The quality of Etawah crossbreed sperm after sexing with different combination of Bovine Serum Albumin concentrations

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Abstract. This research aimed to determine the quality of Etawah crossbreed sperm after sexing with different combination of Bovine Serum Albumin (BSA) concentrations. The parameter of this research were motility, viability, intact plasma membrane (IPM) and intact acrosome cap (IAC) (%). The Completely randomized design (CRD) was applied in this experiment involving 4 treatments of the combination of BSA concentrations (T1= 3%:6%, T2 = 4%:8%, T3= 5%:10%, T4= 6%:12% at upper and lower fraction) and each treatments was repeated 5 times at post chilled and post sexing. Data were analyzed by using analysis of variance (ANOVA), followed by Duncan Multiple Range Test. The result showed that the combination of BSA concentrations affected (P<0.05) motility, viability, IPM and IAC. The highest value of sperm quality in upper and lower fraction was obtained from the combination BSA of 5%:10% (motility 78.60 ± 2.61% and 73.80 ± 2.49%; viability 282 ± 14.30 and 252.8 ± 12.97 hours; IPM-value 79.60 ± 1.98% and 74.70 ± 1.82% and IAC 81.00 ± 1.46% and 76.90 ± 1.29%). Based on the results it can be concluded that the quality of Etawah crossbreed sperm after sexing is affected by the combination of BSA concentrations.

Keywords: Bovine Serum Albumin, sperm quality, sexing, Etawah crossbreed

1. Introduction
The production of sperm sexing can be achieved by inseminating a female with sperm that has been separated through sexing sperm application of Artificial Insemination (AI). This technology will be more efficient if the offspring can be determined by sex according to the purpose of the production, for example in dairy producing goats will expect more females. Several separation methods have been applied, such as the albumin column method with separation using Bovine Serum Albumine (BSA). Bovine Serum Albumin is a cow serum albumin protein that can protect sperm efficiently and can act as an antioxidant that maintain the quality of spermatozoa by protecting the plasma membrane from damage due to free radicals [1]. The successful of sperm sexing technology is the accuracy of combination BSA concentrations in order to maintain optimal sperm quality during the sexing process. The quality of semen sexing that needs to be considered is fresh semen quality before sexing, intact plasma membrane (IPM) and intact acrosome cap (IAC). The evaluation of sperm quality needs to be initially done before the sperm sexing process, because the AI program will be not success if sperm fertility is lower. The integrity of plasma membrane is needed by the sperm, because damaging of the plasma membrane will affect the metabolic processes associated with sperm motility and the fertility [2]. Furthermore [2] stated that the integrity of the acrosome cap also plays an important role in the
fertilization process. Sperm must have an acrosome cap in intacted condition to be able to perform the acrosome reaction function at the right time, release enzymes, and facilitate sperm in penetrating the pellucid zone.

The sperm sexing technique using BSA column should not manipulate sperm excessively, because BSA can protect the plasma membrane and also maintain the integrity of DNA of sperm [3]. The use of BSA is also carried out to protect sperm during storage at a temperature of 5°C, besides that BSA is also protecting the sperm from lipid peroxidase [4]. The content of amino acids or plasma proteins in semen that has been diluted with BSA is expected to substitute the decrease of various substances concentration contained in the semen plasma due to the dilution process, so as to maintain the stability of the sperm cell membrane [5]. Sperm which is capable of fertilizing are motile sperm, viable, have normal morphology, and have an intact chromatin [6]. Chromatin is a protoplasmic material, consisting of protein and DNA. Chromatins strands will shorten and thicken to form chromosomes. This chromosome determines the sex of livestock. Sperm X contains more chromatins in the sperm nucleus contained in its head, so the size of the head of sperm X is larger. Y sperm head size is smaller, lighter and shorter than X sperm, so Y sperm are faster and more mobile and contain less genetic material and DNA compared to sperm X [5]. Several studies on sexing sperm using the BSA column have been conducted. Sperm sexing methods was carrying a female (X) or male (Y) chromosome with the BSA column resulting in the success of the calf by 76% to 89% [7] and goat (sperm X: 74.05%) [8].

