Fertility And Fertile Period in Laying Hens Inseminated With 100 million Kampung Rooster Spermatozoa at Different Insemination Volumes

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Abstract

Artificial insemination (AI) is an essential reproductive biotechnology for accelerating genetic improvement and optimizing breeding efficiency in Indonesian native Kampung chickens. However, standardized protocols defining the optimal total suspension volume at a constant spermatozoal dose remain poorly established for indigenous poultry genotypes. This study investigated the relationship between total insemination volume and reproductive performance in laying hens inseminated with a fixed dose of 100 million Kampung rooster spermatozoa suspended in a skim milk–50 mM glucose extender. Fresh ejaculates were collected from mature Kampung roosters via dorso-abdominal massage and evaluated macroscopically and microscopically for volume, concentration, and progressive motility (~85%) prior to dilution. Pooled semen samples were extended to deliver an identical dose of 100 million spermatozoa across five final insemination volumes: 0.1 mL (P0, serving as the standard positive baseline control), 0.2 mL (P1), 0.3 mL (P2), 0.4 mL (P3), and 0.5 mL (P4). Fifty laying hens were randomly allocated into the five treatment groups (10 hens per group) under a completely randomized design and inseminated intravaginally under standardized management conditions. Eggs were collected daily following AI and incubated to evaluate fertility and the duration of the fertile period. One-way analysis of variance revealed that total insemination volume did not significantly affect either fertility or fertile period ($P > 0.05$). Mean fertility rates were 88.11 ± 11.00%, 90.00 ± 10.95%, 92.00 ± 10.95%, 87.00 ± 10.33%, and 84.00 ± 8.94% for the 0.1, 0.2, 0.3, 0.4, and 0.5 mL treatments, respectively. Corresponding fertile periods were 12.60 ± 2.10, 13.40 ± 1.64, 13.60 ± 1.83, 13.00 ± 2.10, and 12.30 ± 2.16 days. Although the 0.3 mL volume yielded the highest numerical fertility and longest numerical fertile period, these differences were not statistically significant. These findings demonstrate that when a constant dose of 100 million viable Kampung rooster spermatozoa is delivered in a skim milk–50 mM glucose extender, insemination volumes ranging from 0.1 to 0.5 mL support comparable reproductive outcomes. Within this range, total sperm dose and initial semen quality exert a more decisive influence on fertilization efficiency than carrier fluid volume.

Keywords: artificial insemination; chicken; fertile period; fertility; insemination volume.

I. INTRODUCTION

Artificial insemination (AI) is an important reproductive technology in poultry production because it enables controlled pedigree mating, maximizes the genetic contribution of superior sires, overcomes physical or behavioral mating incompatibilities, and improves overall reproductive management efficiency. In Indonesia, native Kampung chickens (*Gallus gallus domesticus*) represent an invaluable indigenous genetic resource prized for their adaptability to tropical environments, disease resilience, and distinct meat and egg characteristics. Nevertheless, the reproductive efficiency of Kampung chickens under conventional natural mating is frequently constrained by low male-to-female mating ratios, behavioral dominance hierarchies among roosters, and variable ejaculate quality.

Implementing standardized AI protocols therefore offers a practical pathway to intensify selective breeding and commercial multiplication of Kampung chickens while maintaining high fertilization rates.

The biological success of poultry AI depends on a complex interplay among semen quality, the total number of viable spermatozoa inseminated, the physicochemical properties of the semen extender, the technical precision of intravaginal deposition, and the physiological capacity of the female reproductive tract to transport, select, and store spermatozoa (Blesbois & Brillard, 2007) .

Unlike mammalian species in which fertilization occurs shortly after mating within the oviductal ampulla, avian females possess specialized anatomical structures capable of prolonged spermatozoal storage. Following intravaginal insemination, spermatozoa must traverse the mucosal folds of the vagina—an immunological and mechanical barrier that eliminates dead, abnormal, or poorly motile cells—before reaching the uterovaginal junction (UVJ). At the UVJ, a selected fraction of spermatozoa enters invaginated tubular structures known as sperm storage tubules (SSTs), which serve as the primary biological reservoir for sustained fertility (BRILLARD, 1993; Hemmings et al., 2015).

