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Effect of Suplementation Tomatoes Juice in Fresh Semen Egg Yolk Citrate Diluent on the Quality of Spermatozoa and the Success of Artificial Insemination in Thin Tailed Sheep

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ABSTRACT

This study aims to determine the effect of adding tomato juice in fresh semen egg yolk citrate diluent to the quality of spermatozoa and the success of artificial insemination in thin tailed sheep. This research was conducted using a completely randomized design research method with 3 treatments and 5 repetitions. The treatments consisted of a solution of egg yolk citrate (P0), egg yolk citrate added 20% tomato juice (P1), and egg yolk citrate added 40% tomato juice (P2). Data analysis on spermatozoa quality included motility, viability, and abnormality using one way anova followed by Duncan's test, while artificial insemination success included Non Return Rate (NRR) and Conception Rate (CR) using descriptive analysis. The results showed that the best diluent was found in P1 with the addition of 20% tomato juice resulting in motility percentages and viability are 75,52 ± 1,92 and 87,70 ± 1,04. It can be concluded that the addition of tomato juice had a significant (P<0,05) effect on the motility and viability of spermatozoa. The addition of 20% tomato juice in egg yolk citrate diluent can improve the quality of spermatozoa. The CR and NRR values obtained from artificial insemination with P1 diluent were 53% and 60%

INTRODUCTION

The potential of sheep farming in Central Java Province is vast. In 2021, the sheep population reached 2,333,425 heads (Badan Pusat Statistik, 2021) and decreased in 2022 to 2,326,859 (Badan Pusat Statistik, 2022). Currently, the availability of sheep has yet to be able to meet the needs of the community. Therefore, it is necessary to improve the genetic quality of sheep. The type of sheep that small and large farmers have widely kept is thin-tailed, often called local sheep. The genetic rate of thin-tailed sheep decreases occasionally due to the high incidence of inbreeding among small farmers. Sheep Thin-tailed sheep are native Indonesian sheep, also called local sheep, which have the characteristics of the tail being thin and small in the male sheep have horns coiled down and not horned in females, and they have white wool fur, down and hornless in females, has a woolly coat that is white and black. The community widely keeps thin-tailed sheep because they have good adaptability and growth adaptability and relatively fast growth (Warastomo et al., 2021).

The genetic quality of thin-tailed sheep has declined over time due to the high incidence of inbreeding among smallholder farmers. Efforts to improve the genetic quality of thin-tailed sheep can be made by crossbreeding between superior livestock and local livestock, one of which can be done by artificial insemination (AI). Artificial insemination (AI) is the insertion or delivery of semen into the female genital tract using artificial tools (Winarso et al., 2004; Salmin et al., 2023). Artificial insemination can be done using liquid semen and frozen semen. Successful artificial insemination is when the acceptor animal is successfully pregnant. Four things determine AI success : inseminator skills, acceptor selection, accuracy of lambing detection by breeders, and semen quality testing (Taufik et al., 2023).

Good semen quality will have a high success rate for artificial insemination. Good fresh semen can be obtained by selecting superior males, checking semen quality visually, and using a microscope. Macroscopic (visual) observation of new semen quality can be seen from the volume, color, consistency, and pH of the semen. Microscopic semen observation (using a microscope) can be observed from motility, concentration, viability, and abnormality. The application of AI with liquid semen has a higher AI success rate because it does not go through the freezing process, and in storage, it only requires a temperature of 40 to 50C, unlike frozen semen using liquid nitrogen, whose temperature touches -1960C.

Fresh semen used in AI programs can be diluted to increase its volume so that many females can use it for a more extended period. Semen diluents that can be used are non-toxic to spermatozoa, have physical and chemical properties similar to semen, are isotonic, contain buffering material as a source of energy, and can prevent the growth and development of bacteria (Susilawati, 2011).

The diluent commonly used is egg yolk citrate. Egg yolk citrate contains lecithin and lipoproteins as a buffer material for semen and prevents cold shock due to sudden temperature drops (Trias, 2001). Egg yolk is an extracellular cryoprotectant material that provides food and energy sources and protects extracellular spermatozoa from cold shock during freezing, and glucose in egg yolk is more dominantly used by spermatozoa for metabolism than fructose in semen (Widiastuti et al., 2018).

