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REVIEW ARTICLE

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Current status of assisted reproductive technologies in buffaloes

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Abstract

Buffaloes are raised by small farm holders primarily as source of draft power owing to its resistance to hot climate, disease, and stress conditions. Over the years, transformation of these animals from draft to dairy was deliberately carried out through genetic improvement program leading to the development of buffalo-based enterprises. Buffalo production is now getting more attention and interest from buffalo raisers due to its socioeconomic impact as well as its contribution to propelling the livestock industry in many developing countries. Reproduction of buffaloes, however, is confronted with huge challenge and concern as being generally less efficient to reproduce compared with cattle due to both intrinsic and extrinsic factors such as poor estrus manifestation, silent heat, marked seasonal infertility, postpartum anestrus, long calving interval, delayed puberty, inherently low number of primordial follicles in their ovaries, high incidence of atresia, and apoptosis. Assisted reproductive technologies (ARTs) are major interventions for the efficient utilization of follicle reserve in buffaloes. The present review focuses on estrus and ovulation synchronization for fixed time artificial insemination, in vitro embryo production, intracytoplasmic sperm injection, cryopreservation of oocytes and embryos, somatic cell nuclear transfer, the factors affecting utilization in various ARTs, and future perspectives in buffaloes.

KEYWORDS
buffalo, embryo and oocytes cryopreservation, estrus synchronization, in vitro embryo production, SCNT

Kanokwan Srirattana and Danilda Hufana-Duran contributed equally.

1 | ESTRUS AND OVULATION SYNCHRONIZATION FOR FIXED-TIME ARTIFICIAL INSEMINATION

Artificial insemination (AI) is considered one of the major and widely used biotechnologies in disseminating superior genetic material of paternal origin in domestic animals. However, buffaloes are generally known to exhibit "silent heat" phenomenon, causing difficulties in estrus detection due to poor expression of estrus resulting in the less precise timing of AI, low conception rate, and ultimately to low calf production (Zicarelli, 1997). Consequently, tremendous research efforts have been exerted to improve pregnancy in buffaloes, focusing on induction of ovulation to achieve more precise timing of AI and increase pregnancy rate. For almost three decades, extensive work and improvement in the efficiencies have been achieved following the development of the original ovulation synchronization protocol: Ovsynch in dairy cattle (Pursely et al., 1995).

1.1 | Estrus synchronization (ES) in buffaloes

Hormonal synchronization of estrus and AI technologies are indispensable reproductive technology tandem for buffalo propagation. Manipulation of luteal phase by hormonal treatment has been applied for shortening or extending this stage of estrous cycle by administration of prostaglandin (PG) and progesterone (P_4), respectively.

The major hormone involved in the synchronization of estrus in luteal phase buffaloes is the PG. Earlier insemination using prostaglandin F2 alpha (PGF2α) and its synthetic analogs in buffaloes have been reported (Brito et al., 2002; Chantaraprateep, 1987; Kamonpatana et al., 1987). PG causes lysis of the corpus luteum (CL) during the responsive phase, especially between 5 days after ovulation and 5 days before the next estrus (Chantaraprateep, 1987), and a consequent decrease in the levels of P_4 within 24 h after administration leading to the development of follicles of the next wave (De Rensis & López-Gatius, 2007; Galina & Orihuela, 2007). Table 1 shows various ES protocols and results enhancing estrus manifestation and detection, thereby facilitating the use of AI for genetic improvement programs. The conception or pregnancy rates vary from 21 to 86% with the highest pregnancy rate achieved in PG double dose 11–12 days apart + hCG protocol (Situmorang & Siregar, 1997).

In buffalo reproduction, difficulty in estrus detection hindered the accurate timing of AI, thus, leading to a low conception rate. Enhancing PG-based ES with either gonadotropin-releasing hormone (GnRH) or human chorionic gonadotropin (hCG) at the time of AI increases the pregnancy rate from 20% to 37% (Atabay et al., 2020). The use of

TABLE 1 Estrus synchronization protocols and results in swamp and riverine buffaloes using luteolytic and luteotropic agents or in combination with other hormones

Breed type	Hormone used	Observed estrus, %	Conception/ pregnancy after AI, %	References
Swamp	PG single dose	43.33–79.10	33.21–41.68	Jiang et al., 2003; He et al., 2005; Liang et al., 2007
	PG single dose + CIDR	86.13–100	37.00–46.03	Jiang et al., 2003; Gabriel et al., 2019
	PG single dose + PMSG	73.01–84.5	43.48–45.6	Jiang et al., 2003
	PG single dose + GnRH or hCG	97.37–100	21.05–38.70	Atabay et al., 2020
	PG double dose 11–12 days apart	100	21.70–83.87	Chantaraprateep et al., 1981; Chirachaikitti et al., 1982; Chantaraprateep et al., 1983; Capitan et al., 1992; Yuan et al., 2008; Sianturi et al., 2012
	PG double dose 11–12 days apart + hCG	81.30–100	50.00–86.6	Situmorang & Siregar, 1997
	PRID	-	47.0	Chantaraprateep et al., 1983.
	PRID + PMSG	6.30–45.60	47.16–50.00	Feng et al., 1990; Jiang et al., 2003
	CIDR	57.5–78.02	56.52	Cai et al., 2011
	CIDR + GnRH	75.0	55.00	Cai et al., 2011
	Norgestomet	36.0	30.77	Virakal et al., 1988
	Norgestomet + PMSG	45.5	39.13	Virakal et al., 1988
Riverine	PG single dose	75.0–100.0	41.5–80.0	Pant & Singh, 1991; Khattab et al., 1996; Ribeiro et al., 1998; Brito et al., 2002
	PG double dose 11–12 days apart	25.0–95.0	22.8–83.0	Chohan et al., 1993; Diaz et al., 1994; Singh & Dabas, 1998; Misra et al., 2003; Srivastava, 2005.
	Norgestomet + E2	60.0–100	30.0–70.0	Phadnis et al., 1994; Patel et al., 2003
	Norgestomet + eCG	86.0–97.6	44.6–66.7	Luthra et al., 1994; Malik et al., 2011
	Norgestomet + PG	100	45.0–66.7	Utage et al., 2010; Chaudhary et al., 2015

Abbreviations: CIDR, controlled internal drug release; E2, estradiol; eCG, equine chorionic gonadotropin; GnRH, gonadotropin releasing hormone; hCG, human chorionic gonadotropin; PG, prostaglandin; PMSG, pregnant mare serum gonadotropin; PRID, progesterone releasing intravaginal device.

GnRH and PGF2α was proven to be very successful in synchronizing estrus in cattle and buffaloes (Amaya-Montoya et al., 2007) for timed insemination.

1.2 | Ovulation synchronization (Ovsynch) and fixed time artificial insemination (FTAI) in buffaloes

Several years after the successful outcome of Ovsynch in dairy cattle (Pursely et al., 1995), FTAI technology has been successfully applied in buffaloes (Baruselli et al., 2002; Baruselli, Madureira, Visintin, et al., 1999). To date, synchronization of estrus and ovulation in tandem with FTAI is widely practiced in buffaloes (Alyas et al., 2013; Atabay et al., 2019; Campanile et al., 2005; Kalwar et al., 2015; Ravikumar & Asokan, 2008). The ovarian activity is manipulated so that the time of ovulation can be predicted. This is achieved by controlling the luteal phase of the estrous cycle or controlling the follicular development and ovulation through hormonal interventions using different combinations of PG, P_4, GnRH, hCG, eCG, and estradiol (E_2) (De Rensis & López-Gatius, 2007). The original Ovsynch protocol is done by administration of GnRH at day 0, PGF2α at Day 7, second GnRH on Day 9, and FTAI 16 h later (Pursely et al., 1995).