Once sequestered within the SSTs, spermatozoa remain quiescent and metabolically supported by the tubular epithelium before being gradually and continuously released into the oviductal lumen, ascending toward the infundibulum where fertilization of the newly ovulated oocyte takes place (Hemmings et al., 2015). Consequently, the efficiency of initial sperm transport into the SSTs governs both immediate egg fertility and the duration of the fertile period—defined as the number of consecutive days following a single insemination during which a hen lays fertile eggs. BRILLARD (1993) demonstrated that oviductal sperm selection following AI is extraordinarily stringent, with fewer than 1% of inseminated spermatozoa successfully gaining entry into the SSTs. Because this selection process relies heavily on intrinsic spermatozoal motility, membrane integrity, and surface interactions with the vaginal and UVJ epithelia, any procedural or environmental factor that impairs sperm transport can substantially diminish reproductive output.

Among quantitative AI parameters, the total number of spermatozoa delivered per insemination dose has been extensively established as a primary determinant of fertility and fertile-period longevity. Brillard & Antoine (1990) demonstrated a direct positive relationship between the number of spermatozoa inseminated, the population of spermatozoa colonized within the UVJ sperm storage tubules, and the number of spermatozoa subsequently trapped on the outer perivitelline layer of laid eggs. Furthermore, (Wishart, 1987) established that the number of perivitelline spermatozoa associated with successive eggs declines exponentially with time post-insemination, and that the duration of the fertile period is directly regulated by the initial size of the oviductal sperm reservoir. When the number of spermatozoa reaching the infundibulum falls below a critical threshold required to penetrate the germinal disc, fertilization ceases and subsequent eggs are infertile.

In addition to total sperm number, the physical volume of the inseminated semen suspension represents a critical yet comparatively underexplored variable in poultry AI. Total insemination volume dictates the concentration of spermatozoa per unit volume of carrier medium as well as the total fluid load introduced into the vaginal lumen. From a biophysical perspective, altering total insemination volume while maintaining a constant sperm dose creates two potentially competing physiological effects. On one hand, an excessively small volume (<0.05–0.1 mL) increases the risk of disproportionate dead-space retention inside the insemination pipette or syringe, uneven dispersion across the vaginal mucosa, and localized viscosity constraints. On the other hand, introducing an overly large volume of fluid into the relatively short avian vagina may exceed luminal retention capacity, triggering muscular contractions and retrograde expulsion (backflow) of the suspension through the cloaca immediately following withdrawal of the insemination cannula. Moreover, higher

dilution ratios inherently reduce the concentration of seminal plasma proteins and protective macromolecules per milliliter of suspension—a phenomenon known as the dilution effect—which can alter local osmotic balance and spermatozoal motility during oviductal transit.

The composition of the semen extender plays a vital buffering and protective role when semen is diluted to achieve specific target volumes. Skim-milk-based extenders have long been recognized in animal reproduction for their ability to protect spermatozoal plasma membranes against cold shock and dilution stress, primarily through the action of casein micelles, while providing a buffered aqueous environment. (Saleh & Susanto, 2025) demonstrated that skim-milk-based extenders effectively preserve the progressive motility, viability, and fertilizing capacity of Indonesian native rooster spermatozoa. Specifically, their evaluation of a skim milk extender supplemented with 50 mM glucose established a balanced formulation in which glucose serves as an exogenous glycolytic energy substrate to sustain flagellar movement without inducing hyperosmotic stress.

In subsequent work, (Saleh et al., 2026) confirmed that Kampung rooster spermatozoa diluted in a skim milk–glucose extender maintains high motility and fertility when inseminated promptly after collection, whereas prolonged storage at ambient temperature progressively degraded motility, fertility, and fertile-period duration. These findings underscore the necessity of standardizing extender formulation and minimizing pre-insemination holding times when evaluating technical AI variables.

