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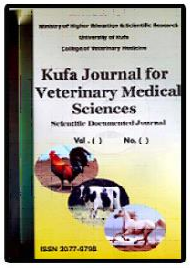

Sperm Membrane and Acrosomal Integrity Affected by using Black Currant

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Abstract

This study was designed in order to investigate and determine the effect of black current (*Prunus cerasus*) extract on semen membrane and acrosome integrity of the rams. Twelve mature healthy rams were used in this study aged between 2-3 years, kept in the animal farm of the College of Veterinary Medicine, Al-Kufa University. Semen ejaculates was collected using electro-ejaculator methods once weekly a total of 36 ejaculates were collected among the period of the study. The black current (*Prunus cerasus*) extract was prepared by using maceration and added to the diluted semen in five doses groups (0.25 gm/ml as G1, 0.5 gm/ml as G2, 1 gm/ml as G3, 2 gm/ml as G4, 4 gm/ml as G5) and the positive control group left without any addition. The treatment samples were evaluated under cooling for five consecutive days, the membrane integrity test was evaluated using hypo-osmotic solution and acrosome integrity was analysis using semen smear stained with eosin–nigrosin stain. The result showed that high concentration of aqueous plant extract give negative result as listed in G3, G4 and G5 in the all preservation days. As found in acrosome integrity at the 5thday of incubation showed decreased at highly significantly sequentially. We conclude that the G1 and G2 of black current extract (*Prunus cerasus*) should be used in chilling process as a part of artificial insemination in sheep due to its protective effect of the membrane and acrosome integrity.

Keywords: Acrosome integrity, black current, membrane integrity, rams, semen.

Introduction:

Reactive oxidative substances (ROS) are produced during the processing of preservation of spermatozoa that exert some physical and chemical changes in the sperm membrane [1]. Therefore, numerous research efforts have evaluated the effect of a various synthetic and natural antioxidants on sperm. Antioxidants are believed to neutralize the free radicals in lipid chains by contributing a hydrogen atom usually from a phenolic hydroxyl group which converts phenolic groups into stable free radicals that do not initiate or propagate further lipid oxidation. The membrane of spermatozoa has high amount of unsaturated fatty acids, and these are so susceptible to oxidative damage, i.e. the endogenous antioxidant defense

mechanism cannot recognize the sperm membrane from the Reactive Oxygen Species (ROS) during semen preservation [2]. The grade of ROS is increased considerably during semen preservation [3]. ROS has a sturdy oxidant effect, and can be produced in living cells during respiration [4], as well from dead and phagocytic cells (*5*). Super oxide anion (O^-_2), hydrogenperoxide (H_2O_2) and hydroxyl radical (OH) are the factors that called ROS [6]. On the other hand, chilling procedure can be induced capacitation- like changes in ram spermatozoa [7] due to ROS [3]. Lipid

peroxidation (LPO) can be defined as an important aspect of oxidation effect in sperm [8], or LPO is a pathophysiological process can occur due to diseases or stress conditions and affecting the cellular components and their function [9]. ROS can cause LPO processes in spermatozoa, and reduce the life time of sperm [10, 11]. Many antioxidants have been used to alleviate or prevent ROS effects on sperm function during and after chilling in almost types of farm animals. For example, α-tocopherol and Vitamin C to enhance equine sperm quality [12,13], rosemary aqueous extract to enhance buck frozen- thawed semen [14], topmo and tempol which are superoxide dismutase analogues on alpaca semen cryopreservation [15], butylatedhydroxytoluene (BHT) on buck semen cryopreservation (16], and even diet supplemented with antioxidants to promote the quality of stallion semen cryopreservation during winter [17]. There are limited studies on the effect of medical plants extract as antioxidant on spermatozoa quality during and after cooling. One of well-known medicinal plants is black currant *(Prunus cerasus*), it has an excellent antioxidant activity [18, 19, 20 and 21] respectively, anti-inflammatory activity [22] as well as anti-carcenogenic activity [23].

