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Developmental Potential and Efficiency of Black Bengal Goat Embryo Production by using Intracytoplasmic Sperm Injection and In Vitro Fertilization

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ABSTRACT

This study aimed to optimize an intracytoplasmic sperm injection (ICSI) for *in vitro* embryo production in black bengal goats and compare its efficiency with *in vitro* fertilization (IVF). A total of 744 oocytes were retrieved from 269 slaughter-house-derived ovaries, with an average recovery rate of 2.76 oocytes per ovary. Only grade I and II oocytes (n = 534) were selected for maturation, of which 376 (70.41%) reached metaphase II. For ICSI, epididymal sperm with broken tails were microinjected into mature oocytes using a 5 µm micropipette, yielding a high survival rate (83.21%) and a low degeneration rate (17.78%, $p < 0.05$). Chemical activation using ionomycin, cycloheximide (CHX), and 6-DMAP resulted in a 74.44% cleavage rate ($p<0.05$). Developmental rates in the ICSI group (n = 90) were: 74.44% (2-cell), 63.33% (4-8 cell), 44.44% (8-16 cell), 23.33% (morula), and 14.44% (blastocyst). A comparative analysis revealed that ICSI resulted in higher early cleavage rates (73.40 ± 3.12%) compared to IVF (64.06 ± 2.44%). In contrast, IVF produced a greater proportion of morulae (37.90 ± 3.15%), while ICSI resulted in 23.20 ± 2.73%. Similarly, blastocyst formation was higher in the IVF group (21.05 ± 2.09%) than in the ICSI group (14.56 ± 1.75%). These findings suggest that ICSI is equally effective as IVF in generating competent embryos. With ICSI achieving higher early cleavage rates and IVF supporting better development at later stages. ICSI can therefore be considered a practical alternative to IVF for goat embryo production; however, it requires further study with larger cohort. Additionally, the study demonstrates that using a fine 5 µm micropipette for ICSI, offers a precise, efficient, and cost-effective technique, especially well-suited for utilizing oocytes from slaughterhouse sources and sperm retrieved from the epididymis.

Keywords: Epididymal Sperm injection, Goat, Embryo Development, *In vitro*, Intracytoplasmic Sperm injection (ICSI), *In Vitro* Fertilization (IVF).

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INTRODUCTION

Reproductive biotechnologies play a crucial role in enhancing genetic improvement in livestock populations by increasing the influence of superior genotypes. Among assisted reproductive technologies, intracytoplasmic sperm injection (ICSI) represents a significant advancement. Recent interest has emerged in utilizing sperm-mediated gene transfer (SMGT) as an efficient and practical approach for generating transgenic animals. For this objective, goat sperm is also being used as DNA carriers in livestock. Sperm quality traits have low heritability values which make traditional genetic selection little efficient to its improvement (Serrano *et al.*, 2021). The different indications of ICSI in livestock species are mostly production focused. ICSI is the recommended ART especially when conventional methods such as IVF fail. However, in ruminants, future improvements are required for ICSI to be more widely used (Briski and Salamone, 2022). While ICSI has been extensively studied in cattle and sheep, its application in goats remains relatively limited. This technique involves the direct microinjection of a single sperm cell into the ooplasm of a mature oocyte using a micromanipulation pipette. By bypassing natural fertilization barriers, such as the cumulus-corona complex, zona pellucida, and oolemma, ICSI enables precise sperm deposition into the ooplasm (Wang *et al.*, 2003). This method provides an effective model for investigating fundamental fertilization processes, including oocyte activation, sperm decondensation, acrosome reaction, and pronuclear formation (Rahman *et al.*, 2007). Slaughterhouse-derived ovaries offer a cost-effective and abundant source of oocytes for in vitro embryo production (IVEP), minimizing genetic loss due to follicular atresia (Hasler, 1998). In a study conducted by Widjiati *et al.* (2020) on Kacang goats, it was observed that the cleavage rate in embryos generated through ICSI was significantly higher, which was attributed to method's enhanced capacity to resist fertilization failure. The objective of the present study was to standardize a protocol for producing ICSI-derived goat embryos from in vitro-matured oocytes and to assess their developmental potential in comparison to embryos generated through conventional IVF specifically within the context of goat breeding practices in India.