Semen dilution materials that can be used as an alternative are fruits. Semen diluents from fruits that have been used such as carrot juice (Barek et al., 2020) and watermelon juice (Bria et al., 2022). Fruits other than carrots and watermelon that can be used are tomato juice. Tomato juice (Lycopersicon escelentum Mill) contains nutrients including carbohydrates, protein, vitamin A, vitamin C, and lycopene, which function as antioxidants (Sumardiono et al., 2009). Tomatoes also contain carbohydrates and antioxidants that act as a source of energy and prevent cell-damaging free radicals (Maulida and Zulkarnaen, 2010).

Based on its content, tomato juice can be used as an alternative diluent. Therefore, a study was conducted to determine the effect of adding tomato juice in fresh semen egg yolk citrate diluent on

spermatozoa quality and the success of artificial insemination.

METHODS

Location and Time

This research was conducted from 11 April 2023 to 20 June 2023 at Yunan Farm Balak Hamlet, Losari Village, Pakis Sub-district, Magelang Regency and Animal Health and Reproduction Laboratory of Agricultural Development Polytechnic of Yogyakarta Magelang, Department of Animal Husbandry.

Research design

Research on liquid semen quality includes motility, viability, and abnormality using the Complete Randomised Design (CRD) method, namely three treatments and five replicates. Completely Randomised Design (CRD) can be used in experimental units with a homogeneous environment such as laboratory experiments (Susilawati, 2015). This study used treatments namely P0 = 80% Na. citrate + 20% egg yolk, P1 = 60% Na. citrate + 20% egg yolk + 20% tomato juice, and P2 = 40% Na. citrate + 20% egg yolk + 40% tomato juice.

Materials And Tools

The livestock used are male Awassi sheep, as much as one head, aged 1.5 years, and healthy and not defective. The ewes of thin-tailed sheep used were 15 tails with a body weight of 23 to 25 kg, had given birth once, were healthy, and not deformed. This study uses materials including tomato fruit, sodium citrate, distilled water, chicken eggs, penicillin, streptomycin, NaCl solution, 2% eosin, 70% alcohol, pregnancy test kit, lubricating gel, aluminum paper, filter paper, pH paper, cotton, tissue, and water.

The equipment used were a research microscope, micropipette, hemocytometer, object glass, cover glass, eppendorf, eppendorf rack, optilab, erlenmeyer tube, erlenmeyer stirring rod, test tube, test tube rack, blender, knife, centrifuge, artificial vagina, cold box, thermometer, refrigerator, vaginal scoop, 3cc syringe, acrylic syringe, plastic sheet, rubber gloves, mobile phone, and stationery.

Research Variables

The observed variables were semen quality consisting of motility, viability, and abnormality, and evaluation of AI success consisting of Conception Rate (CR) and Non-Return Rate (NRR).

Research Procedures

The research procedure to be carried out is as follows:

A. Preparation of Sperm Dilution

1. Preparation of tomato juice

Ripe tomatoes are cut into smaller pieces to make it easier to mash. Then, the tomato fruit is mashed using a blender. The tomato fruit that has been mashed is then filtered using gauze many times. Then, it was put into a test tube with a lid and precipitated using a centrifuge to speed up the precipitation process.

2. Preparation of diluent with the addition of tomato juice in egg yolk citrate diluent

The basic diluent Na Citrate solution is prepared using 2.9 grams of Na citrate powder and dissolved in 100 ml of distilled water. Then, the Na citrate solution was placed in each tube according to the treatment dose and added with egg yolk according to the treatment dose, mixed until homogeneous. Next, as much as a drop each, penicillin and streptomycin solutions that have been diluted with distilled water are added. The egg yolk citrate solution in each tube was added with tomato juice according to each treatment and mixed until homogeneous. The dosage of diluent used can be seen in Table 1.

Table 1. Composition of Diluent Used

Diluent Material	Treatment Group		
	P0 (%)	P1 (%)	P2 (%)
Na. Citrate	80	60	40
Egg Yolk	20	20	20
Fruit Juice Tomato	0	20	40

B. Semen Collection and Semen Evaluation

Semen was collected using an artificial vaginal device. Semen was obtained from one male Awassi sheep in good health, 1.5 years old, and weighing about 70 kg. Semen collection was carried out twice a week in the morning. Fresh semen obtained was immediately observed macroscopically by looking at color, volume, consistency, and pH. After the semen was collected, it was put into an eppendorf coated with aluminium and placed in a cold box in which there were ice cubes coated with tissue. Microscopic tests were carried out in the laboratory which included concentration, motility, viability, and abnormality tests. The semen used in this study was semen with a concentration of more than 600 million/ml, progressive mass motion of more than 70%, and abnormality below 20%.

