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EFFECT OF EGG YOLK TYPE ON THE QUALITY OF POST-THAWING GOAT SEMEN AFTER RESIQUIMOD-BASED SEXING

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ABSTRACT

Egg yolk is an important component of semen extenders for maintaining spermatozoa quality during cryopreservation. This study aimed to evaluate the effects of different egg yolk types in a Tris extender on the abnormality and recovery rate of post-thawing goat spermatozoa following resiquimod-based sexing. A completely randomized design with four treatments commercial chicken (P1), indigenous chicken (P2), quail (P3), and duck egg yolk (P4) and five replications was used. Fresh Sapera goat semen was processed using a resiquimod-based swim-up method and cryopreserved in a Tris extender containing the respective egg yolk types. Spermatozoa abnormality and recovery rate were evaluated in the upper and lower layers after thawing. Data were analyzed using ANOVA followed by Duncan's multiple range test. Abnormality ranged from 0.60–1.40% in the upper layer and 0.60 – 1.10% in the lower layer, with no significant differences among treatments. In contrast, egg yolk type significantly affected recovery rate ($P < 0.05$). The highest recovery rate was obtained with commercial chicken egg yolk (42.56%), followed by indigenous chicken (40.37%), duck (39.97%), and quail egg yolk (31.97%). Commercial chicken egg yolk was therefore the most effective in maintaining post-thaw spermatozoa recovery following resiquimod-based sexing.

Keywords : *egg yolk, cryopreservation, spermatozoa, recovery rate, resiquimod.*

Introduction

Goat (*Capra Hircus*) is very beneficial as an animal protein source either from meat or from milk production. Among dairy goat breeds, the Sapera goat, a crossbred of Saanen and Etawah Crossbred goats, has considerable potential for milk production and good adaptability to tropical environments (Christi *et al*., 2024). The availability of superior breeding female still become limit so the productivity and genetic improvement are not optimum. Coupled with the limitation in the availability of superior males and irregular quality of cryopreserved semen, making the pregnancy rates are low when Artificial Insemination is used (Hermawati & Nuraeni, 2024). With that it will be highly need for the improvement in the reproductive efficiency through reproductive technologies like sperm sexing and semen cryopreservation.

Sperm sexing technology has been widely utilized to control the offspring sex ratio so as to achieve more effective breeding programs. A sperm population consists of spermatozoa carrying X and Y chromosomes in equal numbers. Separation of X and Y bearing spermatozoa needs specific techniques depending on their physical and biological properties (Afriani *et al*., 2011). The swim-up technique has been frequently employed owing to its simplicity and it can efficiently separate highly motile and morphologically normal spermatozoa (Fleming *et al*., 2025). Recently, the use of resiquimod (R848) has been reported, which can inhibit the motility of X bearing spermatozoa specifically through decreasing ATP (Achmad *et al*., 2024; Pauli *et al*., 2021). However, sexing procedure can generate physiological stress and decrease the quality of spermatozoa in terms of motility and membrane integrity.

The cryopreservation is one of the most accepted methods for long term conservation of spermatozoa at sub-zero temperatures. The repeated freezing and thawing process can cause considerable dam-

ages to the sperm cell, such as membrane damage, oxidative stress and structural abnormality (Narwade *et al*., 2017; Oktanella *et al*., 2024). Generation of reactive oxygen species during the cryopreservation can bring about lipid peroxidation of the sperm membrane, which can reduce sperm viability and fertilization ability (Bustani & Baiee, 2021). The successfulness of the cryopreservation is highly dependent on the content of the extender, which protect the spermatozoa from physical and chemical damages.

Egg yolk is the one of the most widely utilized ingredients in semen extenders because it contains large amounts of phospholipids, lipoproteins and cholesterol that help to protect the sperm membrane against cold shock damage (Saieed *et al*., 2018). It is thought that the low density lipoproteins in the egg yolk bind to the plasma membrane of sperm and reduce damage during freezing (Sen *et al*., 2015). Different types of egg yolk have shown varying levels of protective properties on sperm quality The four egg yolk types were selected based on their differences in nutritional and biochemical composition, particularly protein, lipid, and low-density lipoprotein (LDL) contents, which may influence their ability to protect spermatozoa during cryopreservation. Indigenous chicken egg yolk contains approximately 15.7–16.6% protein, 31.8–35.5% fat, 48.2% water, 1.1% ash, and 0.2–1% carbohydrate, with an LDL concentration of 0.047 mmol/L (Ramadhani *et al*., 2018). These components, particularly proteins and lipids, contribute to the stabilization of the sperm plasma membrane and protection against cryo-induced membrane damage. Differences in lipid and phospholipid composition among avian species may consequently result in different cryoprotective capacities (Kulaksız *et al*., 2010; Swelum *et al*., 2022). Therefore, commercial chicken, indigenous chicken, quail, and duck egg yolks were selected to represent different biochemical characteristics and to compare their potential ability to maintain sperm quality during cryopreservation.