Natural antioxidants are compounds that are obtained from natural sources such as herbs, seeds, vegetables, spices and fruits [24]. These natural antioxidants are often phenolic compounds or vitamins that have an appropriate molecular structure allowing them to chemically scavenge free radicals [25]. In plants, phenolic compounds have numerous biological roles such as antioxidants, anti-bacterial and anti-inflammatory [26].

It should be noted that there is no research that was done before to study the effect of black currant *(Prunus cerasus*). Therefore, the present study is to investigate and determine the effect of black currant *(Prunus cerasus*) added to tris-based extender on chilled of ram sperm membrane and acrosome integrity.

Materials and methods:

This study does not require ethical approval according to the guidelines of the College of Veterinary Medicine University of Baghdad.

The extraction of plant was done in the artificial insemination and theriogenology laboratory, inside Veterinary Medicine College, University of Kufa, Iraq. The method of choice for the aqueous extraction was done by using maceration of the crude extracts, due to its beneficial in extracting of the thermolabile and thermostable components [23]. The edible part of the black current Fruits was separated manually from the seeds and chopped into small pieces. The black current plant 200 gram was treated with 300 ml of water and mixed very well at room temperature. The content of bottle then was filtered through a Buchner filtration system and the water was removed by exposure to 45 °C to evaporate the water [27]. The remaining plant was treated again with water and the process was repeated for twice times. The extract was collected and stored in freeze -20C for later use.

The extender (diluted semen and tris) was divided into six aliquots dose of aqueous plant extract as 0% (C), 0.25 mg/ml (G1), 0.5mg/ml (G2), 1 mg/ml (G3), 2 mg/ml (G4) and 4 mg/ml (G5), the solution had normal pH ranged between 6.8-7.2 and 425mOms osmolarity at begin. The membrane integrity test is to assess functional plasma membrane of sperm [28] using hypo-osmotic solution (4.9 g/l sodium citrate, 9 g/l fructose and 100 mosm/kg). To do the test was mixed 10 µl semen with 90 µl of hypo-osmotic solution at 37ºC. 0.01 ml of mixture was placed on slide and covered with cover slip under light microscope a total 400 sperm was evaluated, the sperm with swollen and coiled tail was determined at 400×. While acrosome integrity was investigated using semen smear stained with eosin–nigrosin stain and examined under a phase contrast microscope at 1000× magnification oil immersion. A total of 200 sperm was tested for either detached or intact acrosome.

Membrane and acrosomal integrity was evaluated at day 1,2,3,4, and5.The extender

was stored after preparation at -20 °C. The extender was used in this study is a Tris- based extender includes: (for 100 ml) LDL 8%, tris: 2.40 g, fructose: 1 g, citric acid: 1..50 g, gentamycin 25 mg, penicillin 50000 IU, and D. W. until 100 ml [8]. The extract was added to the extender with different doses as mentioned before and one group kept without adding the extract as a control.

Samples of semen were collected from 12 mature ram aged between 2–3 years using electro-ejaculator method (mini tube; Germany) every two weeks. Thirty-six ejaculates of fresh semen were collected and evaluated at 36 ºC. Selected samples should be ≥70% sperm motility and ≥ 80% normal morphology in order to be used in this study. The extended semen groups were stored in a refrigerator at 5 °C for five days. Sperm membrane and acrosome integrity were evaluated at 1, 2, 3, 4, and 5 days of chilling.

The statistical analysis was done using ANOVA was used to determine the significant differences among means at level of $P<0.05$.

Results:

As can be seen from the Figure (1) which described that comparative of the sperm membrane integrity between all treatment groups and control. It was revealed that G2 group was better than all groups in the first day. In contrast, other days of chilled semen recorded that G1 superiority among all groups. Furthermore, that was no significant different between control, G1 and G2 in the first three days in term of hypo-osmotic swelling test (HOST). The result proved that high concentration of aqueous plant extract give negative result as illustrated in G4 and G5 in the all preservation days and additionally to G3 in 2nd, 3rd, 4th and 5th days.

