



Some Semen Characteristics and Oxidant/Antioxidant Markers of Chilled Diluted Rabbit Semen in Tris-Based Extender Supplemented with Wheat Germ Extracts

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WHEAT germ (WG) was a rich source in antioxidants, and had antimicrobial effects also it proved to have anticold-shock effect in preservation of diluted semen. This study aimed to clarify how various types of wheat germ extracts (including oil, hydroethanolic, and water extracts) in combination with egg yolk contribute to the preservation of buck semen when subjected to the cooling procedure. WG was extracted with petroleum ether (for oil extract), hydroethanol extract and water extract. Chemical analysis of WG extracts was performed by Gas Chromatography to detect saturated and unsaturated fatty acids. Additionally, the antioxidant capacity and metal chelation and reducing power of the hydroethanolic extracts were determined. A pilot experiment was designed to choose the optimum best concentration among 5 concentrations (100-500 µg/ml increment). The concentrations of 200, 300 and 400 µg/ml tris-base were chosen for the study. Diluted semen samples were cooled at 4°C for 48 hours. Sperm motility, viability, abnormality, membrane and acrosome integrity were evaluated in chilled semen during the chilling period. The Malondialdehyde (MDA, nmol/ml) and Catalase (CAT, U/L), in diluted semen, were assessed after 48 hours of chilling. The best extract that conserved a high motile viable sperm with low lipid peroxidation and oxidative stress was the WG oil extract at a concentration of 200 and 300 µg/ml, with egg yolk addition. The WG oil extract was the best in most characteristics specially live sperm (86.33%), acrosome integrity (65.55%), hyperosmotic swelling test (HOST, 56.00%) and CAT (413.12 U/L), while, decrease in abnormal sperm (19.78%) and MDA (35.45 nmol/ml). In conclusion, the use of wheat germ oil extract (200 to 300 µg/ml with less than 5% egg yolk) is suitable for preserving diluted rabbit buck semen for a period of 48 hours during the cooling process.

Keywords: Rabbit; Semen extender; Semen characteristics; Wheat germ extracts; Oxidant state.

Introduction

The maintenance of high productive male rabbit is the target for improving and developing methods for semen conservation. Numerous studies have been carried out on extender structure [1, 2]. Regrettably, the ability of rabbit sperm to stay alive *in vitro* after cold storage [3] is limited. This is owing to lipid peroxidation caused by high level of reactive oxygen species (ROS), which affects sperm membrane lipids, nucleic acids, sugars and proteins [4]. Antioxidants that occurred endogenously in rabbit

semen are insufficient to counteract the lipid peroxidation [5, 6]. Natural extracts from plants were used in semen diluents for conserving animals sperms [7, 8]. The seeds and grains of all plants represent a store of high nutritional elements. Among those high nutritive grains that have influence on the reproductive performance of human and animals, is the wheat germ (WG) [9, 10].

The WG is the embryo of total wheat grain seed. It represents about 2.5–3.8% of the total grain weight. The germ chemical composition contains

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about 10 – 15% lipids, 26 – 35% protein, 17% sugar, 1.5 – 4.5% fiber and about 4% minerals [11]. Major amounts of bioactive compounds are presented in tocopherols (300 – 740 mg/kg DM), phytosterols (24 – 50 mg/kg), policosanols (10 mg/kg), thiamin (15 – 23 mg/kg), carotenoids (4 – 38 mg/kg) and riboflavin (6 – 10 mg/kg) [12]. The WG also contains numerous enzymes related to its embryonic nature [13]. Oil retrieval is achieved by solvent extraction, which recovers about 90% lipids. The WG oil is a good source of triglycerides (57% of total lipids), mainly linoleic (18:2), palmitic (16:0) and oleic (18:1) acids, but significant amounts of sterols, mono- and diglycerides, phospho- and glycolipids are present. The fat-soluble antioxidants tocopherols and carotenoids are also plentiful. The WG meal contains 30–32% protein, represented in albumin (34.5%) and globulin (15.6%), besides a well-balanced amino acid profile. Potassium, magnesium, calcium, zinc and manganese are the main elements in the meal. About 0.35 g rutin equivalent/100 g DM is the major of total flavonoid. The oil has a pharmaceutical and nutritional value and the defatted WG meal is a hopeful source of high-quality vegetable proteins [11].

The reproductive performance of birds had been perfected using the WG oil and their constituents [14]. The WG oil had its powerful effect in rising the reproductive traits of Sinaii gabali rabbit bucks especially on conception rate%, the gestation length of does inseminated with semen of rabbit fed wheat germ oil, also the litter size at birth and weaning, the litter weight at birth and weaning, and decreasing the mortality rate at weaning [15]. The WG oil can mitigate the adverse effect of pollutants on male fertility, and leads to improvement of male reproductive performance [16]. Donoghue and Walker-Simmons [17] reported that the supply of 10% wheat protein extract to semen stored 24 h at 5°C enhanced fertility and hatchability of eggs over semen stored in diluent alone ($P < 0.05$). The heat-soluble proteins removed from wheat seed embryos are capable of defending turkey sperm during *in vitro* preservation and could possibly improve long-term storage of sperm for other species. The WG contains high nutritive value elements and powerful antioxidants scavenging that researchers recommended including it in rations of several animals [18].

Regarding the cryopreservation of semen, Arboud *et al.* [19] enhanced buffalo semen preservation in tris-citrate diluent by incorporating WG methyl alcohol extract. Adding 250 μ l extract to 3.75 ml tris, 500 μ l to 3.5 ml, and 1000 μ l to 3 ml significantly improved semen quality after thawing. Additionally, **EI-Sheshtawy et al.** [10] observed that the inclusion of WG extract in the basic extender for cattle semen led to improved post-thaw sperm characteristics and viability.

The present investigation aimed to study the effect of rabbit basic extender supplementation with wheat germ petroleum ether, hydroethanolic and aqueous extracts on oxidant/antioxidant status and semen quality parameters of diluted semen during chilling for 48 hours.