FTAI program provides an organized approach to the enhanced use of AI, the genetic progress, and the improved reproductive efficiency of dairy and beef cattle (Baruselli et al., 2004; Pursely et al., 1995). In buffaloes, hormonal treatments have been designed to control both luteal and follicular functions, providing exciting possibilities for the synchronization of follicular growth and ovulation that enabled the use of timed artificial insemination (TAI) during the breeding and nonbreeding season (Baruselli, Madureira, Barnabe, et al., 1999; De Rensis et al., 2005). Satisfactory pregnancy rates of approximately 40–60% have been achieved with the Ovsynch protocol in cycling buffalo synchronized during the breeding season (Ali & Fahmy, 2007; Baruselli, Madureira, Visintin, et al., 1999; Berber et al., 2002; Neglia et al., 2003). However, anestrous buffaloes respond poorly to the Ovsynch protocol and have lower pregnancy rates after TAI during the nonbreeding season (Ali & Fahmy, 2007; Baruselli et al., 2007; De Rensis et al., 2005). There are still various factors that limit or reduce the effectiveness of these protocols such as the presence of noncycling cows (seasonal anestrus), asynchronous ovulation, incomplete luteal regression, and luteal sub-function. To rescue acyclic animals, several hormonal protocols have been employed. Previous studies have shown that P_4 concentration during the late luteal phase before insemination is positively associated with conception rates in cattle. In some parts of the Mediterranean countries where riverine and swamp buffaloes are raised, the supplementation of P_4 with Ovsynch protocol to improve the synchronization rate, ovulation rate, and pregnancy rate has been employed with different degrees of efficacy (Barile et al., 2001; Bartolomeu et al., 2002; Chaikhun et al., 2012; Murugavel et al., 2009). One reason for variability in the effectiveness of the Ovsynch protocol is related to asynchrony between induced ovulation and insemination.

Treatment with intravaginal P_4 devices followed by eCG at device removal has been used to increase ovulation rate, CL growth rate, initial P_4 concentration, and pregnancy rate after FTAI in buffalo during the nonbreeding season (Carvalho et al., 2013). Hormonal interventions have been developed to control ovarian dynamics and allow the use of AI without heat detection. Table 2 shows the use and results on percent estrus manifestation and pregnancy obtained in Ovsynch-based protocol by several researchers.

Numerous strategies were developed to evaluate the efficiency of the Ovsynch protocol in buffaloes (Atabay et al., 2019; Baruselli,

TABLE 2 Estrus and conception rates following various Ovsynch-based FTAI protocols in buffaloes

Treatment protocol	Observed estrus, %	Conception after AI, %	References
Ovsynch	46.3–100	28.0–66.6	Ali & Fahmy, 2007; Atabay et al., 2019; Bartolomeu et al., 2002; Berber et al., 2001; Campanile et al., 2005; De Araujo Berber et al., 2002; Derar et al., 2012; Francillo et al., 2005; Hoque et al., 2014; Liang et al., 2007; Neglia et al., 2003; Presicce et al., 2004; Ravikumar et al., 2009; Sathiamoorthy et al., 2007; Sianturi et al., 2012
Ovsynch + FTAI 12 and 24 h after 2nd GnRH	-	18.0–59	Akhtar et al., 2013; Camelo et al., 2002; Chaikhun, Tharasanit, & Rattanatep, 2010; Karen & Darwish, 2010
Presynch + Ovsynch	83.3	-	Chaikhun, Promdireg, & Suthikrai, 2010
Select synch	100	77.14–100	Sianturi et al., 2012; Yendraliza et al., 2011
GnRH+ PGF2α + LH	-	64.2	De Araujo Berber et al., 2002
CIDR-Ovsynch	58.3–100	18.18–66.67	Atabay et al., 2019; Alyas et al., 2013; Campanile et al., 2005; Chaikhun et al., 2012; Kalwar et al., 2015; Murugavel et al., 2009
CIDR-Ovsynch + hCG on day 9	97.0–100	58.04–60.38	Atabay et al., 2019, Tilwani et al., 2019
Norgestomet + Ovsynch	-	71.4	Malik et al., 2010

Madureira, Barnabe, et al., 1999; Baruselli, Madureira, Visintin, et al., 1999; Berber et al., 2002; Chaikhun, Promdireg, & Suthikrai, 2010). These works provided evidence that buffaloes respond to hormonal treatment and that a new follicular wave emergence occurs due to the ovulation of the dominant follicle present at the time of the first GnRH injection. Purohit et al. (2019) emphasized the importance of having a dominant follicle and CL at the start of the treatment, achieving a pregnancy rate of 45–50% in cycling buffaloes during the breeding season.

Carvalho, Vannucci, et al. (2007) documented an increase in pregnancy rates and birth rates with the administration of GnRH 6 days after FTAI in buffaloes on the Ovsynch protocol. This GnRH administration induced the formation of accessory CL to increase the plasmic concentration of P_4 and resulted to a positive effect on the pregnancy rate and birth rate (Campanile et al., 2010; Ferrer et al., 2021). The formation of an accessory CL and supplementation of P_4 after insemination increased the plasma P_4 concentration, which is very important in the preparation of the uterine environment for subsequent development of the embryos to term. When Ovsynch protocol is used during spring and summer when a high incidence of anestrous was observed, 5–35% pregnancy rates were obtained (Atabay et al., 2019; Baruselli et al., 2002; Baruselli, Madureira, Visintin, et al., 1999; Chaikhun, Tharasanit, & Rattanatep, 2010).

The protocol using controlled internal drug release synchronization (CIDR-Synch) is basically the same with Ovsynch except that CIDR is inserted at day 0 during the injection of the first GnRH, and removed at the time of injection of PGF2α at day 7. Chaikhun et al. (2012) reported that CIDR-Synch could induce estrus and ovulation in anestrus swamp buffalo during the breeding season with an average ovulation time after second GnRH injection of 10 ± 5.6 h. Therefore, AI should be applied at the same time as the second GnRH injection in swamp buffalo cows. The P_4 supplementation with Ovsynch protocol produces synchronous follicular wave emergence, large preovulatory follicles and synchronous ovulation and thus the efficacy of timed-AI is improved. In cycling cows, this positive effect of P_4 supplementation can be related to the fact that CIDR acts to maintain elevated blood P_4 concentrations before FTAI (Chaikhun et al., 2012).

A further modification of Ovsynch protocol in buffaloes, which involves the use of exogenous P_4, is the norgestomet implant and intramuscular injection of estradiol benzoate (EB) on a random day of the estrous cycle (Day 0). Five to 9 days later, the implant is removed and intramuscular doses of PGF2α and eCG are administered. Forty-eight hours after the removal (day 7–11), ovulation is induced by the administration of GnRH or hCG. FTAI is performed 16 h after the induction of ovulation, resulting in improved AI efficiencies (Atabay et al., 2019; Baruselli et al., 2003; Carvalho, Nagasaku, et al., 2007).

Moreover, the combination of P_4 and E_2 at the beginning of the protocol (day 0) is effective in inducing the emergence of a new follicular wave due to the suppression of both FSH and LH, which promote the atresia of all follicles present in the ovary in buffalo (reviewed by Baruselli et al., 2007). Previous studies demonstrated that P_4 treatment stimulates an increase in LH pulse frequency during and following treatment period. Treatment of anestrous cows with P_4 results in greater follicular fluid volume and circulating concentrations of E_2, increased pulsatile release of LH, and increased number of LH receptors in granulosa and theca cells in preovulatory follicles (Rhodes et al., 2002). Furthermore, a short period of elevated P_4 concentrations during the anestrous period is important for the expression of estrus and subsequent normal luteal function (McDougall et al., 1992). Gabriel et al. (2019) determined the effects of different PG analogs on P_4 level, follicular growth, estrus manifestation, and pregnancy in dairy buffaloes under CIDR Synch Protocol. Their study revealed that estrus manifestation, follicle size, and pregnancy rate were not significantly different among the animals that received different PG analogs during CIDR-based FTAI program.