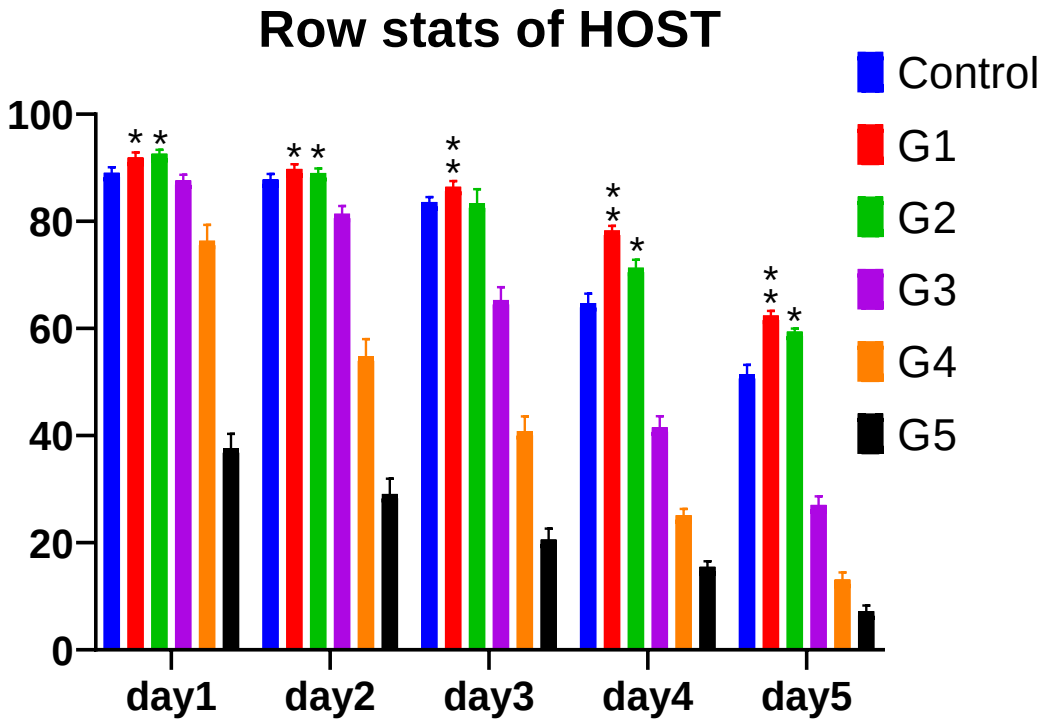

Figure 1. Different between means in 95% confidence intervals of Control and all treatment groups in the 5 days of chilled semen in terms membrane integrity.

- G1 = 0.25 mg/ml of dose of aqueous plant extract of *Prunus cerasus*
- G2 = 0.5 mg /ml of dose of aqueous plant extract of *Prunus cerasus*
- G3 = 1 mg /ml of dose of aqueous plant extract of *Prunus cerasus*
- G4 = 2 mg /ml of dose of aqueous plant extract of *Prunus cerasus*
- G5 = 4m g /ml of dose of aqueous plant extract of*Prunus cerasus*
- Cont = Positive control group; 0 mg (*Prunus cerasus*)

According to Figure (2) the outcomes of the result showed there were no significant differences among acrosomal integrity of treatment and control groups at the first day of incubation, while that was contrast significantly at low level which, in spite of that, there was significant difference since control at the 2nd and 3rd days and other treatment groups. While, in contrast the values are very close to gather except the G4 and G5 in the last day of incubation. As found in the figure (2) at 4th day the value is very close together while in the figure (2) at the 5th day of incubation that showed the acrosomal integrity decreased at highly significantly sequentially