Material and Methods

Animal management and semen collection:

Ten sexually mature and fertile New Zealand White (NZW) male rabbits were used in this study. Rabbits were 26–30 (28 \pm 2) weeks age and 2.371–2.917 (2.644 \pm 0.273) kg initial weight. The experiment was carried out at the experimental lab animal house of National Research Centre, Dokki, Giza-Egypt, started from April to June 2022.

Semen was collected once weekly employing adapted artificial vagina at 40–42 °C. White milky and $\geq 70\%$ motile spermatozoa / ejaculates were considered in the study.

Plant materials and extraction

WG was purchased from Unit of oils, Department of Medicinal and Aromatic Plants Research, National Research Centre, Dokki, Giza, Egypt. One hundred grams of WG was extracted by cold water (3 \times 1L distilled water) in a refrigerator (4 \pm 1°C) to yield 20.45 g. Meanwhile, one kilogram of WG was defatted thrice with petroleum ether 40:60 (analytical grad, 3 \times 2L) to yield 100.50 g. Defatted powder was extracted with hydroethanolic (70%, 3 \times 2L) to yield 178.50 g. Hydroethanolic extract of WG was fractionated by gradual polarity solvent system including, diethyl ether, chloroform, ethyl acetate, and butanol, respectively. Fractions yield of WG were 13.79 g /100 g extract, 7.39 g /100 g extract, and 10.18 g /100 g extract for chloroform, ethyl acetate, and butanol with 68.64 g /100 g extract water residue.

Determination of the chemical composition of WG:

The total phenolic content was determined and expressed as mg gallic acid /g of extract, while total flavonoid content was measured and expressed as mg quercetin /g of extract [20]. According to Onwuka [21] gravimetric method, the alkaloids content was determined and was expressed as mg /g of extract. Tannins content was estimated using Folin–Ciocalteu's reagent according to Rangana [22].

Identification of phenolics composition using HPLC

HPLC analysis was carried out using an Agilent 1260 series. The separation was carried out using Eclipse C18 column (4.6 mm x 250 mm i.d., 5 μ m). The mobile phase DPG consisted of water (A) and 0.05% trifluoroacetic acid in acetonitrile (B) at a flow rate 0.9 ml/min. The mobile phase was

programmed consecutively in a linear gradient as follows: 0 min (82% A); 0–5 min (80% A); 5–8 min (60% A); 8–12 min (60% A); 12–15 min (82% A); 15–16 min (82% A) and 16–20 (82%A). The multi-wavelength detector was monitored at 280 nm. The injection volume was 5 µl for each of the sample solutions. The column temperature was maintained at 40 °C.

Identification of lipid composition of using GC analysis:

The GC conditions for fatty acids:

The GC model 7890B from Agilent Technologies was equipped with flame ionization detector at Central Laboratories Network, National Research Centre, and Cairo, Egypt. Separation was achieved using a Zebron ZB-FAME column (60 m x 0.25 mm internal diameter x 0.25 µm film thickness). Analyses were carried out using hydrogen as the carrier gas at a flow rate of 1.8 ml/min at a split-1:50 mode, injection volume of 1 µl and the following temperature program: 100 °C for 3 min; rising at 2.5 °C /min to 240 °C and held for 10 min. The injector and detector (FID) were held at 250 °C and 285 °C, respectively.

Sample preparation for fatty acids:

The sample was saponified with ethanolic potassium hydroxide, unsaponifiable fraction extracted in petroleum ether.

Sample derivatization:

The unsaponifiable part extracted in petroleum ether was mixed with 50 µL of bis(trimethylsilyl) trifluoroacetamide (BSTFA)+ trimethylchloro-silane (TMCS) 99:1 silylation reagent and 50 µL pyridine for derivatization sample functional groups to trimethylsilyl groups (abbreviated TMS) prior to GC analysis.

Gas chromatography–mass spectrometry analysis (GC-MS) condition for unsaponifiable matter assay:

The GC-MS system (Agilent Technologies) was equipped with gas chromatograph (7890B) and mass spectrometer detector (5977A) at Central Laboratories Network, National Research Centre, Cairo, Egypt. The GC was equipped with HP-5MS column (30 m x 0.25 mm internal diameter and 0.25 µm film thickness). Analyses were carried out using Hydrogen as the carrier gas at a flow rate of 2.0 ml/min at a splitless, injection volume of 2 µl and the following temperature program: 50 °C for 5 min; rising at 5 °C /min to 100 °C and held for 0 min and rising at 10 °C /min to 320 °C and held for 10 min. The injector and detector were held at 280 °C, 320 °C. Mass spectra were obtained by electron ionization (EI) at 70 eV; using a spectral range of m/z 25–700 and solvent delay 6 min. The mass temperature was 230°C and Quad 150 °C.

Identification of different constituents was determined by comparing the spectrum fragmentation pattern with those stored in Wiley and NIST Mass Spectral Library data [23].

Experimental design:

Pilot experiment for selection of useful extract concentrations:

Previous considered ejaculates were pooled. Then divided into 15 aliquots (each of 500 µL), used for WG (WG oil extract (n=5) [100, 200, 300, 400 and 500 µg/ml], hydroethanolic WG extract (n=5), and water WG extract (n=5)). The first aliquots were diluted 1:10 in Tris-citrate-glucose (TCG) basic extender [24].

Five concentrations [100, 200, 300, 400 and 500 µg/ml] were adopted with 3 extracts by [hexane, hydroethanolic, and water extract]. This made 15 diluted samples which were stored in cooling chamber for 2 hours at 10°C. Then, the samples were examined for motility percentage, after 24 and 48 hours. All samples didn't exceed the 48 hours.