2. Materials and methods

2.1. Materials
The object of this study was semen from 1.5-year-old Etawah male goat, which was fed 4 kilograms of grass daily and 300 grams of concentrate. Semen was collected two times a week, on Monday and Thursday at 07.00 am. The semen was then evaluated for macroscopic and microscopic fresh semen quality and in the Laboratory of Animal Reproduction and Artificial Insemination, The Faculty of Animal Husbandry, Universitas Padjadjaran. Bovine Serum Albumine (BSA) (Merck®, Albumin Fraction V) was used as a sperm sexing media.

2.2. Methods
Intact plasma membrane (IPM) of sperm was evaluated using the Hypoosmotic Swelling Test (HOS-test) method. Tail of the sperm that was still intact would look like curved or bent [9]. Observations were made by counting the number of sperms that had an intact plasma membrane of 200 observed sperm. Observation of intact acrosome cap (IAC) was carried out using a NaCl Physiological solution containing 1% formalin. An intact acrosome cap was marked with the tip of a thick black sperm head [10]. Observations were made by counting the number of sperm that have an intact acrosome cap from 200 observed sperms.

2.3. Treatments
Semen of Etawah goats was divided into 4 treatment groups with 5 replications. The treatment consisted of:

- **T1** = BSA concentration 3% in upper fraction and 6% in lower fraction.
- **T2** = BSA concentration 4% in upper fraction and 8% in lower fraction.
- **T3** = BSA concentration 5% in upper fraction and 10% in lower fraction.
- **T4** = BSA concentration 6% in upper fraction and 12% in lower fraction.

Parameters of this study were fresh semen quality, motility, viability, intact plasma membrane (IPM) and intact acrosome cap (IAC).

2.4. Statistical analysis
A completely randomized design (CRD) was used in this experiment involving 4 treatments and 5 replications, so that 20 experimental units were obtained. The collected data were analyzed using
Analysis of Variance, and differences between treatments were tested using Duncan's multiple range test.

3. Results and discussion

3.1. Fresh semen quality of Etawah goat

Fresh semen was initially evaluated using macroscopic and microscopic evaluations for its feasibility before sperm sexing process. The results of macroscopic and microscopic evaluation of fresh semen can be seen in Table 1 below.

| Parameter                        | Collecting |       |       |       |       |
|----------------------------------|------------|-------|-------|-------|-------|
|                                  | 1          | 2     | 3     | 4     | 5     |
| Macroscopic                      |            |       |       |       |       |
| Volume (ml)                      | 0.9        | 1.2   | 1.0   | 0.9   | 1.0   |
| Colour                           | cream      | cream | cream | cream | cream |
| Consistency                      | condensed  | condensed | condensed | condensed |
| pH                               | 6.8        | 6.8   | 6.8   | 6.5   | 6.5   |
| Odor                             | specific   | specific | specific | specific |
| Microscopic                      |            |       |       |       |       |
| Mass-movement                    | +++        | +++   | +++   | +++   | +++   |
| Total Sperm Concentration (10^7 cells sperm/ml) | 281 | 297 | 221 | 249 | 276 |
| Motility (%)                     | 88         | 90    | 90    | 85    | 85    |
| Abnormality (%)                  | 4.5        | 3.5   | 3.5   | 3.0   | 3.0   |
| IPM (%)                          | 86.0       | 89.5  | 87.0  | 88.5  | 89.0  |
| IAC (%)                          | 87.0       | 92.5  | 90.0  | 91.0  | 91.5  |

Based on the results of the macroscopic and microscopic evaluations, fresh semen samples met standard requirements for sperm sexing process and further experiment was continued as being planned. The evaluations results were in accordance with several previous researchers [11];[12];[13];[14];[15];[16].

