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THE EFFECT OF PANAX GINSENG EXTRACT IN EXTENDER ON RAM SPERM PARAMETERS IN VITRO CONDITION

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Abstract:

Sperm survived for a short time in undiluted semen, and semen slow cooling to a temperature of 5°C will cause the death of many spermatozoa. Thus, extender is suitable which sperm parameters preserved during cooling and freezing. The aim of this study was to investigate the effect of panax ginseng extract in extender on sperm motility, morphology and viability of Afshari rams. In this study, four Afshari rams with a mean of 5 ± 50 kg weight and 3 to 4 years, sperm was collected by electro ejaculation. After extraction of the medicinal plant at a concentration of 1%, 3% and 5%, each concentration was added to the sperm extender as separately and one group were considered as a control. Sperm motility, morphology and viability were evaluated at zero time (immediately post ejaculation) and 24 hours post ejaculation by CASA software, papanicolaou and Eosin-Nigrosin staining, respectively. The results of this study shows that progressive motility was significantly increased ($p<0.05$) in treatment groups of 3% and 1% panax ginseng extract compare to control and other treatment groups at time zero and 24 hour post ejaculation, respectively. However, normal sperm morphology was significantly higher ($P<0.05$) in treatment group of 3% extract than control and other treatment groups at zero time and 24 hour post ejaculation, respectively. Percentage of live sperm was significantly higher ($P<0.05$) in treatment group of 3% panax ginseng extract compare to other treatment groups at zero time and also, treatment group of 1% panax ginseng extract was significant higher viability ($P<0.05$) than to other treatment groups. Therefore, the addition of panax ginseng extract in extender was advisable to improve sperm parameters in afshari rams.

Keywords: *Ram Sperm, Panax Ginseng, Extender, Motility, Morphology, Viability.*

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INTRODUCTION:

Artificial insemination potentially has an important role in sheep breeding. It makes the dissemination of genetic material from a small number of superior sires to a large number of females [1].

The development potential for the widespread use of AI across the industry therefore depends on fresh semen insemination. Cervical insemination using fresh semen generally yields around 50-60% conception rates, and the interest in AI using fresh semen has been growing in world following the awareness of the potential of AI for breed improvement during the AI Scheme [2].

However Since a suitable extender is appropriate for sheep sperm, has not been introduced. Also, Research was appeared the increasing use of medicinal plants in cattle diets, but the use of these plants are need in animal sperm as extender. *Panax ginseng*, a member of the *Araliaceae* family, is a slow-growing perennial plant with fleshy roots and grows in Korea and northern China. For at least 2000 years, *P. ginseng*, known as Korean ginseng, has been evaluated as a medicinal plant in traditional oriental medicine [3].

The biological and pharmacological efficacy of Korean ginseng revealed by modern science includes adaptogenic effect, enhanced immune system function, improved sexual functions, as well as anti-oxidative effects [4].

Researchers looking for potential changes in hormones in laboratory animals that were fed North American *ginseng* found no changes in male sex hormones, but instead found that *ginseng* significantly enhanced male libido and copulatory performance. There is also interest in the use of the root in the treatment of sexual dysfunction, such as erectile disorder [5].

Since the beginning of the 20th century, the constituents of ginseng root have been investigated and several classes of compounds have been isolated such as triterpene saponins; essential oil-containing polyacetylenes and sesquiterpenes; polysaccharides; peptidoglycans; nitrogen-containing compounds; and various ubiquitous compounds such as fatty acids, carbohydrates, and phenolic compounds [6]. Ginseng contains an extraordinarily complex mixture.

Sheep farming plays an important role in agrarian economy by providing a major source of livelihood to large number of small, marginal and less farmers in hilly, arid and semi-arid regions of Iran. Afshar is a hardy native sheep breed of the semi-arid tropical environment and is reared for wool and mutton production. One important element for selection of breeding rams for either natural mating or AI relies on semen quality evaluation. Subjective evaluation of semen quality attributes is simple but do not provide accurate estimates for correlating it with fertility [7].