MATERIALS AND METHODS

Oocytes recovery and in *vitro* maturation

Oocytes were aspirated from 2-6 mm follicles of goat ovaries collected from a small animal slaughterhouse. Grade I and II oocytes were matured in Medium 199 (Hyclone, USA) supplemented with Earle's salts, L-glutamine, sodium bicarbonate, 25 mM HEPES, 7.5% (v/v) Fetal Bovine Serum (Hyclone, USA), 10 µg/ml FSH,10 µg/ml LH and 1 µg/ml estradiol. Cumulus-oocyte Complexes (COCs) were matured in groups of 20-25 in 50 µl maturation medium drops in 35 mm petri dishes, over laid with sterile pre-equilibrated mineral oil. Incubation was performed at 38.5°C with 5% $CO_2$and 95% relative humidity in CO_2 incubator for 27 h. Cumulus expansion wasused as a criterion for oocyte maturation.

Semen treatment and oocyte preparation

Goat epididymal semen was collected within 3–4 hours post-slaughter. The epididymis was washed 3–4 times with pre-warmed (37°C) saline containing penicillin (400 IU/ml) and streptomycin (500 µg/ml). After trimming extraneous tissue, it was sequentially rinsed twice with DPBS, followed by 70% ethanol and DPBS. Epididymal sperm were retrieved by making multiple incisions in the cauda epididymides and washing with BO medium in a 15 ml centrifuge tube. The sperm washing medium was prepared by mixing 19 ml BO-A with 9 ml BO-B, 50 µl gentamicin, and 0.3% BSA. Fresh semen (1 ml) was diluted in 9 ml pre-equilibrated BO-A, centrifuged at 1000 rpm for 10 min, resuspended in 5 ml fresh medium, and centrifuged again. The motile sperm fraction was collected using the swim-up method, resuspended in 2 ml fertilization medium (8 ml BO-A, 4 ml BO-B, 50 µl gentamicin, 0.6% BSA, 30 µg/ml heparin), and incubated at 38.5°C with 5% CO_2 for 1 hour for capacitation. After in vitro maturation, Grade I and II COCs with expanded cumulus were partially decumulated by pipetting for IVF. For ICSI, oocytes were fully denuded using 0.5 mg/ml hyaluronidase. Oocytes with the first polar body were selected and maintained in IVM medium at 38.5–39°C with 5% CO_2 until further use.

In vitro fertilization

Matured oocytes were collected from IVM drops, washed 2-3 times in fertilization medium and transferred to 50 µl of fertilization medium drops in 35 mm dishes, covered with sterile mineral oil. Oocytes (15-20 per drop) were co-incubated with sperm in a fertilization medium containing heparin, BSA, and essential components. The final sperm concentration was adjusted to 1-2 million sperm/ml, based on live sperm percentage. Fertilization was conducted at 38.5°C and finally incubated at 5 percent CO_2 at 38.5° for 18 hours.

Intracytoplasmic sperm injection

ICSI was performed in a 35 mm petri dish under an inverted microscope (Nikon, Japan) with Narishige hydraulic micromanipulators. The holding and injection pipettes had inner diameters of 15 µm and 5 µm, respectively, with a 35° bevel angle. A 1 µl washed sperm suspension was mixed with 5 µl injection medium (TCM-199, 10% FBS, 10% PVP). Matured oocytes were placed individually in 5 µl microdrops under mineral oil. Sperm immobilization was done by aspirating the head into the injection pipette and breaking the tail. Each metaphase II oocyte, aligned with its polar body at 6 or 12 o'clock, was injected with sperm at 9 o'clock after zona pellucida penetration at 3 o'clock. Cytoplasm aspiration confirmed membrane rupture before sperm injection (<5 pl medium). Sham injections used vehicle medium as controls.

Activation of goat oocytes

Briefly, oocytes were exposed to 5 mM ionomycin in TCM-199 (Sigma, USA) supplemented with 10% FBS for 5 min, washed thrice in the same medium and transferred to TCM-199 containing 2 mm 6-DMAP and 2 mM cycloheximide for 4 h (Ongeri *et al.*, 2001). The oocytes were then washed and placed into 50 µl drops of IVF medium.

