The role of low-density lipoprotein diluents to spermatozoa motility on limousine liquid semen during storage at 5°C

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**Abstract.** The length of the spermatozoa storage process can experience a decrease in quality, so it is not feasible to be disseminated. This study aimed to reduce the declining rate of quality of bovine spermatozoa during storage at 5°C after the addition of egg yolk and Low-Density Lipoprotein (LDL) on diluent medium. The study design used a Completely Randomized Design with a factorial pattern (4 x 5) with replications 3 times, where factor A is an LDL thinner (A1: 20% whole egg yolk (control); A2: 7%; A3: A %; A4: 9%) factor B is the length of storage (B1: first day; B2: second day; B3: third day; B4: fourth day; B4: fifth day). The results showed that the spermatozoa motility of Limousine bovine with low-density lipoprotein diluents had high motility compared to spermatozoa diluted with egg yolk diluents during storage at 5°C, the treatment of low-density lipoprotein can reduce the rate of spermatozoa decreased during storage compared to 20% egg yolk. The best LDL concentration is 8% during storage. The conclusion of this study shows that the administration of LDL could minimize the rate of deterioration in the quality of Limousine bovine spermatozoa during storage compared to egg yolk.

1. **Introduction**
Optimizing the use of artificial insemination (IB) is to strive for each parent bovine to be able to produce children every year with the sex as desired, namely male or female. The male bull is the choice of breeders to be maintained considering the ability to grow and develop body cells faster than females so that it is very appropriate if male calves are cultivated for fattening purposes; while female livestock is maintained because of their ability to produce children and milk [1].

Efforts to produce livestock population are by processing spermatozoa to extend the ability of spermatozoa to transform capacitation through reproductive technology such as Artificial Insemination. However, the spermatozoa can experience a decrease in quality during the treatment process (dilution, storage in the form of liquid semen at a specific temperature until the IB process is carried out). Previous studies reported that storage of spermatozoa at cold temperatures of 5°C decreased the motility and percentage of life carried out on the acquisition of 0.2; in bovine, sexing yields 56% and rate of living 67.9% [1].

The use of diluents is important in packing semen in the form of straw or frozen ampoules. It is expected that semen quality and viability of spermatozoa during the freezing process can be maintained. The use of diluents is intended to guarantee the physical and chemical needs of spermatozoa so that the quality of spermatozoa can be kept incapacitation ability primarily. The function of other diluents is to increase the volume of semen so that each ejaculate can be used to
inseminate more artificial female bovine. Another report states that the diluent function is: (1) increasing the volume of semen; (2) protect spermatozoa from cold shock; (3) providing food substances as an energy source for spermatozoa; (4) provides a buffer to maintain pH, osmotic pressure and electrolyte balance [2]. The use of egg yolk center tris aminomethane is more able to keep the quality of spermatozoa compared to tris aminomethane without egg yolk [3].

One ingredient that has been used is chicken egg yolk, which has been a standard component of thinners for freezing spermatozoa from different livestock types over the past 60 years. It has been shown that egg yolk can help in resistance to cold stress and improvement in the fertility ability of spermatozoa [4]; [5]; [6]. Also, the action of protecting yolk is identified widely in the presence of low-density lipoprotein (LDL) [7]; [8]; [9].

The presence of the substance in the yolk in protecting the liquid spermatozoa until it has freezing has reinforced the use of LDL extracted from the yolk in the diluent, compared to whole egg yolk. [8] reported remarkable results about motility and some characteristics of spermatozoa movement when replacing egg yolk with 8% LDL in frozen spermatozoa diluents. LDL can protect/maintain male spermatozoa and improve fertility ability after freezing until re-melting [6]. 8% LDL supplementation gave the highest spermatozoa motility (55.8%) [7]. Other reports that diluents containing LDL can increase/enhance spermatozoa motility, acrosome reaction and plasma membrane integrity, DNA Boer integrity after freezing until re-melting and can be used as a frozen protective medium [10]. A possible component of LDF (low-density fraction) - lipoprotein from diluted egg yolk that interacts with seminal plasma protein (BSP protein) and this may represent a common mechanism of protection for spermatozoa with egg yolk [4] and [5].

