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Short Communication

POLYPHENOLS AS AN ANTIOXIDANT AGENT IMPROVES CHICKEN SPERM QUALITIES DURING COLD STORAGE

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ABSTRACT

An *in vitro* study was carried out to assess the addition of polyphenols in the chicken seminal plasma on sperm quality and antioxidant status at different storage periods. Twenty healthy breeding roosters of White Leghorn chicken having similar body weight were randomly selected. The polyphenols in the pomegranate peels were extracted using methanol as a solvent. Samples were split equally into three portions for inclusion of different concentrations of 0, 50 and 100 ppm of polyphenol**s,** respectively. Estimation of catalase, inhibition of lipid peroxidation, reduced glutathione and superoxide dismutase was done at 0, 24 and 48 h intervals. The sperm motility invariably increased in all treatment groups (50, 100 ppm) than control in both storage conditions. Other physical attributes such as sperm concentration, live and dead spermatozoa did not differ over control regardless of concentrations and time periods of storage. Inhibition of lipid peroxide action was found significant at the end of 48 h of storage in both the polyphenol treatment groups than control, while there was no change at 0 and 24 h. Polyphenols from pomegranate peels improved spermatozoal motility and antioxidant activity in seminal plasma and it could be concluded that the polyphenols has capability to protect the spermatozoa against oxidative damage.

Keywords: Pomegranate peels, phenols, white leghorn cocks, antioxidants, physical attributes.

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INTRODUCTION

The ultimate aim of poultry breeder operations is the production of fertile eggs as it has great economic importance in determining the profitability of production. Proper selection and maintenance of the proficient breeding cockerels is essential since a great deal of importance is attached with their seminal quality as one sire covers a large number of hens. Any impairment in their reproductive efficiency will adversely affect the economic returns. The avian sperm itself has unique structure and composition unlike mammalian species, having its own merits and challenges as follows. The lipid portion of the spermatozoal membrane is highly enriched with polyunsaturated fatty acids, highlighting its function in permeability, membrane fluidity and flexibility for sperm movement and adhesion with the egg cell (Surai *et al*., 2001). On the contrary, this initiates the risk of lipid peroxidation accompanied by free radicals resulting in morphological deformities, poor motility, membrane degradation and inferior capacity to fuse oocyte causing male infertility (Wishart, 1984). Further, due to low volume cytoplasmic contents with antioxidants makes the avian spermatozoa to lose the fertilizing ability rapidly (Donoghue and Wishart, 2000). Surai (2002) postulated that under physiological conditions, the natural antioxidants (vitamin E, ascorbic acid, glutathione) coupled with the enzymatic antioxidants (superoxide dismutase, glutathione peroxidase) in sperm and seminal plasma counteract the harmful effects of reactive oxygen species (ROS). However, when the ROS generation exceeds the defense mechanisms, oxidative stress develops due to disparity between the pro and anti-oxidants. Some of the important ROS present in seminal plasma include the superoxide anion, hydroxyl radical and hydrogen peroxide (Urso and Clarkson, 2003).

With the growing technique of artificial insemination (AI) in the poultry sector, short term storage of diluted semen from few hours to days around 0 to 5°C is routinely practiced. It has been revealed that over the course of *in vitro* semen storage, there was a significant reduction in the total lipid content and the fraction of phospholipids along with decline in sperm quality and fertility rate (Blesbois *et al*., 1999). In an effort to ameliorate the seminal traits and antioxidant protection of seminal plasma, numerous works have been recommended through incorporation of antioxidants of different means such as Vitamin E (Tabatabaei *et al*., 2011) organic selenium (Jafari Ahangari *et al*., 2013) and L-Carnitine (Partyka *et al*., 2017). However, there has been an emerging interest in the application of phytogenic derivatives as an antioxidant additive to augment the semen quality in humans and animals (Seddiki and da Silva, 2017). Bioactive principles like polyphenols are known to have antioxidant properties through its role in scavenging free radical, metal chelation, stimulating antioxidant enzymes, reduction of

alpha tocopherol radicals and inhibition of oxidases (Surai, 2014). Polyphenols in grape pomace, olive oil, sage, *S. Khuzistanica*, rosemary and green tea (Rahman *et al*., 2018) have been explored to be a potent antioxidant with varying responses and still considerable studies are being conducted with the possible sources.

Evidence also propose that peels of pomegranate (*Punica granatum*) accounting 60% of the fruit, comprise several polyphenolic principles such as flavanoids, ellagitannins and proanthocyanidins and are expected to mitigate the oxidative stress as well (Rahmani *et al*., 2017). Hence, the current work has been aimed to evaluate the efficacy of different concentrations of polyphenols on seminal traits and antioxidant status of cockerel semen at different storage periods.