Treatment with eCG at the time of device removal increases the follicular diameter, ovulation rate, CL diameter, P_4 concentrations, and pregnancy rate (Carvalho et al., 2013). These results confirm the necessity of eCG in Ovsynch protocols for FTAI during the nonbreeding season. Similarly, replacing second GnRH with hCG as ovulatory hormones resulted in satisfactory follicular response, ovulation rate, and pregnancy rate during the nonbreeding season (Atabay et al., 2019; Carvalho et al., 2012). In addition, the use of Ovsynch protocol during the breeding season and P_4 + EB, $PGF_{2,}$ and eCG protocol during the nonbreeding season resulted in a pregnancy rate of approximately 50% in a single FTAI. Therefore, the FTAI program can be used throughout the year to efficiently schedule conception and the calving period in buffalo.

In a recent study, a third GnRH injection was given to swamp buffaloes during the normal AI time (AI must be done 24 h after the second GnRH injection) as part of the Ovsynch protocol. The ovulation rate and pregnancy rate were improved from 80 to 100% and 34% ($n = 50$) to 50% ($n = 45$), respectively (Chaikhun-Marcou et al., unpublished data). This research is ongoing in generating more data. In other research, kisspeptin-10 administration was compared with GnRH administration to see which one produced higher LH levels during luteal phase in swamp buffalo cows, and the result showed that the LH concentration level with GnRH administration was greater than kisspeptin-10 (Chaikhun-Marcou et al., 2019).

Lastly, the efficiency between pre-synch protocol and with Ovsynch protocol was compared in Argentinean buffaloes; however, no statistical differences were found between the treatment groups (Konrad et al., 2010). Essentially, the success of the application of certain protocol is affected by various factors, and this must be seriously taken into consideration in the implementation of timed AI in water buffaloes.

2 | IN VITRO EMBRYO PRODUCTION

The multiple ovulation and embryo transfer (MOET) in buffaloes resulted in very low recovery of embryos from the nonsurgical collection. The average number of embryos collected from a donor was 1.0 (Drost et al., 1988; Vlakhov et al., 1986), 2.0 (Cruz et al., 1991) 2.7 (Misra, 1993), 4.5 (Kasiraj et al., 1993), 1.6 (Kandil et al., 2012), 2.7

(Qin et al., 2012), and 5.8 (Singh et al., 2015). These outputs cover the trials made in Bulgaria, India, Philippines, Egypt, and China (Hufana-Duran & Duran, 2015). This technology is one of the biotechnologies of reproduction that is supposed to be most utilized in the world to produce a high number of in vivo embryos. In the buffalo species, however, the application meets several difficulties and the embryo recovery rate is definitely lower than that recorded in cattle (Neglia & Bifulco, 2017). The state of art of MOET in buffaloes and analysis of the factors that limit and influence its efficiency were elaborated earlier (Hufana-Duran & Duran, 2015; Neglia & Bifulco, 2017). Due to the scarce results of in vivo embryo recovery in superovulated buffaloes, the association of ovum pick-up (OPU) with in vitro embryo production (IVEP) represents an alternative method of exploiting the genetics of high yield buffaloes (Baruselli et al., 2018). With the above considerations, the production of buffalo embryos using IVEP technique become an alternative to MOET.

The pioneering works in the production of buffalo embryos from follicular oocytes by in vitro maturation (IVM), in vitro fertilization (IVF), and in vitro culture (IVC) have resulted in the birth of calves out of embryo transfer (ET) using freshly produced river buffalo embryos (Madan et al., 1994), crossbred 50:50 river: swamp embryos (Ocampo et al., 2000), and vitrified in vitro produced river buffalo embryos both in the river (Galli et al., 2012; Hufana-Duran et al., 2004) and swamp (Hufana-Duran et al., 2007) buffalo recipients including twins (Hufana-Duran et al., 2008).

2.1 | Laboratory and culture conditions

In carrying out the IVEP in buffalo, studies showed that a complete aseptic condition is necessary and in vitro manipulation procedures were found best at an appropriate temperature (35–37°C), pH (7.1 to 7.4), osmolarity (280–300 mOsmol), minimum exposure to UV light, and IVC in a humidified CO_2 incubator at 38–39°C (Hufana-Duran, 2008; Ravindranatha et al., 2003). It was found that oocytes from juvenile donors lack the developmental requirements while those from adult donors have a high incidence of chromosome abnormalities. The sequential steps involved in IVEP are collection of oocytes by retrieval from abattoir-derived ovaries or by ovum pick up (OPU) from live donors, selection of developmentally competent oocytes, and IVM to mature the oocyte, sperm capacitation and IVF, and IVC for embryo development.

2.2 | Sources of oocytes

2.2.1 | Collection from abattoir ovaries

Collection of ovaries from the local abattoir is a requisite of IVEP research and the common ovary storage used was physiological saline with (Hufana-Duran et al., 2004) or without antibiotics (Abdoon et al., 2001). Developmental competence of oocytes was found affected by the ovary storage temperatures; within 6 h is best stored at 25–33°C (Hufana-Duran, 2008) and beyond 6 h, 15°C is preferred (Atabay, Atabay, Aquino, et al., 2010). Oocyte recovery from ovaries is best done by follicular aspiration using an 18-gauge needle (Mehmood et al., 2011) and to preserve oocyte viability Tissue Culture Medium-199 (TCM199) with 10% fetal calf serum (FCS) buffered with 25-mM HEPES and 5-mM sodium bicarbonate (Gasparrini, 2002), prewarmed modified phosphate-buffered saline (m-PBS) with 3 mg/ml bovine serum albumin (BSA) (Hufana-Duran et al., 2008) or 5% (v/v) heat-inactivated FCS (Hegab et al., 2009) were used as holding and washing media.

2.2.2 | Collection by transvaginal ultrasound-guided ovum pick up

To produce embryos in vitro from oocytes of live donors, OPU is used. OPU involves ultrasound-guided follicle aspiration for the recovery of oocytes that allows great use of genetically valuable females. Studies showed that repeated OPU has no major impact on the health of female donors (Boni et al., 1996) and can be applied to cycling and lactating postpartum buffaloes (Promdireg et al., 2005), donors of all ages from two-month-old calves to very old cows with exception of pregnant animals after the third or fourth month of pregnancy (Duran et al., 2013), and animals with severe ovarian hypoplasia or during the immediate postpartum period (Galli et al., 2001).

The technique of OPU in buffaloes is the same in cattle with the step-by-step procedure described earlier (Hufana-Duran & Duran, 2015). Antral follicles ≥2 mm in diameter are aspirated from each ovary using stainless steel needle of 50 cm (Aquino et al., 2013) to 55 cm (Manjunatha, Ravindra, et al., 2008) long with a 0.1-cm diameter or 18-gauge to minimize mechanical damage to the oocyte. The ultrasound echo tip is 5-MHz (Manjunatha, Ravindra, et al., 2008) to 9-MHz (Sakaguchi et al., 2019) micro-convex transvaginal transducer to aspirate the oocytes from the follicles using a negative pressure of 40 mmHg (Liang et al., 2008; Neglia et al., 2011), 68 (Ferraz et al., 2015), 55–70 mmHg (Sakaguchi et al., 2019), or 110 mmHg (Manjunatha, Gupta, et al., 2008) depending on the machine. Studies showed that checking the quality of the cumulus cells surrounding the oocytes is needed for each machine.