With this research in evaluating the influence of LDL on motility, the life percentage of cow spermatozoa with the addition of LDL at various concentrations of cattle spermatozoa thinners stored at a temperature of 5°C gives better results Compared to the addition of 20% whole egg yolks. It is a crucial case in processing liquid semen until freezing storage so that it can support in animal reproduction technology, especially artificial insemination.

2. Methods

2.1. Research Materials

The equipment used is a set of artificial vaginal devices, test tubes, measuring cups, volume pipettes, drop pipettes, centrifuges, spoits, thermometers, pumpkin Erlenmeyer, water bath, stirrer, propipet, aluminum foil, spoit, filter, cotton, label paper, microscope, glass, and deck glass object, tube rack, cool box, thermos, tissue, soft cloth, and coarse cloth.

The material used is semen is accommodated using the artificial vaginal method (VB), 20% egg yolks, thinners with a composition consisting of 2.42 g Tris; 1.48 g of citric acid; 1.00 g fructose; 6.6 ml glycerol; Gentamicin 25 mg; 50,000 IU; penicillin, different LDL concentrations (7–9% w/v) for 100 ml of sterile non-pyrogenic water, 70% alcohol, eosin, 0.9% NaCl, aquades.

2.2. Research Method

The study used a completely randomized design (CRD) with a factorial model (4 x 5) with 3 replications, where factor A is a low-density lipoprotein thinner (A1: 20 % whole egg yolk (control); A2: 7%; A3: A%; A4: 9%) factor B is the length of storage (B1: first day; B2: second day; B3: third day; B4: days ahead; B4 : fifth day) The procedure for conducting research consists of several stages, namely:

2.2.1. Extraction of Low-density Lipoprotein (LDL)

LDL is an egg yolk extraction. It is in line with the describing method by [8]. Egg yolk is diluted twice or thrice with isotonic salt solution (0.17M NaCl) and stirred using stirring magnetic equipment for one hour before centrifugation at 10,000 x g for 45 minutes at 4°C. The supernatant is separated from the sediment (granule). The plasma is centrifuged again for Avoiding the presence of pullets. The
supernatant is then mixed with 40% ammonium sulfate to accelerate livetins. After 1 hour stirring at 4°C, the mixture was centrifuged at 10,000 x g for 45 minutes to separate the supernatant from the sediment. The sediment is removed, and the supernatant is dialysis (purified) for about 12 hours in distilled water (distillates) to remove ammonium sulfate. After complete removal of ammonium sulfate, the solution was centrifuged again at 10,000 x g for 45 minutes at 4°C, and the residual/remaining LDL fatty residue was collected [8].

2.2.2. Making Diluent Medium
The diluent solution used in this study consisted of 2.42 g Tris, 1.48 g citric acid, 1.00 g fructose, 6.6 ml glycerol, 25 mg gentamicin, 50,000 IU penicillin for 100 ml of sterile non-pyrogenic water. One by one, the ingredients are put into the distilled water while stirring using a magnetic stirrer for 20 minutes. After the diluent is available, then each different LDL concentration (7-9%, w/v) and 20% egg yolk is added as a control group dilution solution. Then diluent solutions are available with various concentrations of LDL treatment, and 20% of egg yolk is stored at 5°C.

2.2.3. Processing of Spermatozoa
At first, semen collection. Semen from ejaculation is obtained from bovines using an artificial vagina. Sperm used in this study for the preservation process was carried out by assessing volume, spermatozoa concentration, and percentage of motile spermatozoa and having requirements with motility > 70% and normal sperm morphology > 85%. Then the ejaculated semen used is divided equally between different diluent solutions.

Then, packaging and storage of spermatozoa at 5°C for five days. After the spermatozoa are available, then the resultant spermatozoa are cooled from 37°C to 5°C and then placed in a refrigerator for five days at 5°C.

At last, motility Assessment. Semen is dropped on the glass object and covered with a glass deck and then observed under a microscope with a magnification of 40 x 10. The assessment of mass movements is set with a score of 0; 1; 1+; 2; 2+; 3; 3+ (11). This assessment was carried out in two stages, namely on fresh semen, spermatozoa after adding LDL which was stored for five days at 5°C.