MATERIALS AND METHODS

Extraction of polyphenols from pomegranate peels: Fresh pomegranate peels were collected from nearby local market and dried under shade for five days. The polyphenols in the peels were extracted as per the method described by Althunibat *et al*. (2010) using methanol as a solvent with slight modification. The extract was dried under incubator at temperature around 40ºC till the methanol residues were evaporated from the contents. The total phenolic content in the extract was determined calorimetrically (Çam and Hişil, 2010).

Experimental birds and collection of semen: Twenty healthy breeding roosters belonging to the same hatch of White Leghorn (WLH) chicken having similar body weight were randomly selected and caged individually under uniform managemental conditions. They were provided with normal breeder ration and water *ad libitum* with a constant photoperiod (14L: 10D/day). All the experimental birds were trained to ejaculate semen on every other day through abdominal massage method (Burrows and Quinn, 1937). Care was taken to prevent any contamination through feces and urates in the collection process.

Physical analysis of semen samples: Semen samples, thus, collected from 20 roosters were pooled and processed for the analysis of physical characteristics and antioxidant status. The fresh, pre storage semen was initially subjected to physical evaluation. For Mass Motility analysis, a drop of semen (4-5 µL) was placed on a dry, clean glass slide, spread uniformly by a cover slip and examined under light microscope (10x). Based on the activity of swirls, scoring was done as per Wheeler and Andrews (1943). The percent live and dead spermatozoa were quantified as per Lake and Stewart (1978) method. The sperm concentration ($x10^6$/ml) was determined from the standard calibration curve established between absorbance and sperm concentration in a double beam UV-VIS spectrophotometer at 550 nm (Brillard and McDaniel, 1985). The sample was diluted with patented CARI poultry semen diluent (1:2) and divided equally into three aliquots for incorporation of 0 (control), 50 and 100 ppm of polyphenol respectively. The effect of polyphenols under storage condition was studied by storing the diluted semen at 4°C for 24 and 48h. The percent motility, live and dead sperms were recorded in the similar manner as mentioned above at 24 and 48 h intervals.

Assessment of antioxidant status and lipid peroxidation: For the assessment of antioxidant profile, seminal plasma was collected through refrigerated centrifugation (4°C) of semen at 5000 rpm for 10 minutes. The seminal plasma was split equally into three portions for inclusion of different concentrations of 0, 50 and 100 ppm of polyphenol respectively. The estimation of catalase, inhibition of lipid peroxidation, reduced glutathione (GSH) and superoxide dismutase was done at 0, 24 and 48 h intervals with the storage temperature of -20°C through following protocols.

Catalase (Bergmeyer, 1983): The seminal plasma (2.5 µL) was mixed with 247.5 µL of phosphate buffer. To initiate the reaction, 50 µL of hydrogen peroxide was added under dark condition to make up the volume of 300 µL and absorbance was immediately measured at 240 nm in kinetic mode of 4 min at 30 sec interval using ELISA reader (SpectraMax, Molecular devices Ltd., Chennai). The time (seconds) required for the fall in initial absorbance by 0.05 was recorded

Lipid peroxidation inhibition (Placer *et al.*, 1966): The seminal plasma (50 µL) was mixed with 325 µL of Tris KCl buffer and 375 µL of thiobarbituric acid (TBA). Blank was prepared by replacing the seminal plasma with distilled water. All samples were heated in a boiling water bath (10 min) followed by brief cooling. Finally, 750 µL of pyridine-butanol solution (3:1) and 250 µL of sodium hydroxide (1M) solution were added with proper mixing. Absorbance value was recorded at 548nm against blank.

Concentration of reduced glutathione (GSH) (Prins and Loos, 1969): The seminal plasma (50 µL) was well mixed with 1000 µL of 0.08N H2SO4 in micro-centrifuge tube and maintained at room temperature for 10 min. About 125 µL of tungstate solution was added, vortexed and allowed to stand in room temperature for 5 min. The sample was centrifuged at 2000 rpm for 20 min at 4°C and the supernatant thus obtained was mixed with Tris buffer in the ratio of 1:1.25. Blank was prepared by replacing the supernatant with distilled water. Then 50 µL of DTNB reagent was added in all the samples except blank and the absorbance was recorded at 412 nm in spectrophotometer.