Parameters such as abnormality and recovery rate are widely used to evaluate the quality of post-thawed spermatozoa. Abnormal spermatozoa are due to morphological abnormality in spermatogenesis, and the handling of spermatozoa and during cryopreservation particularly secondary abnormali-ties due to thermal and osmotic stress (Kusumawati, 2015). High rate of abnormal spermatozoa might lead to a poor fertilizing capacity of the spermatozoa, and normally less than 20% abnormal spermatozoa rate are considered to be acceptable for Artificial Insemination (Zakaria *et al*., 2020). Recovery rate is a parameters to explain the ability of post-thawed spermatozoa to retain the mobility compared to the initial, whereby a high recovery rate is the indication of its resistance to the freezing process and high possibility in fertilization (Solihati *et al*., 2018). The relationship between different type of egg yolk used in sexing of semen then subsequently cryopreservation in Sapera goats using resiquimod-based methods have not been widely studied. Differences in the lipid content in egg yolk might lead to the capability of egg yolk as a cryoprotectant to be different, therefore resulting in different post-thawed abnormalities and recovery rates.

Materials and Methods

Dilluent and Sexing Media

Tris-citrate medium was made up by dissolving 30mM tris(hydroxymethyl)aminomethane, 9.5mM citric acid monohydrate and 2.8mM glucose in to distilled water and was gently mixed until all dissolved. This was then pH tested. To fill each tube with 3mls medium we first took 27L out and then added R848 to a final volume of 0.9M.

Another Tris-citrate medium was then loaded with Egg Yolk to a final volume of 50ml (10% of the final volume). The mixture was then blended and the penicillin (3l) and streptomycin (10l) were added and blended. Then 0.6 ml medium was replaced with 0.6 ml glycerol and blended for 30 min until all had combined.

Sperm Collection and Sexing

The Sapera buck used in this study was approximately 2–3 years old, with an estimated body weight of 30 kg, based on the condition of its teeth, which had developed three pairs (six teeth). The buck was maintained at the Faculty of Animal Husbandry, Universitas Padjadjaran, Ciparanje, Jatinangor, and was fed with King grass (*Pennisetum purpupoides*) at 3 kg in the morning, corn silage at 3 kg in the afternoon, and concentrate at 150 g in both the morning and afternoon before the forage and corn silage were provided; drinking water was supplied *ad libitum*. Semen

collection was conducted once a week, every Tuesday at 07:00 WIB, using an artificial vagina, with the buck allowed to perform three false mounts before ejaculation was collected on the fourth mount.

Fresh semen was obtained from Sapera goats and analyzed before processing. Only semen that fulfilled minimum criteria for quality (motility 70%, abnormality 20%) was utilized. The semen was then processed for sexing of the sperm using a resiquimod (R848)-based swim-up procedure. Semen was diluted in Tris-citrate-glucose extender and incubated with resiquimod at final concentration of 0.1 mM to suppress the motility of X-bearing spermatozoa. Following the incubation, the samples were held vertically to facilitate the migration of motile spermatozoa into the upper fraction. After 30 minutes of incubation at 37°C, the upper (Y-enriched) and lower (X-enriched) fractions were then collected independently.

Sperm Diluted and Freezing

Each sperm fraction was washed by centrifugation to remove residual resiquimod and then diluted in Tris extender supplemented with egg yolk according to the treatment combinations: P1AR, P2AR, and P3AR for commercial chicken egg yolk at 5%, 10%, and 20%, respectively; P1AK, P2AK, and P3AK for indigenous chicken egg yolk at 5%, 10%, and 20%; P1P, P2P, and P3P for quail egg yolk at 5%, 10%, and 20%; and P1I, P2I, and P3I for duck egg yolk at 5%, 10%, and 20%. The four egg yolk sources were selected because they have different biochemical characteristics, particularly in their lipid, protein, lipoprotein, and cholesterol composition, which may result in different cryoprotective capacities. Therefore, the egg yolk sources were not assumed to have identical compositions; rather, these compositional differences were intentionally evaluated as part of the treatment factor to determine their effects on sperm quality after cryopreservation. The diluted semen was equilibrated at 4°C for 4 hours, transferred into 0.25-mL mini straws, and cryopreserved at −196°C by immersion in liquid nitrogen. The semen was thawed at 37°C for 30 seconds. The experiment employed a nested completely randomized design, consisting of four egg yolk sources with three concentration levels (5%, 10%, and 20%) and five replications, resulting in 12 treatment combinations.