Computer Assisted Semen Analysis (CASA) quantifies wide range of parameters of sperm motility and provides a rapid and objective method for assessing the motility of ram spermatozoa [8].

The aim of study was conducted In vitro effects of *Panax ginseng* in semen extender on sperm characteristics of adult Afshar rams by the computer-aided sperm analysis (CASA) technique.

MATERIAL AND METHODS:

All chemical reagents were obtained from Merck, (Darmstadt, Germany) unless otherwise noted.

Semen collection, evaluation and sample preparation:

Semen samples were collected from 4 mature Afshar rams (3 to 4 years) maintained at the Animal Breeding Center Farm of Islamic Azad University, Branch of Isfahan (Khorasgan), Iran. The rams were feed 0.91 kg of concentrate daily and good quality hay and water were supplied ad libitum. After semen collection, the raw semen samples were transferred to the laboratory immediately, and kept in a water bath at $37^{\circ C}$ for examination. Ejaculates were collected from the rams using the electrical ejaculator (imv, Frances) twice a week during the breeding season (autumn to early winter). Sperm concentration was measured with a hemocytometer. Semen samples were mixed in a pool, balancing the sperm contribution of each ram to eliminate individual differences that containing a semen volume that varied between 1 and 2 ml, spermatozoa with >70% progressive motility and concentrations higher than 2.5×10^9 spermatozoa/ ml [9].

Extender preparation:

Extender (10 ml) was prepared in autoclaved double distilled water (prepared in Biotechnology Laboratory of Islamic Azad University of Khorasgan branch) containing 0.244 g TRI vS, 0.136 g citric acid, 0.082 g D-fructose and 20 % v/v egg yolk [10].

Extract preparation:

To accomplish this, we use the method of extraction by using Percolation (prepared in GOLDARU, Pharmaceutical Co). Then put the *Panax*

ginseng extract for 48 hours at 30 to 40 °*C* and completely isolated environment free from microbes (oven). After the extracts were dried and then weighting using a Sodium chloride serum injection 9% can hold volume.

Experimental design:
In this study, semen samples were dividing to control group and 3 treatment groups with *Panax ginseng* extract concentration 1% (v/v), 3% (v/v) and 5% (v/v) at 3 replication. Each group individually was added to the basic semen extender and there was evaluated at zero time (immediately after ejaculation) and 24 hours post ejaculation. For evaluation of semen samples at 24 hour post ejaculation, whole sample were maintained to 4$^{°C}$ temperature.

Semen motility:
Spermatozoa motility was evaluated using computer automated semen analysis (CASA analyzer, video sperm test 2.1), an Olympus BX40 microscope under 100× magnifications and at 37 $^{°C}$. The sperm motility variable was measured as below:

Fast forward moving sperm (A motility), slow forward moving sperm (B motility), not forward moving sperm (C motility), non-moving sperm (D motility), percentage of progressive motility (PM %) and percentage of total motility (TM %).

Also, the other motility values were recorded: VSL (straight linear velocity, µm/s), VCL (curvilinear velocity, µm/s), VAP (average path velocity, µm/s), ALH (lateral head displacement, µ), STR= VSL/VAP×100 (sperm track straightness, %) and LIN= VSL/VCL×100 (linearity, %). For each evaluation, 10 microscopic fields were analyzed to include at least 300 sperm cells [In keeping with WHO 2010 standards].

Semen viability:
Eosin and Nigrosin (E&N) staining was carried out according to WHO standards [WHO 2010]. Briefly, 1% eosin (Merck) and 10% nigrosin (Merck) was prepared in distilled water. A 1:2 volume of semen was mixed with 1% eosin. After 30 second, an equal volume of nigrosin was added to this mixture, then thin smears were prepared and observed by light microscopy at 1000X magnification.Viable sperm remained colorless while nonviable sperm stained red.