Despite substantial progress in characterizing sperm dose requirements and extender formulations for indigenous roosters, empirical evidence regarding the optimal total insemination volume at a fixed sperm dose in Kampung chickens remains scarce. In practical AI operations, technicians frequently adjust dilution rates based on ejaculate concentration without knowing whether varying the final insemination volume between 0.1 and 0.5 mL alters oviductal sperm retention or SST filling. Therefore, the present study was conducted to evaluate fertility and fertile-period duration in laying hens inseminated with a constant dose of 100 million Kampung rooster spermatozoa across five total insemination volumes (0.1, 0.2, 0.3, 0.4, and 0.5 mL) using a standardized skim milk–50 mM glucose extender. The objective was to determine whether total fluid volume within this practical range significantly influences reproductive outcomes or whether a specific volume optimizes fertilization efficiency.

II. METHOD

A. Experimental Animals

The experiment used mature, clinically healthy, kampung roosters with proven semen production served as semen donors and laying hens in active egg production were utilized as recipient females. A total of 50 mature, productive laying hens were used as experimental animals. The hens consisted of 45 newly procured hens and five hens retained from the previous study. The 50 hens were randomly allocated into five treatment groups, with 10 hens assigned to each treatment.

B. Semen Collection and Evaluation

Fresh semen was collected from the donor Kampung roosters using the standard non-invasive dorso-abdominal massage technique. Before collection, the cloacal area was gently cleaned with dry tissue paper to prevent contamination from feces, urates, or cloacal fluid. Prior to dilution, fresh semen was evaluated. Sperm quality evaluation included sperm concentration and progressive motility. Semen used for artificial insemination had approximately 85% sperm motility. Only ejaculates exhibiting creamy-white appearance, absence of fecal or blood contamination, and progressive sperm motility of approximately 85% were pooled and utilized for extender dilution and artificial insemination. The semen

was diluted so that each insemination dose contained a constant total of 100 million spermatozoa.

C. Preparation of skim milk–glucose extender

A skim milk–glucose extender was used for preparation of the insemination doses. The extender contained skim milk supplemented with glucose at a final concentration of 50 mM. The use of skim milk as a semen extender was based on previous research demonstrating its suitability for maintaining the quality and fertility of Kampung rooster spermatozoa (Saleh & Susanto, 2025) .

D. Insemination Treatments

The total sperm dose was held strictly constant at 100 million spermatozoa per hen across all experimental groups, while total insemination volume (comprising neat semen plus skim milk–50 mM glucose extender) served as the sole independent treatment factor. Based on the measured spermatozoal concentration of the pooled fresh semen, precise aliquots of semen and extender were combined so that each designated treatment volume delivered exactly 100 million spermatozoa.

Table 1. Experimental treatments detailing total insemination volume, fixed sperm dose, and resulting spermatozoal concentration in the inseminated suspension

Treatment	Total insemination volume	Sperm dose
P0	0.1 mL	100 million spermatozoa
P1	0.2 mL	100 million spermatozoa
P2	0.3 mL	100 million spermatozoa
P3	0.4 mL	100 million spermatozoa
P4	0.5 mL	100 million spermatozoa

In the experimental design, the 0.1 mL treatment (P0) served as the positive baseline control, reflecting the conventional insemination volume widely adopted in routine poultry artificial insemination protocols. An uninseminated negative control group or an extender-only sham control group was excluded because laying hens housed in individual battery cages without male contact naturally produce 0% fertile eggs, rendering such groups biologically uninformative for evaluating volume-dependent sperm transport. Furthermore, an undiluted neat semen control was not feasible because fresh Kampung rooster semen exhibits high sperm density; consequently, delivering a fixed dose of 100 million spermatozoa without dilution would require an extremely minute volume (<0.05 mL), which cannot be accurately metered or deposited without substantial dead-space retention inside the insemination pipette.

E. Artificial Insemination

Artificial insemination was performed by depositing the prepared semen suspension into the reproductive tract of each laying hen. The same insemination procedure was applied to all treatment groups. Particular care was taken during insemination to minimize loss of the inseminated suspension.