Culture of zygotes

After 18 h of co-incubation, IVF-derived oocytes and activated ICSI oocytes showing male and female polar bodies (identified by sperm tail presence) were transferred to 50 µl drop of pre-equilibrated RVCL media (Cook®, USA) with 1% fattyacid-free bovine serum albumin (FAF-BSA) in 35 mm petri dish covered with mineral oil. Dishes were incubated in humidified chamber at 38.5°C with 5% CO_2 for 72 h. Embryo development was monitored and only cleaved embryos were transferred to blastocyst media (Cook®, USA) with 1% BSA for additional 5-7 days. Embryonic developmental stages were recorded throughout the period.

All plasticwares were purchased from Falconware, Becton-Dickinson (Bedford, MA, USA) and chemicals/biochemicals were sourced from Sigma Chemical co. (St. Louis, MO, USA), unless otherwise specified.

Statistical analysis

The results are presented as means with standard error of the means (Mean ±SEM). Statistical comparison among the IVF, ICSI groups were conducted using Chi- square test. A p-value <0.05 was considered statistically significant. Statistical analysis was performed using GraphPad Prism Version 5.00 (Graph Pad Software, San Diego, California).

RESULTS AND DISCUSSION

A total of 744 oocytes were retrieved from 269 goat ovaries obtained from a slaughterhouse, yielding average retrieval rate of 2.76 oocytes per ovary. Among these, 287 (38.58%) were considered as grade I, 247 (33.20%) as grade II and 206 (27.69%) as grade III.Four numbers (0.53%) lost in processing and grading. Only grade I and II oocytes (n=534) were selected for *in vitro* maturation. After culture, 376 oocytes (70.41%) exhibited the first polar body formation, indicating successful maturation. The efficiency rate of ICSI was optimized using sharp microinjection pipette of 5 µm diameter (Figure 1), ensuring minimal mechanical damage to oocytes. A low proportion of oocytes (16.79%, n=90) were damaged during injection, while a high survival rate (83.21%, n=90) was observed post-injection. Additionally, the degeneration rate remained low (17.78%, n=90) during 13-21 hours of *in vitro* culture, indicating minimal cellular damage due to fine microinjection pipette. Post-ICSI, chemical activation was necessary for successful oocyte activation and cleavage. Chemical activation was induced using ionomycin, cycloheximide, and 6-DMAP, resulted in a high cleavage rate (74.44%, n=90), demonstrating the efficacy of this activation protocol. The fine microinjection pipette showed high survival rate (83.21%) than the proportion of damaged oocytes (16.79%). Furthermore, the degeneration rate (17.78%) was lower in surviving oocytes compared to those discarded further supporting the precision of the technique. Activated zygotes exhibited higher activation rate (74.44%) than the degenerated (17.78%) or non-activated oocytes (21.11%), highlighting the effectiveness of the chemical activation protocol. However, no remarkable differences were observed between degenerated (17.78%) and non-activated oocytes (21.11%) indicating that while ICSI is efficient, some oocytes fail to respond to activation.

The developmental potential of goat embryos generated by ICSI, IVF and sham injection is shown in table 1. In the ICSI group (n=90 oocytes), cleavage to the 2-cell stage was observed in 67 (74.44%). Further culturing the cleaved oocytes led to development of 57 (63.33%) 4-8 cell embryos, 40 (44.44%) 8-16 cell embryos, 21 (23.33%) morulaeand13 (14.44%) blastocyst. In the IVF group (n=155 oocytes), 101 oocytes (65.16%) cleaved to the 2-cells stage, with subsequent development into 89 (57.42%) 4-8 cell embryos, 76 (49.03%) 8-16 cell embryos, 60 (38.71%)

morulae and36 (23.23%) blastocysts. In sham injected control group, no cleavage or embryonic development was observed in 22 oocytes shown in figure 2. When comparing ICSI and IVF groupsembryos the proportion of morulae was higher in IVF group (37.90±3.15%) than in the ICSI group (23.20±2.73%). Although the ICSI group initially exhibited a higher proportion of early-stage embryos, no significant differences ($p<0.05$) were observed between the two techniques at the later developmental stages. Both ICSI and IVF techniques yielded similar proportions of embryos that were developmentally distinct and equally competent.