The efficiency of OPU is affected by various factors such as the frequency and length of the collection. Females submitted to OPU every 14 to 15 days had a larger ($P < 0.001$) number of ovarian follicles suitable for puncture (15.6 ± 0.7 vs. 12.8 ± 0.4) and an increased ($P = 0.004$) number of cumulus-oocyte complexes (COCs) recovered (10.0 ± 0.5 vs. 8.5 ± 0.3) compared with 7-day interval (Konrad et al., 2017). From the retrieved COCs, average of 5.2 ± 3.9 are selected to continue the in vitro maturation process with 3.1 ± 2.6 COCs/animal/aspiration session considered viable according to the morphological characteristics of the COCs (Di Francesco et al., 2012). A twice-a-week collection allows for the maximum recovery of oocytes of suitable quality for embryo production (Yindee et al., 2011) while a once-a-week collection results in the recovery of a smaller number of oocytes (of lower quality) that have already undergone

cumulus expansion and atresia (Duran et al., 2013). Gupta et al. (2006) found that OPU has no side effects even after twice-a-week collections for over a year. In some cases, though, hardening of the surface of the ovaries occurred after several months of repeated collections, a decline in the follicle recruitment, and oocytes collection with a drop in developmental competence after the first 2 months of recovery (Neglia et al., 2011). It was reported that a combination of superovulation with OPU to recover the oocytes before the onset of estrus can be repeated at best every 2 weeks (Galli et al., 2001). Prior stimulation in buffaloes with gonadotrophins (Promdireg et al., 2005) or bovine somatotropin (Ferraz et al., 2015) or pharmacologically synchronized follicular waves (Gimenes et al., 2015) before OPU is known to increase the number of medium and large-sized follicles. OPU during the breeding season yielded a better oocyte recovery and better-quality embryos after IVEP (Abdoon et al., 2014) and has its own therapeutic effect on infertile donors, especially those affected by ovarian cysts (Duran et al., 2013). In vitro produced embryos from OPU resulted in births of live calves after embryo transfer (Aquino et al., 2013; Galli et al., 2012; Prasad et al., 2013) demonstrating the potential of the in vitro embryo production as a tool in the production of genetically valued water buffaloes and in overcoming the various reproductive problems that affect the reproduction of this animal species.

The mean number of good oocytes collected from a buffalo ovary ranges from 0.43 to 3.3 oocytes/ovary (Sharma et al., 2013). Competence and efficiency of the person doing the aspiration, breed and health condition of donor, size of the ovary, number of follicles present in the ovary, presence or absence of CL, the season of the year (Manjunatha, Ravindra, et al., 2008), and the inherent low follicular reserve in buffaloes (Smith, 1990) affect the number of oocyte retrieval.

2.3 | Oocyte selection and in vitro maturation

Oocyte selection is critical for IVEP in water buffalo and the selection is based on the compaction of the cumulus-corona investment and homogeneity of the ooplasm (Hufana-Duran, 2008). The time required for complete nuclear maturation of oocytes in vitro is from 18 to 24 h (Gasparrini et al., 2008) and the length can be determined by the appearance of the surrounding cumulus cells where oocytes with a compact cumulus cell mass require a longer period of IVM while those with loose cumulus mass require a shorter period for optimum blastocyst development (Hufana-Duran, 2008).

The quality of oocytes is important in ensuring the production of viable embryos in vitro. A decrease in developmental competence is due to insufficient nuclear and cytoplasmic maturity brought about by the limitations in the IVC environment. Increased oxidative stress was found as a major factor affecting in vitro embryo development (Gasparrini et al., 2000). Ultrastructural studies showed an abundance of cytoplasmic granules characterized by significant lipid content (Hufana-Duran, 2008) that probably renders the buffalo oocytes and embryos more sensitive to oxidative damage.

The critical factor in the IVM environment is the provision of the support needed for signals that enhances the mechanisms to acquire developmental competence by the oocyte. Culture media and its components play an important role and can be categorized into simple and complex (Gasparrini, 2002). TCM 199, Ham's F-10, CR1aa, and CR2aa, MEM, mSOF, and RPMI-1640 are used as basic media and made complex by the supplementation with either serum (Hufana-Duran, 2008) or follicular fluid (Gupta et al., 2002), growth factors (Chauhan et al., 1998), hormones (Abdoon et al., 2001), antioxidants (Gasparrini et al., 2006), and a controlled level of antibiotics to provide protection from bacterial contamination.

2.4 | Sperm capacitation and in vitro fertilization

The success of IVF in water buffalo is significantly influenced by bull fertility, the medium used, and the duration of IVF (Suresh et al., 2009). Separation of live sperm cells for IVF by swim-up procedures (Jamil et al., 2007), ion-exchange filtration (Mustafa et al., 1998), or centrifugation using discontinuous density gradients of percoll (Purohit et al., 2005) or silica particles (Hufana-Duran et al., 2005) improved success rate. Appropriate sperm concentration is necessary as too high could result in polyspermy while too few sperm could result in a low fertilization rate. Sperm capacitation necessary for the sperm to penetrate and fertilize an egg can be enhanced using various media such as Brackett & Oliphant medium with 2.5-mM caffeine and 10 μg/ml heparin (Hufana-Duran et al., 2004; Madan et al., 1994; Nandi et al., 1998) or theophylline (Hufana-Duran, 2008) or a mixture of phenylephrine, hypotaurine, and epinephrine (Purohit et al., 2005), or osteopontin (Boccia et al., 2013). Oocytes partly freed from cumulus cells enhance sperm penetration and promote a higher fertilization rate. Sperm-oocyte co-culture for IVF is carried out for 6 to 18 h depending upon the composition of the IVF medium. It is necessary to examine the best duration of sperm-oocyte co-culture as differences exist depending on the IVF media formulation, sperm concentration, and bull used (Gasparrini et al., 2008). The use of sex-sorted sperm cells for IVF in water buffalo was successfully demonstrated resulting in the birth of calves of predetermined sex (Liang et al., 2008; Lu et al., 2007). Accuracy of sexing is around 90% in water buffalo, and a 4% difference in DNA contents between X- and Y-chromosome-bearing spermatozoa was observed (Lu et al., 2007). Embryos produced from IVF using silica gel isolated sperm cells (Hufana-Duran et al., 2005) and sex-sorted sperm cells (Liang et al., 2008; Lu et al., 2007) resulted in the birth of live healthy calves.

2.5 | IVC and blastocysts development

The culture of embryos in vitro requires the necessary nutrients and appropriate environment (temperature, humidity, gas) so that the fertilized oocytes can undergo cleavage divisions and be able to reach the blastocyst stage of development. Several culture media have

been tested in the culture of buffalo embryos and all resulted in the development of blastocysts: CR1aa, CR2aa, TCM-199, MEM, RPMI-1640, and mSOF media (Gasparrini, 2002; Suresh et al., 2009). The effectiveness of each medium formulation depends mainly on providing the appropriate combination of antioxidants, co-culture, growth factors, and gas phases. The methods used in IVC of bovine embryos by co-culture with cumulus cells (Hamano & Kuwayama, 1993) and the sequential media system containing pyruvate and lactate and different concentrations of serum and presence of glucose (Hufana-Duran, 2008) resulted in full-term development after embryo transfer (Hufana-Duran et al., 2004, 2007, 2008). Glucose has been required by buffalo embryos for their proper development from the earliest cultivation (Kumar et al., 2012). It was observed that removal of the uncleaved oocytes during IVC promotes a better quality of embryos that reach the blastocyst stage with blastocyst development ranging from 22% to 32% (Gasparrini, 2002; Hufana-Duran, 2008; Suresh et al., 2009). Metabolism of water buffalo embryos is different from cattle evidenced by the 12 to 24 h earlier development than bovine embryos both under in vitro and in vivo conditions (Galli et al., 2001; Ocampo et al., 2000). Table 3 presents the breakthroughs in IVEP of water buffalo.