3.2. Motility sperm after sexing

Table 2 shows the average motility of sperm after sexing treated by the different BSA combination at upper and lower fractions.
Table 2. Average motility of sperm after sexing.

| Replication | Treatment | Upper fraction | Lower fraction |
|-------------|-----------|----------------|----------------|
|             |           | T1  | T2  | T3  | T4  | T1  | T2  | T3  | T4  |
| 1           | 74.00     | 76.00 | 80.00 | 76.00 | 72.00 | 70.00 | 74.00 | 67.00 |
| 2           | 73.00     | 76.00 | 78.00 | 73.00 | 69.00 | 71.00 | 73.00 | 67.00 |
| 3           | 74.00     | 70.00 | 78.00 | 70.00 | 71.00 | 68.00 | 76.00 | 69.00 |
| 4           | 77.00     | 74.00 | 82.00 | 74.00 | 76.00 | 72.00 | 76.00 | 71.00 |
| 5           | 74.00     | 72.00 | 75.00 | 76.00 | 71.00 | 69.00 | 70.00 | 72.00 |
| Total       | 372.00    | 368.00 | 393.00 | 369.00 | 359.00 | 350.00 | 369.00 | 346.00 |
| Average     | 74.40±    | 73.60± | 78.60± | 73.80± | 71.80± | 71.00± | 70.00± | 69.20± |
|             | 1.52a     | 2.61a  | 2.61b  | 2.49a  | 2.59a  | 1.58a  | 2.49b  | 2.28a  |

*T1 = BSA concentration 3% in upper fraction and 6% in lower fraction.
*T2 = BSA concentration 4% in upper fraction and 8% in lower fraction.
*T3 = BSA concentration 5% in upper fraction and 10% in lower fraction.
*T4 = BSA concentration 6% in upper fraction and 12% in lower fraction.

*Different superscript within row show significant different

Based on the results, it was proved that the combination treatment of BSA concentrations had a significant effect (P < 0.05) on the sperm motility of sexed sperm Etawah crossbreed goat. The results of the study occurred due to age, feed, health, time of semen colecting, and sexing processes. The motility has decreased than the fresh sperm, indicating that further treatment will decrease the motility. This was according to [8] stated that the reducing of nutrition reserves and electrolyte fluid imbalance due to sperm metabolism can cause damage of the sperm cell membrane. This damage is due to the exchange of intracellular and extracellular solutions between the diluent and sperm due to differences of concentration. The process of diluting the semen can cause damage to the plasma membrane and reduce their motility. Treatment T3 (5% and 10%) had the highest motility, compared to other treatments. This was shows that T3 has a maximum function on sperm quality. The level of inhibition of lipid peroxidation at T1, T2, and T4 was not maximal so that it affected the protection of the sperm cell membrane and the motility of the sperm itself. The BSA treatment was carried out to replace of the egg white albumin which was not have uniform quality. This is in accordance with the opinion of [5] that BSA is a granular protein (globular) with a molecular weight of 66 kDa, and has a composition of 20 amino acids. In terms of amino acid content, BSA has a more complete content than seminal plasma. In addition, according to [3] stated that using of BSA as a sperm sexing medium is possible because BSA could be protected the plasma membrane and maintain the integrity of DNA. Furthermore, BSA can affect to the sperm motility because it has the function of binding Ca\(^{2+}\) in accordance with the opinion of [12] that BSA is a macromolecule that acts to bind Ca\(^{2+}\), prevents the entry of excess Ca\(^{2+}\) into the cytosol, allows the membrane to more effectively regulate the movement of calcium across the membrane and inhibits intracellular Ca\(^{2+}\) accumulation to levels that are toxic to sperm, resulting in viability and motility of sperm.

Separating sperm process using a medium, in terms of motility, it requires energy to maintain its normal condition to continue metabolic processes. The metabolic process will increase secretion and production of lactic acid, the more lactic acid will damage membrane of sperm. This is in accordance with [11] who stated that increase of lactic acid would affect the increase of osmotic pressure in plasma semen, thereby reducing the permeability of the membrane sperm and would increasing damaging of membrane. Finally, will affect to the activity of sperm.
3.3. Viability of sperm after sexing

Table 3 shows the average viability of sperm after sexing treated by the different BSA combination at upper and lower fractions.

