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REVIEW ARTICLE

IN VITRO PRODUCTION OF GOAT EMBRYO IN BANGLADESH- A SHORT REVIEW WITH SPECIAL REFERENCE TO BLACK BENGAL GOAT

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ABSTRACT

In vitro production (IVP) of embryo is one of the most propitious assisted reproductive technology (ART) adopted in modern livestock industry. Bangladesh, a developing country has been working enormously to adopt this technique in various livestock species. As goat is the most promising small ruminant of this country, several research work has been ongoing to adopt IVP on this species, more specifically on Black Bengal goat (BBg), the only recognized breed of Bangladesh. For conservation, improvisation and increased production of this goat breed ART like IVP is a major pre requisite. IVP of embryos usually includes retrieval of oocytes from the ovarian follicles, in vitro maturation (IVM), sperm capacitation, in vitro fertilization (IVF) of oocytes and in vitro culture (IVC) of presumptive zygotes to morula or blastocyst stage. Research showed, the source and quality of the ovary, oocyte and the cumulus-oocyte-complexes (COCs), semen quality, oocyte recovery time and procedure, culture condition, supplements and the subsequent factors plays a critical role in the maturation and subsequent development of the embryo which can be varied from lab too lab upon researcher's choice. A short review on the IVP of goat embryos is discussed here in the context of recent encouraging data which will aid future researchers aiming at successful embryo production of BBg in vitro.

KEYWORDS

Assisted reproductive technology (ART), Black Bengal goat (BBG), in vitro culture (IVC), in vitro fertilization (IVF), In vitro production (IVP) and in vitro maturation (IVM).

1. INTRODUCTION

In vitro production of embryo is an emerging concern in the livestock industry today. This technology has accelerated the genetic improvement and enhanced production potential of livestock and poultry. Goat is one of the most profitable livestock species especially in the developing countries like Bangladesh. Among the Asiatic countries, Bangladesh has fourth highest population of goats (DLS, 2008) and the population is about 26.1 million (FAO, 2018). It is estimated that more than 90% of the goat population in Bangladesh comprised the Black Bengal, while the remainder being Jamunapari and their crosses (Husain, 1993). Black Bengal goat, a precious germplasm of Bangladesh, is more popular for its high fertility, prolificacy, short generation period, early sexual maturity, delicious meat and superior skin, resistant to disease and found in almost all villages of Bangladesh (Devendra and Burns, 1983). As well as, it plays a major role in the economy of Bangladesh by contributing 1.66% of the GDP (Gross Domestic Product) (DLS, 2017). As a result, the demand of this goat breed is increasing at a dramatic trend this days in Bangladesh which focus on the increase production of this local breed.

In vitro production (IVP) of goat embryos is a rapidly advancing field and it has been improved greatly during the past two decades (Han *et al*., 2006; Katska-Ksiazkiewicz *et al*., 2007). This technology has contributed a lot in production of embryos with high developmental competence that are used in agricultural and biomedical research, and animal biotechnology (Hansen and Block 2004). In Bangladesh, in vitro techniques in goat is an emerging concept and a great deal of work has been done regarding evaluation and grading of ovaries, collection of cumulus oocytes complexes (COCs) from slaughterhouse ovaries and grading of oocytes followed by IVM, IVF of the oocytes and IVC (Ferdous, 2006; Islam *et al*., 2007; Mondal *et al*., 2008; Hoque, 2009).

The IVP of embryos usually includes retrieval of oocytes from the ovarian follicles of a female, in vitro maturation (IVM), sperm capacitation, in vitro fertilization (IVF) of oocytes and in vitro culture (IVC) of presumptive zygotes to the morula or blastocyst stage (Brackett *et al*., 1982). Requirements for in vitro capacitation of mammalian spermatozoa have been investigated extensively. In vitro maturation and fertilization of goat oocytes and subsequent in vitro culture of zygotes and embryos is discussed in the context of recent encouraging data. Mass production of embryos produced by IVP in the future will have a great impact on goat production and goat breeding plan in Bangladesh.