Experimental design for selected concentration of extract enriched extender:

Three selected extract concentrations for WG from each method of extractions (oil, hydroethanolic, and water extracts) from the pilot experiment were engaged in a chilling experiment. The selected pooled sample was splitted in 10 subsamples (each of 500 µL) as follows:

1. The first aliquot was diluted 1:10 in TCG basic extender (control).
2. The other 9 aliquots were diluted 1:10 in the TCG extender enriched with the selected concentrations of the WG extracts that were obtained from the pilot experiment (Table 1).
3. WG extract extenders were used with less than 5% egg yolk addition.

Semen assessment:

Sperm motility, viability, morphology, acrosome and membrane integrities were assessed after 2, and 48 h post-chilling.

Sperm motility:

Sperm motility will be assessed by using phase contrast hot stage microscope set at magnification of 400 × and equipped with a heating plate (37°C).

Sperm morphology and viability:

Stained smear was prepared as soon after ejaculation using an eosin nigrosine staining mixture at 1:4 dilution rates [25].

Sperm membrane integrity: Hypo-osmotic swelling test (HOST):

The Sperm membrane integrity was evaluated by

HOST test according to Amorim, *et al.* [26], where, the swollen coiled tail sperm was measured to have an intact plasma membrane.

Acrosome integrity:

Giemsa staining technique was used to stain the acrosome with dark purple [27].

Diluted semen oxidant/antioxidant chemical analysis:

Catalase (CAT) [U/L]

The CAT activity is founded on the enzyme reaction with H_2O_2 [28]. After one minute the reaction will be stopped with CAT inhibitor. In the presence of horse radish peroxidase (HRP), the H_2O_2 residue reacts with a chromogen (3,5-Dichloro 2-hydroxybenzene sulfonic acid) and 4-aminophenazone to yield a chromophore with a colour intensity inversely proportional to the activity of CAT in the original sample. In brief, 50 μ l of standard (0.5 mM/L H_2O_2) or sample was incubated with 500 μ l of chromogen buffer and 50 μ l of diluted substrate (H_2O_2 (0.5 mM/L) diluted 1000 times immediately prepared before use) for exactly one minute at 25°C. 100 μ l of CAT inhibitor and 500 μ l of HRP were added to the previous mixture. Incubation for 10 minutes at 37°C and read the absorption of standard and samples against distilled water at 510 nm (500 – 520 nm).

Lipid peroxidation (nmol/ml)

The principle is based on the reaction of Thiobarbituric acid (TBA) with the malondialdehyde (MDA) [an organic compound resulted from lipid peroxidation of polyunsaturated fatty acids and used as a marker indicator for the oxidative stress in biological fluids and tissues] in acidic medium with catalytic temperature 95°C for 30 minutes. A pink reactive product is measured at 534 nm [29].

Statistical analysis:

Simple one-way ANOVA was performed to determine the effect of extract and the concentrations of each extract on semen parameters, MDA, and catalase using the SAS computerized program v. 9.2 [30]. Duncan's Multiple Range test was used to compare between different means at $P < 0.05$

Results

Physical examination and organoleptic characters of the WG extract

Germ powder contains 10.50% oil, distributed as 81.62% saponifiable matter (fatty acids) and 1.37% unsaponifiable matter. Germ powder was extracted by 70% ethanolic solution and yielded 18.75%. When the hydroethanolic extract was fractionated by

polarity graduated solvent system yielded; 13.79% chloroform fraction, 7.39% ethyl acetate fraction, and 10.18% butanol fraction, and about 68.64% residual remained.

The hydroethanolic extract is light-brown, like-germ odor, and is sugary condition. The extract contains total polyphenols (95.33±0.02 mg gallic acid /g extract), total flavonoids (22.97± 4.33mg quercetin /g extract), total tannins (45.85± 1.35 mg tannic /g extract), and total alkaloids (113.40± 6.33 mg alkaloid/ g extract).

Saponifiable matter composition of WG oil.

Germ oil's fatty acid composition contains several fatty acids, including saturated and unsaturated fatty acids. Germ oil is rich in unsaturated fatty acids. The saturated fatty acids act about 24.71% that distributed as palmitic acid (18.46%), stearic acid (3.93%), arachidic acid (1.36%), and behenic acid (0.96). On the other hand, germ oil is rich in unsaturated fatty acids (75.30%) that are classified as mono-unsaturated fatty acids, 20.29%, and polyunsaturated fatty acids, 55.01%. The mono-unsaturated fatty acids are palmitoleic acid, 2.60%, and oleic acid, 17.69. And polyunsaturated fatty acids are linoleic acid, 49.56, and linolenic acid, 5.45. In addition, the germ oil is rich in ω -6 fatty acids that cat for about 49.56%, which is the highest fatty acid in the oil.

Germ oil's unsaponifiable matter composition (table 1) contains several components, including sterols and esters. Germ oil is rich in sterols. The sterols act about 64.15% that distributed as Campesterols (16.48%), fagarasterols (3.03%), and γ -sitosterols (44.64%). On the other hand, germ oil contains Thymol, TBDMS derivative (3.96%).

Antioxidant capacities of WG extract

Antioxidant capacities of WG extract were determined by several ways including, radical scavenging (DPPH), ROS scavenging (H_2O_2), Fe^{2+} chelating, and reducing capability. The extract was evaluated by comparison with two reference materials: Vitamin C (as a natural antioxidant) and Trolox (as a synthetic antioxidant).

DPPH scavenging capacity of WG petroleum ether, hydroethanolic (70%) and residual extract were presented as IC_{50} compared to vitamin C as a natural antioxidant and Trolox as a synthetic antioxidant. WG and reference materials were tested at 125, 250, 500, and 1000 μ g/ml. WG materials gave low DPPH scavenging capacity with residual fraction (IC_{50} = 21292.43 μ g/ml) while it was high with the ether extract (IC_{50} = 456.05 μ g/ml). The ether extract DPPH scavenging capacity (IC_{50} =456.05 μ g/ml) was near to vitamin C (IC_{50} =232.54 μ g/ml).

TABLE 1. Chemical composition of unsaponifiable matter of germ oil using GC.