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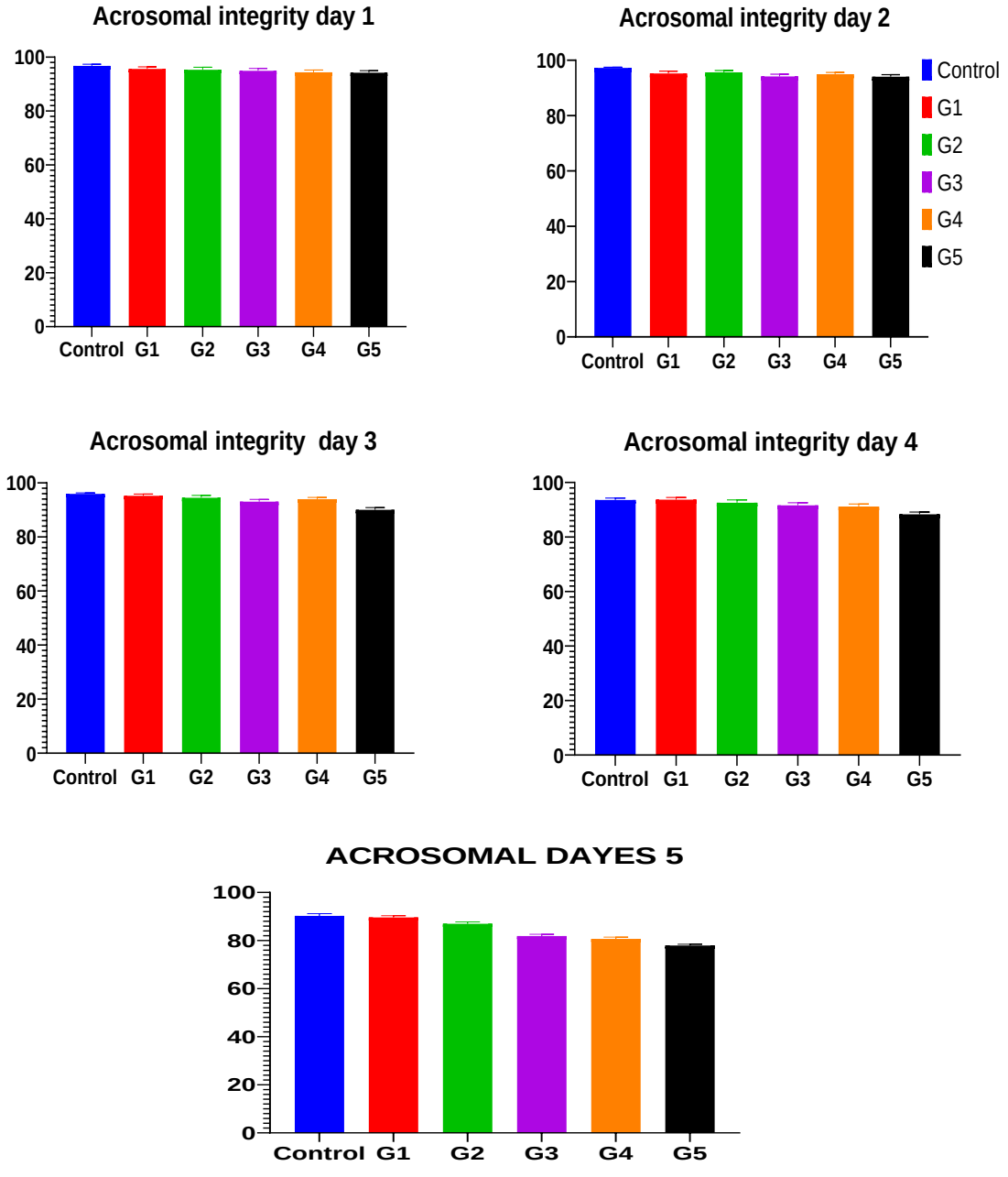

Figure 2. Different between means in 95% confidence intervals of Control and all treatment groups in the 5 days of chilled semen in terms acrosomal integrity.