Over the recent years, researchers have been trying to determine which conditions are needed during IVM, IVF, and in vitro development (IVD) processes (Joanna Maeia *et al*., 2014) and which supplementation and positive factors are beneficial to enhance embryo production to optimize the technique, allowing increasing its adoption in Bangladesh in near future. This is an short review of the research works done on Black Bengal

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goat which would be an aid in future research works regarding IVP of goat embryos.

1.1 Oocyte retrieval

A pair of goat ovaries contain thousands of oocytes, but only a fraction of them (0.01 percent) can be used during the female's reproductive lifetime, while the remaining follicles become atretic during their development and maturation (Carroll *et al*., 1990). For in vitro embryo production, oocytes from ovaries can be collected from live animals or from dead and slaughtered animal (Anna *et al*., 2013). From live goat, the best way to collect oocytes non-invasively by LOPU techniques (Bladassarre *et al*., 1996, 2002; cognie *et al*., 2004) but this procedure is costly, and the amount of oocytes retrieved per ovary is insignificant (Pawshe *et al*., 1994). Whereas, the cheapest and most plentiful source of primary oocytes for large-scale development of embryos by (IVM) and (IVF) are the ovaries of slaughtered animals (Agrawal *et al*., 1995). To retrieve oocytes from slaughterhouse ovaries, several methods have been established of which, mainly 3 methods have been followed in case of domestic animals namely, follicular aspiration (Datta *et al*., 1993; Boediono *et al*., 1995), slicing the ovaries (Carolan *et al*., 1992; Mogas *et al*., 1992; Pawshe *et al*., 1994), and puncture of visible surface follicles (Wani *et al*., 1999; Shirazi *et al*., 2005). In studies on goat, it was inferred that puncture and slicing yielded a substantially higher amount of total COCs per ovary (4.22 and 4.14, respectively) than aspiration (3.28) (Hoque *et al*., 2011; Rahman *et al*., 2016; Dadok *et al*., 2020). Since goat ovaries are smaller than bovine ovaries, oocytes remain tightly bound to small to medium sized follicles prior to cumulus expansion, making aspiration difficult (Asad *et al*., 2016). In the case of puncture, the whole ovarian surface was punctured with a hypodermic needle, and in the case of slicing, incisions were made around the entire ovarian surface using a scalpel blade, resulting in the harvesting of all sizes of surface follicles. However, with respect to quality COCs, aspiration (2.48) yielded a slightly higher (p 0.01) amount of regular COCs per ovary than puncture (1.85) and slicing (1.91) (Dadok *et al*., 2020; Rahman *et al*., 2016; Hoque *et al*., 2011). Differently, Shirazi et al. (2005) reported that in case of ewe lambs, the amount of oocytes per ovary for slicing (4.0) and aspiration (3.7) did not differ significantly. Regardless oocyte recovery technique, efficacy of oocytes recovery assessed based on which type of goat used for oocyte collection and observed that, greater amount of COCs recovered from pubertal BBG (88%) than from their prepubertal (77%) counterparts (Khatun *et al*., 2011).