Semen morphology:
Morphology was evaluated with direct microscopic examination by using Papanicolaou staining technique according to strict criteria [11]. Normal and abnormal evaluated by CASA software after shooting about 200 sperm samples from each group the following results were obtained.

Statistical analysis:
The analysis of this study uses SPSS (VER.21, 2012) data software package, statistical model based on project ANNOVA were analyzed and compared between the mean and LS Means LSD test at 5% significance level was used.

RESULTS AND DISCUSSION:

Effect of ginseng extract on sperm motility in zero times:
In this study, the impacts of *Panax Ginseng* extract in extender were evaluated on ram sperm motility, morphology and viability in vitro condition. Table 1 shows that maximum of motility spermatozoa was related to treatment group of 3% extract (10.4 ± 7.05) in A motility at time zero but wasn't significant difference compare than the control and other treatment groups.

Table 1: Effects of *Ginseng* extract on sperm motility in afshari ram measured by CASA at zero time (immediately after ejaculation).

Level of extract in extender	*Ginseng* extract			
Motility Parameters	1%	3%	5%	control
A%	9.12 ± 6.8	10.4 ± 7.05	6.7 ± 3.6	7.16 ± 1.1
B%	39.12 ± 6.2^{a}	38.85 ± 2.7^{a}	28.97 ± 6.4^{b}	19.53 ± 2.1^{c}
C%	44.4 ± 6.1^{a}	43.37 ± 5.7^{a}	45.22 ± 3.6^{a}	27.1 ± 4.9^{b}
D%	7.72 ± 6.5^{c}	7.37 ± 3.2^{c}	19.1 ± 10.2^{b}	46.2 ± 5.8^{a}
PM%	48.25 ± 10.6^{a}	49.25 ± 8.9^{a}	35.67 ± 9.4^{b}	26.7 ± 1.1^{c}
TM%	92.27 ± 6.5^{a}	93 ± 3.2^{a}	80.9 ± 10.2^{b}	53.8 ± 5.8^{c}
LIN%	74.8 ± 2.29^{a}	72.87 ± 3.39^{a}	43.1 ± 3.5^{b}	74.06 ± 3.02^{a}
STR%	97.3 ± 0.6^{a}	97.3 ± 0.8^{a}	93.47 ± 1.2^{b}	98 ± 0.2^{a}
BCF (Hz)	7.32 ± 0.6^{b}	7.3 ± 0.8^{b}	8.1 ± 0.2^{a}	6.8 ± 0.4^{b}
ALH (µm/s)	1.8 ±0.4	1.8 ± 0.7	1.47 ± 0.2	2.13 ± 0.45

VCL (µm/s)	59.02 ± 6.7	56.42 ± 12	58.55 ± 3.6	59.26 ± 3.8
VSL (µm/s)	42.85 ± 5.5 [a]	39.67 ± 6.9 [a]	22.35 ± 1.3 [b]	42.93 ± 3.8 [a]
VAP (µm/s)	44.27 ± 5.7 [a]	41.02 ± 7.4 [a]	24.4 ± 1.7 [b]	43.73 ± 3.9 [a]

Mean percentages ± SD of motility of fresh ram spermatozoa in *Ginseng* extract on extender
Different letters in the same Rows indicate a statistical difference (P < 0.05).

All treatment groups were significantly higher than the control group (P<0.05) at B motility. Also, treatment group of 1% extract had the highest B motility than to other treatment. All treatment groups were significant increased at C motility compare to the control group (P<0.05), and the highest level was related to treatment group of 5% extract. Treatment group of 5% extract was significantly higher than to other treatment groups (P<0.05) and it was significantly lower than the control group (P<0.05) at D motility.