- G1 = 0.25 mg/ml of dose of aqueous plant extract of *Prunus cerasus*
- G2 = 0.5 mg /ml of dose of aqueous plant extract of *Prunus cerasus*
- G3 = 1 mg /ml of dose of aqueous plant extract of *Prunus cerasus*
- G4 = 2 mg /ml of dose of aqueous plant extract of *Prunus cerasus*
- G5 = 4m g /ml of dose of aqueous plant extract of*Prunus cerasus*
- Cont = Positive control group; 0 mg (*Prunus cerasus*)

Discussion:

In the present study result increased in intact membranes of sperm in hypo-osmotic test which it tends to increase chances of pregnancy, this supports evidence from previous observations [29] which storage with gradually lowered extender pH impaired sperm motility and decreased defect sperm under hypo-osmotic condition test.

In the present study, higher concentration addition of aqueous plant extract of *Prunus cerasus*was found to decrease the quality of spermatozoa similar results were also obtained where higher concentrations of fructose [30], trehalose [31] and sucrose [32] were shown to be toxic for spermatozoa of ram and in the cattle bull as illustrated in the figure 2 since the HOST percentage of G3, G4 and G5 group decreased significantly lower the G1, G2 and control.

As desecrated previously the plasma membranes of sperm get stress during sperm preservation that might occur due to alter in the asymmetry of the phospholipid bilayer and changed the functional state of the sperm membranes due to chilling temperature and period of preservation [33].

Previous studies, that proved the addition of natural antioxidant as natural honey [34], garlic [34] and fish oil [36] was optimum to obtain improved semen quality during chilling and cryopreservation. Moreover, researcher found the addition of Melatonin [37] vitamins [38], cyclodextrins and cholesterol [39] improve the semen parameters including membrane integrity.

Generally, according to the present data it was observed a stability of the acrosomal integrity and absence of acrosome defect through two mechanisms can be proposed for protective mechanism one that it is incorporated into the spermatozoal membrane and increases the fluidity of membrane thus protecting it. Alternatively, it prevents the damaging activity of lipid peroxyl radicals by converting them to hydroperoxides [33]. The previous studies illustrated that accretion of natural anti-oxidant to the semen extender showed massive effects on the amounts of reactive oxygen species (ROS), lipid peroxidation and DNA injuries in frozen semen [40], while nonetheless that other studies noted decreased in parameters that extender contains no anti-oxidant [41]. It was known that sperm membranes of mammalian contain large quantities of poly-unsaturated fatty acids thus; sperm membranes are strongly sensitive to the oxidative [42]. Reactive oxygen species are increased post-thawed of semen compared to fresh or chilled semen [43]. Which generation the level of H_2O_2 followed chilling could be decreased by addition an anti-oxidant (catalase) to the semen extender, therefore, the anti-oxidants levels are not able to counteract the damaging effect of reactive oxygen species of post-thawed semen. It is well-known that the aquatic extract is highly anti-oxidant effect [20] as *Prunus cerasus*rich with poly-phenolic compounds [44]. Which phenolic compounds can share to anti-oxidant activity due to its properties to act as hydrogen donors lead to reducing agents and singlet oxygen quenchers [44, 45]. All evidence supported our results obvious in Figure (2). Also the season in Iraq had a significant negative effect upon animal testis specially the summer season which show the lowest reproductive activity [46].

On other hand, the result in the 5^{th}day illustrated that were negative effect of addition high concentration of aqueous plant extract of *Prunus cerasus* at G2, G3, G4 and G5 which did not improve semen quality was concomitant with previous studies where high concentration of disaccharide has been used to limit their dosages effect on sperm quality, On the other hand, [47] found that glucose (69.38mM) was better than sucrose and trehalose (33.04mM) supplementation but not fructose (69.38mM).

Conclusion

The black currant (*Prunus cerasus*) extracts had a remarkable effect on sperms membrane and acrosome integrity of the rams on 0.25 and 0.5 gm/ml during cooling process with good longevity effect, and it should be used in artificial insemination programs of sheep.

Conflict of interest

The authors declared that they have no potential conflict of interest with respect to the authorship and/or publication of this article.