1.2 Selection and grading of retrieved oocytes

Whenever the oocytes have been retrieved and assembled for in vitro maturation, the direct association with the individual oocyte and its special follicular atmosphere and physiological context is lost. Even though connection to the originating follicle is unquestionably fundamental for subsequent oocyte development (Vassena *et al*., 2003). Therefore, assessing oocyte ability to undergo in vitro blastocyst stage is pertinent in every IVF program. Evaluation of morphological features of oocyte is the only non-invasive quality assessment criterion available at this time. Prior to IVM, the morphology of the cumulus investment is widely used as a selection criterion, as it has a significant impact on the maturity of goat oocytes. The presence of more and compact layers of cumulus cells is deemed better (Rahman *et al*., 2008). Cumulus cells are essential for the maturation of oocytes as they provide energy substrates (Sutton *et al*., 2003), nutrients (Haghight and Van Winkle, 1990), and act as messenger molecules (Buccione *et al*., 1990). In most of the IVM studies, oocytes were classified into 4 grades on the basis of cumulus cells and nucleus as described by Khandoker *et al*. (2001), briefly; Grade A: Oocytes completely surrounded by cumulus cells; Grade B: Oocytes partially surrounded by cumulus cells; Grade C: Oocytes not surrounded by cumulus cells and Grade D: Degeneration observed in both oocytes and cumulus cells. The grade A and B were considered as normal and grade C and D as abnormal COCs (Khandoker *et al*., 2001). Rahman *et al*. (2006) stated that higher percentages of goat oocytes were matured as they were encircled by more than five layers of CCs than those with less than five CC layers and denuded oocytes. In other studies, Hoque *et al*. (2012) found that oocytes with good quality COCs had a better success rate and embryo development after fertilization. The size of an oocyte is also relevant for the attainment of maturation. De Smedt *et al*. (1992) showed that 86% of goat oocytes from follicles 2 to 6 mm in diameter progressed to MII, whereas only 24% of oocytes from follicles 1-1 .8 mm attained that stage. Dadok *et al*. (2020) conducted a study based on association between oocyte diameter and oocytes quality revealed that more diameter containing oocytes bear thicker CCs which denotes for better quality oocytes and, as a result, facilitate in vitro oocyte maturation.

1.3 In vitro maturation of oocytes

IVM (In vitro maturation) is the first and one of the most crucial step towards successful fertilization in vitro. IVM is very much dependent on the quality of ovary, ovarian follicles and oocytes (Rahman *et al*., 2007; 2008; Hoque *et al*., 2011, 2012). Finding the most preferable technique it was revealed that, aspiration of 2 to 6 mm diameter follicles is the effective technique for oocyte recovery goat ovaries (Ferdous, 2006; Islam *et al*., 2007; Mondal *et al*., 2008) and there was no significant ($p > 0.05$) effect of cumulus oocyte complexes (COCs) collection techniques on in vitro maturation and fertilization in goats (Brackett *et al*., 1982) while COCs were collected only by aspiration of 2 to 6 mm diameter follicles.

Maturation of mammalian oocytes is defined as the sequence of events occurring from the germinal vesicle stage to completion of the second meiotic division with formation of the first polar body (McGaughey, 1983). In vitro maturation of oocyte is divided into nuclear and cytoplasmic processes. Nuclear maturation involves resumption of meiosis and progression to metaphase-II stage (Rahman *et al*., 2008). Cytoplasmic maturation encompasses a variety of cellular processes that must be completed for the oocytes to be fertilized and developed into a normal embryo and offspring (Eppig, 1996) where cumulus cells (COCs) surrounding the oocyte plays a key role.

It is indispensable that the in vitro maturation mechanism for oocytes need to be improved with the goal of establishing in vitro conditions that are more analogous to those found in the body (Wang *et al*., 2007). Several IVM media for goat oocyte IVM have been designed in various laboratories. Generally buffered Tissue Culture Medium-199 (TCM199) is used as a basic medium for IVM of goat oocytes (Rahman *et al*., 2007). Normal quality COCs (A and B grade) are usually washed 2-3 times separately in D-PBS and finally into the maturation media (TCM-199), placed into the droplets containing TCM-199 and eventually droplets were then kept in a CO_2 incubator at 38.5 ˚C with 5% carbon dioxide in humidified air for 22-27 hours (Mondal *et al*., 2008; Hoque *et al*., 2011; Asad *et al*., 2017, 2018). After the culture of COCs in maturation medium, the level of nuclear maturation was checked by staining of oocytes and examined for germinal vesicle break down (GVBD), metaphase-I (M-I) and metaphase-II (M-II) stage (Hoque *et al*., 2012). Level of cumulus cells expansion might be considered as another way which is used to justify oocyte maturation morphologically. Degree of cumulus cell expansion could be leveled as level-1: indicating less expansion of COCs; level-2: indicating moderate expansion and level-3: indicating marked expansion of cumulus cells with a compact layer or chorona radiata. For in vitro fertilization it is expected to choose moderate to marked expanded cumulus cells denotes fully matured oocytes (Rahman *et al*., 2003). Supplementation with Follicular Fluid (FF), Bovine Serum Albumin (BSA) and fetal calf serum (FCS) is practiced very occasionally for optimum maturation of oocyte which got positive impact (Saha *et al*., 2014; Asad *et al*., 2017, 2018) in maturation. Previously, maturation of oocytes is carried out in culture medium generally containing mediun-199 with FSH, LH, estradiol-17β, BSA, estrus goat serum etc. (Cognié *et al*., 2003). The maturation rate of goat oocytes was found around 40 to 68% [Hoque, 2009; Asad *et al*., 2017, 2018].