Peak	RT	Area Sum %	Area	
1	14.903	3.26	530346.59	Palmitic acid
2	18.108	4.6	747724.55	Linoelaidic acid
3	18.193	5.2	845339.42	Linoleic acid
4	20.9	5.77	938004.39	Ricinoleic acid methyl ester
5	25.048	5.16	839159.08	Phthalic acid, bis(2-ethylhexyl) ester
6	33.866	2.39	389582.38	Cholest-22-ene-21-ol, 3,5-dehydro-6-methoxy-, pivalate
7	36.973	16.48	2681095.46	Campesterol
8	39.262	3.03	493622.31	Fagarasterol
9	39.64	44.64	7262633.61	γ -Sitosterol
10	42.094	2.96	482144.47	1-Heptatriacotanol
11	46.409	2.55	414712.03	W-18
12	51.93	3.96	644305.17	Thymol, TBDMS derivative

H₂O₂ scavenging capacity of WG petroleum ether, hydroethanolic (70%) and residual extract were presented as IC₅₀ compared to vitamin C as a natural antioxidant and Trolox as a synthetic antioxidant. Germ and reference materials were tested at 125, 250, 500, and 1000 μ g/ml. WG materials gave low H₂O₂ scavenging capacity with residual fraction (IC₅₀= 2548.85 μ g/ml) while it was high with the ether extract (IC₅₀= 583.25 μ g/ml). The ether extract H₂O₂ scavenging capacity (IC₅₀= 583.25 μ g/ml), compared to vitamin C (IC₅₀= 346.19 μ g/mL) and Trolox (IC₅₀= 37.66 μ g/mL).

Metal chelation ability of WG petroleum ether, hydroethanolic (70%) and residual extract were presented as IC₅₀ compared to vitamin C as a natural antioxidant and Trolox as a synthetic antioxidant. WG and reference materials were tested at 125, 250, 500, and 1000 μ g/ml. WG materials gave low metal chelation ability compared to vitamin C and Trolox. Petroleum ether extract recorded the highest activity among WG materials (IC₅₀= 788.19 μ g/mL), compared to vitamin C (IC₅₀= 656.93 μ g/mL) and Trolox (IC₅₀= 135.34 μ g/mL).

Reducing power capacity of WG petroleum ether, hydroethanolic (70%) and residual extract were presented as IC₅₀ compared to vitamin C as a natural antioxidant and Trolox as a synthetic antioxidant. WG and reference materials were tested at 125, 250, 500, and 1000 μ g/ml. WG materials gave low reducing power with residual fraction (IC₅₀=2287.74 μ g/ml) while it was high with the hydroethanolic extract (IC₅₀=94.38 μ g/ml). The hydroethanolic extract reducing power (IC₅₀=94.38 μ g/mL) was

potent compared to vitamin C (IC₅₀=17.96 μ g/mL) and Trolox (IC₅₀=36.60 μ g/mL)

The semen characteristics and antioxidant analysis of diluted rabbit semen in basic TCG diluent enriched with different WG extracts:

Data output in table 2, after 48 hours chilling period, the overall means of sperm motility for the concentrations 200 to 400 μ g/ml in the three WG extracts were significantly (P<0.0008) differed from the concentrations 100 and 500 μ g/ml.

Wheat germ hydro-ethanolic extract (WGH) deteriorated the sperm motile % (P<0.0001), live sperm % (P<0.0001), acrosome integrity (P<0.05), and increased the abnormal sperm % (P<0.0001) compared to the control semen extender, the semen extender supplemented with wheat germ oil extract (WGO), and that supplemented with wheat germ aqueous extract (WGA) for 2 hours chilling (Table 3). Compared to control, WGO insignificantly increased MDA and maintained the CAT activity. 2 h after chilling rabbit semen supplemented with both WGH and WGA increased MDA (P<0.01) and declined CAT activity (P<0.0001). WGO extract improved the sperm motile % (P>0.05), live sperm % (P>0.05), acrosome integrity (P<0.05), and HOST (P<0.05) with decreasing the abnormal sperm % (P<0.05) compared to the control semen extender, the semen extender supplemented with WGH, and that supplemented with WGA for 48 hours chilling (Table 3). The lowest MDA (P<0.01) and the highest CAT activity (P<0.0001) was recorded after supplementing rabbit semen diluent with WGO compared to control, WGH, and WGA (Table 3).

TABLE 2. The pilot experiment for choosing the best concentrations to enrich the r-TBE after 48 hours of cooling.

	Concentration of extract in the diluent					Overall mean	P-value
	$\mu\text{g/ml}$						
	100	200	300	400	500		
WGO	45.00 \pm 2.89	53.75 \pm 3.75	51.25 \pm 3.75	47.50 \pm 4.79	30.00 \pm 2.48	45.50 ^a	
WGH	23.75 \pm 2.39	42.50 \pm 3.57	38.75 \pm 3.75	37.50 \pm 2.50	16.25 \pm 1.25	31.75 ^c	0.0111
WGA	30.00 \pm 2.89	46.25 \pm 3.15	43.75 \pm 3.75	41.25 \pm 3.84	30.00 \pm 2.55	38.25 ^b	
Overall mean	26.88 ^B	40.83 ^A	41.04 ^A	39.58 ^A	21.42 ^C		
P-value	0.0008						

Means with different superscripts (A,B,C) within row (a,b,c) within column indicate significance at $P < 0.05$. WGO= oil extract; WGH= hydroethanolic extract; WGA= Aqueous extract

TABLE 3. Mean \pm SEM of semen characteristics, CAT and MDA in rabbit buck semen chilled for 2h and 48h supplemented with wheat germ oil, hydroethanolic and aqueous extract