Table 3. Average viability sperm after sexing.

| Replication | Upper fraction | Lower fraction |
|-------------|----------------|----------------|
|             | T1  | T2  | T3  | T4  | T1  | T2  | T3  | T4  |
| 1           | 242 | 242 | 294 | 241 | 221 | 220 | 267 | 220 |
| 2           | 217 | 222 | 266 | 222 | 196 | 217 | 244 | 196 |
| 3           | 244 | 222 | 294 | 244 | 218 | 218 | 243 | 195 |
| 4           | 220 | 218 | 267 | 218 | 194 | 194 | 243 | 196 |
| 5           | 217 | 244 | 289 | 217 | 194 | 222 | 267 | 194 |
| Total       | 1140| 1148| 1410| 1142| 1023| 1071| 1264| 1001|
| Average     | 228.0±13.77 | 229.6±12.36 | 282.0±14.30 | 228.4±13.05 | 204.6±13.67 | 214.2±11.45 | 252.8±12.97 | 200.2±11.10 |
|             | a   | a   | b   | a   | a   | a   | a   | a   |

*Different superscript within row show significant different

$T_1$ = BSA concentration 3% in upper fraction and 6% in lower fraction.
$T_2$ = BSA concentration 4% in upper fraction and 8% in lower fraction.
$T_3$ = BSA concentration 5% in upper fraction and 10% in lower fraction.
$T_4$ = BSA concentration 6% in upper fraction and 12% in lower fraction.

Based on the analysis of variance, that the difference in the combination of BSA concentrations on sperm viability had an significant effect on the viability of sperm ($P < 0.05$). The results of the Duncan Multiple Range Test regarding the viability of sperm contained in the upper fraction were $T_3$ (282 ± 14.30 hours) significantly different ($P < 0.05$) higher than $T_1$ (228 ± 13.77 hours), $T_2$ (229.6 ± 12.36 hours) and $T_4$ (228.4 ± 13.05 hours). This results was same with the lower (Table 3). This is related to the results of motility in several treatment, the large percentage of motility will correlate with sperm viability. This is appropriate with [13] stated that the evaluation of the percentage of viability of sperm is usually carried out in conjunction with the evaluation of sperm motility because there is a positive correlation between motility and live sperm. Treatment $T_3$ (5% and 10%) had the highest survival rate compared to other treatments ($T_1$, $T_2$, and $T_4$). This shows that $T_1$ treatment has a maximum function on sperm quality. The level of inhibition of lipid peroxidation at $T_1$, $T_2$, and $T_4$ was not maximal so that it affected the protection of the sperm cell membrane and the motility of the sperm itself. The high of BSA concentration makes the sperm expend more energy to penetrate the BSA layer. [14] stated that sperm in the lower fraction uses more energy because the sperm has passed two layers, therefore the sperm in the lower fraction of the sperm was lower of survival rate.

According to [15] stated that the 12% BSA concentration was not effective for separating X and Y sperm because the energy required for sperm would be greater to penetrate the layer and would cause a decrease in sperm motility. This is in accordance with the opinion of [16] stated that the reduced amount of sperm nutrition is caused by using of energy for mechanical (motion) and chemical (biosynthetic) activities. The results in this study shows that the viability of sperm between 200.2 - 282 hours up to 0% motility. This is in accordance with the opinion of [17] stated that the storage of sperm at 5°C.

3.4. Viability of sperm after sexing

Membrane integrity is an important factor for sperm because damaged of membrane affects the metabolic processes associated with sperm motility and fertility. Evaluation of intact plasma membranes
(IPM) using the Hypoosmotic Swelling Test (HOST Test). Table 4 shows the average IPM of sperm after sexing treated by the different BSA combination at upper and lower fractions.