C. Spermatozoa Motility Examination

Checking the motion of spermatozoa that walk straight ahead is done with an object glass that is dripped with 10 to 15 µl of semen and then coated with cover glass. Motility checking can be done using a 200 times magnification microscope. The motility of spermatozoa is known by observing spermatozoa that move forward and calculated as a percent of the total spermatozoa.

D. Spermatozoa Viability Check

Spermatozoa were examined with 2% eosin. One drop of semen was mixed with 2% eosin and made a review preparation and dried. Observations were made with a 400 times magnification microscope. Live spermatozoa have white heads, while dead heads will turn red in color.

E. Spermatozoa Abnormality Check

Examining spermatozoa abnormality is using 2% eosin observed with a 400 times magnification microscope. Abnormality observations were reviewed from abnormal spermatozoa shapes such as no head, large head, broken tail, and circular tail.

F. Artificial Insemination

ArtificiaI Insemination was carried out with fresh semen that had been diluted using egg yolk citrate diluent and tomato juice to check the motility, viability, and abnormality of the best spermatozoa. According to Pratiwi et al., (2015) the dose of diluent used is calculated by the following formula:

$$V_t = \frac{Vo \; x \; concentration \; x \; motility}{AI \; dose}$$

$$V_p = V_t - V_o$$

Notes:

Vo = sperm volume before dilution

Vt = volume after dilution

Vp = volume of diluent

G. Pregnancy Detection

Pregnancy detection can be carried out on the 21st day after insemination using pregnancy detection materials through cattle urine with a pregnancy test kit. The workings of the test kit are as follows:

a. 3 ml of cattle urine is collected.
b. Administer three drops of the pregnancy test kit.
c. Observe the color change in the urine. If the cattle are pregnant, the color is clear, while those who are not are cloudy.

Data Analysis

Data from liquid semen quality, including motility, viability, and abnormality, were analyzed with a one-way ANOVA pattern in SPSS. If the data showed a significant effect, it was followed by the Duncan test. Meanwhile, data obtained from CR and NRR values were analyzed descriptively to determine the success rate of pregnancy. Descriptive analysis is used to collect, summarise, and present data so that information is easily understood (Wijayanti et al., 2022).

RESULTS AND DISCUSSION

Quality of Fresh Semen of Awassi Sheep

Fresh semen for liquid semen samples can be checked first on volume, color, pH, consistency, motility, mass movement, concentration, viability (live spermatozoa), and abnormality. The results of the quality check of the fresh semen of Awassi sheep are in Table 2.

Table 2. Average Fresh Semen Quality of Awassi Sheep

Parameters	Observation Results
Volume (ml)	0,85 ± 0,42
Color	Whitish beige
Consistency	Viscous
Mass movement	+++
Ph	6,8 ± 0,45
Motility (%)	75,00 ± 1,58
Concentration (10^9/ml)	3,12 ± 0,14
Live Spermatozoa (%)	86,40 ± 0,89
Abnormality (%)	9 ± 0,71

Source : Primary Data

Semen collection of sheep is done using an artificial vagina tool. The average volume of semen produced by Awassi sheep 5 times taken in this study was 0.85 ± 0.42. This result was obtained by Rizal and Herdis (2008), who stated that the average semen in sheep ranges from 0.3 to 2 ml. Many things influence the average value of sheep semen volume. According to Barek et al. (2020) things that affect the volume of semen are age, environmental temperature, nation, frequency of collection, feed, testicle size, and body. In addition, the collection method also affects the volume of semen produced. According to Hafez (2004) collection with an electroejaculator has more semen volume than using an artificial vagina.

The color of the semen obtained is standard. The average semen obtained is whitish beige. This is similar to research conducted by Rosmaidar et al. (2013) and Wahyuningsih (2013), who reported that the semen produced from ejaculation is milky white and beige. Dark red to bright red semen indicates fresh blood with different volumes in the semen. A brownish-brown color indicates the presence of decomposed blood. A light brown or greenish color indicates that the semen may be contaminated with feces (Toelihere, 1985).

