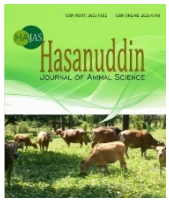

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Effect of Egg Yolk Powder as An Alternative Extender on The Quality of Bali Bull Spermatozoa

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ABSTRACT

Semen extender is closely related to the success of artificial insemination to achieve maximum results. Extender aims to increase the volume of semen, provide food substances as a source of energy for spermatozoa provide buffers to maintain pH, osmotic pressure, and electrolyte balance, and prevent the possibility of germ growth. This study aims to determine the effect of extender on the quality of Bali bull spermatozoa through an alternative approach, one of which uses Tris-Egg Yolk powder extender, which is efficient and effective. The study was designed randomized, with three treatments and five replications. The treatment combinations were P0 (Tris-Fresh Egg Yolk 20%), P1 (Tris Oven Egg Yolk 20%), and P2 (Tris-Commercial Egg Yolk 20%). The results of analysis of variance showed that in the parameters of progressive motility, viability, abnormality and intact plasma membrane, treatment of P0 (Tris-Fresh Egg Yolk 20%) tended to be similar as treatment P1 (Tris-Oven Egg Yolk 20%) ($P>0.05$), but in P2 (Tris-Commercial Egg Yolk 20%) was significantly lower ($P<0.05$) than P0 and P1. It can be concluded that oven egg yolk powder mixed with tris solution can maintain sperm quality after dilution.
Keywords: Oven egg yolk powder, commercial egg yolk powder, buffer solution

INTRODUCTION

Tris-based egg yolk extender is a type of diluent often used for bovine semen [1]. Tris-based extender has good buffering properties. TRIS + egg yolk effectively preserved the functional parameters of cooling semen for up to 48 hours [2]. The glucose content is an energy source, and the egg yolk content is a source of amino acids for spermatozoa [3]. The yolk components responsible for the cryoprotectant effect are lecithin, phospholipids, and lipoprotein fractions [4]. Besides being quite economical, the materials to make a Tris-based egg yolk extender are also readily available [5]. However, the use of Tris based-egg yolk extender is also a problem when the buffer solution has been mixed with fresh egg yolk at the time of use [6]; fresh egg yolk as a diluent is not durable [7], and fresh egg yolk cannot be stored for more than 24 hours because it can become toxic to spermatozoa in the semen [8]. The condition of

spermatozoa that are easily damaged during treatment [9] and the low quality of semen obtained after thawing require diluents to maintain quality during storage [10]. Semen diluents increase volume, provide nutrients, protect sperm cells during processing, provide buffers, and protect sperm from bacteria [11] [12] [13] [14] [15]. The various components of semen fused in a perfect medium are needed so that the semen can maintain its natural properties and extend its life from the initial process to the freezing and post-thawing process [16]. Tris-based Egg yolk diluent is an energy source, osmotic pressure buffer, and extracellular cryoprotectant that can coat the plasma membrane to protect sperm from cold shock and maintain plasma membrane stability during semen storage [17] [18]. A common problem in the supply of diluents is that commercial diluents are expensive and difficult to obtain.

Fresh egg yolks have been processed into egg yolk powder using freeze drying. Freeze drying to dry matter levels that exceeded 96% stabilized the proteins and lipoproteins by immobilizing the matrix in the vitrified state [19]. Tris Freeze dry-egg yolk powder can maintain the quality of spermatozoa up to 50% longer motility percentage than fresh egg yolk tris [20]. However, using the freeze-drying method takes a long time and is relatively expensive.

One of the efforts made to make egg yolk powder through the action process using an oven is the atomization pasteurization process. Using an oven can maintain the quality of the drying results for a long time and protect against microbial growth of low water activity. When freeze-drying occurs below 30 °C, the proteins preserve all or most of their initial biological activity in the dry state [21]. Egg yolk powder has a longer shelf life; egg yolk flour has qualities, protein stability, and activates the level of antibody resistance [22] [23]. Therefore, this study was conducted to make, prove, and compare egg yolk powder (oven) and egg yolk powder (oven) with fresh egg yolk and commercial egg yolk powder on the quality of Bali cattle spermatozoa.