1.4 In vitro fertilization and in vitro culture

First kid was born using the IVF techniques on ovulated oocytes by (Hanada, 1985). The very first reported pregnancy in goats by embryo transfer after IVF of goat oocytes (Younis *et al*., 1991) was in 1991. Consequently, the first goat kid from in vitro maturation and fertilization of goat oocytes was produced in 1993 (Crozet *et al*., 1993).

In Bangladesh, IVF has been practiced with successful production of blastocyst (Mondal *et al*., 2008; Hoque, 2009; Khatun *et al*., 2011, Asad *et al*., 2017, 2018) but there is no record of successful goat embryo transfer till date. In most experiments, COCs was proceed to fertilization with frozen or fresh semen in Brackett and Oliphant (BO) medium in CO_2 incubator at 38.5 ˚C with 5% carbon dioxide in humidified air for 5 to 6 hours (Mondal *et al*., 2008; Hoque, 2009; Khatun *et al*., 2011; Saha *et al*., 2014; Asad *et al*., 2017, 2018). Fertilization can be checked directly by staining their pronuclei formation (Asad *et al*., 2018).

Research showed, the Percoll gradient sperm separation technique may be more expedient than the swim-up method for IVF in pubertal black Bengal goats (Khatun *et al*., 2011). Previous report in pubertal goats said that, Percoll density-gradient centrifugation was found to be superior to the swim-up and glass-wool methods for separating spermatozoa from frozen-thawed semen for IVF (*Rho et al*., 2001). Pubertal goat's oocytes were more competent for IVF due to their higher IVM rate respectively (Khatun *et al*., 2011) where the normal fertilization rate was about 37% (Khatun *et al.*, 2011). Ionomycin was used as a sperm capacitating agent in goat IVF media by some researchers, who indicated a higher fertilization rate in response to the inclusion of ionomycin with heparin in the fertilization medium. However, Khatun *et al.* (2011) reported that the same dosage of ionomysin (10 g/mL heparin and 200 nM ionomycin) prescribed by Wang *et al.* (2002), had no effect on the in vitro fertilization rate of Black Bengal goats.

In comparison between the fresh and frozen semen, higher percentage of normal fertilization was observed in fresh semen (36.01%) than in frozen semen (34.72%) (Saha *et al*., 2014) while the fertilization rate was found a bit higher with frozen semen of 37% in different experiment (Khatun *et al*., 2011). Though previous study reported, 57.1% of in vitro matured goat oocytes were normally fertilized of fresh semen (De *et al*., 1992). Comparatively higher number of cleavage rate was obtained from fresh semen (26.19%) than that of frozen semen (21.42%) (Saha *et al*., 2014). Significantly higher number of compact morula and early blastocysts were obtained from the study of (Khatun *et al*., 2011) (25.64% and 12.82%, respectively). It was (Keskintepe *et al*., 1994) found 31.4% and 18.6% morula and blastocyst stages of goat oocytes which were very much similar the result of (Khatun *et al*., 2011). Though it was contradicted with the findings of (John *et al*., 2000; Katska *et al*., 2004) who obtained blastocyst yield up to 37.3% after IVF with fresh sperm capacitated without heparin.