Observed Variables

Abnormality

Morphologically abnormal sperm was evaluated to quantify the percentage of abnormal sperm following sexing and freezing thawing process, which is the main issue because physical and osmotic shock induced by cryopreservation process and subsequent treatments can induce secondary abnormality in sperm, which affects fertilizability of sperm (Yendraliza & Rahman, 2023). Lower percentage of abnormality in sperm shows that the structures of sperm were better protected during the process. The formula used for evaluate sperm morphology and calculates percentage of abnormality is:

$$Sperm\ Abnormality\ (\%) = \frac{Total\ of\ Abnormal\ Sperm}{Sperm\ Sample\ that\ Was\ Observed} \times 100\%$$

Recovery Rate

The percentage of recovery rate, as determined from the calculation: shows how well motile sperm can be preserved when compared with pre-sexing value, i.e., the sensitivity of sperms against the stress of sexing process, freezing, and thawing. A higher value of recovery rate is related to higher success of sperm function and semen cryopreservation and artificial insemination (Hoa *et al*., 2022). The recovery rate is calculated with the formula as measurement for the treatment efficiency:

$$Sperm\ Recovery\ Rate\ (\%) = \frac{Post\ Thawed\ Sperm\ Motil}{Fresh\ Sperm\ Motil} \times 100\%$$

Analysis Statistic

Sperm abnormality and sperm recovery data were examined in relation to changes in post-thawed sperm quality in response to variations in egg yolk type. Duncan (1955) Specify that data were subjected to a completely randomized design. Significance was assessed by ANOVA and means of significant treatment differences ($P < 0.05$) were ranked using the Duncan's Multiple Range Test (DMRT) with the formula:

$$LSD_{Duncan} = r_{\alpha} \times \sqrt{\frac{MSE}{n}}$$

Notes :
r_α : Duncan's significant range value
MSE : Mean square error from ANOVA
n : number of replication

Results and Discussion

Fresh Sperm Assessment

Semen was analyzed before usage for its quality standards. Macro and micro characteristics were determined before usage immediately after semen collection. Macro characteristics included volume, color, odor, consistency, pH while micro characteristics included mass movement, sperm concentration, motility, viability and abnormalities. The fresh Sapera goat semen characteristics are shown in Table 1.

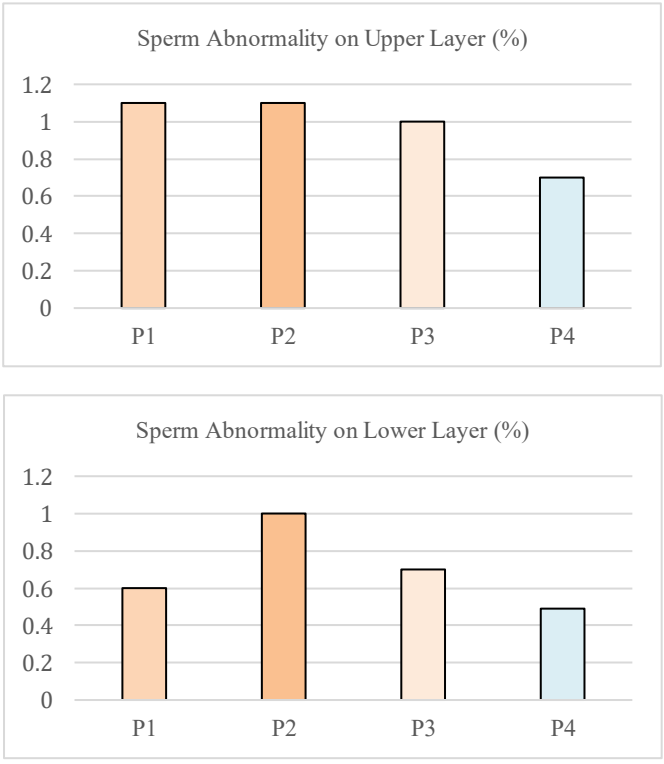
Table 1. Fresh Semen Examination Results of Sapera Goats