F. Experimental Design

The experiment was arranged in a completely randomized design (CRD) comprising five treatments with 10 laying hens (replicates) assigned randomly to each treatment group (N =

50). The total sperm dose was held strictly constant at 100 × 106 (100 million) spermatozoa per hen across all experimental groups, while total insemination volume (comprising neat semen plus skim milk–50 mM glucose extender) served as the sole independent treatment factor. The linear statistical model applied for the completely randomized design was:

$$Yij = \mu + Ti + \varepsilon ij$$

where Yij represents the observed reproductive response (fertility or fertile period) of the j-th hen receiving the i-th insemination volume treatment, μ is the overall population mean, Ti is the fixed effect of the i-th total insemination volume (i = 1, 2, 3, 4, 5), and εij is the random experimental error associated with the ij-th observation, assumed to be independently and normally distributed with a mean of zero and constant variance σ2.

G. Fertility Determination

Eggs produced from artificial inseminations were collected and examined for fertility. Eggs displaying a distinct embryonic vascular network ("spider web" blood vessels) and dark embryonic spot were classified as fertile. Clear eggs lacking visible vascular development were broken open (egg break-out examination) to differentiate true infertile blastodiscs from early embryonic mortality; eggs exhibiting early blastodermal development were scored as fertile. Fertility was calculated using the following equation:

$$\text{Fertility (\%)} = (\text{number of fertile eggs} / \text{total number of eggs examined}) \times 100$$

The treatment fertility values represent the arithmetic mean and standard deviation of the 10 individual hens within each treatment group.

H. Fertile Period Determination

The fertile period was defined as the duration of sustained fertilization following the single artificial insemination, expressed as the number of days from the day after insemination until the last fertile egg was laid prior to the onset of consecutive infertile eggs The values were expressed as mean ± standard deviation for each treatment.

I. Statistical Analysis

Data on percentage fertility and fertile-period duration were checked for normality and homogeneity of variance prior to analysis. Because percentage fertility values were uniformly high (>80%) and exhibited homogeneous variances across groups, data were analyzed directly using one-way analysis of variance (ANOVA) under the completely randomized design. Statistical significance was declared at $P < 0.05$. Because neither fertility nor fertile period differed significantly among treatments ($P > 0.05$), post-hoc multiple comparison tests were not required, and observed variations among treatment means were interpreted descriptively alongside biological and physiological context.

III. RESULTS AND DISCUSSIONS

A. Result

Baseline Semen Quality and Dilution Consistency

Pooled fresh ejaculates collected from mature Kampung roosters met all pre-established quality thresholds prior to extension, exhibiting a creamy-white color, absence of contaminants, and progressive individual motility of approximately 85%. Dilution with the

skim milk–50 mM glucose extender proceeded smoothly across all five dilution ratios, yielding homogenous suspensions delivering a standardized dose of 100 million spermatozoa per hen.

Fertility

The mean fertility rates of laying hens inseminated with 100 million Kampung rooster spermatozoa across total insemination volumes ranging from 0.1 to 0.5 mL are summarized in Table 2.

Table 2. Fertility of laying hens inseminated with 100 million Kampung rooster spermatozoa at different total insemination volumes

Treatment	Total volume (mL)	Fertility (%)
P0	0.1	88.11 ± 11.00
P1	0.2	90.00 ± 10.95
P2	0.3	92.00 ± 10.95
P3	0.4	87.00 ± 10.33
P4	0.5	84.00 ± 8.94
Significance		**ns**

Notes: Values are expressed as mean ± standard deviation (n = 10 hens per treatment); ns = not significant (P > 0.05).

Across all five treatment groups, overall fertility was consistently high, ranging from 84.00% to 92.00%, with an overall grand mean of 88.22%. One-way ANOVA demonstrated that total insemination volume had no statistically significant effect on egg fertility (P > 0.05).

Descriptively, fertility exhibited a clear curvilinear numerical trend across the tested volume gradient. Mean fertility increased gradually from 88.11 ± 11.00% in the standard baseline control group (P0, 0.1 mL) to 90.00 ± 10.95% at 0.2 mL (P1), reaching a numerical peak of 92.00 ± 10.95% at 0.3 mL (P2). Beyond 0.3 mL, mean fertility declined numerically to 87.00 ± 10.33% at 0.4 mL (P3) and reached its lowest numerical value of 84.00 ± 8.94% at 0.5 mL (P4). Notably, within-treatment variability—reflected by standard deviations ranging from 8.94% to 11.00%—was relatively consistent across all groups, indicating comparable hen-to-hen variation regardless of the suspension volume administered.