3 | INTRACYTOPLASMIC SPERM INJECTION (ICSI)

A micromanipulation technique that involves the injection of a single spermatozoon into the cytoplasm of a mature oocyte is called ICSI. As the egg will theoretically be fertilized using only a single sperm, this method is considered a standard way to produce normal diploid embryos. With regard to buffalo, ICSI became an alternative fertilization technique for research and production purposes. Frozen buffalo spermatozoa sometimes show immobility after thawing (Muer et al., 1988), which may cause reduced fertility. This technique has successfully been applied to buffaloes since 2006 (Lu et al., 2006).

3.1 | Application of buffalo ICSI

ICSI was applied in buffalo for the first time using the sex-sorted sperm to produce sex-specific buffalo embryos (Lu et al., 2006). It is beneficial in the preservation and conservation of endangered buffalo species using frozen–thawed sperm or oocytes. With ICSI, reproduction of males with motility and fertilizing capacity problems as observed in sex-sorted sperm cells and some sperm cells after cryopreservation can be made possible as it can improve pronuclear formation and cleavage rate (Liang, Ye, et al., 2011) compared with IVF (Liang et al., 2020). IVF with sex-sorted sperm that resulted in poor embryo development can be improved via ICSI. ICSI-derived in vitro production of buffalo embryos can reach 17–29% blastocyst rates (Liang, Phermthai, et al., 2011).

3.2 | Factors affecting ICSI in buffalo

Chemical activation of oocytes is considered a key factor in buffalo ICSI (Liang, Ye, et al., 2011). These authors found that without chemical activations, none of the buffalo oocytes could be fertilized by sperm injection. This means that additional activation treatment is necessary for meiosis completion, pronuclear formation, and embryo development. In their report, the highest rate of second polar body extrusion occurred at 3 h of activation with ethanol (EtOH) found as the best chemical for activation when compare with ionomycin (Io) (Liang, Ye, et al., 2011). After that, the oocytes showed second polar body from both EtOH and Io groups were cultured in either 6-dimethylaminopurine (6-DMAP) or cycloheximide (CHX) and then in vitro embryo cultured to examine embryo development to

TABLE 3 Success rate on calf production of in vitro matured, fertilized, and cultured water buffalo embryos

Embryo production method	Nature of sperm	Resultant embryo status before ET	Embryo breed	Embryo recipient breed	Calf production rate, % (calf/recipient)	References
Abattoir ovary, IVM, IVF, IVC	Frozen–thawed	Frozen–thawed	Riverine ($2n = 50$)	Riverine ($2n = 50$)	23.1 (9/39)	Kasiraj et al., 1993
Abattoir ovary, IVM, IVF, IVC	Frozen–thawed	Transferred fresh	Riverine ($2n = 50$)	Riverine ($2n = 50$)	25.0 (4/16)	Madan et al., 1994
Abattoir ovary, IVM, IVF, IVC	Frozen–thawed	Vitrified-warmed	Riverine ($2n = 50$)	Riverine ($2n = 50$)	10.9 (6/55) 26.9 (7/26)	Hufana-Duran et al., 2004, 2008
Abattoir ovary, IVM, IVF, IVC	Frozen–thawed	Vitrified-warmed	Riverine ($2n = 50$)	Swamp ($2n = 48$)	10.0 (4/40)	Hufana-Duran et al., 2007
Abattoir ovary, IVM, ICSI, IVC	Sex-sorted	Transferred fresh	Riverine ($2n = 50$)	Riverine ($2n = 50$)	200.0 (2/1) (twins)	Lu et al., 2007
OPU, IVM, ICSI, IVC	Sex-sorted	Transferred fresh Frozen–thawed	Riverine ($2n = 50$)	Riverine ($2n = 50$)	20.6 (7/34) 9.0 (4/43)	Liang et al., 2008

Abbreviations: IVC, in vitro culture; IVF, In vitro fertilization; IVM, In vitro maturation; OPU, Ovum pick up.

blastocyst stage. The combination of Io + 6-DMAP showed highest (29%) blastocyst rates but no significant differ with EtOH + CHX (24%) (Liang, Ye, et al., 2011).

3.3 | Frozen–thawed buffalo oocytes affecting the developmental ability of ICSI-derived embryo

After cryopreservation or treatment with cryoprotectant (CPA), structural changes in the zona pellucida (ZP) have been shown to reduce fertilization rates (Carroll et al., 1990; Vincent et al., 1990), but this ZP hardening could be overcome by ICSI (Carroll et al., 1990; Karlsson et al., 1996; Kazem et al., 1995; Mavrides & Morroll, 2002; Porcu et al., 1997). Liang reported buffalo oocytes vitrified by the microdrop method and activated by EtOH and CHX after ICSI (Liang, Phermthai, et al., 2011). In this study, the blastocyst rates in the ICSI control groups (23%) were significantly higher than those of vitrified groups (11%). In another oocyte vitrification study, oocytes that extruded the second polar body after ICSI and activation revealed that only a minority (7–20%) of the vitrified oocytes compared with 46–48% of the control oocytes also had two pronuclei, indicating that normal activation is compromised by vitrification (Liang, Srirattana, et al., 2012).

4 | CRYOPRESERVATION OF OOCYTES AND EMBRYOS

The scarcity of oocytes is a major drawback for exploiting embryo technologies in buffaloes. Therefore, cryopreservation can be a useful technique to avail buffalo oocytes for various reproductive technologies. There are two methods applied to the cryopreservation of oocytes and embryos: controlled slow freezing, which was favored in early procedures, and ultra-rapid cooling by vitrification, which is now a widely used method. Conventional slow freezing was first introduced in 1971, which has become the cornerstone of slow freezing of embryos (Whittingham, 1971). This method basically involves the use of a single cryoprotectant in low concentrations (approximately 1 to 2 M) minimizing chemical and osmotic toxicity. However, during slow cooling, ice crystal formation occurs, which is the major cause of cryoinjury and cell death following cryopreservation (Fuller & Paynter, 2004). Meanwhile, over the past several years, vitrification has become an alternative method for oocyte/embryo cryopreservation that minimizes cellular damage wherein ice crystal formation is prevented by the viscosity of the high concentrations of cryoprotectants in vitrification media (Vajta, Holm, et al., 1997). Vitrification is generally defined as the glass-like solidification of solutions at low temperatures due to the increased concentration of cryoprotectant during cooling, without the formation of intracellular ice crystals (Rall & Fahy, 1985). It is being described as an inexpensive, fast, and simple procedure (Stachecki et al., 2008) compared with the slow freezing method. The principle of slow freezing and vitrification of oocytes and embryos has been extensively reviewed elsewhere (Hwang & Hochi, 2014; Konc et al., 2014; Leibo & Songsasen, 2002; Mandawala et al., 2016).

4.1 | Cryopreservation of buffalo embryos

Both slow freezing and vitrification techniques are used for buffalo embryo cryopreservation, and pregnancies as well as live calves from slow-freezing (Galli et al., 2011) and vitrified–warmed embryos (Hufana-Duran et al., 2004, 2007, 2008) have been achieved. To date, cryopreservation of buffalo embryos is mainly carried out by vitrification, as shown in Table 4.