Table 4. Average IPM of sperm after sexing.

| Replication | Treatment | Upper fraction | Lower fraction |
|-------------|-----------|----------------|----------------|
|             | T<sub>1</sub> | T<sub>2</sub> | T<sub>3</sub> | T<sub>4</sub> | T<sub>1</sub> | T<sub>2</sub> | T<sub>3</sub> | T<sub>4</sub> |
| 1           | 78.00     | 76.00          | 81.00          | 73.00          | 71.00          | 70.00          | 73.50          | 69.00          |
| 2           | 73.50     | 74.50          | 77.00          | 71.50          | 69.50          | 70.50          | 72.50          | 67.00          |
| 3           | 75.50     | 74.00          | 78.50          | 74.50          | 72.50          | 73.50          | 76.00          | 70.00          |
| 4           | 76.00     | 78.50          | 80.00          | 75.00          | 74.00          | 74.00          | 77.00          | 71.00          |
| 5           | 77.50     | 75.50          | 79.50          | 73.50          | 72.50          | 71.50          | 74.50          | 69.50          |
| Total       | 380.50    | 378.00         | 396.00         | 367.00         | 359.00         | 359.50         | 373.00         | 346.50         |
| Average     | 76.10±    | 75.60±         | 79.20±         | 73.50±         | 71.80±         | 75.60±         | 79.20±         | 73.50±         |
|             | 1.78<sup>a</sup> | 1.56<sup>b</sup> | 1.52<sup>c</sup> | 1.37<sup>a</sup> | 1.68<sup>b</sup> | 1.78<sup>b</sup> | 1.82<sup>c</sup> | 1.48<sup>a</sup> |

<sup>T<sub>1</sub></sup> = BSA concentration 3% in upper fraction and 6% in lower fraction.
<sup>T<sub>2</sub></sup> = BSA concentration 4% in upper fraction and 8% in lower fraction.
<sup>T<sub>3</sub></sup> = BSA concentration 5% in upper fraction and 10% in lower fraction
<sup>T<sub>4</sub></sup> = BSA concentration 6% in upper fraction and 12% in lower fraction
*Different superscript within row show significant different

According to Table 4, there was a decreased of IPM values after sexing at upper and lower fraction. This was caused by the treatment during the sexing process could be the sperm losing the ability to maintain intracellular fluid. There is a difference between the average IPM value of the upper and lower fractions, where the upper fraction has an average value greater than the lower fraction. This is caused by the higher viscosity of the solution in the lower fraction, and sperm that can penetrate the lower fraction have used a lot of energy so that it will ultimately affect sperm metabolism and reduce its ability to move. This is appropriate with the statement of [18] that the greater the gradient viscosity, the frictional force of sperm cells towards the gradient is greater. As a result, protein binding and membrane lipids unsteady and interfere with sperm membrane permeability.

The results of the variance analysis showed that differences combination of BSA concentrations had a significant effect (P <0.05) on the IPM of the upper and lower fractions. Data were then further analyzed using Duncan's distance test to find out the differences between each practice and which treatment gave the highest results. Based on the results of Duncan's tests, the IPM of T<sub>3</sub> (5%:10%) was significantly higher (P <0.05) compared to other treatments. This shows that the combination of 5% BSA concentration in the top fraction and 10% in the lower fraction gives optimum results to protecting sperm during sperm sexing process compared with other combinations (3%: 6%, 4%: 8%, and 6%: 12%). The optimum BSA concentration minimize the occurrence of lipid peroxidation, as well as maintaining membrane stability without disrupting the permeability and motility of the sperm itself as a result of too high viscosity. This is according to the opinion of [1] albumin protein can protect the plasma membrane from the outside so that there is no contact between the plasma membrane and Reactive oxygen species (ROS), so that plasma membrane of sperm was not damaged and the transport of ions and molecules through the membrane runs normally. ROS is naturally produced in the cellular metabolism of sperm itself and ROS can attack polyunsaturated fatty acids in cell membranes resulting in damage to cell membranes (lipid peroxidation). This is in line with the opinion of [19] that high levels of ROS in cells can oxidize lipids, proteins, and DNA. The IPM value at T<sub>1</sub> (3%: 6%) and T<sub>2</sub> (4%: 8%) is smaller than the T<sub>3</sub> (5%:10%), because the concentration of BSA in this treatment is not optimal to protected of sperm. [20] states that dilution of semen can cause decreasingly of the concentration of
substances contained in the semen plasma such as amino acid levels, and ions which can change the balance of osmotic pressure on diluents which can later affect motility and vitality. [1] also stated that bovine serum albumin can provide energy and maintain osmotic pressure on both sides of the plasma membrane by maintaining the balance of ions and small molecules. Based on this, the concentrations used in T1 and T2 are not optimal for sperm sexing media because the albumin protein to maintain the stability and permeability of the plasma membrane is still relatively low. [21] stated, that if BSA concentrations are low, BSA is not enough to provide enough energy and antioxidant protection for spermatozoa.