The PM% of sperm that main criteria of selection for fertility sperm in the WHO, all treatment groups were significantly increased compare to control group (P<0.05), and the highest of the PM% was observed in treatment group of 3% extract.

However, the TM% of sperm was significantly increased at all treatment groups compare to control group, and treatment group of 3% extract was highest between other treatment groups at zero time, but no significant.
Other sperm parameters, LIN and STR parameters were significantly lowest in treatment group of 5% extract than other treatment and control groups (P<0.05). Also, BCF parameter was significantly higher in treatment of 5% extract than the control group (P<0.05). ALH and VCL parameter weren't significant difference between control and treatment groups. On the other hand, VSL and VAP parameters were significantly decreased compared to the control group (P<0.05) at treatment group of 5% extract.

Effect of ginseng extract on sperm motility in 24 hours post ejaculation:
Table 2 shows that the treatment group of 1% extract was significant difference with treatment group of 5% extract (P<0.05) in A motility at 24 hours post ejaculation. According to the table, A motility was reduced with the increasing concentration of 1% to 5% level *Ginseng* extract. Also, it was significant difference between control group and treatment group of 5% extract.

The treatment group of 1% extract was significantly highest (P<0.05) then the control and other treatment groups at B motility.

Table 2: Effects of *Ginseng* extract on sperm motility in afshari ram measured by CASA at 24 hours post ejaculation

Level of extract in extender	*Ginseng* extract			
Motility Parameters	1%	3%	5%	control
A%	7.46 ± 1.12 [a]	6.4 ± 0.65 [a]	4.63 ± 1.02 [b]	6.33 ± 1.52 [a]
B%	31.3 ± 1.41 [a]	17.63 ± 1.33 [b]	16.56 ± 0.66 [b]	18.23 ± 1.07 [b]
C%	21.56 ± 2.5 [c]	24.03 ± 0.6 [b]	31 ± 0.5 [a]	16.43 ± 5.94 [d]
D%	39.66 ± 1.5 [d]	51.93 ± 1 [b]	48.26 ± 1.2 [c]	59 ± 7.9 [a]
PM%	39.1 ± 3.01 [a]	24.03 ± 0.7 [b]	21.2 ± 1.3 [c]	24.56 ± 2.3 [b]
TM%	60.33 ± 1.5 [a]	48.06 ± 1 [c]	52.6 ± 1.1 [b]	38.33 ± 3.5 [d]
LIN%	72 ± 2	73.35 ± 2.1	73.75 ± 5.8	74.56 ± 3.5
STR%	96.82±0.8	96.67 ± 0.9	95.8 ± 2.5	96.96 ± 1.9
BCF (Hz)	6.95 ± 0.3	6.67 ± 0.1	7.42 ± 0.6	7 ± 0.4
ALH (µm/s)	2 ± 0.2	2.32 ± 0.2	1.5 ± 0.5	1.96 ± 0.5
VCL (µm/s)	58.12 ± 1.5	63.37 ± 5.4	54.75 ± 9.4	56.4 ± 6.2
VSL (µm/s)	42.25 ± 1.8	45.1 ± 2.7	37.85 ± 3	40.76 ± 6.1
VAP (µm/s)	42.67 ± 1.6	46.77 ± 3.4	38.95 ± 2.6	42.06 ± 5.6

Mean percentages ± SD of motility of fresh ram spermatozoa in *Ginseng* extract on extender
Different letters in the same Rows indicate a statistical difference (P < 0.05).

The C motility, the treatment group of 5% extract was significant increased compare to the control and other treatment groups (P<0.05).
All treatment groups were significantly reduced compare to control group (P<0.01) at D motility. However, the treatment group of 3% extract was significantly higher than other treatment groups (P<0.05).
The treatment of 1% extract was significantly increased (P<0.05) compare to the control and other treatment groups at the PM%.

All treatment groups were higher than control groups at the TM% but, treatment group of 1% was significantly higher (P<0.05) than control and other treatment groups. The highest of TM% was at the level of 1% extract (60.33 ± 1.5 %).