MATERIALS AND METHODS

Materials of the Study

The material used in the study was one Bali bull of 4 years of age. The tools used included an artificial vagina, scale tubes, stationery (pens, labels), test tubes, measuring tubes, measuring cups, Petri dishes, syringes, latex gloves, mini tubes, water bath, drying and sterilization oven, CASA (Computer-assisted sperm analysis) computer, SDM 6 photometer, hot plate, cuvette, centrifuge, oven, microscope, electric scales, macro and micropipettes, micropipette tips, tally counters, tweezers, Petri dishes, object glass, and cover glass. Supporting materials included sterile aqua bidestilata, commercial egg yolk powder, fresh egg yolk (free-range chicken eggs), tris hydroxyl methylamine, citric acid, fructose, penicillin, streptomycin, glycerol, eosin 2%, alcohol 70%, vaseline, parafilm, tissue, pH scale paper 0.25, filter paper, aluminum foil.

Procedures of the Study

The preparation of egg yolk and its effect on the quality of Bali cattle spermatozoa consisted of several procedures: preparation of egg yolk, preparation of semen dilution, semen collection, semen evaluation, and frozen semen preparation.

Preparation of Egg Yolk Powder

The egg yolk used was the fresh egg yolk of native chickens with the criteria that the egg was intact, oval, thick, not cracked or dirty, and without red spots on the eggshell. When the shell is broken, the yolk is still intact and light yellow [20]. The yolk was separated from the egg white by placing it on a filter paper, and the egg white is discarded. The vitelline membrane was also separated from the yolk using a syringe and put into a petri dish. The yolks were baked at 45-50°C for days. The temperature used for pasteurization was below the boiling point temperature using an oven not to cause denaturation of the protein in the egg yolk because the protein in the egg yolk is denatured at 65°C [24]. Egg yolks were sieved/grinded using a blender to become egg yolk powder.

Preparation of Semen Diluent

The preparation of tris buffer solution was done by putting 3.03 grams of tris (trishydroxyl methylamine), 1.25 grams of crystal fructose, 1.78 grams of citric acid into a measuring flask, then adding 100 ml of aquabidest, and then homogenized for 15 minutes. Furthermore, the solution was heated to boiling and left in a room with a temperature of 37°C [9]. Next, tris buffer solution was substituted into the treatments: fresh egg yolk (P0), egg yolk powder (oven) (P1), and commercial egg yolk powder (P2) each by 20%, then homogenized using a vortex. The solution was centrifuged for 30 minutes at 3000 rpm, and each diluent was added with penicillin and streptomycin antibiotics at a dose of 1000 IU/mL and 1 mg/mL, respectively [20].

Semen Collection and Semen Evaluation

Semen collection was carried out using an artificial vagina with a frequency of collection twice a week. Semen that has been collected was immediately taken to the laboratory for evaluation. Semen evaluation was conducted both macroscopically and microscopically. Semen that met the macroscopic evaluation requirements was then evaluated microscopically, including motility, viability, abnormality, and intact plasma membrane.

Frozen Semen Preparation

Frozen semen was made when fresh semen tested macroscopically met the requirements and continues with microscopic tests. After macroscopic and microscopic evaluation, the following process was to dilute, after which equilibration was carried out with the addition of glycerol, then continued filling and sealing, namely the process of inserting semen that has been mixed with diluent into the straw. After filling and sealing, the straw was arranged on the straw rack and put into the refrigerator for 4 hours at a temperature of 0-5°C, then pre-freezing was done by placing the straw ±10 cm above the surface of liquid nitrogen for 15 minutes with a temperature of -140°C. The following process was freezing by putting the straw into a goblet and immersing it in liquid nitrogen at a temperature of -196°C. To determine the success of freezing semen, the frozen semen was thawing by inserting the straw into the thawing device for 45 seconds; then microscopic observations were made.

Data Analysis

Data obtained from examining spermatozoa quality were statistically analyzed using analysis of variance (ANOVA). Treatments that affect the variables evaluated were then compared using the Least Significant Difference Test. All calculation were performed using the SPSS Program for Window.

RESULTS AND DISCUSSION

Table 1 shows the results of the study of the effect of egg yolk powder on the quality of Bali bull spermatozoa.