Consistency is the degree of viscosity of the semen. Consistency can be obtained by tilting the collection tube. If the tube is tilted and then returned to its original position, there is still a lot of remaining semen in the thick category, the remaining semen is slightly included in the medium category, and no remaining semen is included in the liquid type. The consistency of the semen obtained is thick on average. This is to the results of research by Barek et al. (2020) that the semen produced has a moderately thick consistency. According to Faridah (2016) good quality semen has a viscosity level similar to or slightly thicker than milk.

The degree of acidity can be measured using pH paper. The average degree of acidity obtained from the Awassi sheep semen collection is 6.8 ± 0.45. This is based on the research of Feradis (2007), who found that the pH of fresh sheep semen received is 6.8. Toelihere (1985) states that the pH of neutral sheep semen is 6.8. Meanwhile, Solihati et al. (2018) research shows that the sheep semen obtained has a pH of 7.22. Differences in pH can be influenced by different breeds and differences in complex buffer systems in semen plasma (Yendraliza et al., 2015).

Calculation of concentration was done using a hemocytometer. The concentration is calculated by sucking 0.5 ml of semen with a hemocytometer pipette and adding NaCl solution up to 101 ml and shaking it to make it homogeneous, and then dropping it into the counting chamber. Checking is done using a 400 times magnification microscope by counting at five points according to the diagonal direction (Manehat el al., 2021). The average concentration obtained was 3.12 ± 0.14 x 109/ml. This is by Toelihere (1985), who states that fresh semen to be diluted has a minimum concentration of 2000 million per milliliter of semen. This result is higher than that reported by Hartanti and Karja

(2014) who obtained a fresh semen concentration of Garut sheep of 2,865 million/ml. Individual influences and conditions of the experimental animals can cause a difference in the results obtained. In addition, according to Rosmaidar et al. (2013) differences in concentration can be influenced by individual variations, maintenance patterns, and age.

In addition, temperature also affects libido and spermatozoa production produced in the tropics (Toelihere, 1993). The average mass movement of spermatozoa obtained from the semen collection of Awassi sheep is +++, which means very good. The percentage of motility and viability of spermatozoa from fresh semen of Awassi sheep brought is 75.00 ± 1.58 and 86.40 ± 0.89. These results are by Toelihere's statement (1985) that the requirements of fresh semen to be diluted have a minimum motility of 70%, viability of 75%, and mass movement ++/++++.

The percentage of spermatozoa that have an abnormal shape is 9.00 ± 0.71. These results are by Toelihere (1985), who states that fertile sheep semen is not more than 15%.

Spermatozoa Motility Percentage by Addition of Tomato Fruit Juice in Egg Yolk Citrate Diluent Fresh Semen

Spermatozoa motility plays a vital role in the success of artificial insemination. Each species has a different speed of movement of spermatozoa and varies according to the place's conditions and the environment's temperature (Toelihere, 1985). The motility of fresh semen spermatozoa after dilution can be seen in Table 3.

Table 3. Average Percentage of Spermatozoa Motility by Addition of Tomato Fruit Juice in Egg Yolk Citrate Diluent Fresh Semen

Parameters	Treatment		
	P0 (0%)	P1 (20%)	P2 (40%)
Motility (%)	69,63 ± 2,83[a]	75,52 ± 1,92[b]	67,60 ± 2,30[a]

Source : Primary Data

Based on Table 3, the results of statistical analysis on the percentage of spermatozoa motility showed that the addition of tomato juice in egg yolk citrate diluent had a significant effect ($P < 0.05$) on the motility of Awassi sheep spermatozoa. The average percentage of motility produced was 69.63 ± 2.83a (P0); 75.52 ± 1.92b (P1); and 67.60 ± 2.30a (P2). The average percentage of spermatozoa motility on the addition of 20% tomato juice in fresh semen egg yolk citrate diluent (P1) is significantly different ($P <0.05$) with P0 and P2. P0 showed a significantly higher percentage of motility ($P>0.05$) than P2.