Intact plasma membrane in the T4 (6%:12%) have the lowest spermatozoa membrane integrity value compared to other treatments, this is due to the decreasingly in spermatozoa membrane function due to the high viscosity of the solution because the concentration of BSA was too high. BSA concentrations that are too high cause more sperm to release energy to penetrate the layer. Sperm that release more energy will conduct higher metabolic activities and will produce more ROS so that sperm are no longer able to maintain the integrity of the plasma membrane. [22] stated that higher BSA concentrations can reduce plasma membrane integrity. This decreasing condition is due to an increase in the osmolarity which can damage sperm cells. Damaging of the sperm cell membrane will have an impact on membranes that initially have a semipermeable nature no longer able to select the entry and exit of substances. Based on this the plasma membrane eventually becomes damaged and increases Ca^{2+} that enters through the plasma membrane and can result in early acrosome reactions. [23] stated that the acrosome reaction is characterized by an increase in Ca^{2+} concentration in the equator region head of the spermatozoa membrane so that the spermatozoa become unstable with the release of enzymes in the acrosome.

3.5. Intact Acrosome Cap (IAC) after sexing

The integrity of the acrosome plays an important role in the process of fertilization, because in the acrosome there are enzymes needed to facilitate spermatozoa in penetrating the zona pellucida. The condition of the acrosome cap can be observed by mixing semen with physiological NaCl plus 1% formalin. Table 5 shows the average IAC of sperm after sexing treated by the different BSA combination at upper and lower fractions.

Based on Table 5, the IAC values was bigger than IPM values. The bigger of IAC because of the location of the acrosome which is still protected by the plasma membrane. The results of the variance analysis showed that the combination of BSA concentrations significantly affected (P <0.05) on the IAC in upper and lower fractions. Data were then analyzed using Duncan's distance test, and showed that the IAC value at T3 (5%:10%) was significantly (P <0.05) higher compared to other treatments. The combination of 5% BSA concentration in the upper fraction and 10% in the lower fraction gives optimum results to protected sperm during sexing process compared with other combination of BSA concentrations (3%: 6%, 4%: 8%, and 6%: 12%). Similar with IPM values, the IAC values at T1 and T2 is smaller than T3, this is because the protein content contained in BSA is still relatively low to protect sperm.

As we know that sperm sexing process requires the diluent that are able to protect and provide an optimal environment for sperm, so that the quality of spermatozoa can be maintained. [24] states that addition of the diluent by BSA as an antioxidant can protect the acrosome membrane and the integrity of the sperm membrane from lipid peroxidation.
### Table 5. Average IAC of sperm after sexing.