Fertile Period

The duration of the fertile period following a single artificial insemination across the five volume treatments is presented in Table 3.

Table 3. Fertile period of laying hens inseminated with 100 million Kampung rooster spermatozoa at different total insemination volumes

Treatment	Total volume (mL)	Fertile period (days)
P0	0.1	12.6 ± 2.10
P1	0.2	13.4 ± 1.64
P2	0.3	13.6 ± 1.83
P3	0.4	13.0 ± 2.10
P4	0.5	12.3 ± 2.16
Significance		**ns**

Notes: Values are expressed as mean ± standard deviation (n = 10 hens per treatment); ns = not significant (P > 0.05).

Consistent with the fertility findings, one-way ANOVA indicated no statistically significant differences in the duration of the fertile period among the five insemination volumes ($P > 0.05$). Treatment means spanned a narrow range of 1.30 days, from 12.30 to 13.60 days, with an overall experimental mean of 12.98 days.

The numerical pattern observed for the fertile period mirrored that of percentage fertility. Starting from 12.60 ± 2.10 days in the 0.1 mL baseline control (P0), the fertile period increased numerically to 13.40 ± 1.64 days at 0.2 mL (P1) and attained its longest numerical duration of 13.60 ± 1.83 days at 0.3 mL (P2). Thereafter, the fertile period shortened slightly to 13.00 ± 2.10 days at 0.4 mL (P3) and 12.30 ± 2.16 days at 0.5 mL (P4). Standard deviations across groups remained tightly clustered between 1.64 and 2.16 days, demonstrating uniform persistence of oviductal sperm storage across all tested volumes.

B. Discussion

The present study demonstrated that administering a fixed dose of 100 million Kampung rooster spermatozoa suspended in a skim milk-50mM glucose extender produced consistently high fertility (84.00-92.00%) and prolonged fertile periods (12.30-13.60 days) across total insemination volumes ranging from 0.1 to 0.5 mL. Crucially, statistical analysis revealed no significant effect of total insemination volume on either reproductive parameter ($P > 0.05$). Under the standardized conditions of this experiment, varying the carrier fluid volume fivefold did not significantly alter the fertilizing efficiency or storage longevity of Kampung rooster spermatozoa once the total sperm number delivered per hen was fixed at 100 million.

These results underscore the fundamental principle in avian reproductive physiology that total viable sperm dose and intrinsic semen quality outweigh moderate variations in carrier fluid volume. Because the experimental protocol standardized initial sperm motility (~85%), extender composition (skim milk supplemented with 50 mM glucose), pre-insemination holding time, and intravaginal deposition depth across all treatments, the experiment successfully isolated total suspension volume as the sole independent variable. The lack of significant divergence among treatments indicates that the reproductive tract of the laying hen possesses sufficient physiological adaptability to accommodate insemination volumes between 0.1 and 0.5 mL without impairing the recruitment of spermatozoa into the oviductal storage reservoirs.

Role of the 0.1 mL Baseline Control and Rationale for Experimental Control Selection

An important methodological aspect of the present investigation is the function of the 0.1 mL treatment (P0) as the positive baseline control. In both commercial poultry production and experimental avian reproduction, an insemination volume of approximately 0.1 mL is widely adopted as the standard reference protocol. In our study, P0 achieved a high mean fertility of 88.11 ± 11.00% and a fertile period of 12.60 ± 2.10 days, confirming that the conventional 0.1 mL volume containing 100 million Kampung rooster spermatozoa in a skim milk–50 mM glucose extender provides a robust physiological benchmark. The absence of statistically significant differences between P0 and the higher-volume treatments (P1 through P4, spanning 0.2 to 0.5 mL) demonstrates that increasing the volume of carrier fluid up to five times above the standard control level does not confer a statistically superior reproductive advantage.

Physiological Mechanisms of Sperm Selection, UVJ Storage, and Fertile Period Persistence

The high fertility and sustained fertile periods observed across all volume treatments align closely with the established biology of sperm transport and storage in the avian female reproductive tract. Following intravaginal deposition, spermatozoa do not swim directly to the site of fertilization in large numbers; instead, they undergo rigorous physiological selection within the vagina before entering the sperm storage tubules located predominantly at the uterovaginal junction (BRILLARD, 1993; Hemmings et al., 2015).