This study successfully optimized a manual ICSI protocol for in vitro embryo production in goats, utilizing slaughterhouse-derived oocytes and epididymal sperm. Pawshe and Totey (2023) used slaughterhouse ovaries to establish optimal culture condition for in vitro fertilization of goat. The use of 5 µm microinjection pipette proved to be efficient for sperm injection and yielded high survival rate (83.21%), with minimal oocyte damage (16.78%). The degeneration rate (17.78%) during 13–21 hours of in vitro culture was also low, confirming the efficiency of the injection technique. These results align with Palermo *et al.* (1992), where optimized sperm handling significantly increased fertilization rates.

Table 1. Developmental potency of intracytoplasmic sperm injection, in vitro fertilization and sham injection group of goat embryos

Groups	*N* of oocyte Taken for evaluation	2 cells (%)	4-8 cells (%)	8-16 cells (%)	Morula (%)	Blastocyst (%)
In vitro fertilization[a]	90	64.06 ±2.44	56.81 ±3.05	48.45 ±2.58	37.90 ±3.15	21.05 ±2.09
Intra Cytoplasmic sperm injection[b]	155	73.40 ±3.12	65.46 ±5.12	44.09 ±3.92	23.20 ±2.73	14.56 ±1.75
Sham injection	22	0	0	0	0	0

[ab]Test groups having different superscript within columns are significantly different ($p<0.05$)

Chemical activation with ionomycin, cyclohexamide and 6-DMAP led to 63.33% activation rate which was found to be higher than 38.6% reported by Jiménez-Macedo *et al.* (2005). Additionally, the non-activation rate (21.11%) was lower than their reported 42.8%. Sham-injected controls (n=22) showed no embryonic development, confirming that fertilization, not parthenogenesis, drove embryo formation. A comparative analysis of ICSI vs. IVF-derived embryos revealed key developmental differences. ICSI resulted in a higher cleavage rate (73.40 ± 3.12%) compared to IVF (64.06 ± 2.44%), indicating superior early-stage embryo formation. However, IVF embryos exhibited significantly higher development at later stages, with morula formation rates of 37.90 ± 3.15% in IVF vs. 23.20 ± 2.73% in ICSI and blastocyst rates of 21.05 ± 2.09% in IVF vs. 14.56 ± 1.75% in ICSI. These results suggest that while ICSI enhances early cleavage efficiency, IVF provides a more supportive environment for morula and blastocyst formation. This aligns with Jiménez-Macedo *et al.* (2005), who reported higher 8–16 cell embryo formation in ICSI (22.8%) than in IVF (10.3%) and higher zygote formation in ICSI (40%) vs. IVF (25.1%).

Table 2: Micromanipulation and chemical activation (ionomycin+cyclohexamide+6-dimethylaminopyridine) efficiency of intracytoplasmic sperm injection

Trial *n*	*N* of oocytes injected	*N* of (%) oocytes damaged By injection	*N* of (%) oocytes surviving The injection	*N* of (%) oocytes discarded	*N* of (%) intact oocytes taken for chemical activation	*N* of (%) Oocytes Degenerated During 13-21 hours Culture	*N* of (%) activated zygote (fertilized)	*N* of (%) oocytes not activated
1	13	3 (23.08)	10 (76.92)	1 (7.69)	9 (69.23)	1 (11.11)	6 (66.67)	2 (22.22)
2	15	4 (26.67)	11 (73.33)	1 (6.67)	8 (53.33)	1 (12.50)	6 (75.00)	3 (37.50)
3	16	5 (31.25)	11 (68.75)	2 (12.50)	10 (62.50)	1 (10.00)	7 (70.00)	1 (10.00)
4	11	2 (18.18)	9 (81.82)	1 (9.09)	8 (72.72)	2 (25.00)	4 (50.00)	2 (25.00)
5	13	2 (15.38)	11 (84.62)	2 (15.38)	9 (69.23)	2 (22.22)	4 (44.44)	3 (33.33)
6	11	1 (9.09)	10 (90.91)	2 (18.18)	9 (81.82)	1 (11.11)	6 (66.67)	1 (11.11)