| Replication | Treatment | Upper fraction | Lower fraction | % |
|-------------|-----------|----------------|---------------|---|
|             | T1       | T2             | T3            | T4 | T1 | T2 | T3 | T4 |
| 1           | 78.00    | 76.50          | 80.00         | 76.00 | 74.50 | 75.00 | 77.50 | 73.50 |
| 2           | 77.50    | 75.50          | 79.00         | 74.00 | 72.00 | 71.00 | 75.00 | 69.00 |
| 3           | 80.00    | 78.00          | 81.50         | 77.00 | 75.00 | 74.00 | 76.50 | 72.00 |
| 4           | 79.50    | 79.00          | 82.50         | 78.00 | 76.50 | 74.50 | 78.00 | 73.00 |
| 5           | 78.50    | 77.50          | 82.00         | 76.50 | 73.50 | 73.00 | 77.00 | 71.00 |
| Total       | 393.50   | 386.50         | 405.00        | 381.50 | 371.50 | 367.00 | 384.50 | 358.50 |
| Average     | 78.7 ±   | 77.30 ±        | 81.00 ±       | 76.30 ± | 74.30 ± | 73.50 ± | 77.00 ± | 71.70 ± |
|             | 1.04b    | 1.35ab         | 1.46c         | 1.48a  | 1.68b | 1.58ab | 1.29c  | 1.79a  |

T<sub>1</sub> = BSA concentration 3% in upper fraction and 6% in lower fraction.
T<sub>2</sub> = BSA concentration 4% in upper fraction and 8% in lower fraction.
T<sub>3</sub> = BSA concentration 5% in upper fraction and 10% in lower fraction
T<sub>4</sub> = BSA concentration 6% in upper fraction and 12% in lower fraction

*Different superscript within row show significant different

The integrity of the sperm acrosome cap is needed to support the successful of fertilization process. [23] states that when undergoing an acrosome reaction at the right time and place, spermatozoa must be able to last long enough and intracellular K<sup>+</sup> concentrations are kept high and at the same time intracellular Na<sup>+</sup> and Ca<sup>2+</sup> concentrations are kept low, because it is very important for survival spermatozoa. According to [25] albumin binds calcium ionophores from the plasma membrane thereby allowing the membrane to more effectively regulate the movement of Ca<sup>2+</sup> across the membrane and inhibit intracellular Ca<sup>2+</sup> accumulation the toxic levels for spermatozoa. This minimizes spermatozoa to capacitation and early acrosome reactions. Higher of intracellular Ca<sup>2+</sup> concentrations that enter through the membrane are triggering factors for capacitation of sperm. Higher of ion Ca<sup>2+</sup> concentration before fertilization is not expected because it causes death of sperm. [26] states that if an acrosome reaction occurs before spermatozoa reach the site of fertilization, the ability of spermatozoa to fertilize of oocytes will lose. The acrosome is protected by a plasma membrane in term of serving first defense of cell from the outside that can damage of cells. The plasma membrane is protected from mechanical or ROS damage so the acrosome cap is also protected. The spermatozoa IPM and IAC values increase when BSA concentrations added to the diluents are optimal for sperm, but doses higher than the amount required make the IPM and IAC values decrease. The treatment 4 (T<sub>4</sub>) that is used is too concentrated, the sexing of sperm expends a lot of energy to penetrate the layer that is too concentrated, it is difficult to maintain normal physiological conditions. [21] states that too high a BSA concentration will create a hyperosmotic condition in the diluent, make a big pressure on the membrane and can damage the sperm membrane. [27] states that increased sperm metabolism will produce lactic acid and reactive oxygen species (ROS) derived from the process of mitochondrial respiration. High levels of ROS will no longer be balanced by antioxidants eventually causing lipid peroxidation. If the plasma membrane is damaged, the transport of molecules through the plasma membrane cannot run normally and can increase Ca<sup>2+</sup> that enters through the plasma membrane and sperm are more susceptible to acrosome damage. Although T<sub>4</sub> has the lowest IAC value (71.70%), this combination is still in good criteria for artificial insemination program. [28] states that the percentage of intact acrosome caps of 58-63% is still optimal for Artificial Insemination program.
4. Conclusion
Based on the results, the quality of Etawah crossbreed sexed sperm was affected from combination of BSA concentrations.

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