2.3. Research Parameters
The parameters measured in this study are (1) Macroscopic assessment that includes the volume of semen, pH, color, and consistency. This assessment is done once, which is on fresh semen and has not received any treatment, (2) Microscopic assessment that includes evaluation of motility carried out twice. The first assessment of fresh semen included mass motility, individual motility, concentration, and percentage of life — the second assessment of spermatozoa that received Low-density lipoprotein (LDL) treatment with different levels.

2.4. Data Analysis
All experiments were carried out at least four replications. For each group (control and experimental group, each LDL concentration for each spermatozoan that was stored for five days at 5°C. Data obtained were analyzed by analysis of variance with treatments that showed a significant effect, then tested using test The Smallest Significant Difference (LSD) with the ANOVA procedure.

3. The Results of Research

3.1. Characteristics of Limousin Bovine Fresh Semen
Based on the research conducted on the fresh semen of Limousin bovine, the results shown in Table 1. The assessment of fresh sperm in Limousin bovine is carried out in two ways, namely macroscopically and microscopically. It is by the opinion expressed by [12] that the assessment of semen quality is carried out macroscopically (pH, color, consistency) and microscopically (motility, the percentage of life, concentration and morphology of spermatozoa).
Table 1. Characteristics of fresh semen limousin bovine

| Parameters                  | Average     | Researchers*                          |
|-----------------------------|-------------|---------------------------------------|
| Makroskopis:                |             |                                       |
| • Volume (cc)               | 7 ± 0,82    | [1] was 6-10 ml                       |
| • Colour                    | Beige       | [1] was Beige                         |
| • Concentration             | Medium      | [1] was medium to thick                |
| • pH                        | 5,8         | [1] was 7                             |
| Mikroskopis:                |             |                                       |
| • Motility                  | 70 ± 0      | [1] was >70% (ranging from 70 - 90%)   |
| • Mass motility             | 3+          | [1][13] was 2+ up to 3+               |
| • Concentration (Milion/ml) | 1200        | [1] was ranges from 800-1160 million/ml|
| • Percentage of life (%)    | 80,2±0,31   | [14] was normal (spermatozoa are 80-95%)|

3.2. Motility of Spermatozoa Limousine Bovine

3.2.1. The interaction between low-density lipoprotein (LDL) with storage time at 5°C to spermatozoa motility of Limousine.

The results of the variance analysis showed that the interaction of low-density lipoprotein (LDL) with storage time at 5°C had a very significant effect (P <0.01) on the motility of spermatozoa. The reaction of low-density lipoprotein (LDL) with storage time at 5°C can be seen in Figure 1.

![Figure 1. Interaction of low-density lipoprotein (LDL) with storage time at 5°C to spermatozoa motility of Limousine.](image)

During the at 5°C storage, the lowest decrease in motility occurred in the addition of low-density lipoprotein (LDL) diluents with a concentration of 8%, while the highest reduction in motility happened in the treatment of 20% egg yolk as treatment control. Although the rate of decline in motility during the preservation stage for all procedures (20% egg yolk; 7%, 8%, and 9% LDL) showed the same pattern of decline, the concentration of 8% LDL was more capable and competent in minimizing the rate of decrease in sperm motility.

The inability to reduce the rate of decrease in motility in the 7 and 9% LDL concentrations, it is assumed that the level of 7% LDL is quite low in playing a role as a protection for spermatozoa, as well as a concentration of 9% too high in LDL so that LDL is collected at high levels during storage of spermatozoa. As [8] who experimented with the use of LDL in frozen spermatozoa that an increase in LDL concentrations above 10% in diluents led to a decrease in spermatozoa performance after
freezing and thawing again. This observation can be related to diluent osmotic pressure. Furthermore, [8] that osmotic pressure decreases when the concentration of LDL fraction in thinners increases.

3.2.2. The effect of low-density lipoprotein (LDL) diluents on motility spermatozoa.
Low-density lipoprotein (LDL) diluents have a very significant effect (P < 0.01) on spermatozoa motility. The level of influence of the use of low-density lipoprotein (LDL) concentrations (7%, 8%, 9%, and controls) showed significant differences (P < 0.05).