1.5 Effect of follicular fluid

Follicular fluid (FF) is being successfully incorporated in IVM media of cattle (Alia et al., 2004), human (Chi *et al.,* 1998), sheep (Shabankareh *et al*., 2008) and pigs (Ito *et al*., 2008), buffalo (Nandi *et al*., 2004), and goats (Cognié *et al*., 2004). FF contains growth factors some of which have been suggested to play a key role in the ability of oocytes to undergo nuclear and cytoplasmic maturation (Driancourt and Thuel, 1998). Supplementation of FF from non-atretic or gonadotrophin-stimulated large follicles (>4mm) had some positive effect in maturing goat oocytes (Martino et al., 1995; Cognié *et al*., 2004). It was reported that, this beneficial effect on goat oocyte maturation may be due to the presence of growth factors, hormones and intra-ovarian peptides in more physiological proportions in FF (Cognié *et al*., 2004). In the experiment of (Hoque *et al*., 2012) , the results of the cumulus cell expansion of COCs cultured in TCM-199 supplemented with different levels of goat follicular fluid showed that the rate of cumulus cell expansion in level-3 could be significantly ($P<0.01$) increased from 51.5% to 60.3% by supplementing 5% level of follicular fluid (FF) to control media while expansion rate could further be significantly ($P<0.01$) increased to 73.9% by increasing the FF supplementation up to 10% but no significant improvement (75.0%) was observed when the level of follicular fluid increased to 15%. Significant differences ($P<0.01$) were found in the oocytes classified as M-II stages between follicular fluid supplemented (5%) and control group (Hoque *et al*., 2012; Asad *et al*., 2018). Consequently, significantly higher percentage of normal fertilization (formation of 2 pronuclei) occurred in the oocytes matured in TCM-199 media supplemented with 10 and 15% goat (Hoque *et al*., 2012; Asad *et al*., 2018). The rate of development of morula should be increased when the maturation and culture media would be supplemented with 10% gFF (Asad *et al*., 2018). Previous studies reported that, 10% level of goat FF could be used to supplement TCM-199 media for in vitro production of embryos in Black Bengal goat (Hoque *et al*., 2012; Asad *et al*., 2018).

1.6 Right and left ovary

Several studies was conducted to determine the effect of right and left ovary on oocyte collection, maturation and subsequent embryo production. In different categories of ovaries the mean weight, length and width were distinctly higher in right ovaries compared to that of left ovaries (Islam et al., 2007). The mean weight, length and width found higher in right ovaries than those of left ovaries (Islam et al., 2007). Normal physiological explanation of ovarian activity is that right ovaries are more active than left ones, according to previous reports (Singh et al., 1974; Rahman et al., 1977; Sarkar, 1993). Total numbers of COCs were found almost similar in both ovaries with a mean of 1.85 COCs per ovary (Islam et al., 2007) which was lower than the 4.00 oocytes per ovary reported by (Wahid et al., 1992) in sheep, 2.17 oocytes per ovary by (Das et al., 1996) in sheep and 2.75 oocytes per ovary by (Datta et al., 1993) in bovine. Though the higher numbers of normal COCs were recorded in left than that of right ovary (Islam et al., 2007).

1.7 Presence of corpus luteum (cl)

It was observed that the mean weight, length, width and height of ovaries with CL in both goat and sheep were significantly higher than those of ovaries without CL (Hoque et al., 2016). As corpus luteum is an extra cellular material within the ovary which made the differences of its weight and dimensions it was stated that, presence of CL increased the weight and dimensions of ovary (Islam et al., 2007; Hoque et al., 2011, 2016). According to (Islam et al., 2007) CL-absent group ovaries comprise higher number as well as superior quality of COCs than CL-present group ovaries. Total number of COCs was found significantly higher in CL-absent group ovaries with a mean of 2.55 COCs per ovary in his experiment. Several research suggested that, CL-absent group ovaries can be used to collect the quality COCs for IVP of goat embryos (Islam et al., 2007; Mondal et al., 2008, Sathi, 2019).