Table 1. Quality of Bali Bull Spermatozoa after Dilution and Freezing (Post Thawing)

Parameter	Stage	P0	P1	P2
Progressive Motility (%) (±SD)	PD	71.18±7.18[a]	75.61±3.76[a]	25.81±5.81[b]
	PT	33.90±5.84	34.45±6.19	
Viability (%) (±SD)	PD	86.20±3.96[a]	86.17±3.24[a]	23.00±10.14[b]
	PT	32.10±14.64	27.40±11.60	
Abnormality (%) (±SD)	PD	14.78±3.21[a]	13.80±2.08[a]	20.10±5.24[b]
	PT	37.40±5.45	36.80±15.79	
Plasma Membrane Intact (%) (±SD)	PD	86.59±5.71[a]	85.20±4.64[a]	8.10±2.88[b]
	PT	44.20±6.82	41.70±14.61	

Description: PD (Post-dilution), PT (Post-thawing), P0 (Tris-Fresh Egg Yolk 20%), P1 (Tris-Oven Egg Yolk 20%), P2 (Tris-Commercial Egg Yolk 20%).
[a,b,c]Different superscripts indicate significant different ($P<0.05$).

Progressive Motility

Table 1 shows the percentage of progressive motility after dilution in fresh egg yolk tris diluent (P0) with an average of 71.18±7.18%, lower than and not significantly different from oven egg yolk tris-powder (P1) 75.61±3.76% ($P>0.05$). Still, both are higher than commercial egg yolk tris-powder (P2), 25.81±5.81% ($P<0.05$). At the post-thawing stage, the percentage of progressive motility in tris-fresh egg yolk diluent (P0) averaged 33.90±5.84%, lower than and not significantly different ($P>0.05$) from tris-oven egg yolk powder (P1) 34.45±6.19% ($P>0.05$).

The percentage of progressive motility after dilution in tris-powder of oven egg yolk (P1) tended to be higher than that of fresh egg yolk (P0). This results is in line with the opinion of Ariantie et al. [20] that the percentage of progressive motility of diluents using egg yolk powder is higher than that of fresh egg yolk diluents. The high percentage of spermatozoa motility using oven yolk powder is thought to be due to the ability of egg yolk lecithin to protect the plasma membrane in liquid semen, which acts as an extracellular cryoprotectant. It has been suggested

that the replacement of phospholipids in sperm membranes, with a reduction in freezing point, is the cause of the lecithin protective mechanism. Additionally, it might create a shield around the sperm to prevent ice crystals from forming and, as a result, shield the sperm membranes from physical harm [25]. Egg yolk as a semen diluent functions as an energy source, osmotic pressure buffer, and extracellular cryoprotectant that can cover the plasma membrane to protect sperm from cold stress and maintain plasma membrane stability during semen preservation [25].

Viability

The percentage of viability of spermatozoa after dilution in fresh tris-yolk diluent (P1) could survive with an average of 86.20±3.96% and was not significantly different from oven egg yolk tris-powder (P1) 86.17±3.24% (P>0.05). Still, both are higher than commercial egg yolk tris-powder (P2), 23.00±10.14% (P<0.05). At the post-thawing stage, the percentage of viability in fresh tris-yolk diluent (P0) averaged 32.10±14.64%, higher and not significantly different (P>0.05) from tris-oven egg yolk powder (P1) 27.40±11.60%.

The percentage of spermatozoa viability after dilution tended to be the same in the fresh egg yolk treatment with oven yolk powder. However, the commercial egg yolk powder experienced a drastic decrease. It is suspected that some substances in egg yolk have inhibited cell respiration and, therefore, caused a reduction in the number of motile cells and their viability [26]. The decrease in parameters using egg yolk powder could be due to the increased viscosity of the medium; the high temperature reached during the pasteurization process denatured the egg yolk proteins, inducing them to have a gel-like consistency in the medium after reconstitution, causing high diluent viscosity [27].

Abnormality

The percentage of spermatozoa abnormalities after dilution in tris-fresh egg yolk diluent was higher with an average of 14.78±3.21% and not significantly different from tris-oven egg yolk 13.80±2.08% (P>0.05), but both are lower than tris-commercial egg yolk meal (P2) 20.10±5.24% (P<0.05). At the post-thawing stage, the percentage of abnormalities in fresh egg yolk tris-powder diluent (P0) averaged 37.40±5.45%, higher and not significantly different from oven egg yolk tris-powder (P1) 36.80±15.79% (P>0.05).

The abnormality of spermatozoa after dilution using oven egg yolk powder and fresh egg yolk is still within the normal range. Spermatozoa abnormalities exceeding 20% can reduce fertility [28]. The diluent processing and cooling factors influence the increase in the percentage of semen abnormality [29].