	Control	WGO	WGH	WGA	P-value
After 2 hours					
Motility %	86.67 \pm 1.67 ^b	87.22 \pm 1.21 ^b	72.22 \pm 3.02 ^a	75.00 \pm 2.64 ^a	0.0001
Live sperm %	88.33 \pm 0.17 ^b	88.00 \pm 1.21 ^b	83.00 \pm 0.58 ^a	89.67 \pm 0.91 ^b	0.0001
Abnormal sperm %	18.00 \pm 0.58 ^c	15.78 \pm 0.52 ^b	20.00 \pm 0.65 ^d	14.00 \pm 0.50 ^a	0.0001
Acrl %	92.67 \pm 1.17 ^b	91.33 \pm 0.82 ^b	88.67 \pm 0.47 ^a	91.44 \pm 0.80 ^b	0.017
HOST %	64.00 \pm 1.12	60.22 \pm 3.82	61.11 \pm 2.75	57.00 \pm 2.36	0.346
MDA (nmol/ml)	33.02 \pm 2.52 ^a	39.89 \pm 1.93 ^{ab}	41.21 \pm 3.35 ^{bc}	47.65 \pm 1.97 ^c	0.002
CAT (U/L)	419.17 \pm 15.37 ^b	398.58 \pm 25.52 ^b	318.79 \pm 21.12 ^a	299.38 \pm 21.97 ^a	0.0001
After 48 hours					
Motility %	37.44 \pm 1.05 ^b	34.44 \pm 1.49 ^{ab}	32.00 \pm 1.68 ^a	37.22 \pm 1.40 ^b	0.06
Live sperm %	88.67 \pm 1.48 ^b	86.33 \pm 1.25 ^{ab}	84.67 \pm 1.09 ^{ab}	83.44 \pm 1.85 ^a	0.082
Abnormal sperm %	23.56 \pm 1.24 ^b	19.78 \pm 0.66 ^a	20.22 \pm 0.95 ^a	21.00 \pm 1.03 ^{ab}	0.05
Acrl %	60.22 \pm 2.90 ^{ab}	65.55 \pm 2.17 ^b	60.33 \pm 2.69 ^{ab}	53.55 \pm 2.84 ^a	0.029
HOST%	56.78 \pm 1.12 ^b	56.00 \pm 3.20 ^{ab}	48.89 \pm 2.78 ^a	49.33 \pm 2.21 ^a	0.047
MDA (nmol/ml)	43.39 \pm 1.03 ^b	35.45 \pm 2.92 ^a	41.62 \pm 2.59 ^{ab}	46.03 \pm 2.24 ^b	0.014
CAT (U/L)	303.19 \pm 15.44 ^a	413.12 \pm 18.05 ^c	291.54 \pm 11.38 ^a	354.65 \pm 22.19 ^b	0.0001

Acrl= Acrosome integrity; HOST = hypo-osmotic swelling test; MDA= Malondialdehyde; CAT= catalase; WGO= oil extract; WGH= hydroethanolic extract; WGA= Aqueous extract. Different superscripts (a,b,c,d) within row are significantly different at $P < 0.05$.

In the oil extract of wheat germ (WGO), the percent of motile sperm, abnormal sperm, acrosome integrity, and HOST (Table 4) did not vary 2 hours after supplementing semen diluents with 0, 200, 300, and 400 $\mu\text{g/ml}$ WGO. However, the live sperm % decreased ($P < 0.01$) after supplementing semen diluents with 300 $\mu\text{g/ml}$ WGO with slight non-significant increase after supplementing 200 $\mu\text{g/ml}$ WGO. Two hours after chilling rabbit semen supplemented with 300 and 400 $\mu\text{g/ml}$ WGO increased MDA ($P < 0.01$) compared to control and diluents supplemented with 200 $\mu\text{g/ml}$ WGO. CAT activity declined after supplementing semen diluents with 200 $\mu\text{g/ml}$ WGO compared to control (Table 4). The chilling semen for 48h using diluent supplemented with 200 $\mu\text{g/ml}$ WGO preserved the motile sperm % similar to control ($P < 0.05$). The percent of live sperm, abnormal, acrosome integrity, and HOST did not vary 48 hours after supplementing semen diluents with 0, 200, 300, and 400 $\mu\text{g/ml}$

WGO. The MDA decreased greatly ($P < 0.0001$) and CAT activity increased ($P < 0.0001$) after supplementing semen diluents with 200 $\mu\text{g/ml}$ WGO compared to control (Table 4).

The chilling rabbit semen for 2 h slightly declined the live sperm % ($P < 0.01$) and tended to decrease the percent of motile sperm ($P > 0.05$) and the abnormal sperm % kept the same value ($P > 0.05$) after adding 200 $\mu\text{g/ml}$ WGH compared to control (Table 5). The acrosome integrity, and HOST (Table 5) did not vary 2 hours after supplementing semen diluents with 200, 300, and 400 $\mu\text{g/ml}$ WGH. Two hours after chilling rabbit semen supplemented with 300 and 400 $\mu\text{g/ml}$ WGH increased MDA ($P < 0.01$) compared to control and diluents supplemented with 200 $\mu\text{g/ml}$ WGH. The lowest CAT activity is observed after supplementing semen diluents with 200 $\mu\text{g/ml}$ WGH compared to control, 300, and 400 $\mu\text{g/ml}$ WGH (Table 5). The chilling semen for 48h using diluents supplemented with 0, 200, 300, 400 $\mu\text{g/ml}$ WGH

have no influence on motile sperm %, live sperm, abnormal sperm, acrosome integrity, and HOST. The MDA ($P>0.05$) tended to decreased and CAT activity declined after supplementing semen diluents with 300 $\mu\text{g/ml}$ WGH compared to 0, 200, 400 $\mu\text{g/ml}$ WGH (Table 5).