Superoxide dismutase (Minami and Yoshikawa, 1979): The assay mixture was prepared in a total volume of 3 ml consisting of 50 mM Tris cacodylic buffer, diluted samples and 0.2 mM of pyrogallol. Blank was made using distilled water in place of enzyme. The increase in absorbance due to auto oxidation of pyrogallol was recorded at 425 nm in spectrophotometer.

Statistical analysis: The data obtained under various physical and antioxidant activities were subject to analysis of variance for single factor using Statistical Package for Social Scientists (SPSS) version 20.0. The means were compared for significance at P≤0.05 using Tukey Multiple Range test (1949).

RESULTS AND DISCUSSION

The poultry semen is highly concentrated one which needs dilution before going for any form of storage or preservation. During the process of the dilution and subsequent storage there is loss in fertilizing ability of spermatozoa. This *in vitro* study assessed the sperm motility, livability and antioxidant status of the semen. The freshly collected semen samples were diluted, added with polyphenols and stored at refrigeration temperature (4±1ºC) for 24 and 48 hours.

Physical attributes of semen: The effect of polyphenol addition on physical characteristics of White Leghorn chicken spermatozoa during *in vitro* storage (4±1ºC) is presented in Table 1. The sperm motility invariably increased (P≤0.05) in all the treatment groups (50, 100 ppm) than control in both the storage conditions (24, 48 h). Nevertheless the other physical attributes *viz*., sperm concentration, live and dead spermatozoa did not produce differences over control regardless of the concentrations and time periods of storage. The beneficial impact on spermatozoa motility can be attributed to the antioxidant property of polyphenols in protecting the integrity and motility of the lipid rich sperm from the damaging effects of ROS production. Researchers have also demonstrated improved motility of spermatozoa upon incorporation of similar antioxidants in chicken and other species (Wittayarat *et al*., 2013). However, studies pertaining to the use of polyphenols as antioxidant agent is spare. The addition of polyphenols as an antioxidant agent improved the semen quality without affecting the physical attributes of semen in chicken.

Antioxidant status: Among the antioxidants, catalase is one of the enzymatic defenses commonly found in seminal plasma that help in prevention of ROS formation by converting hydrogen peroxide to oxygen and water (Halliwell, 2001). The antioxidant status, as shown in table 2, the addition of polyphenol at 100 ppm significantly improved the catalase activity of chicken semen than the control, while 50 ppm treatment showed intermediate response, after 24 h of storage. In contrast, at the end of 48 h, the catalase activity was significantly higher at 50 ppm than 100 ppm when compared with control group showing the least activity. Incorporation of polyphenols improved the catalase activity at different doses (50, 100 ppm) at varying time periods (24, 48h). Papadopoulou *et al.* (2017) affirmed the enhanced catalase enzyme activity in chicken red blood cells over administration of polyphenols in drinking water.

On the other hand, inhibition of lipid peroxide action was found significant at the end of 48 h of storage in both the polyphenol treatment groups than control, while there was no change at 0 and 24 h as shown in table 2. Lipid peroxides are known to be detrimental for the binding of spermatozoa to zona pellucida. Wishart (1984) remarked that the chicken seminal plasma has a moderate ability to impede peroxide formation in sperm membranes upon storage. Nevertheless the inbuilt defense of seminal plasma against lipid peroxides becomes suboptimal with dilution and in vitro storage, necessitating the need for antioxidant additives (Donoghue and Donoghue, 1997). The present work exhibit the chain breaking effect of polyphenol on lipid peroxidation with increase in storage time, thereby preserving the semen quality (Rahman *et al*., 2018).

The experiment showed the levels of reduced glutathione activity to be significantly high at 100 ppm polyphenol treatment than the control, with 50 ppm treatment showing intermediate response at both the time intervals of 24 (P≤0.05) and 48 h (P≤0.01) as shown in table 3. The possible modes for the increase of GSH by the bioactive polyphenols at higher concentration can be constituted as raise in enzymes accountable for GSH production; GSH that is retained after direct free radical scavenging and enhanced glutathione reductase activity from which GSH is resynthesized (Aquilano *et al*., 2014; Papadopoulou *et al.,* 2017). It has been expressed that reduced glutathione which is synthesized in all mammalian cells is entailed in antioxidation, detoxification and alteration of cell propagation (Lu, 2009). Shamiah *et al*. (2017) has demonstrated higher fertility rate in roosters through inclusion of GSH in semen diluent. The dilution rate of 1:2 and above might impair the fertility ~~the~~ percent in chicken which could ~~be~~ possibly due to ~~the~~ reduced GSH levels (Gadea *et al*., 2011). In our study, the antioxidant activity of superoxide dismutase was significantly higher at 100 ppm polyphenol than the other groups at both the storage periods. SOD was established to be an essential antioxidant enzyme of seminal plasma in scavenging the superoxide anions. These anions are converted to hydrogen peroxide by SOD which is further turned to water by catalase and glutathione peroxidase (Lin *et al*., 2005). Polyphenols obtained from sage leaves has also revealed the predominant inhibitory effect on superoxide radicals (Lu and Yeap Foo, 2001). De Lamirande and