The results of this study prove that adding tomato juice to egg yolk citrate diluent affects the motility of Awassi sheep spermatozoa. This can be caused by the interaction of semen diluent and spermatozoa, and the content of egg yolk citrate diluent with tomato juice affects the metabolism and physiological conditions of spermatozoa (Rosmaidar et al., 2013).

This study proves that adding tomato juice as much as 20% in egg yolk citrate diluent is the best. The percentage of spermatozoa motility with the addition of 20% tomato juice is higher than the control due to the nutritional content of tomatoes, which contain a lot of vitamin C, vitamin E, and lycopene, which are antioxidants. The antioxidant content in tomatoes can bind to oxygen radicals contained in spermatozoa to prevent the formation of lipid peroxidation in the mitochondrial membrane, inhibiting glycolysis and motility.

Percentage of Live Spermatozoa by Addition of Tomato Fruit Juice in Egg Yolk Citrate Diluent Fresh Semen

The percentage of live spermatozoa can be determined using 2% eosin liquid. Living spermatozoa will not absorb color, and dead spermatozoa will absorb color. This is because, in

living spermatozoa, the attachment of eosin to sodium with the sodium pump mechanism will be pushed out. Whereas in dead spermatozoa, there is no potential difference in sodium and potassium ions between inside and outside the cell, so the eosin that binds to sodium will quickly diffuse and change the color of the spermatozoa head (Rosmaidar et al., 2013). The percentage of live spermatozoa after dilution can be seen in Table 4.

Table 4. Average Percentage of Live Spermatozoa with the Addition of Tomato Fruit Juice in Egg Yolk Citrate Diluent Fresh Semen

Parameters	**Treatment**		
	P0 (0%)	**P1 (20%)**	**P2 (40%)**
Viability (%)	$84,60 \pm 2,41^{b}$	$87,70 \pm 1,04^{b}$	$76,80 \pm 3,96^{a}$

Source : Primary Data

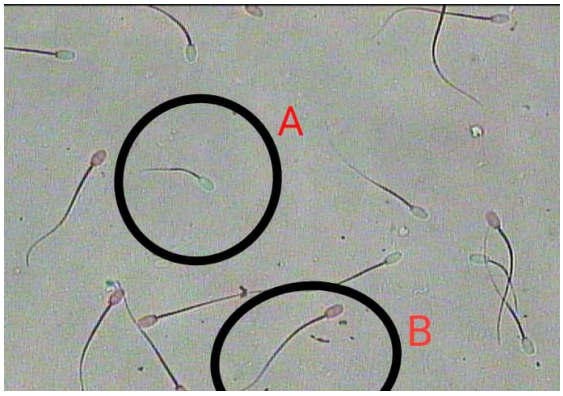

Figure 1. Live Spermatozoa White (A) and Dead Spermatozoa Red (B)

Based on Table 4, the results of statistical analysis of the percentage of live spermatozoa showed that the addition of tomato juice in egg yolk citrate diluent had a significant effect ($P < 0.05$) on the percentage of live spermatozoa of Awassi sheep. The average percentage of viability resulting from the dilution of Awassi sheep semen with the addition of tomato juice in egg yolk citrate diluent is 84.60 ± 2.41b (P0); 87.70 ± 1.04b (P1) and 76.80 ± 3.96a (P2). P1 treatment was not significantly different ($P>0.05$) with P0 and significantly different ($P<0.05$) with P2.

The results of this study prove that adding tomato juice as much as 20% can maintain the condition of the diluent so that it can maintain the percentage of live spermatozoa. This may occur because tomatoes contain high levels of vitamins C, E, and lycopene, which have antioxidant properties. In addition, the content of vitamin C, vitamin E, and lycopene can bind oxygen radicals in the diluent to avoid lipid peroxidation that can damage the plasma membrane of spermatozoa cells. According to Lubis et al. (2013), adding vitamin C as much as 200mg/100 ml diluent can protect the plasma membrane of Boer goat spermatozoa from damage by lipid peroxidation.

Adding 40% tomato juice produces a lower percentage of live spermatozoa of Awassi sheep. The percentage of live spermatozoa in adding 40% tomato juice was significantly lower ($P < 0.05$) than the control and P2. This may be due to the higher concentration of vitamin C in tomato juice in the diluent, which will make the fructolysis rate faster and increase lactic acid. Elevated lactic acid content can reduce pH, resulting in a decrease in the activity of metabolic enzymes due to insufficient energy needs. The reduction in pH can also increase the concentration of H+, which will react with radicals to form lipid peroxidation so that it can damage the plasma membrane and inhibit glycolysis, and the

mortality rate becomes higher (Rosmaidar et al., 2013).