Other sperm parameters including LIN, STR, BCF, ALH, VCL, VSL and VAP wasn't significant difference between control and other treatment groups.

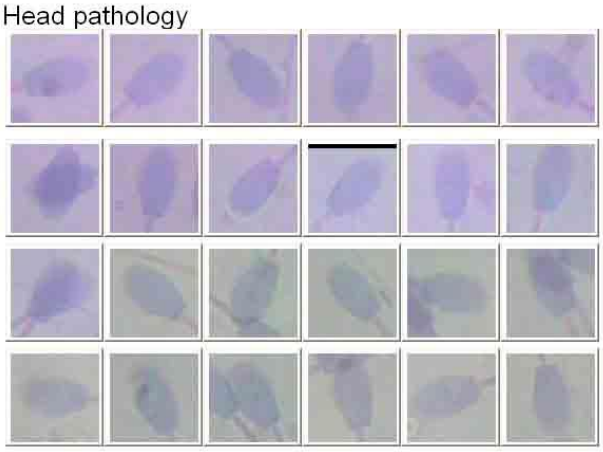
Effect of ginseng extract on sperm morphology:
The result of this study (Table 3) shows that normal sperm was significant higher (73% and 52%) in treatment group of 3% extract compare to control and other treatment groups at zero time and 24 hour post ejaculation (P<0.05). however, abnormal sperm was highest (39% and 60%) in treatment group of 5% extract compare to control and other treatment groups at zero time and 24 hour post ejaculation.

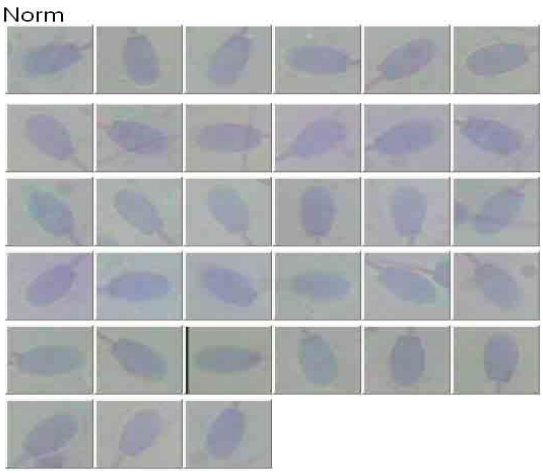
Therefore, treatment group of 3% *Ginseng* extract had the best effect on ram sperm morphology parameters.

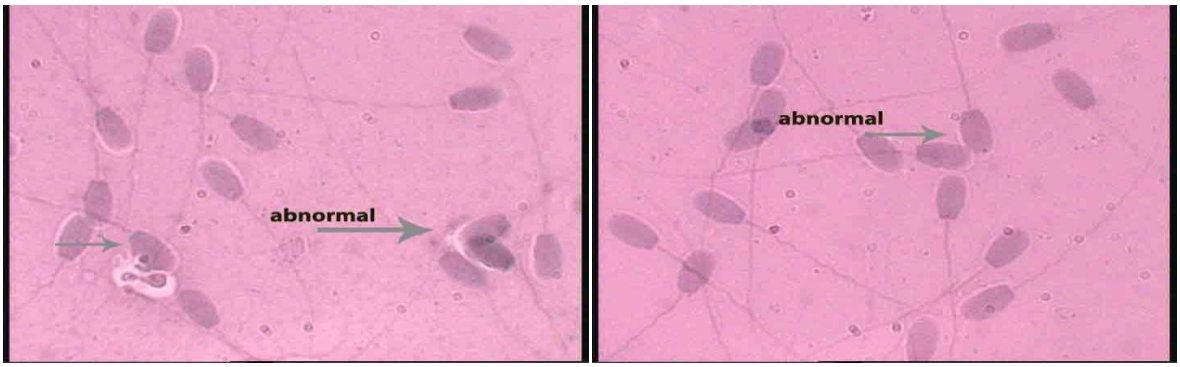
Table 3: Effect of *ginseng* extract on afshari rams sperm morphology