This finding is consistent with the biological characteristics of sperm transport and storage in the hen. Following AI, only a small proportion of inseminated spermatozoa is selected for transport to the sperm storage tubules. (BRILLARD, 1993) demonstrated that less than 1% of inseminated spermatozoa successfully traverse the vagina and colonize the SSTs, highlighting the highly selective barrier function of the lower oviduct. Because this selection mechanism filters spermatozoa primarily on the basis of progressive motility, membrane integrity, and surface biochemical compatibility rather than total fluid volume, delivering 100 million high-motility (~85%) spermatozoa ensured that an adequate subpopulation of competent cells reached the UVJ across all treatments.

The sustained fertile period of 12.30 to 13.60 days observed across groups reflects the gradual, regulated release of spermatozoa from the UVJ sperm storage tubules toward the infundibulum over successive ovulatory cycles (Hemmings et al., 2015). Brillard & Antoine, (1990) demonstrated a strong quantitative correlation between the number of spermatozoa inseminated, the number of spermatozoa stored within the UVJ tubules, and the density of spermatozoa subsequently trapped on the perivitelline layer of laid eggs. Furthermore, Wishart, (1987) established that the number of spermatozoa associated with consecutive eggs declines progressively with increasing time after insemination, and that the duration of the fertile period is directly governed by the rate at which oviductal sperm reserves deplete below the minimum fertilization threshold. In the present study, maintaining a constant insemination dose of 100 million spermatozoa across P0–P4 likely resulted in comparable initial colonization of the UVJ sperm storage tubules, thereby producing statistically indistinguishable fertile-period durations across all five volumes.

Interpretation of the Numerical Optimum at 0.3 mL and Fluid Dynamics at Higher Volumes

Although statistical analysis confirmed no significant differences among treatments, the dataset exhibited a coherent, parallel curvilinear pattern in both reproductive traits. Specifically, mean fertility rose numerically from 88.11% at 0.1 mL to 90.00% at 0.2 mL and peaked at 92.00% at 0.3 mL, before tapering off to 87.00% at 0.4 mL and 84.00% at 0.5 mL. Exactly the same trajectory was observed for the fertile period, which lengthened numerically from 12.60 days at 0.1 mL to 13.40 days at 0.2 mL and peaked at 13.60 days at 0.3 mL, followed by declines to 13.00 days at 0.4 mL and 12.30 days at 0.5 mL.

This parallel numerical trend suggests that an intermediate total insemination volume of 0.3 mL may provide particularly favorable physical conditions for spermatozoal distribution and retention within the hen's vagina. At 0.1 mL, a small fraction of the suspension may remain adhered to the insemination cannula or disperse less broadly across the mucosal folds leading toward the UVJ, although the high fertility achieved in P0 (88.11%) confirms that 0.1 mL remains highly effective when insemination technique is properly executed. Increasing the volume to 0.2 and 0.3 mL may facilitate gentler, more uniform dispersion of the 100 million spermatozoa along the vaginal lumen, potentially enhancing contact with the UVJ orifice without overwhelming vaginal capacity.

Conversely, the numerical declines observed at 0.4 mL (87.00% fertility; 13.00 days) and 0.5 mL (84.00% fertility; 12.30 days) suggest that expanding the fluid volume beyond 0.3 mL provides no additional benefit and may introduce mild physical constraints. Two plausible physiological mechanisms could account for this slight downward numerical shift at higher

volumes. First, the avian vagina is a muscular, folded tubular organ with a finite luminal capacity; introducing 0.4 to 0.5 mL of liquid may stimulate local stretch receptors and smooth-muscle contractions, increasing the likelihood of partial retrograde fluid expulsion (backflow) through the cloaca immediately after withdrawal of the insemination straw. Any loss of fluid prior to spermatozoal migration into the UVJ would proportionally reduce the effective number of spermatozoa available for SST colonization. Second, increasing the total volume from 0.1 to 0.5 mL at a fixed sperm dose reduces spermatozoal concentration fivefold (from 1,000 × 106 to 200 × 106 cells/mL) and dilutes native seminal plasma components fivefold. Excessive dilution of seminal plasma proteins and ions can slightly alter local fluid viscosity and surface interactions within the vaginal mucus. Nevertheless, because post-insemination fluid backflow and luminal sperm retention were not directly quantified in this study, these mechanisms should be regarded as physiological hypotheses rather than confirmed causes. Equally important, because the numerical reductions at 0.4 and 0.5 mL were not statistically significant ($P > 0.05$), larger volumes within the tested range should not be interpreted as biologically detrimental.