Gagnon (1995) substantiated that higher concentrations of SOD was associated with the prevention of abnormal sperm motility. The activity of all the enzymatic antioxidants showed no significant differences at 0 h storage irrespective of the treatment groups.

Table 1. Effect of polyphenols on physical characteristics of white leghorn chicken semen during *in vitro* storage at 4±1ºC.

Group	Motility (%)	Live Spermatozoa (%)	Dead Spermatozoa (%)
Pre-storage	85.34±2.54	90.24±4.21	9.76±3.39
Post-storage – 24h			
Control	80.14^{b}±1.05	84.54±2.09	15.46±4.03
50 ppm	84.35^{a}±1.24	89.63±2.34	10.37±4.18
100 ppm	83.56^{ab}±1.39	87.37±3.46	12.63±4.71
P value	**0.011**	**0.349**	**0.299**
Post-storage – 48h			
Control	70.45^{b}±2.29	80.42±1.67	19.58±3.87
50 ppm	77.36^{a}±2.49	83.54±2.05	16.46±4.26
100 ppm	75.61^{a}±2.03	82.16±4.36	17.84±4.71
P value	**0.029**	**0.148**	**0.334**

The sperm concentration in the sample is 2.57±0.37 (x10^{6}/ml).
^{a,b}Means bearing different superscript within column differ significantly (P≤0.05).

Table 2. Effect of polyphenols on catalase activity and lipid peroxidation inhibition (%) in white leghorn chicken seminal plasma during *in vitro* storage at 4±1ºC.

Group	Catalase			Lipid peroxidation inhibition (%)		
	0 h	24h	48h	0 h	24h	48h
Control	2.26±0.16	3.42^{b}±0.16	1.56^{c}±0.16	9.94±1.33	11.68±1.33	7.78^{b}±0.81
50ppm	2.25±0.10	5.09^{ab}±0.12	8.19^{a}±0.39	8.64±0.29	10.09±0.29	14.27^{a}±0.50
100ppm	2.14±0.09	6.89^{a}±1.01	6.68^{b}±0.31	8.34±0.40	13.28±0.90	13.40^{a}±1.32
P value	**0.738**	**0.003**	**0.001**	**0.365**	**0.089**	**0.001**

^{a,b}Means bearing different superscript within column differ significantly (P≤0.05)

Table 3. Effect of polyphenols on reduced glutathione and superoxide dismutase activity in white leghorn chicken seminal plasma during *in vitro* storage at 4±1ºC.

Group	Reduced glutathione			Superoxide dismutase			
	0 h	24h	48h	0 h		24h	48h
				Semen	Spermatozoa		
Control	1.49±0.09	1.60^{b}±0.06	1.56^{b}±0.08	15.50±0.43	15.55±0.12	14.24^{b}±0.43	11.57^{c}±0.43
50ppm	1.67±0.18	1.76^{ab}±0.17	1.84^{ab}±0.17	13.98±0.67	16.08±0.70	17.38^{b}±0.67	20.07^{b}±0.67
100ppm	1.85±0.04	2.00^{a}±0.03	2.06^{a}±0.03	22.97±0.69	14.96±0.95	23.34^{a}±1.47	25.50^{a}±1.47
P value	**0.127**	**0.051**	**0.019**	**0.111**	**0.301**	**0.001**	**0.001**

^{a,b}Means bearing different superscript within column differ significantly (P≤0.05).

Conclusion: Polyphenols extracted from pomegranate peels has been found to improve spermatozoal motility and antioxidant mechanisms of seminal plasma in terms of catalase, lipid peroxidation inhibition, GSH and SOD levels, evidencing its potentiality to defend sperm against oxidative stress. With the parameters being time and dose dependant, substantial research on the molecular mechanisms responsible for this antioxidant activity of polyphenols is essential, as these bioactive phytochemicals have immense scope in the effective preservation of spermatozoa.

Conflict of Interest: All the authors declare that there is no conflict of interest in the work.

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