Goat ovaries containing a corpus luteum yielded a lower number of oocytes and also a lower proportion of usable oocytes than ovaries without a corpus luteum (Agrawal, 1992). According to (Kumar et al., 1997) the oocyte recovery rate decreased when ovaries had a corpus luteum. The possible cause might be due to the restriction of follicular development as lutein cells occupy most of the ovary (Nandi et al., 2000).

1.8 Supplementation with bovine serum albumin

BSA improves maturation, fertilization, blastocyst formation and hatching rates In Vitro (Visconti et al., 2000). It has been widely used in medium for the capacitation of sperm and the acrosome reaction.

BSA has a nutritional role to play by supplementing amino acids after hydrolysis; thereby maintaining the intracellular amino acid pools. BSA also provide undefined embryo trophic (e.g. citrate, steroids) compounds, functioning as a heavy metal ion chelator/free radical scavenger, protecting cellular constituents against the effect of toxins, which enhance the fertilization of oocytes (Asad at al., 2017). Asad at al. (2017) stated that, the maturation rate of caprine oocyte could be significantly increased by supplementing 2 to 4 mg per ml level of BSA while the same amount of BSA supplementation has showed positive effect on in vitro fertilization rate of goat embryos.

1.9 Age of the goat

Age of the goat has been reported to affect the maturation of oocytes from juvenile or prepubertal does (Izquierdo et al., 2002). Palomo et al. (1999) reported that, oocytes derived from prepubertal does had high rates of polyspermy. Oocyte collected from prepubertal goats showed low blastocyst production rate (Izquierdo et al., 2002) and high percentage of haploid embryos (Villamediana et al., 2001). More oocytes with higher IVM rates can be recovered from pubertal black Bengal goats than from their prepubertal counterparts and oocytes collected from pubertal goats were more competent for IVF due to their higher IVM rate (Khatun et al., 2011).

There are reports that oocytes from prepubertal goats show higher percentages of polyspermic fertilization and lack of sperm head decondensation up to the male pronucleus stage (Mogas et al., 1997), low percentage of blastocysts formation (Izquierdo et al., 2002) and production of high percentages of haploid embryos (Villamediana et al., 2001). Pubertal oocytes were found to be of superior quality when compared to prepubertal oocytes in cows (Keskintepe et al., 1994; Lonergan et al., 1994) and similar finding was also found in the experiment of (Wright et al., 1976) for ewe oocytes.

2. Conclusions

IVF in goat is a promising ART throughout the world. In Bangladesh, IVM

of goat oocytes shows a great deal of promise aimed towards the better production of this small ruminant. Accumulating various research work done for in vitro goat embryo production, it was observed that, both fresh and frozen semen can be used on in vitro fertilization of goat oocytes and subsequent development of goat embryos while the source of the ovary, ovary, oocyte and the quality cumulus-oocyte-complexes (COCs) plays a critical role in the maturation and subsequent development of the embryo. Found that, superior quality oocytes are cultured in presence of CO, (5%) in air and humidity (95%) at 38 to 39°C. TCM-199 is the main base medium which can be supplemented with hormones, serum, carbohydrates and other components as required depending on the experiments while the protocol and the choice of supplementation may varies from lab too lab upon the researcher's choice. Actually, due to indiscriminate breeding, the black Bengal goat has already lost its genetic merit and fertility. As a result, it is necessary to determine the nuclear status and IVM rate of black Bengal goat oocytes collected from slaughterhouses under Bangladesh climate conditions to enable more use of assisted reproductive biotechnology which will eventually contribute a lot in increasing production potential and genetic diversity of this goat breed.

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