References

1. Baghshahi, H.; Riasi, A.; Mahdavi, A.H. and Shirazi, A. Antioxidant effects of clove bud (*Syzygium aromaticum*) extract used with different extenders on ram spermatozoa during cryopreservation. Cryobiology, 2014, 69 (3): 482-487.
2. Kasimanickam, R.; Pelzer, K.D.; Kasimanickam, V.; Swecker, W.S. and Thatcher, C.D. Association of classical semen parameters, sperm DNA fragmentation index, lipid peroxidation and antioxi-dant enzymatic activity of semen in ram-lambs. Theriogenology,2006, 65: 1407–1421.
3. Peris, S.; Bilodeau, J.; Dufour, M. and Bailey, J. Impact of cryopreservation and reactive oxygen species on DNA integrity, lipid peroxidation and functional parameters in ram sperm. Mol Reprod Dev,2007, 74: 878–892.
4. Henkal, R. The impact of oxidants on sperm function. Andrologia, 2005, 37.6: 205-206.
5. Pandya, J.J. The leukocytic reaction of the human uterine cervix to spermatozoa. Fertil Steril,1985, 43: 417–421.
6. Aitken, R.J. Free radicals, lipid peroxidation and sperm function. Reprod Fertil Dev,1995, 7: 659–668.
7. Bailey, J.L.; Bilodeau, J.F. and Cormier, N. Semen cryopreservation in domestic animals: a damaging and capacitating phenomenon. J Androl,2000, 21: 1–7.
8. Baiee, F.H.; Wahid, H.; Rosnina, Y.; Ariff, O.M.; Yimer, N.; Salman, H. and Khumran, A.M. Hypo-Osmotic Swelling Test Modification to Enhance Cell Membrane Integrity Evaluation in Cryopreserved Bull Semen. Pertanika Journal of Tropical Agricultural Science,2017, 40 (2).
9. Aitken, R.J.; Clarkson, J.S. and Fishel, S. Generation of reactive oxygen species, lipid peroxidation and human sperm function. Biol Reprod,1989, 40: 183–197.
10. Moussa, M.; Martinet, V.; Trimeche, A.; Tainturier, D. and Anton, M. Low density lipoproteins extracted from hen egg yolk by an easy method: cryoprotective effect on frozen–thawed bull semen. Theriogenology, 2002, 57 (6): 1695-1706.
11. Amirat-Briand, L.; Bencharif, D.; Vera-Munoz, O.; Pineau, S.; Thorin, C.; Destrumelle, S.; Desherces, S.; Anton, M.; Jouan, M.; Shmitt, E. and Tainturier, D. In vivo fertility of bull semen following cryopreservation with an LDL (low density lipoprotein) extender: Preliminary results of artificial inseminations. Animal reproduction science, 2010, 122 (3): 282-287.
12. Sikka, S.C. Role of oxidative stress and antioxidants in andrology and assisted reproductive technology. J Androl, 2004, 25 (1): 5-18.
13. Almeida, J. and Ball, B.A. Effect of a-tocoferol and tocopherol succinate on lipid peroxidation in equine spermatozoa. Anim Reprod Sci, 2005, 87 (3-4): 321-337.
14. Boe-Hansen, G.B.; Morris, I.D.; Ersboll, A.K.; Greve, T. and Christensen, P. DNA integrity in sexed bull sperm assessed by neutral Comet assay and sperm chromatin structure assay. Theriogenology,2005, 63: 1789–1802.
15. Muin˜o, R.; Pen˜a, A.I.; Rodrı´guez, A.; Tamargo, C. and Hidalgo, C.O. Effects of cryopreservation on the motile sperm subpopulations in semen from Asturiana de los Vallesbulls. Theriogenology,2009, 72: 860–868.
16. Naijian, H.R.; Kohram, H.; Shahneh, A.Z.; Sharafi, M. and Numan, M. Bucak Effects of different concentrations of BHT on microscopic and oxidative parameters of Mahabadi goat semen following the freeze–thaw process. Cryobiology,2013, 66: 151–155.
17. Schmid-Lausigk, Yvonne; Aurich, Christine. Influences of a diet supplemented with linseed oil and antioxidants on quality of equine semen after cooling and cryopreservation during winter. Theriogenology, 2014, 81.7: 966-973.
18. Piccolella, S.; Fiorentino, A.; Pacifico,

S.; D'Abrosca, B.; Uzzo, P. and Monaco, P. Antioxidant properties of sour cherries (*prunus cerasus* l.): Role of color less phytochemicals from the methanolic extract of ripe fruits. J Agric Food Chem,2008, 56 (6): 1928–1935.