The most commonly used vitrification protocol applied to any embryo stage in buffaloes is the two-step equilibration in a combination of permeating cryoprotective agents (CPAs), most often ethylene glycol (EG) and dimethyl sulfoxide (DMSO). The *protocol* was reported to be effective for buffalo embryos with good post-thaw in-vitro development using in-straw vitrification (Manjunatha, Gupta, et al., 2009) and solid-surface vitrification (SSV) method (Rahangdale et al., 2021). High survival rates of compact buffalo morula and blastocyst development were achieved following the use of EG as sole vitrification solution or in combination with DMSO (Rahangdale et al., 2021). In contrast, lower cryosurvival rate of buffalo morula stage embryos was reported if compared with blastocyst stage embryos (Manjunatha, Gupta, et al., 2008; Manjunatha, Ravindra, et al., 2009). Hufana-Duran et al. (2004), however, demonstrated no significant differences in the hatching rates (75–90%) among vitrified-thawed embryos at the morula, early blastocyst, blastocyst, and expanded blastocyst stages, following vitrification with EG-based solution. As to the type of vitrification containers, several devices have been applied for buffalo embryo vitrification such as French straw (Hufana-Duran et al., 2004), Cryotop (Laowtammathron et al., 2005), open pulled straw (OPS) (Sirisha et al., 2013), and SSV (Rahangdale et al., 2021). High hatching blastocyst rate (90%) and birth of live calves were reported following vitrification of buffalo embryos at the early stage (Hufana-Duran et al., 2004). Meanwhile, somatic cell nuclear transfer (SCNT) buffalo blastocysts were found more tolerant to vitrification yielding a higher survival rate than bovine blastocysts using Cryotop (Laowtammathron et al., 2005). In addition, OPS was found to be superior over slow freezing for the cryopreservation of zona-free cloned buffalo blastocysts with improved cryosurvival rates at post-warming (Sirisha et al., 2013). Meanwhile, OPU technique has been successfully applied to buffaloes (Atabay et al., 2008; Boni et al., 1996; Galli et al., 2014). Vitrified blastocysts derived from OPU oocytes resulted in significantly higher blastocyst hatching rates (53%) than vitrified blastocysts derived from slaughterhouse oocytes (40%) (Manjunatha, Gupta, et al., 2008). Essentially, though vitrification technologies have been applied on buffalo embryos more successfully than slow freezing methods (Sirisha et al., 2013), buffalo embryos are found more cryosensitive compared with bovine or ovine embryos, thus optimization of the protocol considering several factors can improve embryonic development.

TABLE 4 Vitrification of buffalo embryos using various protocols and devices

Embryo source	Stage of embryos	Equilibration solution	Vitrification solution	Devices	Survival rate	References
IVF	Morula Early BL BL Expanded BL	10% EG 2 min	40% EG + 18% Ficoll 1 min	Straw	Morula 91% Early BL 80% BL 75%, Expanded BL 90%	Hufana-Duran et al., 2004
SCNT	BL	10% EG + 10% DMSO 2 min	20% EG + 20% DMSO 30 s	Cryotop	87%–89%	Laowtammathron et al., 2005
IVF	BL	10% EG + 10% DMSO 4 min	25% EG + 25% DMSO 45 s	Straw	SH-derived oocytes 40% OPU-derived oocytes 53%	Manjunatha, Ravindra, et al., 2008
IVF	Morula BL	a.10% EG b.10% GLY + 10% EG c.10% EG + 10% DMSO 2, 4, 6 min	a.40% EG b.25% GLY + 25% EG c.25% EG + 25% DMSO 45 s	Straw	2 and 4 min in group c yielded reasonable results Mor. 46%–51% BL. 68%–72%	Manjunatha, Gupta, et al., 2009
IVF	Morula BL	10% EG + 10% DMSO 4 min	25% EG + 25% DMSO 45 s	Straw	Mor. 45% w/o CB 53% w/CB BL. 66% w/o CB 75% w/CB	Manjunatha, Ravindra, et al., 2009
SCNT	BL	7.5% EG + 7.5% DMSO 1 min	15% EG + 15% DMSO 30 s	Straw	Conception rate: 11%–25%	Saha et al., 2013
SCNT	BL	8.5% EG + 8.5% DMSO 5 min	16% EG + 16% DMSO 35–40 s	OPS	71%	Sirisha et al., 2013
SCNT	BL	8.5% EG + 8.5% DMSO 5 min	16% EG + 16% DMSO 35–40 s	OPS	Domestic buffalo as donor: 50% Wild buffalo as donor: 38%	Priya et al., 2014
IVF	Morula	4% EG 15 min	35% EG + 0.5 M sucrose + 0.5% PVP 45 s.	SSV	Compact morula 59.0 ± 1.94, blastocyst 32.0 ± 1.10	Rahangdale et al., 2021
		7.5% EG + 7.5% DMSO 4 min	15% EG + 15% DMSO+ 0.5 M sucrose 45 s	SSV	Compact morula 49.0 ± 1.63, Blastocyst 29.0 ± 1.63	

Abbreviations: BL, blastocysts; CB, cytochalasin B; DMSO, dimethyl sulfoxide; EG, ethylene glycol; GLY, glycerol; OPS, open-pulled straw; OPU, ovum-pick-up; PVP, polyvinyl pyrrolidone.

4.2 | Cryopreservation of immature buffalo oocytes

To date, there has been no consistent oocyte cryopreservation method established in buffaloes, unlike in other livestock species such as in cattle (Vajta, Hyttel, & Callensen, 1997). The development of procedures for decreasing the detrimental effects of vitrification on buffalo oocytes is needed to increase oocyte availability for reproductive technologies.

Oocytes collected from slaughterhouse-derived ovaries are at the germinal vesicle (GV) stage in which the genetic material is contained within the nucleus. Because this stage has no spindle present, GVs are assumed to be less prone to chromosomal and microtubular damage during cryopreservation. Possible damages of the meiotic spindle, and other cytoskeletal elements and zona hardening, which could have occurred during vitrification of buffalo oocytes at metaphase of the second meiotic division (MII), can be overcome by cryopreservation of buffalo immature oocytes (Chen et al., 2003) involving optimized cryodevices and CPAs (Table 5). Earlier works using traditional French straws on GV stage buffalo oocytes reported a high postwarming survival but poor maturation rates (Dhali et al., 1999, 2000; Wani, Maurya, et al., 2004). Wani, Misra, and Maurya (2004) reported the first successful production of buffalo blastocysts from vitrified immature GV-stage oocytes. High concentration of the CPAs has proven to be more efficient, wherein higher cleavage and blastocyst rates were obtained from oocytes cryopreserved in 6- and 7-M DMSO, EG, propanediol (PROH), and glycerol (GLY) compared with oocytes cryopreserved in lower concentrations (3.5–5 M). Subsequent works reported higher maturation rates (40%, 43%, 40%, and 24%) from buffalo immature oocytes vitrified in 7-M DMSO, EG, PROH, and GLY (Wani, Misra, & Maurya, 2004). Furthermore, a high blastocyst rate (15%) from vitrified-warmed GV-stage buffalo oocytes with the combination of CPAs such as EG, DMSO, and trehalose was described (Abd-Allah, 2009). Recently, different vitrification solutions and various cryodevices were compared with assess the developmental competence of buffalo cumulus oocyte-complexes vitrified at GV

TABLE 5 Vitrification of buffalo immature oocytes using various protocols and devices