Protective Role of the Skim Milk–50 mM Glucose Extender and Pre-Insemination Handling

A critical factor enabling high fertility even at the highest dilution ratio was the use of a standardized skim milk–50 mM glucose extender combined with prompt insemination after semen collection. When rooster spermatozoa are diluted into larger fluid volumes, they become more vulnerable to dilution-induced membrane destabilization and energy depletion if the carrier medium lacks adequate macromolecular protection and metabolic substrates. In skim-milk-based extenders, heat-treated casein micelles interact with the spermatozoal plasma membrane to stabilize lipid bilayers and buffer against ionic shifts, while the supplementation of 50 mM glucose provides a readily metabolizable glycolytic substrate to support flagellar adenosine triphosphate (ATP) synthesis during transit through the vagina.

Our findings build directly upon prior investigations by Saleh & Susanto (2025) who demonstrated that skim milk supplemented with 50 mM glucose effectively preserves motility, membrane viability, and fertilizing potential in Indonesian native rooster spermatozoa.

Furthermore, Saleh et al., (2026) showed that Kampung rooster spermatozoa diluted in a skim milk–glucose extender maintain high fertility and extended fertile periods when inseminated shortly after collection, whereas prolonged exposure to room temperature causes progressive declines in motility and reproductive performance. By performing all inseminations promptly after semen collection and extension, the present experiment prevented time-dependent thermal or oxidative deterioration, ensuring that the physiological competence of the 100 million spermatozoa remained uniform across all five volume groups.

Practical Implications

From an applied breeding and farm management perspective, the findings of this study offer valuable operational flexibility for Kampung chicken AI programs. Because insemination volumes between 0.1 and 0.5 mL yielded statistically comparable fertility (84.00–92.00%) and fertile-period durations (12.30–13.60 days) at a constant dose of 100 million spermatozoa, AI technicians can adjust dilution volumes within this range according to ejaculate concentration and syringe calibration without compromising flock fertility. Where extender economy and rapid preparation are prioritized, the standard 0.1 mL volume (P0) remains completely sufficient, delivering nearly 90% fertility and a fertile period exceeding 12.5 days. Conversely, when working with highly concentrated ejaculates where slightly larger volumes facilitate easier pipetting and dosing accuracy, a volume of 0.2 to 0.3

mL—particularly 0.3 mL, which achieved the highest numerical fertility (92.00%) and longest fertile period (13.60 days)—represents an attractive practical choice.

IV. CONCLUSION

Total insemination volumes of 0.1, 0.2, 0.3, 0.4, and 0.5 mL, each containing a standardized dose of 100 million Kampung rooster spermatozoa suspended in a skim milk–50 mM glucose extender, did not significantly affect egg fertility or fertile-period duration in laying hens ($P > 0.05$). Overall fertility remained high across all groups, ranging from 84.00% to 92.00%, while the fertile period ranged from 12.30 to 13.60 days. The 0.1 mL positive baseline control (P0) achieved reliable reproductive performance (88.11 ± 11.00% fertility and 12.60 ± 2.10 days fertile period), whereas the 0.3 mL treatment (P2) yielded the highest numerical fertility (92.00 ± 10.95%) and longest numerical fertile period (13.60 ± 1.83 days). These findings demonstrate that within the 0.1 to 0.5 mL range, total insemination volume exerts minimal influence on reproductive outcomes provided that a sufficient dose of 100 million viable spermatozoa is accurately deposited into the female reproductive tract. Based on its favorable numerical response, 0.3 mL represents the most promising suspension volume for further investigation in Kampung chicken artificial insemination protocols.

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