19. Kirakosyan, A.; Seymour, E.M.; Noon, K.R.; Llanes, D.E.U.; Kaufman, P.B. and Warber, S.L. Interactions of antioxidants isolated from tart cherry (*Prunus cerasus*) fruits. Food Chem, 122 (1): 78–83.

20. Damar, I. and Eksi, A. (2012). Antioxidant capacity and anthocyanin profile of sour cherry (*prunus cerasus* l.) juice. Food Chem,2010, 135 (4): 2910–2914.

21. Xu, Y.; Cai, F.; Yu, Z.; Zhang, L.; Li, X.; Yang, Y. and Liu, G. Optimisation of pressurised water extraction of polysaccharides from blackcurrant and its antioxidant activity. Food chemistry,,2016 194: 650-658.

22. Desjardins, J.; Tanabe, S.; Bergeron, C.; Gafner, S. and Grenier, D. Anthocyanin-rich black currant extract and cyanidin-3-O-glucoside have cytoprotective and anti-inflammatory properties. Journal of medicinal food, 2012, 15 (12): 1045-1050.

23. Al Alawi, R.; Alhamdani, M.S.S.; Hoheisel, J.D. and Baqi, Y. Biomedicine & Pharmacotherapy, 2020,121: 109522.

24. Velasco, V. and Williams, P. Improving meat quality through natural antioxidants. Chilean Journal of Agricultural Research, 2011, 71(2): 313-322.

25. Liang, J.; Li, J.K.; Zhao, W. and Liu, Y.L. Effect of pomegranate peel polyphenols on lipid peroxidation in vitro. Journal of Food Science and Biotechnology, 2012, 31: 159–165.

26. Manthey, J. and Grohmann, K. Phenols in Citrus Peel Byproducts. Concentrations of Hydroxycinnamates and Polymethoxylated Flavones in Citrus Peel Molasses. Journal of Agricultural and Food Chemistry,2001, 49(7): 3268-3273.

27. Bustani, G.S.A.A. Effect of *Phoenix dactylifera* extracts supplemented to tris on preserved sperm parameters and pregnancy rates in bull,2020, Thesis of Master Science in Physiology- College of Veterinary Medicine- University of Kufa.

28. Revell, S.G. and Mrode, R.A. An osmotic resistance test for bovine semen. Anim Reprod Sci,1994, 36: 77–86.

29. Nalley, W. M. M., &Arifiantini, R. I. The hypo-osmotic swelling test in fresh garut ram spermatozoa. Journal of Indonesian Tropical Animal Agriculture,2013, 38(4), 212-216.

30. Rasad, S. D., &Simanjuntak, L. C. The Effect of Fructose Addition in Semen Extender on Quality of Separation of Garut Ram Sperm in Several Storage Length. Animal Production,2009, 11(3).

31. Bucak, M. N., Keskin, N., Ili, P., Bodu, M., Akalın, P. P., Öztürk, A. E., ... &Dursun, ŞDecreasing glycerol content by co-supplementation of trehalose and taxifolin hydrate in ram semen extender: Microscopic, oxidative stress, and gene expression analyses. Cryobiology,0220, 96, 19-29.

32. El-Sheshtawy, R. I., Gamal, A., & El-Nattat, W. S. Effects of pomegranate juice in Tris-based extender on cattle semen quality after chilling and cryopreservation. Asian Pacific Journal of Reproduction,2016, 5(4), 335-339.

33. Bustani, G.S. and Baiee, F.H. Semen extenders: An evaluative overview of preservative mechanisms of semen and semen extenders. Veterinary World,2021, 14(5): 1220.op089

34. Banday, M. N., Lone, F. A., Rasool, F., Rather, H. A., & Rather, M. A. Does natural honey act as an alternative to antibiotics in the semen extender for cryopreservation of crossbred ram semen?. Iranian journal of veterinary research,2017, 18(4), 258.