Parameter	Average
Volume (mL)	1.19 ± 0.32
pH	6.15 ± 0.32
Mass Movement	+++
Sperm Concentration Total (million/ml)	404.8
Motility (%)	92.50 ± 3.17
Viability (%)	91.85 ± 4.72
Abnormality (%)	0.75 ± 0.34

The results of the initial semen quality assessment showed that the fresh Sapera goat semen met the minimum quality criteria for further processing, with high sperm motility and viability, adequate sperm concentration, and low sperm abnormality. Semen were pre-selected with >70% motile which is an essential characteristic in artificial insemination, the characteristics of semen generally show normally in color creamy, odor characteristic, consistency viscid, with near neutral pH (Susilawati, 2011). The volume of semen was slightly higher as compared to Hidayati *et al.* (2018) while pH was still in the normal physiological limit. The quality evaluation on the mass movement (+++), sperm concentration, motility (>90%) and viability (>90%) were higher compared to the earlier report. While abnormality (<1%) was considerably lower and better, perhaps this was influenced by difference in individuals and handling process.

Sperm Abnormality

The above fraction represents sperm that have migrated to the upper fraction during swim-up and is mainly enriched with Y-bearing sperm which also has a greater motility and better physiological characters compare to the sperm in the lower fraction (Setiawan *et al*., 2024). Post thaw sperm abnormality values of upper and lower fraction under each treatment are shown in Ilustration 1 below,



Ilustration 1. Sperm Abnormality on Upper and Lower Layer

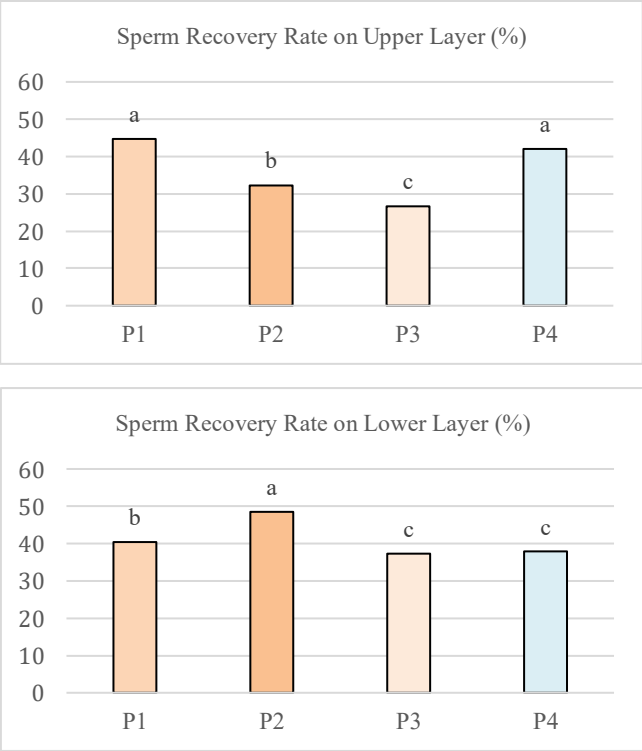
Notes :
P1 = Commercial Chicken Egg Yolk,
P2 = Indigenous Chicken Egg Yolk,
P3 = Quail Egg Yolk,
P4 = Duck Egg Yolk

The sperm abnormality in the upper layer ranged from 0.50 – 2.50%, with mean values of 1.10 ± 0.37% for commercial chicken, 1.10 ± 0.73% for indigenous chicken, 1.00 ± 0.55% for quail, and 0.70 ± 0.24% for duck egg yolk. In the lower layer, abnormalities ranged from 0.50 – 2.00%, with means of 0.60 ± 0.20%, 1.00 ± 0.55%, 0.70 ± 0.24%, and 0.90 ± 0.49%, respectively. ANOVA showed no significant differences among egg yolk types in either layer ($P \geq 0.05$), indicating that sperm abnormality was not in-

fluenced by the sexing layer or treatments. This suggests that the treatments maintained sperm morphology without causing structural damage. These findings are consistent with Siswanto *et al*. (2024), who reported no significant differences in sperm abnormalities among different egg yolk types, and Zakaria *et al*. (2020), who stated that sperm abnormality is more strongly influenced by intrinsic factors than external treatments. Similarly, Oktanella *et al*. (2024) reported that post-thaw sperm quality is mainly affected by oxidative stress and freezing damage.