Equilibration solution	Vitrification solution	Devices	Survival rate	Maturation rate	BL rate	References
2.25 M EG + 1.7 M DMSO 1 or 3 min	4.5 M EG + 3.4 M DMSO 2 min	Straw	1 min: 88% 3 min: 98.4%	1 min: 22% 3 min: 32%	NA	Dhali et al., 1999
a. 2.25 M EG + 1.7 M DMSO 1 or 3 min b. 1.75 M EG 1 or 3 min	a. 4.5 M EG + 3.4 M DMSO 2 min b. 3.5 M EG 2 min	Straw	a. 1 min: 89% a. 3 min: 96% b. 1 min: 92% b. 3 min: 92%	a. 1 min: 28% a. 3 min: 32% b. 1 min: 24% b. 3 min: 33%	NA	Dhali et al., 2000
1.5 M DMSO, EG, PROH, and glycerol, respectively, 5 min	3.5, 4, 5, 6, and 7 M DMSO, EG, PROH, and glycerol, respectively, 5 min	Straw	82%–96%	27%–43%	6 and 7 M DMSO, EG, PROH, and glycerol: 10%–15.4%	Wani, Maurya, et al., 2004
a. 1.5 M DMSO 5 min + 3.5 M DMSO 2 min b. 1.5 M EG 5 min + 3.5 M EG 2 min c. 1.5 M PROH 5 min + 3.5 M PROH 2 min d. 1.5 M G 5 min + 3.5 M G 2 min	a. 7 M DMSO 30–40 s b. 7 M EG 30–40 s c. 7 M PROH 30–40 s d. 7 M G 30–40 s	Straw	NA	a. 40.3% b. 42.5% c. 40.4% d. 23.5%	NA	Wani, Misra, & Maurya, 2004
a. 3 M EG b. 1.5 M EG + 1.5 M DMSO c. 1.5 M EG + 1.5 M glycerol d. 1.5 M DMSO + 1.5 M glycerol 45 s each	a. 6 M EG b. 3 M EG + 3 M DMSO c. 3 M EG + 3 M glycerol d. 3 M DMSO + 3 M glycerol 25 s each	Straw, OPS	a. 80.6% b. 82.3% c. 76.4% d. 71.6%	a. 28% b. 41.5% c. 19% d. 17.8%	NA	Mahmoud et al., 2010
10% EG 5 min with 10% EG + 0.3 M trehalose 5 min	40% EG + 0.3 M trehalose + 20% PVP 1 min	Straw	81%	82%	15%	Abd-Allah, 2009
a. 7.5 mg/ml CB 15 min + 10% EG + 10% DMSO 1 min (with CB) b. 10% EG + 10% DMSO 1 min (without CB)	20% EG + 20% DMSO 30 s	Cryotop, SSV	CT(-CB): 86% CT(+CB): 82% SSV(-CB): 80% SSV (+CB): 71%	CT(-CB): 32% CT(+CB): 22% SSV(-CB): 23% SSV (+CB): 13%	CT(-CB): 1.4% CT(+CB): 1% SSV(-CB): 0.6% SSV(+CB): 0%	Liang, Rakwongrit, et al., 2012
10% EG + 10% DMSO (ES1) 3 min 10% EG + 10% GLY (ES2) 3 min	20% EG + 20% DMSO (VS1) 60s 20% EG + 20% GLY (VS2) 60s	Straws OPS SSV	Straw VS1:71.8% Straw VS2:73.6% OPS VS1: 73.9% OPS VS2: 88.2% SSV VS1: 96.3% SSV VS2: 96.7%	36.3% 35.7% 43.8% 44.1% 70.0% 75.0%	1.9% 5.0% 6.5% 7.1% 12.3% 24.0%	El-Shalofy et al., 2017
7.5% EG + 7.5% DMSO 5 min	15% EG + 15% DMSO + 0.5 M sucrose and (0, 5, 10% PVP) 1 min	Cryotop	0% PVP: 40% 5% PVP: 93% 10% PVP:2%	0%PVP: 0% 5%PVP: 40% 10% PVP: 0%	NA NA NA	Jannatul et al., 2020

Abbreviations: CB, cytochalasin B; CT, cryotop; DMSO, dimethyl sulfoxide; EG, ethylene glycol; OPS, open-pulled straw; PROH, propylene glycol (1, 2-propanediol); PVP, polyvinyl pyrrolidone; SSV, solid surface vitrification.

stage (El-Shalofy et al., 2017). The highest survival rate (97%), maturation rate (76%), cleavage rate (47%), and blastocyst development rates (24%) of the COCs were achieved in SSV group compared with those vitrified using traditional straws or those vitrified using OPS. In addition, the use of VS1 solution (20% EG plus 20% DMSO) was found more effective than VS2 solution (20% EG plus 20% GLY). This work clearly shows that the combination of SSV and 20% EG + 20% DMSO could be used effectively to vitrify GV stage buffalo COCs (El-Shalofy et al., 2017). While DMSO has been used for the vitrification of oocytes in buffalo (Wani, Maurya, et al., 2004), it has been reported however that DMSO adversely affects the developmental processes of oocytes (Vincent et al., 1990). Most recently, the replacement of DMSO with 5% PVP on EG + sucrose vitrification solution protects buffalo oocytes from cryoinjury and supports the meiotic progression of oocytes in-vitro after vitrification and warming (Jannatul et al., 2020).

Pretreatment of immature buffalo oocytes with cytochalasin B (CB) for their cryopreservation in SSV and cryotop vitrification methods failed to increase the viability, maturation, or embryo development of vitrified oocytes (Liang, Rakwongrit, et al., 2012). Sharma and Loganathasamy (2007) provided evidence that the meiotic stage affects survival rates of buffalo cumulus-oocyte complexes submitted to vitrification and/or warming with higher survival for matured oocytes compared with immature ones. The low efficiency of vitrified GV stage buffalo oocytes can be attributed to the lower penetrability of GV-stage membrane compared with MII stage emphasizing the effect of cryopreservation on uncoupling of cumulus cells and oocytes, leading to poor maturation rates of vitrified immature oocytes.

4.3 | Cryopreservation of matured buffalo oocytes

Comparative evaluation of efficiency between slow freezing and vitrification of invitro matured buffalo oocytes resulted in successful embryo development following vitrification (Atabay, Atabay, de Vera, et al., 2010; Gautam et al., 2008). In the MII stage oocyte, the cumulus cells surrounding the oocyte are expanded, microfilaments of actin are involved in cell shape and movements, and microtubules form the spindle apparatus (Manjunatha, Gupta, et al., 2008; Manjunatha, Ravindra, et al., 2008). Accordingly, the oocyte stage (GV or MII) affects the composition and permeability of the plasma membrane, which determines its sensitivity to cryopreservation (Agca et al., 1998; Le Gal et al., 1994). In most species, MII stage oocytes survive cryopreservation at higher rates than GV stage ones (Otoi et al., 1995; Somfai et al., 2012). Although vitrification of MII stage buffalo oocytes also worked better than that of GV stage ones (Sharma & Loganathasamy, 2007), the subsequent embryo-development competence was still lower than the fresh oocytes. To overcome problems of container volumes, several devices have been applied for buffalo MII oocyte vitrification by using very small amounts of solution and submerging the sample quickly into the LN_2 (Table 6). This includes SSV (Atabay et al., 2013; Boonkusol et al., 2007; Gasparrini et al., 2007; Liang, Rakwongrit, et al., 2012), Cryoloop (Gasparrini et al., 2007), Cryotop (Atabay et al., 2013; Attanasio, Boccia, et al., 2010; Liang, Rakwongrit, et al., 2012; Muenthaisong et al., 2007; Wang et al., 2016), straws (Gautam et al., 2008), and microdrop (Liang, Srirattana, et al., 2012). Cryotop has been successfully applied for IVM oocytes and SCNT blastocysts (Laowtammathron et al., 2005; Parnpai et al., 2004, 2016). On the other hand, parthenote blastocysts were obtained from invitro matured buffalo oocytes vitrified using SSV and French straw, which resulted in less damage and better blastocyst development (Boonkusol et al., 2007).

