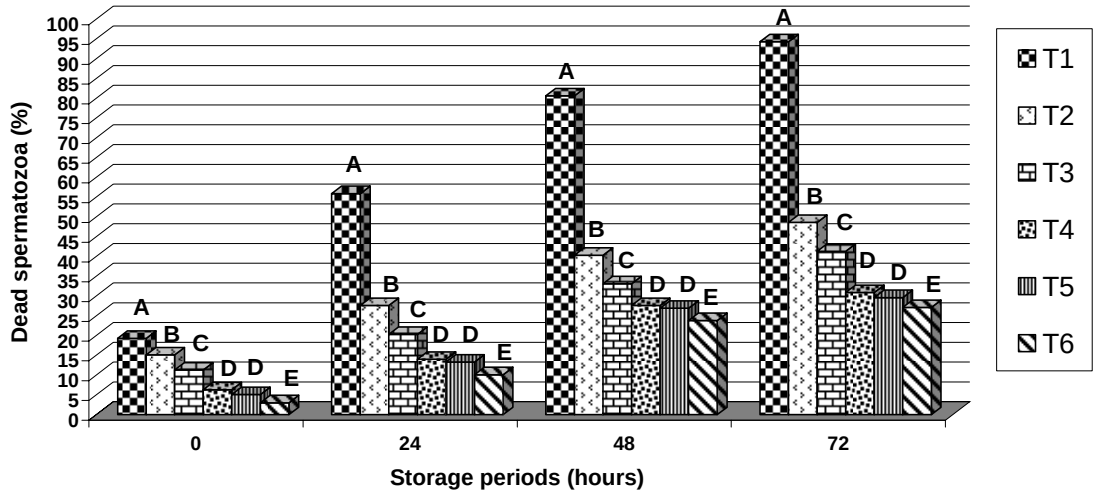
T1 = fresh semen, T2 = semen diluted with AD2D alone, T3 – T6 =semen samples diluted with AD2D supplemented with 2 ml, 4 ml, 6 ml or 8 ml of olive oil / 100 ml of diluent, respectively. AD2D = Al – Daraji 2 diluent.
Bars with different superscripts differ significantly ($p < 0.05$).
Each value represented the mean of 20 measures that conducted during 10 consecutive weeks.

Fig. 1 Effect of diluent supplementation with olive oil on mass activity of aged roostes' semen *in vitro* stored for certain periods



T1 = fresh semen, T2 = semen diluted with AD2D alone, T3- T6 = semen samples diluted with AD2D supplemented with 2 ml, 4 ml, 6 ml or 8 ml olive oil / 100 ml of diluent, respectively. AD2D = Al – Daraji 2 diluent.
Bars with different superscripts differ significantly ($p < 0.05$).
Each value represented the mean of 20 measures that conducted during 20 consecutive weeks.

Fig. 2 Effect of diluent supplementation with olive oil on individual motility of aged roosters' semen in vitro stored for certain periods

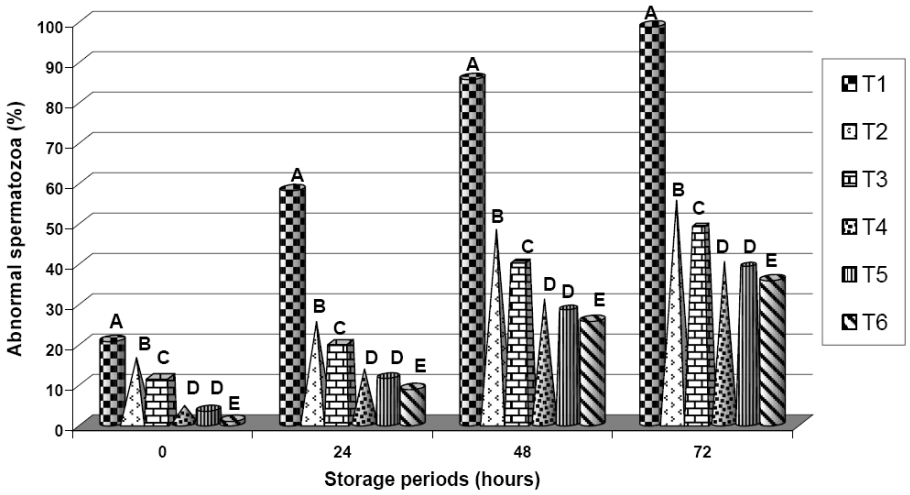


T1 = fresh semen, T2 = semen diluted with AD2D alone, T3 – T6 =semen samples diluted with AD2D supplemented with 2 ml, 4 ml, 6 ml or 8 ml of olive oil / 100 ml of diluent, respectively. AD2D = Al – Daraji 2 diluent.