35. Jerez-Ebensperger, R., Gil, L., Gonzalez, N., & De Blas, I. The combined use of honey, garlic (Allium Sativum L.) and skimmed milk as an extender for chilling sheep semen. CryoLetters, 101536(4),

243-251.

36. Masoudi, R., Sharafi, M., Shahneh, A. Z., Towhidi, A., Kohram, H., Zhandi, M., ... &Shahverdi, A. Effect of dietary fish oil supplementation on ram semen freeze ability and fertility using soybean lecithin–and egg yolk–based extenders. Theriogenology, 2016,86(6), 1583-1588.

37. Ashrafi, I., Kohram, H., Naijian, H., Bahreini, M., &Poorhamdollah, M. Protective effect of melatonin on sperm motility parameters on liquid storage of ram semen at 5C. African Journal of Biotechnology,2011, 10(34), 6670-6674.

38. Allai, L.; Benmoula, A.; da Silva, M.M.; Nasser, B. and Al Amiri, B. Supplementation of ram semen extender to improve seminal quality and fertility rate. Animal Reproduction Science, 2018, 192: 6-17.

39. Benhenia, K., Lamara, A., Fatmi, S., &Iguer-Ouada, M. Effect of cyclodextrins, cholesterol and vitamin E and their complexation on cryopreserved epididymal ram semen. Small Ruminant Research, 2016, 141, 29-35.

40. Paudel, K. P., Kumar, S., Meur, S. K., & Kumaresan, A. Ascorbic Acid, Catalase and Chlorpromazine Reduce Cryopreservation-induced Damages to Crossbred Bull Spermatozoa a. Reproduction in domestic animals, 2010, 45(2), 256-262.

41. Arslan, S., Kadayifçilar, S., &Samur, G. The potential role of dietary antioxidant capacity in preventing age-related macular degeneration. Journal of the American college of nutrition, (2019). 38(5), 424-432.

42. Khumran, A. M., Yimer, N., Rosnina, Y., Ariff, M. O., Wahid, H., Kaka, A., ... &Sarsaifi, K.Butylated hydroxytoluene can reduce oxidative stress and improve quality of frozen–thawed bull semen processed in lecithin and egg yolk based extenders. Animal reproduction science, 2015, 163, 128-134.

43. Gürler, H., Malama, E., Heppelmann, M., Calisici, O., Leiding, C., Kastelic, J. P., &Bollwein, H. Effects of cryopreservation on sperm viability, synthesis of reactive oxygen species, and DNA damage of bovine sperm. Theriogenology, 2016, 86(2), 562-571.

44. Kimble, R., Keane, K. M., Lodge, J. K., Cheung, W., Haskell-Ramsay, C. F., &Howatson, G. Polyphenol-rich tart cherries (Prunus Cerasus, cv Montmorency) improve sustained attention, feelings of alertness and mental fatigue and influence the plasma metabolome in middle-aged adults: a randomised, placebo-controlled trial. British Journal of Nutrition, 2022, 1-31.

45. Negri, G., Teixeira, E. W., Florêncio Alves, M. L. T. M., Moreti, A. C. d. C. C., Otsuk, I. P., Borguini, R. G., &Salatino, A. Hydroxycinnamic acid amide derivatives, phenolic compounds and antioxidant activities of extracts of pollen samples from Southeast Brazil. Journal of agricultural and food chemistry, 2011, 59(10), 5516-5522.

46. Naoman, U. D. Effect of Ivermectin on semen characteristics of Iraqi Awassi ram. *Al*-Anbar J. Vet. Sci,2012, 5(2), 129-133.

47. Naing, S. W., Wahid, H., Azam, K. M., Rosnina, Y., Zuki, A. B., Kazhal, S., ... & San, M. M. Effect of sugars on characteristics of Boer goat semen after cryopreservation. Animal reproduction science,2010, 122(1-2), 23-28.