Sperm Recovery Rate

The upper fraction contains more motile spermatozoa which did survive through the sexing process and hence are of better quality and more stress resistant. Recovery rates of post-thaw sperm in the top fraction for each treatment are given in Ilustration 2.



Ilustration 2. Sperm Recovery Rate on Upper and Lower Layer

Notes :
P1 = Commercial Chicken Egg Yolk,
P2 = Indigenous Chicken Egg Yolk,
P3 = Quail Egg Yolk,
P4 = Duck Egg Yolk

From the Ilustration above, the recovery rates after thawing are different in each treatment. High recovery rate of sperm can be generally seen in commercial chicken, indigenous chicken and duck, and rather stable result can be seen in quail egg yolk. ANOVA result revealed that the type of egg yolk had a significant influence on the recovery rate, ($P \leq 0.05$), so Duncan's test was employed to obtain the best type of egg yolk and significant difference between treatments. Recovery rate of spermatozoa in the upper layer differed significantly among egg yolk types, where P1 showed the highest value, followed by P4, P2, and P3 ($P < 0.05$). The superior performance of chicken egg yolk (P1) can be attributed to its adequate cholesterol content and the presence of low-density lipoprotein (LDL), which effectively protects the sperm plasma membrane by stabilizing the lipid bilayer and preventing membrane damage during post-sexing processing (Langerová *et al*., 2026; Ramadhani *et al*., 2018). Compared with quail egg yolk (P3), which contains higher cholesterol levels, chicken egg yolk provides sufficient membrane protection without exposing spermatozoa to excessive lipid accumulation that may interfere with cellular function (Singh *et al*., 2020). In addition, the balanced composition of proteins, lipids, and LDL in chicken egg yolk supports the maintenance of membrane integrity and facilitates optimal recovery of sperm cells after thawing (Ngongo *et al*., 2024). These findings indicate that the biochemical characteristics of the egg yolk source, particularly its lipid and lipoprotein profile, play an important role in determining the recovery rate of sexed frozen-thawed goat spermatozoa.

The post thaw sperm recovery rate in lower layer of sexed goat semen using resiquimod shows varied within each different kind of egg yolk and different concentration. Maximum value shows in some mixture such as indigenous chicken (48.51 ± 1.74) and commercial chicken (40.41 ± 1.37), minimum value shows mostly in Duck Egg Yolk (37.91 ± 1.44) and Quail Egg Yolk (37.30 ± 3.77). Duncan's multiple range test was applied to analyze difference between groups ($P \leq 0.05$) at ANOVA shows egg yolk kind significantly effected on the recovery rate and difference of concentration.

Treatment P2 produced the highest recovery rate in the lower layer, followed by P1, while P4 and

P3 showed the lowest values and were not significantly different (P ≥ 0.05). The superior performance of the indigenous chicken egg yolk treatment (P2) and commercial chicken egg yolk (P1) may be related to its relatively high protein and lipoprotein content, which provides effective membrane protection for X-bearing spermatozoa in the lower layer. These spermatozoa experience deliberate metabolic suppression through TLR7/8 activation, resulting in reduced ATP production and increased susceptibility to cryoinjury during freezing and thawing (Ren *et al*., 2021). The cholesterol and LDL fractions present in indigenous chicken egg yolk help stabilize membrane fluidity, prevent phospholipid loss, and maintain cellular integrity under cold stress conditions (Langerová *et al*., 2026; Wulansari & Ducha, 2019). In contrast, quail egg yolk (P3) and duck egg yolk (P4), despite having higher lipid and cholesterol contents, may create a denser extracellular environment that limits cellular recovery after thawing, particularly in metabolically compromised spermatozoa. Therefore, the results suggest that the balanced lipoprotein composition of indigenous chicken egg yolk provides more protective environment for the recovery of X-bearing spermatozoa than the lipid-rich profiles of quail and duck egg yolks.

Conclusion

There was no significant change in abnormality of post thaw sperm on type of egg yolk for both layers, but highly significant on recovery rate on egg yolk concentration and on type of egg yolk on recovery rate. The most effective treatments were commercial chicken in general and in layer formation, commercial chicken egg yolk on upper layer and indigenous chicken egg yolk on lower layer.

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