Bars with different superscripts differ significantly ($p < 0.05$).

Each value represented the mean of 20 measures that conducted during 10 consecutive weeks.

Fig. 3 Effect of diluent supplementation with olive oil on dead spermatozoa of aged roosters' semen in vitro stored for certain periods

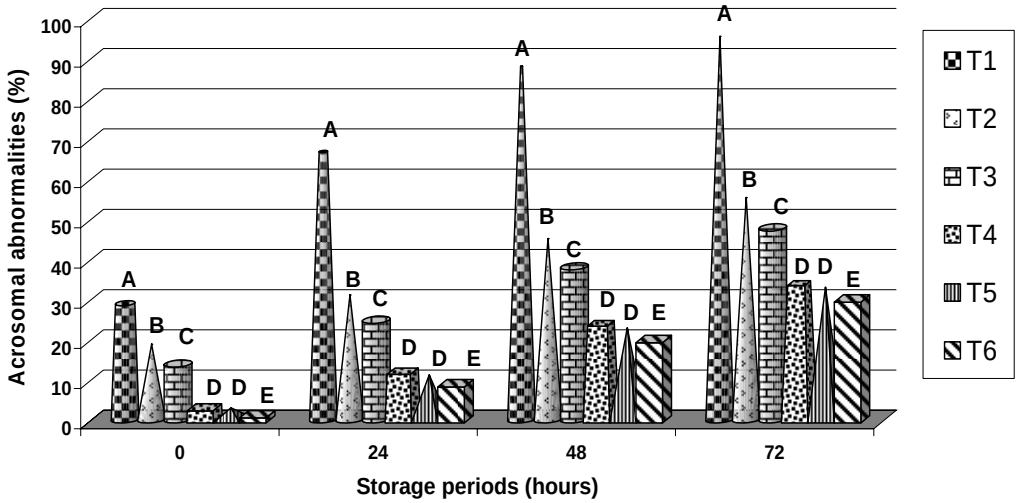


T1 = fresh semen, T2 = semen diluted with AD2D alone, T3 – T6 =semen samples diluted with AD2D supplemented with 2 ml, 4 ml, 6 ml or 8 ml of olive oil / 100 ml of diluent, respectively. AD2D = Al – Daraji 2 diluent.

Bars with different superscripts differ significantly ($p < 0.05$).

Each value represented the mean of 20 measures that conducted during 10 consecutive weeks.

Fig. 4 Effect of diluent supplementation with olive oil on abnormal spermatozoa of aged roosters' semen in vitro stored for certain periods



T1 = Fresh Semen, T2 = Semen Diluted with Ad2d Alone, T3 – T6 =Semen Samples Diluted with Ad2d Supplemented with 2 Ml, 4 Ml, 6 Ml Or 8 Ml Of Olive Oil / 100 Ml Of Diluent, Respectively. Ad2d = Al – Daraji 2 Diluent.
Bars with Different Superscripts Differ Significantly ($P < 0.05$).
Each Value Represented the Mean of 20 Measures that Conducted During 10 Consecutive Weeks.

Fig. 5 Effect of diluent supplementation with olive oil on acrosomal abnormalities of aged roosters' semen in vitro stored for certain periods

IV. Conclusion

The present data in conjunction with that previously reported indicated that the antioxidant / prooxidant balance in chicken semen is an important element in maintaining membrane integrity and functions including sperm motility and viability during *in vitro* storage. Therefore, developing a defense system against lipid peroxide damage is of practical importance to improving the extended liquid storage of roosters' semen. The present study demonstrated improved motility, survival, and membrane and acrosome integrity after cold storage of roosters' semen with olive oil antioxidants that scavenge reactive oxygen species in the lipid membrane. Ultimately, the fertilizing ability of spermatozoa is most important and future studies will evaluate the influence of olive oil as antioxidant on this spermatozoa function.

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