7	9	1 (11.11)	8 (88.89)	1 (11.11)	5 (55.56)	3 (60.00)	3 (60.00)	1 (20.00)
8	10	2 (20.00)	8 (80.00)	3 (30.00)	7 (70.00)	1 (14.28)	2 (28.57)	2 (28.57)
9	17	1 (5.88)	16 (94.11)	3 (17.65)	14 (82.35)	2 (14.29)	9 (64.29)	2 (14.29)
10	16	1 (6.25)	15 (93.75)	1 (6.25)	11 (68.75)	2 (18.18)	10 (90.91)	2 (18.18)
Total	131	22 (16.79)	109(83.31)	17 (12.98)	90 (68.70)	16 (17.78)	57 (63.33)	19 (21.11)

Values within the parenthesis indicate the percentage in each group; N (%) of activated zygotes (fertilized), not activated and degenerated are calculated from number of intact oocytes taken for chemical activation.

Sperm permeabilization and decondensation are crucial for ICSI success, typically achieved via tail cutting (Kasai *et al.*, 1999). In this study, tail breaking using a microinjection pipette was successfully employed, reducing procedural complexity and increasing efficiency. Unlike Wang *et al.* (2003), who required piezo-drill-assisted penetration, our manual technique achieved comparable developmental rates in a cost-effective manner. Immobilizing sperm by breaking the tail before ICSI increased blastocyst yield (Eloriaga *et al.*, 2024). Furthermore, sperm motility was not essential for ICSI success. Consistent with Kimura and Yanagimachi (1995), motile sperm caused oocyte degeneration post-injection due to persistent movement within the ooplasm, preventing normal development beyond cleavage. The high elasticity of goat metaphase II oocytes (Wang *et al.*, 2003; Gagne *et al.*, 1995) posed challenges for oolemma penetration, leading to sperm expulsion from the ooplasmic furrow. To address this, we optimized the injection pipette dimensions (5 µm inner, 7 µm outer diameter), minimizing oocyte damage (17.78%). Successful penetration was confirmed via cytoplasm aspiration and reinjection. We also observed challenges in oolemma penetration, attributed to the high elasticity of goat metaphase II oocytes (Wang *et al.*, 2003; Gagne *et al.*, 1995). By optimizing the injection pipette dimensions (5 µm inner, 7 µm outer diameter) and confirming penetration via cytoplasm aspiration, we minimized oocyte expulsion and damage (17.78%). For in vitro embryo production (IVEP), selecting an appropriate culture system is critical. Using RVCL-blast-BSA sequential media, ICSI-derived embryos exhibited 74.44% cleavage (67/90), 63.33% (4–8 cell), 44.44% (8–16 cell), 23.33% morula, and 14.44% blastocyst formation, aligning with manual ICSI studies (Jiménez-Macedo *et al.*, 2005; Rahman *et al.*, 2007). However, blastocyst rates were lower than Wang *et al.* (2003) (21.1%), possibly due to differences in culture media composition. In case of cryopreserved black Bengal buck semen, RVCL media have competence to produce embryos and could be used for embryo development through in vitro fertilization (Kumar *et al.*, 2020).

In conclusion, this study optimized a manual ICSI protocol for goat embryo production, showing that using a fine injection pipette enhances oocyte survival and reduces mechanical damage. Chemical activation improved cleavage rates, addressing activation challengein goats. Unlike previous piezo-assisted injection methods, our approach offers a cost-effective alternative with comparable early-stage development outcomes. ICSI is found to be as effective as IVF, with higher early cleavage rates and better later-stage development in IVF. ICSI can therefore be considered a practical alternative to IVF for goat embryo production. Where conventional IVF failed due to low sperm motility, low sperm count ICSI is generally adopted for in vitro embryo production. ICSI is a promising tool for genetic rescue of endangered and wild species (Salamone *et al.*, 2017). The findings bridge a critical gap in optimizing ICSI for small ruminants, offering a viable method for embryo production using slaughterhouse-derived oocytes and epididymal sperm.

CONCLUSION

It can be concluded that immobilized sperm with breaking tail microinjected by sharp injection pipette after chemical activation is an efficient approach for goat embryo production by ICSI. Chemical activation with Ionomycin, CHX and 6-DMAP was found to be effective. ICSI with sharp micropipette of 5 µm diameter can be applied to assisted reproduction for *in vitro* goat embryo production.

CONFLICT OF INTEREST

None

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