Plasma Membrane Intact

The percentage of intact plasma membrane (MPU) of spermatozoa after dilution in fresh egg yolk tris diluent (P0) with an average of 86.59±5.71% was higher and not significantly different from oven egg yolk tris-powder (P1) 85.20±4.64% (P>0.05), higher than commercial egg yolk tris-powder (P2) 8.10±2.88% (P<0.05). At the post-thawing stage, the percentage of intact plasma membranes in fresh egg yolk tris-powder diluent (P0) averaged 44.20±6.82%, higher and not significantly different from oven egg yolk tris-powder (P1) 41.70±14.61% (P>0.05).

Intact plasma membranes in diluents using oven egg yolk powder can maintain the quality of spermatozoa after dilution; this is because the components of egg yolk that play a role in protecting spermatozoa during dilution include phospholipids, low-density lipoprotein (LDL), and cholesterol is believed to be able to associate with plasma membranes to form a protective layer on the surface and can replace plasma membrane phospholipids and maintain plasma membrane integrity [30]. Plasma membrane functionality and integration are essential for evaluating sperm quality because an intact plasma membrane is a critical limit in sperm cell survival [31].

CONCLUSION

Oven egg yolk powder can be an alternative to fresh egg yolk and potentially maintain sperm quality after dilution. Commercial egg yolk powder cannot maintain the quality of spermatozoa after dilution, so the freezing stage cannot be carried out using commercial egg yolk powder diluent.

REFERENCES

[1] D. Das, M. Mahbubul, M.Y. Ali, M.S. Kobir, M.A. Kabir, S.M.J. Hossain, and A.S. Apu, "Morphometric Features, Seminal Profile and Diluters Effect on Post-Thaw Semen Quality of Munshiganj Cattle in Bangladesh", Heliyon, Vol. 9, no. 12:, p. e21967, 2023. https://doi.org/10.1016/j.heliyon.2023.e21967

[2] R.P. Santos, S.S.J. Moreira, L.G.P. Bezerra, Y.C.S. Cavalcante, A.G. Pereira, T.M. Matos, M.F. Oliveira, and A.R. Silva, "Comparison Between Commercial Media and TRIS-Egg Yolk Extender in The Refrigerated Storage of Collared Peccary Semen at 17 °C", Anim. Reprod. Sci., Vol. 265, p. 107478, 2024. DOI: 10.1016/j.anireprosci.2024.107478

[3] B. Rostami, D. Ebrahimi, H. Sadeghipanah, R. Masoumi, and M.H. Shahir, "Effects of Supplementation of Tris-Egg Yolk Extender with Different Sugars and Antioxidants on Freezability of Ram Semen. Cryobiology, Vol. 92, pp. 62–66, 2020. DOI: 10.1016/j.cryobiol.2019.10.198

[4] R.I. El-Sheshtawy, G.A. El-Sisy, and W.S. El-Nattat, "Effects of Pomegranate Juice in Tris-Based Extender on Cattle Semen Quality After Chilling and Cryopreservation", Asian Pacific J. Reprod., Vol. 5, no. 4, pp.335–339, 2016. https://doi.org/10.1016/j.apjr.2016.06.001

[5] S.S. Layek, T.K. Mohanty, A. Kumaresan, and J.E. Parks, "Cryopreservation of Bull Semen: Evolution from Egg Yolk Based to Soybean Based Extenders", Anim. Reprod. Sci., Vol. 172, pp. 1–9, 2016. DOI: 10.1016/j.anireprosci.2016.04.013

[6] A.A. Tarig, H. Wahid, Y. Rosnina, N. Yimer, Y.M. Goh, F.H. Baiee, A.M. Khumran, H. Salman, and M. Ebrahimi, "Effect of Different Concentrations of Egg Yolk and Virgin Coconut Oil in Tris-Based Extenders on Chilled and Frozen-Thawed Bull Semen", Anim. Reprod. Sci., Vol, 182, pp. 21–27, 2017. DOI: 10.1016/j.anireprosci.2017.03.024

[7] B. Koç, M. Koç, and E.F. Kaymak, "Spray Drying of Egg Components, In: Spray Drying for the Food Industry", Elsevier, p. 223–241, 2024.