Sperm evaluation (hour		*Ginseng* extracts concentration (%)			
		1	3	5	Control
0	Normal %	71 [b]	73 [a]	61 [b]	65 [b]
	Abnormal %	29	27	39	35
24	Normal %	49 [b]	52 [a]	41 [b]	44 [b]
	Abnormal %	51	48	60	56

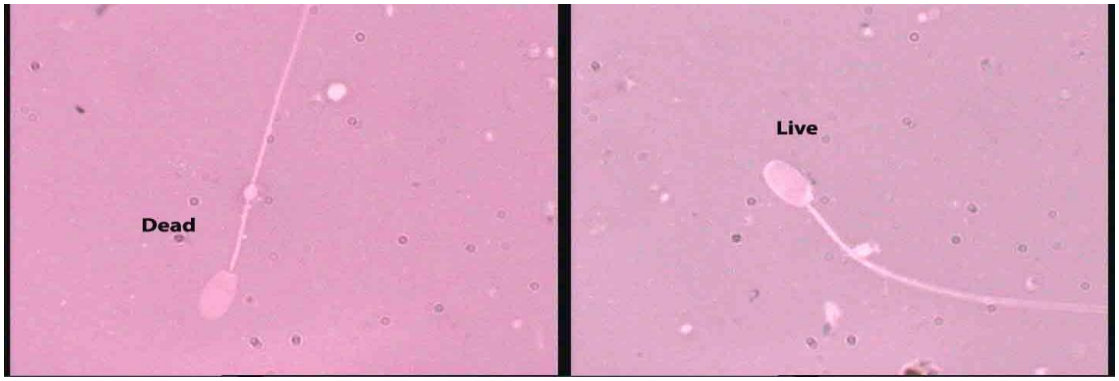
Different letters in the same Rows indicate a statistical difference (P < 0.05).







Figures 1: Pictures has been taken by CASA from head of ram sperm



Figures 2: Pictures has been from sperm viability of ram

Effect of ginseng extract on sperm viability:
Viability of sperm is dependent on health cell membrane. According to the table 4, treatment group of 3% *Ginseng* extract used in semen extender was significantly higher viability (87%) compare to other treatment groups at time zero (P<0.05) but, no significant difference to control group. Treatment group of 1% extract was significant higher viability (66%) than to other treatment group (P<0.05) but no significant difference to control group at 24 hour post ejaculation.

Table 4: Effect of *ginseng* extract on afshari rams sperm viability

Sperm evaluation (hour)		*Ginseng* extracts concentration (%)			
		1	3	5	Control
0	Live %	82 [b]	87 [a]	76 [b]	89 [a]
	Dead %	18	13	24	11
24	Live %	66 [a]	57 [b]	39 [b]	70 [a]
	Dead %	34	43	61	30

Different letters in the same Rows indicate a statistical difference (P < 0.05).

In this study, sperm motility was significant higher in treatment group of 3% extract than to control group at zero time. This was observed after treatment finding provided a valuable evidence for past researches on antioxidanti properties and rule of saponin in *ginseng*. Saponin was including a three parts as below: *panaxosides*, *ginsenosides* and *cheikusetsusaponins*. In the other hand, *ginseng* enhances the nitric oxide (NO) synthesis in the endothelium, and nitric oxide has a protective role as an antioxidant [12].

Indeed, *ginsenosides* have contain L-arginine that it is the source of NO [13] and a constitutive NO synthase appears to be involved in sperm motility , metabolism , capacitation , and acrosome reaction [14].