The motility of spermatozoa in the four treatment concentrations decreased, but with the use of diluents containing low-density lipoprotein (LDL) (7, 8 and 9%) more able to reduce the decrease in sperm motility (41.58; 46.23 and 43, respectively). 31%) compared to the treatment of 20% egg yolk in this case as control, which only has a 38.21% motility. Thus, the treatment of 20% of egg yolk experienced the most significant decrease in spermatozoa motility during storage, which was 32.7%, followed by 7% LDL (26.7%), 9% LDL (23.7%) and 8% LDL (18.6%). It proves that Low-density lipoprotein (LDL) derived from egg yolk plays an essential role in the process of preserving spermatozoa, especially spermatozoa, which will experience freezing to be protected from cold stress. It is in line with the opinion [15]; [16] that the source of lipids (egg yolk in general) or lipoprotein (milk in general) or a combination of both is needed to protect spermatozoa from cold stress. Furthermore, [15] states that severe anxiety is a process in which the spermatozoa lipid layer changes, due to rapid temperature changes.

The low motility of spermatozoa in diluents containing 20% of egg yolk is thought to be due to the presence of elements in the yolk which do not play a role in maintaining the motility of the spermatozoa. As [17] suggests that egg yolks have other constituents and do not directly participate in spermatozoa. Furthermore, [18], which separates egg yolks in two lipoproteins, namely LDL and high-density lipoprotein (HDL contained in granules). If LDL provides better protection for Boer spermatozoa than egg yolk, HDL and when compared with egg yolk, HDL decreases spermatozoa motility significantly. It can be hypothesized that HDL in egg yolk can have a detrimental effect on its protective characteristics.

3.2.3. The fact of low-density lipoprotein (LDL) is on the motility of spermatozoa
The storage time for five days at 5°C had a very significant linear and quadratic effect (P<0.01). It means that the difference in decreased spermatozoa motility during storage is substantial, as seen in the results of the smallest real difference test indicating that during the preservation day there were significant differences (P < 0.05) on spermatozoa motility.

Generally, The motility of spermatozoa during five days of storage at 5°C decreases every day. The first day was 56.79%, which subsequently continued to decline on the second day to the fifth day (41.552% respectively; 38.275%; 32.726%, 27.506%). It shows a relatively high rate of decline from the first day to the second day. The percentage of decrease in sperm motility during storage is most likely due to metabolic processes. So that the amount of energy used to move causes decreased motility every day of storage and the possibility of physiological changes during the storage of spermatozoa in the diluent medium. As such [19] that during the process of processing the spermatozoa, it will affect the metabolic process which causes the supply of energy (from diluents) to decrease more and more which causes the motility of the spermatozoa decrease even longer. The same is stated by [20] that the presence of metabolic processes in aerobic conditions (fructolysis) continuously will cause accumulation of lactic acid and result in a decrease in pH which occurs in decreased spermatozoa motility.

During storage of spermatozoa, cold stress occurs which causes the integrity of the spermatozoa membrane to decrease and even damage occurs, pH decreases as the storage day lasts at 5°C, so the motility decreases and ends in death in spermatozoa. It is consistent with that stated by [1] that more specifically, the integrity of the membrane can be affected by sudden cooling and thawing if the spermatozoa are frozen, osmotic changes and changes in pH.
The related research with the utilization of LDL can help the process of processing spermatozoa better. Thus, it is expected that the processing of spermatozoa of the beef limousine can use the diluents containing LDL, either in the spermatozoa stored at a temperature of 5°C in the form of liquid or storage in the way of frozen spermatozoa. The objectives can eventually produce competent quality spermatozoa during the implementation of reproductive technology in the form of artificial insemination that can support the development program of the number of livestock populations in an area of livestock production.

4. Conclusions
This research concluded that the motility of Limousin bovine spermatozoa which was stored for five days at 5°C has better motility in the treatment of addition of low-density lipoprotein (LDL) diluents. LDL can prevent a decrease in motility during storage days compared to 20 % egg yolk. The best LDL concentration is a concentration of 8% during preservation days on the motility of spermatozoa compared to other treatments during storage

5. References
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