[8] J. Zhang, and Y. Ma, "Formation Mechanism and Inhibition Methods of Frozen Egg Yolk Gelation: A review", Trends in Food Science & Technology, Vol. 148, p. 104491, 2024. https://doi.org/10.1016/j.tifs.2024.104491

[9] A. Noblanc, C. Damon-Soubeyrand, B. Karrich, J. Henry-Berger, R. Cadet, F. Saez, R. Guiton, L. Janny, H. Pons-Rejraji, J.G. Alvarez, J.R. Drevet, and A. Kocer, DNA Oxidative Damage in Mammalian Spermatozoa: Where and Why is the Male Nucleus Affected?", Free Radic. Biol. Med., Vol. 65, pp. 719–723, 2013. DOI: 10.1016/j.freeradbiomed.2013.07.044

[10] A. Salman, E. Fernández-Alegre, R. Francisco-Vázquez, R. Gómez-Martín, A. Fernández-Fernández, H. Areán-Dablanca, J.C. Dominguez, J.R. Gonzalez-Montana, J.N. Caamano, and F.Martinez-Pastoer, "Extension of the Equilibration Period up to 24 h Maintains the Post-Thawing Quality of Holstein Bull Semen Frozen with OPTIXcell®. Anim. Reprod. Sci., Vol. 250, p. 107209, 2023. https://doi.org/10.1016/j.anireprosci.2023.107209

[11] E.M. Murphy, C. Murphy, C. O'Meara, G. Dunne, B. Eivers, P. Lonergan, and S. Fair, "A Comparison of Semen Diluents on the In Vitro and In Vivo Fertility of Liquid Bull Semen", J. Dairy Sci., Vol. 100, no. 2, pp.1541–54, 2017. DOI: 10.3168/jds.2016-11646

[12] D.H. Yang, N.T. Standley, and Z.Z. Xu, "Application of Liquid Semen Technology Under the Seasonal Dairy Production System in New Zealand", Anim. Reprod. Sci., Vol. 194, pp. 2–10, 2018. https://doi.org/10.1016/j.anireprosci.2018.01.004

[13] M. Wiebke, B. Hensel, E. Nitsche-Melkus, M. Jung, and M. Schulze, "Cooled Storage of Semen from Livestock Animals (Part I): Boar, Bull, and Stallion", Anim. Reprod. Sci., Vol. 246, p. 106822, 2022. https://doi.org/10.1016/j.anireprosci.2021.106822

[14] A. Saberivand, R. Mehrabi, S.Z. Peighambarzadeh, M. Saberivand, M. Shirafkan, T. Haghgouei, F. Sarvarzadeh, P. Safaei, M. Saki, K.J. Siahpoush, and T. Alizadeh, "The Cryoprotective Effect of Guar Gum Co-Supplemented with Ethylene Glycol and Glycerol in Simmental Bull Semen", Anim. Reprod. Sci., Vol. 259, p. 107362, 2023. https://doi.org/10.1016/j.anireprosci.2023.107362

[15] Y. Li, S. Qin, W. Cui, F. Zhao, M. He, and Z. Jiang, "Progress on the Roles of Zinc in Sperm Cryopreservation", Theriogenology, Vol. 211, pp. 134–41, 2023. https://doi.org/10.1016/j.theriogenology.2023.08.014

[16] B. Singh, K.P. Singh, M. Hoque, and S. Mondal, "Biotechnology in Livestock Reproduction. In: Emerging Issues in Climate Smart Livestock Production", Elsevier, p. 371–413, 2022. https://doi.org/10.1016/B978-0-12-822265-2.00013-2

[17] P. Singh, M.K. Bedi, S. Singhal, A.K. Singh, A. Kumar, and M. Honparkhe, "Effect of Graphene Oxide as Cryoprotectant on Post-Thaw Sperm Functional and Kinetic Parameters of Cross Bred (HF X Sahiwal) and Murrah Buffalo (Bubalis bubalus) Bulls", Cryobiology, Vol. 106, pp. 102–112, 2022. DOI: 10.1016/j.cryobiol.2022.03.002

[18] D.M. Shokry, M.R. Badr, A.A.M. Sakr, A.M. Elmesiry, M.M. Assy, Z. Rawash, and M.A.A. Eldaim, "Enhancement Potential of Moringa Oleifera Leaves Extract on Buffalo Bull Cryopreserved Semen Quality and Fertilization Capacity", Anim. Reprod. Sci., Vol. 262, pp. 107414, 2024. https://doi.org/10.1016/j.anireprosci.2024.107414

[19] S. Thierau, H. Keil, I. Seuss-Baum, and O. Hensel, "Effects of Heat Treatment and Freeze-Drying on Theprotein and Lipoprotein Structures of Egg Yolk as Determined Using Differential Scanning Calorimetry (DSC)", Agric. Commun., Vol. 2, no. 3, pp.27–37, 2024.