In the current study, it was found that *ginseng* extract improved morphology sperm and decreased abnormal sperm [15] was found that *ginseng* extracts scavenge oxidative species; also, [16] was indicated that *ginseng* extracts attenuate lipid peroxidation. That is, it may be related to saponins which play a major role in antioxidant activities. In addition, *ginsenoides* which are important components heavily present in *ginseng* production of powerful antioxidant activities other than radical scavenging activities by stimulating gene expression of antioxidant enzymes and enhancing their activities. The anti-inflammatory and antioxidative effects of *ginseng* could improve the shape and concentration of spermatozoa [17]. However, L-arginine, L-carnitine, and acetyl-L-carnitine could enhance sperm motility and function by stimulating the activity of endothelial nitric oxide (NO) synthase [18]. In addition, the anti-inflammatory and antioxidative effects of *ginseng* may have an important role in semen quality recovery with regard to sperm concentration. Several studies showed a significant reduction of oxidative stress biomarkers such as F_2-isoprostane and 8-hydroxy-deoxyguanosine in healthy subjects after oral administration of *ginsenoside*-enriched *Panax quinquefolius ginseng* extract [19].

These results were in agreement with Hwang in 2004 who report that *Panax ginseng* improves survival, sperm quality and percentage of sperm with progressive motility in guinea pigs which exposed to 2, 3, 7, 8-tetrachlorodibenzop-dioxin. Also Hwang was reported that *Panax ginseng* improves senile testicular function in rats and progressiveness and VCL were increased markedly in the *ginseng* group compared with the control group. Aziza et al. [20] was reported that ginseng alone or in combination caused a significant decrease in total chromosomal aberrations, sperm abnormalities and increase in testosterone level, sperm counts and motility. Ginseng saponins were found to compete strongly with estradiol and R5020 (a synthetic progestin) for estrogen and progesterone binding sites in the human myometrial cytosol [21].

Saponins have been shown to have both positive and negative effects on the viability of human sperm cells in vitro that some ginseng saponins resulted to increase motility as well as progression of sperm [22]. It is debatable how relevant these in vitro effects are for dietary saponins as there are relatively few reports in this regard [23].

The dried rhizomes of *ginseng* and extract`s contain many physiologically important constituents. These comportment have been isolated and characterized that include *ginseng* saponins, *ginseng* oils and phytosterol, carbohydrates and sugars, organic acids, nitrogenous substances, amino acids and peptides, vitamins and minerals, and definite enzymes [24]. The main function of carbohydrates is to supply the life energy to the spermatozoa in the form of an easily glycolyzable material, semen uses fructose which is the naturally available sugar .It should be pointed out, however, that although normally spermatozoa utilize fructose they are well able to glycolyze other sugars as well If glucose or mannose, for instance, is added to the semen, this offers the spermatozoa a choice of several glycolyzable substrates and they actually make use of all of them so that whereas in untreatedsemen the entire lactic acid would have been the outcome of fructolysis, in presence of additionalsugar only a certain proportion of lactic acid is derived from the seminal fructose and the rest is the result of glycolysis of the extraneous sugar [25].

Ram spermatozoa were incubated in sperm motility inhibiting extender for 48 h phospholipids levels decreased constantly and triglycerides levels during the first 24 h of incubation indicating that spermatozoa utilize lipids as energy resources. After 24 h triglycerides levels started to re-increase indicating a change in sperm metabolism, in particular the onset of triglycerides synthesis by the fatty acid synthase complex. In the incubation period from 0 to 24 h glucose levels were constant, and decreased thereafter. Glycogen levels did not change at all. Semen contained also considerable amounts of sialic acid, glucuronic acid and hexosamines, components of mucopolysaccharides [26], so by increasing the extract, the expected results of sperm motility and viability were observed.

CONCLUSIONS:

Therefore, the addition of Panax ginseng extract in extender was advisable to improve sperm parameters in afshari rams.

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Conflict Of Interest:
Authors claim that there is no conflict of interest.

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