The percent of motile sperm, live sperm %, the acrosome integrity, and HOST (Table 6) were not influenced by adding 200, 300, 400 $\mu\text{g/ml}$ WGA to semen diluents chilled for two hours. Adding 200,

300, 400 $\mu\text{g/ml}$ WGA declined ($P<0.05$) abnormal sperm % and CAT activity ($P<0.0001$) but increased MDA ($P<0.0001$). The percent of motile sperm, live sperm %, abnormal sperm %, the acrosome integrity, and HOST (Table 6) were not influenced by adding 200, 300, 400 $\mu\text{g/ml}$ WGA to semen diluents chilled for 48 hours. The MDA ($P>0.05$) tended to decreased but CAT activity increased ($P<0.001$) after supplementing semen diluents with 300 $\mu\text{g/ml}$ WGA compared to 0, 200, and 400 $\mu\text{g/ml}$ WGH (Table 6).

TABLE 4. Effect of concentrations of WG oil extract on semen characteristics, CAT and MDA activity of chilled rabbit buck semen after 2h and 48h

	0	200	300	400	P-value
After 2 hours					
Motility %	86.67 \pm 3.33	88.33 \pm 1.67	86.67 \pm 1.67	86.67 \pm 3.33	0.95
Live sperm %	88.33 \pm 0.33 ^b	91.00 \pm 0.58 ^b	84.00 \pm 1.16 ^a	89.00 \pm 1.73 ^b	0.011
Abnormal sperm %	18.00 \pm 1.16	15.00 \pm 0.58	17.00 \pm 0.58	15.33 \pm 1.20	0.15
AcrI %	92.67 \pm 2.33	93.33 \pm 0.67	90.00 \pm 1.16	90.67 \pm 1.76	0.45
HOST%	64.00 \pm 2.31	50.67 \pm 4.81	68.00 \pm 6.11	62.00 \pm 6.11	0.173
MDA (nmol/ml)	31.82 \pm 4.31 ^a	30.91 \pm 1.99 ^a	44.25 \pm 3.14 ^b	44.54 \pm 0.33 ^b	0.002
CAT (U/L)	431.77 \pm 17.15 ^b	299.77 \pm 16.34 ^a	379.19 \pm 20.83 ^b	516.78 \pm 33.49 ^c	0.0001
After 48 hours					
Motility %	38.33 \pm 1.67 ^b	38.33 \pm 1.67 ^b	36.67 \pm 3.33 ^b	28.33 \pm 1.67 ^a	0.035
Live sperm %	89.00 \pm 2.89	88.00 \pm 1.54	86.00 \pm 3.46	85.00 \pm 1.73	0.67
Abnormal sperm %	24.00 \pm 2.31	20.00 \pm 1.15	20.33 \pm 1.76	19.00 \pm 0.58	0.21
AcrI%	60.33 \pm 5.78	64.67 \pm 4.81	67.33 \pm 4.33	64.67 \pm 3.53	0.768
HOST%	57.67 \pm 1.45	52.67 \pm 3.93	64.00 \pm 6.11	51.33 \pm 4.66	0.24
MDA (nmol/ml)	42.73 \pm 1.52 ^c	23.03 \pm 2.45 ^a	34.55 \pm 2.51 ^b	48.79 \pm 2.86 ^c	0.0001
CAT (U/L)	296.05 \pm 26.67 ^a	481.73 \pm 31.49 ^b	337.06 \pm 8.74 ^a	420.58 \pm 12.04 ^b	0.0001

AcrI= Acrosome integrity; HOST = hypo-osmotic swelling test; MDA= Malondialdehyde; CAT= catalase.

Different superscripts (a,b,c) within row are significantly different at $P<0.05$.

TABLE 5. Effect of concentrations of WG hydroethanolic extract on semen characteristics, CAT and MDA activity of chilled rabbit buck semen after 2h and 48h

	0	200	300	400	P-value
After 2 hours					
Motility %	86.67 \pm 3.33 ^b	78.33 \pm 4.41 ^{ab}	66.67 \pm 6.01 ^a	71.67 \pm 4.41 ^{ab}	0.070
Live sperm %	88.33 \pm 0.33 ^b	84.00 \pm 1.16 ^a	82.00 \pm 1.16 ^a	83.00 \pm 0.58 ^a	0.004
Abnormal sperm %	18.00 \pm 1.16	18.00 \pm 1.16	21.00 \pm 0.58	21.00 \pm 0.58	0.065
AcrI %	92.67 \pm 2.33 ^b	89.33 \pm 0.67 ^{ab}	89.33 \pm 0.67 ^{ab}	87.33 \pm 0.67 ^a	0.103
HOST%	64.00 \pm 2.31	65.00 \pm 5.13	58.33 \pm 4.91	60.00 \pm 5.29	0.703
MDA (nmol/ml)	31.82 \pm 4.32 ^a	30.00 \pm 1.99 ^a	58.46 \pm 5.29 ^b	35.00 \pm 3.48 ^a	0.0001
CAT (U/L)	431.79 \pm 17.15 ^c	210.29 \pm 12.25 ^a	402.68 \pm 18.79 ^c	343.40 \pm 12.66 ^b	0.0001
After 48 hours					
Motility %	35.67 \pm 2.33	31.67 \pm 1.67	30.00 \pm 2.89	34.33 \pm 4.26	0.55
Live sperm %	88.00 \pm 3.05	84.00 \pm 2.31	84.00 \pm 2.31	86.00 \pm 1.53	0.60
Abnormal sperm %	22.67 \pm 2.67 ^b	22.00 \pm 1.15 ^{ab}	17.00 \pm 0.58 ^a	21.67 \pm 1.20 ^{ab}	0.12
AcrI %	60.00 \pm 5.77 ^{ab}	68.67 \pm 2.91 ^b	53.33 \pm 3.38 ^a	59.00 \pm 2.52 ^{ab}	0.11
HOST%	55.00 \pm 2.89	50.00 \pm 4.62	42.67 \pm 3.53	54.00 \pm 5.03	0.21
MDA (nmol/ml)	42.72 \pm 1.52 ^{ab}	40.60 \pm 4.52 ^{ab}	35.15 \pm 3.25 ^a	49.09 \pm 4.15 ^b	0.078
CAT (U/L)	296.05 \pm 26.67	301.27 \pm 25.96	256.52 \pm 5.79	316.83 \pm 14.89	0.220