[20] O.S. Ariantie, A. Amrozi, T.L. Yusuf, N.T. Rochman, and B. Purwantara, "The Production of Freeze-Dried Egg Yolk Powder and Its Effect on the Quality of Garut Ram Liquid Semen," Jurnal Kedokteran Hewan, Vol. 15, no. 2, pp. 37–46, 2021. DOI: https://doi.org/10.21157/j.ked.hewan.v15i2.19669

[21] S. Alcay, E. Gokce, M.B. Toker, N.T. Onder, B. Ustuner, E. Uzabacı, Z. Gul, and S. Cavus, "Freeze-Dried Egg Yolk Based Extenders Containing Various Antioxidants Improve Post-Thawing Quality and Incubation Resilience of Goat Spermatozoa", Cryobiology, Vol. 72, no. 3, pp. 269–73, 2016. https://doi.org/10.1016/j.cryobiol.2016.03.007

[22] Z. Wang, X. Duan, G. Ren, J. Guo, J. Ji, Y. Xu, W. Cao, L. Li, M. Zhao, Y. Ang, X. Jin, B. Bhandari, L. Zhu, and Y. Zhang, "Improving Effect of Disaccharides and Maltodextrin on Preparation of Egg Yolk Powder by Microwave-Assisted Freeze-Drying: Functional Properties, Structural Properties, and Retention Rate of Active IgY", Food Chemistry, Vol. 404, p. 134626, 2023. https://doi.org/10.1016/j.foodchem.2022.134626

[23] M. Primacella, N.C. Acevedo, and T. Wang, "Effect of Freezing and Food Additives on the Rheological Properties of Egg Yolk", Food Hydrocolloids, Vol. ;98, pp. 105241, 2020. https://doi.org/10.1016/j.foodhyd.2019.105241

[24] D. Bermudez-Aguirre, and B.A. Niemira, "A Review on Egg Pasteurization and Disinfection: Traditional and Novel Processing Technologies", Compr. Rev. Food Sci. Food Saf., Vol. 22, no. 2, pp. 756–784, 2023. https://doi.org/10.1111/1541-4337.13088

[25] M. Mehdipour, H.D. Kia, G. Moghaddam, and H. Hamishehkar, Effect of Egg Yolk Plasma and Soybean Lecithin on Rooster Frozen-Thawed Sperm Quality and Fertility", Theriogenology, Vol. 116, pp. 89–94, 2018. DOI: 10.1016/j.theriogenology.2018.05.013

[26] A. Kumar, A. Kumar, V. Sachan, J.K. Agrawal, and A. Saxena, "Effect of Egg Yolk Powder on Seminal Attributes During Cryopreservation of Hariana Bull Semen", Veterinary Clinical Science, Vol. 7, no. 4, pp. 50–52, 2029.

[27] J. Zhang, M. Zhang, W. Liang, Z. Geng, and X. Chen, "Green Tea Powder Supplementation Increased Viscosity and Decreased Lysozyme Activity of Egg White During Storage of Eggs From Huainan Partridge Chicken", Italian Journal of Animal Science, Vol. 19, no. 1, pp. 586–592, 2020. https://doi.org/10.1080/1828051X.2020.1769512

[28] J.F. Taylor, R.D. Schnabel, and P. Sutovsky, "Identification of Genomic Variants Causing Sperm Abnormalities and Reduced Male Fertility", Anim. Reprod. Sci., Vol. 194, pp. 57–62, 2018. DOI: 10.1016/j.anireprosci.2018.02.007

[29] M. Ansari, B. Rakha, and S. Akhter, "Cryopreservation of Nili-Ravi Buffalo (*Bubalus bubalis*) Semen in AndroMed® Extender In Vitro and In Vivo Evaluation", "Reprod. Domest. Anim., Vol. 52, no. 6, pp. 992–997, 2017. DOI: 10.1111/rda.13008

[30] F. Sicchieri, A.B. Silva, V.P. Santana, M.A.C. Vasconcelos, R.A. Ferriani, A.A. Vireque, and R.M.D. Reis, "Phosphatidylcholine and L-acetyl-carnitine-based Freezing Medium can Replace Egg Yolk and Preserves Human Sperm Function", Transl. Androl. Urol., Vol. 10, no. 1., pp. 397–407, 2021. DOI: 10.21037/tau-20-1004

[31] A. Amaral, B. Lourenço, M. Marques, and J. Ramalho-Santos, "Mitochondria Functionality and Sperm Quality", Reproduction, Vol. 146, no. 5, pp. R163–74, 2013. DOI: 10.1530/REP-13-0178