The percentage of live spermatozoa is higher than the percentage of motility. This could indicate that many spermatozoa are still alive but are not motile (not moving) or are only moving abnormally (shaking). According to Toelihere (1985) the percentage of live spermatozoa is usually 10% higher than the percentage of motility.

Percentage of Spermatozoa Abnormality of Awassi Sheep with Tomato Fruit Juice Addition in Egg Yolk Citrate Diluent Fresh Semen

The abnormality of sheep spermatozoa can be known using 2% eosin liquid and observed with a 400 times magnification microscope. The percentage of spermatozoa abnormalities after dilution can be seen in Table 5.

Table 5. Average Percentage of Sperm Abnormality with the Addition of Tomato Fruit Juice in Egg Yolk Citrate Diluent Fresh Semen

Parameters	Treatment		
	P0 (0%)	P1 (20%)	P2 (40%)
Abnormality(%)	8,10 ± 0,42[a]	7,90 ± 0,42[a]	8,50 ± 0,50[a]

Source : Primary Data

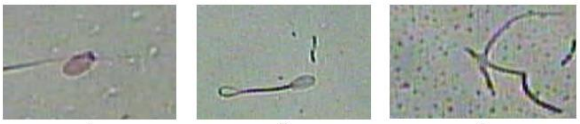

Figure 2. Spermatozoa Abnormality Severed Tail (A), Circular Tail (B), and No Head (C)

Based on Table 5, the results of statistical analysis on the percentage of spermatozoa abnormalities showed that the addition of tomato juice in egg yolk citrate diluent had no significant effect (P>0.05) on the percentage of spermatozoa abnormalities of Awassi sheep. The average percentage of abnormality produced was 8.10 ± 0.42a (P0); 7.90 ± 0.42a (P1); and 8.50 ± 0.50a (P2). The average percentage of abnormality between treatments showed no significant difference (P>0.05).

Abnormalities of spermatozoa found were spermatozoa without tails, no head, and circular tails. Abnormal conditions are still in low numbers because they are still below 20%, so it is still feasible for them to be inseminated into livestock. This is the opinion of Kartasudjana (2001) that semen can be inseminated into livestock if it has an abnormality value of no more than 20%.

Percentage Conception Rate (CR)

Conception Rate (CR) shows the number of pregnant animals after the first artificial insemination. Artificial Insemination (AI) was performed using fresh semen with the best diluent, namely P1 with the addition of 20% tomato juice in egg yolk citrate diluent. CR observations were made on 15 sheep using a pregnancy test kit.

The number of sheep that successfully got pregnant in the first IB was 8 sheep from the ewe and 15 sheep that had been AI, resulting in a CR percentage value of 53%. Fanani (2013) stated that the CR rate in Indonesia of 40 to 50% is good considering natural conditions, management, and livestock distribution.

Percentage Non Return Rate (NRR)

Non-Return Rate (NRR) indicates the number of animals that were not requested to be re-bred. Artificial insemination was performed using fresh semen with the best diluent which is the addition of 20% tomato juice in egg yolk citrate diluent (P1). NRR observations were made on day 28 to 35 days after the sheep were inseminated by observing the condition of the livestock.

The number of sheep that did not show signs of lambing again after artificial insemination was as many as 9 heads or in percentages of 60%. This is based on the statement of Rosita et al. (2014) that the NRR value above 50% is in the good category.

CONCLUSION

Based on the research results, it can be concluded that:

1. The addition of tomato juice in egg yolk citrate diluent of fresh semen had a significant effect ($P<0.05$) on the motility and viability of spermatozoa. However, adding tomato juice in egg yolk citrate diluent of fresh semen had no significant effect ($P>0.05$) on spermatozoa abnormality. Adding 20% tomato juice in egg yolk citrate diluent of fresh semen can improve the quality of spermatozoa.
2. The success rate of IB seen from the Conception Rate (CR) value was obtained with a percentage of 53% and Non Return Rate (NRR) of 60%.

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