AcrI= Acrosome integrity; HOST = hypo-osmotic swelling test; MDA= Malondialdehyde; CAT= catalase. Different superscripts within row (a, b, c) are significantly different at $P<0.05$

TABLE 6. Effect of concentrations of WG aqueous extract on semen characteristics, CAT and MDA activity in chilled rabbit buck semen after 2h and 48h

	0	200	300	400	P-value
After 2 hours					
Motility %	86.67±3.33 ^b	80.00±5.00 ^{ab}	70.00±5.00 ^a	75.00±2.89 ^{ab}	0.10
Live sperm %	88.33±0.33	89.00±1.73	90.00±2.31	90.00±1.16	0.84
Abnormal sperm %	18.00±1.16 ^b	15.00±0.58 ^a	14.00±1.16 ^a	13.00±0.58 ^a	0.023
AcrI %	92.67±2.33	93.67±0.88	90.00±1.16	90.67±1.33	0.35
HOST%	64.00±2.31	57.33±2.48	57.00±5.00	56.67±5.46	0.58
MDA (nmol/ml)	31.82±4.32 ^a	56.82±0.83 ^c	40.45±0.83 ^b	46.82±2.82 ^b	0.0001
CAT (U/L)	431.77±17.15 ^c	344.52±15.52 ^b	353.47±27.77 ^b	182.33±3.67 ^a	0.0001
After 48 hours					
Motility %	38.33±1.67	36.67±3.53	38.33±1.67	36.67±2.73	0.93
Live sperm %	89.00±2.89	83.33±1.76	84.00±4.61	83.00±1.04	0.61
Abnormal sperm %	24.00±2.31 ^b	23.00±1.73 ^{ab}	22.00±1.15 ^{ab}	18.00±1.15 ^a	0.13
AcrI %	60.33±5.78	53.33±3.52	46.67±4.41	60.67±4.06	0.17
HOST%	57.67±1.45	48.67±4.67	50.00±4.16	49.33±4.37	0.38
MDA (nmol/ml)	42.73±1.52 ^{ab}	50.27±3.57 ^b	40.30±3.41 ^a	49.09±3.47 ^{ab}	0.096
CAT (U/L)	296.05±26.68 ^a	264.73±15.33 ^a	410.19±38.88 ^b	401.89±15.38 ^b	0.001

Means with different superscripts (a, b, c) are significantly different at P<0.05,

AcrI= Acrosome integrity; HOST = hypo-osmotic swelling test; MDA= Malondialdehyde; CAT= catalase

Discussion

The use of plants in the alternative medicine had its sound in the reproductive field especially for the semen preservation. In the last decades, the artificial insemination domain had reported good results via the enrichment of the tris-base diluent with natural extracts (vegetable and fruits extracts, also bee's products such as honey and propolis, and other natural stuffs) in cattle [31], buffalo [8, 32], rabbit [33], ram [34] and horse [35].

The reduction of 30-80% in sperm quality is the consequence of the oxidative stress. The lipid composition of sperm cells membrane, including multiple unsaturated fatty acids, plasminogen, and sphingomyelins is responsible for elasticity and liveability of the sperm cells. However, these lipid components proved essential substrates for membrane peroxidation and deterioration [36]. The later resulted due to ROS that causes injury to sperm DNA. Sperm produces ROS in very small physiological amounts under normal conditions. This is essential for capacitation, ovarian activation, acrosome reaction, motility, fertility, and conception [37]. However, when ROS levels exceed the antioxidant capacity, mitochondrial energy production is reduced and glycolysis enzymes deactivated leading to a decrease in sperm motility. Therefore, a balance between free radicals and antioxidants in the body is critical. The antioxidants from natural sources decrease free radical levels through their scavenging effect [38]. Herbal antioxidants defend sperm against oxidative stress and assist their survival [39].

The antioxidant activity of WG was demonstrated by Liaqat *et al.* [40]. They reported that WG had

anti-free radicals scavenging and reducing power activities. These activities associated with the ability of antioxidant materials to donate hydrogen or electron. Phenols, flavonoids, alkaloids have this ability [41]. The present study demonstrated that WG contains these Phenols, flavonoids, and alkaloids. Therefore, we can refer the antioxidant capacities of WG to these compounds. The oil% in WG powder was near to that registered by Çetinkaya and Öz [42] and Liaqat *et al.* [40]. They found that WG contained 9.72 oil%. Total phenol and flavonoids contents of hydroethanolic extract was higher than that obtained by Liaqat *et al.* [40] (80.95 ± 0.55 mg gallic acid/ g extract and 17.54 ± 0.35 mg quercetin / g extract, respectively).

Our results concerning the fatty acids composition were near to those obtained by Salem and Khalifa [43]. Their record about fatty acids composition of WG was comparable to our results. They reported that unsaturated fatty acids content in WG oil was higher than the saturated content. Also, the highest fatty acid was the linolic acid (50.90%). Concerning the unsaponifiable matter, for the WG oil, our results agreed with Marzocchi *et al.* [44], who reported that WG oil contains campesterols (18.5%) and sitosterol (18.8%).

The chemical analysis proceeded in this study showed that WG had high nutritive values proved by their contents of oils, saturated and unsaturated fatty acids, polyphenols and flavonoids, and high antioxidant capacity. Several studies were performed to demonstrate the effect of nutrients on the reproductive performance of male animals [9, 34]. The antioxidant effect of feed stuffs was the pivot for incorporating the whole grains or their extracts in

improving the reproductive performance of male.

WG offers nutrients of superior biological value having prebiotic effects [45]. El-Sisy *et al.* [9] reported that the use of 4 g whole WG grains as food additives for rabbit bucks had improved their reproductive performance and semen characteristics. While, Halawa [15] reported that feeding male rabbits with WG oil gave good results in semen characteristics, conception rate, litter size and litter weight. On the other hand, the use of WG extracts in enrichment of semen extender was performed by Arboud *et al.* [19] in cryopreservation of cattle semen. They found that the methanol extract of WG had overcome the oxidative stress during the cryopreservation and thawing of cattle semen and maintained the sperm motility and other semen characteristics. There was a limit concentration for incorporation of WG in cattle Tris-extender.

Our results showed that the oil and water extracts of WG in case of addition of less than 5% egg yolk extender were the best in preserving buck semen for 48 hrs. Precisely, the oil and water extracts had maintained motility in best performance, the water extract was the best for live sperm, all the three extracts gave the worst abnormal morphology, no significant difference between the three extraction methods concerning acrosome integrity, they had bad effect on plasma membrane integrity, and they lowered CAT activity. Although, the oil and hydroethanolic extracts gave the lowest lipid peroxidation.

Sperm is sensitive to ROS damage due to the relatively high content of unsaturated fatty acids in the phospholipids of the sperm membrane [46]. As known, MDA measurement is widely used as an indicator of lipid peroxidation in a different cell type, including spermatozoa [47]. The present results explained that, the supplementation of the extender with 200 – 300 µg/ml WG extract resulted in significant reduction of MDA levels in comparison with control group. In addition, CAT enzyme activities increased with different doses of WG extract (200 – 300 µg/ml). These results confirm the hypothesis that one of the most beneficial effects of antioxidant during the cryopreservation is the reduction of the membrane lipid peroxidation. On the same consent, feeding of albino rats with WG oil significantly reduced lipid peroxidation of testicular tissue and improved the antioxidant defences [48]. In addition, El-Sheshtawy *et al.* [10] recorded that addition of WG extract improved post-freeze thaw sperm characteristics and viability index. This improvement could be explained by the significantly higher total antioxidants and the decrease in the level of MDA. In this aspect, Lone *et al.* [49] recorded that, at post-thaw stage, total antioxidants were positively correlated with sperm motility, sperm

membrane integrity and viability and that these correlations are considered as predicting for bull fertility. These findings are also compatible with that of Alyethodi *et al.* [50] who recorded an elevated unsaturated fatty acids peroxidation and superoxide anions and inferior total antioxidant capacity (TAC) in the inferior freeze-semen samples compared to the superior freeze-samples with the better semen characteristics. WG extract has high antioxidant potential relative to its elevated percent of unsaturated lipids, minerals and vitamins that ameliorated the value of cryopreserved semen by elimination of the over oxygen free radicals as indicated by decreased MDA [10].

Conclusion

In conclusion, we can use the WG oil rich in antioxidant and nutritive value at a concentration averaged between 200 – 300 µg/ml (in addition to less than 5% egg yolk) for 48 hrs in preserving chilled rabbit buck semen.

Ethical Approval

The experimental plan was approved by the Medical Research Ethics Committee of the National Research Centre, Dokki, Egypt and its registration number is 19/144 and its date is 2/2/2020.

Conflict of interest

The authors announce that, there isn't any conflict of interest.

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بعض خصائص السائل المنوي وعلامات الأكسدة/مضادات الأكسدة لسائل المنوي للأرنب المخفف والمبرد في المخفف القائم على التريس ومكمل بمستخلصات جنين القمح

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يعتبر جنين القمح من المصادر الغنية بمضادات الأكسدة، ولها تأثيرات مضادة للميكروبات، كما أنه قد ثبت أن له تأثير مضاد للصدمة الباردة في الحفاظ على السائل المنوي المخفف. وقد أوضح البحث الحالي دور المستخلصات المختلفة لجنين القمح (مستخلصات الزيت، والهيدروايتانول والماء) مع صفار البيض في الحفاظ على السائل المنوي أثناء عملية التبريد. تم استخلاص جنين القمح باستخدام الإيثر البترولي (لاستخلاص الزيت)، ومستخلص الهيدروايتانول والمستخلص المائي. تم إجراء التحليل الكيميائي لمستخلص جنين القمح بواسطة كروماتوجرافيا الغاز للكشف عن الأحماض الدهنية المشبعة وغير المشبعة. أيضًا، القدرة المضادة للأكسدة واستخلاص المعادن وقوة الاختزال في المستخلصات الهيدروايتانولية. صممت تجربة استرشادية لاختيار أفضل تركيز من بين ٥ تركيزات (١٠٠، ٢٠٠، ٣٠٠، ٤٠٠، ٥٠٠ ميكروغرام / مل). وقد تم اختيار ٢٠٠ و ٣٠٠ و ٤٠٠ ميكروغرام / مل التريس الأساسي للدراسة الحالية. تم تبريد عينات السائل المنوي المخفف عند ٤ درجة مئوية لمدة ٤٨ ساعة. وتم تقييم حركة الحيوانات المنوية، حيويتها، تشوهاتها، وسلامة الغشاء البلازمي والغشاء القمي أثناء فترة التبريد. تم تقييم المالوندايالدهيد والكاتاليز في السائل المنوي المخفف بعد ٤٨ ساعة من التبريد. أفضل مستخلص حافظ على الحركة العالية للحيوانات المنوية هو مستخلص زيت جنين القمح بتركيز ٢٠٠ و ٣٠٠ ميكروغرام / مل، مع إضافة صفار البيض. وقد خلصت الدراسة إلى أنه يمكن استخدام مستخلص زيت جنين القمح الغني بمضادات الأكسدة والقيمة الغذائية بتركيز متوسط يتراوح بين ٢٠٠-٣٠٠ ميكروغرام / مل بالإضافة إلى أقل من ٥٪ من صفار البيض لمدة ٤٨ ساعة في حفظ السائل المنوي المبرد لتيوس الارانب.

الكلمات الدالة: الارانب، مخفف السائل المنوي، خصائص السائل المنوي، مستخلص جنين القمح، حالة التأكد.