Cryotop method has resulted in excellent survival and developmental rates in human and bovine oocytes (Kuwayama et al., 2005). Attanasio, De Rosa, et al. (2010) reported the blastocyst production after IVF of vitrified matured oocytes, proving the feasibility of Cryotop in buffalo. To date, Cryotop has been successfully proven to vitrify buffalo embryos and MII-stage oocytes, which retain the capability to develop into blastocyst following parthenogenetic activation, SCNT, IVF, and ICSI (Atabay & Atabay, 2017; Liang, Srirattana, et al., 2012). Most recently, a more advanced form of Cryotop method, known as Cryotech has been widely used in the human oocyte (Kuwayama et al., 2005) and in bovine embryo vitrification with 47% pregnancy rate (Gutnisky et al., 2013). Report on the use of Cryotech in buffaloes has not been available so far. On the other hand, microdrop was effective in terms of buffalo oocyte recovery, survival, and embryo developmental rates (Liang, Srirattana, et al., 2012).

On the type of permeable cryoprotectants, a mixture of EG and DMSO has been widely used for buffalo MII oocyte vitrification (Atabay et al., 2013; Attanasio, Boccia, et al., 2010; Gautam et al., 2008; Liang, Srirattana, et al., 2012; Muenthaisong et al., 2007). The exposure time in CPAs is considered a critical factor that requires balancing between preventing the formation of intracellular ice and preventing toxic injury. Evidence showed that buffalo oocytes exposed in 7.5% EG and 7.5% DMSO (without cooling) for 4 min gave a similar blastocyst rate (22%) as that of control (23%) but not in the 7- and 10-min exposure groups with 14%–15% blastocyst rates, respectively (Muenthaisong et al., 2007). The low development rate after warming has been attributed to the high lipid content in buffalo oocytes, cytoskeleton damage during freezing, and plasma membrane enriched with cholesterol or unsaturated fatty acids, making it more sensitive to chilling injuries. Most recently, supplementation with 0.6 mg/ml L-carnitine during IVM improves the buffalo oocytes' survival, IVF rates, and subsequent embryo development, which had been associated with improved mitochondrial activity, enhanced β-oxidation, and reduced levels of reactive oxygen species (Liang et al., 2020). Finally, the addition of cytoskeleton stabilizers, such as CB has been demonstr5ated to reduce injury to oocytes during vitrification. Treatment with 8 mg/ml CB prior to vitrification had a positive effect on the developmental capacity of vitrified buffalo oocytes (Wang et al., 2016).

TABLE 6 Vitrification of buffalo matured oocytes using various protocols and devices

Equilibration solution	Vitrification solution	Devices	Survival rate	BL rate	Reference
SSV: 4% EG 12–15 min CLV: 7.5% EG + 7.5% DMSO 3 min	SSV: 35% EG + 5% PVP + 0.4 M trehalose 25–30 s CLV: 16.5% EG + 16.5% DMSO 25 s	SSV, CLV	SSV + cumulus cell: 95.8% SSV - cumulus cell: 84.6% CLV + cumulus cell: 98.5% CLV - cumulus cell: 81.4%	SSV + cumulus cell: 1.5% SSV cumulus cell: 7% CLV + cumulus cell: 0% CLV cumulus cell: 2.8%	Gasparrini et al., 2007
SSV: 4% EG 5–10 min Straw: 4% EG 5–10 min	SSV: 35% EG + 5% PVP + 0.4 M Trehalose 25–30 s Straw: 40% EG + 5% PVP + 0.4 M Trehalose 1 min + LN2 vapor 3 min	SSV, straw	SSV: 89.3% straw: 81.8%	SSV: 13.6% straw: 5.5%	Boonkusol et al., 2007
7.5% EG + 7.5% DMSO 4 min 7 min 10 min	15% EG + 15% DMSO 1 min	Cryotop	MII/vitrified: 66%–71% enucleated/vitrified: 69%–71%	MII/vitrified: 4 min: 10% 7 min: 9% 10 min: 8% enucleated/vitrified: 4 min: 9% 7 min: 7% 10 min: 7%	Muenthaisong et al., 2007
a. 10%, 25%, 40% EG each 1 min b. 10%, 25%, 40% DMSO each 1 min c. 10% EG + 10% DMSO 1 min d: 10% EG + 10% PROH 1 min	a: 40% EG 1 min b: 40% DMSO 1 min c: 20% EG + 20% DMSO 1 min d: 20% EG + 20% PROH 1 min	Straw	a. 85% b. 92% c. 96% d. 95%	a. 1.66% b. 2.29% c. 5.49% d. 2.74%	Gautam et al., 2008
a. 7.5% EG + 7.5% DMSO 3 min b. 10% EG + 10% DMSO 3 min	a. 16.5% EG + 16.5% DMSO 20–25 s b. 20% EG + 20% DMSO 20–25 s	Cryotop	a. 84% b. 85.6%	a. 6.4% b. 7.8%	Attanasio, Boccia, et al., 2010
10% EG + 10% DMSO 3 min	20% EG + 20% DMSO 20–25 s	Cryotop	86%–92%	1.4%–8.0%	Attanasio, De Rosa, et al., 2010
10% EG + 10% DMSO 1 min	20% EG + 20% DMSO 30 or 45 s	Microdrop	30 s: 96% 45 s: 91%	30 s: 11% 45 s: 7%	Liang, Phermthai, et al., 2011
VA: 10% EG + 10% DMSO 1 min VB: 4% EG 12–15 min	VA: 20% EG + 20% DMSO 30 s VB: 35% EG + 50 mg/ml PVP 30 s	Microdrop Cryotop	VA + microdrop: 93% VA + Cryotop: 97% VB + microdrop: 79% VB + Cryotop: 81%	VA + microdrop: 8% VA + Cryotop: 10% VB + microdrop: 5% VB + Cryotop: 11%	Liang, Rakwongrit, et al., 2012
7.5% EG + 7.5% DMSO 5 min	15% EG + 15% DMSO + 0.5 M sucrose	Cryotop SSV	CTP cumulus (+): 85.93% CTP cumulus (−): 82.67% SSV cumulus (+):87.74% SSV cumulus (−): 81.38%	CTP cumulus (+): 10.46% CTP cumulus (−): 4.29% SSV cumulus (+):12.41% SSV cumulus (−): 3.00%	Atabay et al., 2013
10% DMSO and 10% EG 1 min. (with pre-treatment with CB 8 μg/ml for 30 min)	20% DMSO, 20% EG and 0.5 M sucrose 30 s	Cryotop	2nd polar body formation: CB + Cryotop:51.16% Cryotop: 43.88%	17.08% 10.21%	Wang et al., 2016
10% EG + 10% DMSO	20%EG + 20% DMSO	Cryotop	0 mg/ml L-carnitine 96% 0.3 mg/ml L-carnitine 97% 0.6 mg/ml L-carnitine 97% 1.2 mg/ml L-carnitine 96%	0 mg/ml L-carnitine 4% 0.3 mg/ml L-carnitine 4%	Liang et al., 2020

(Continues)

TABLE 6 (Continued)

Equilibration solution	Vitrification solution	Devices	Survival rate	BL rate	Reference
			Fresh control 100%	0.6 mg/ml L-carnitine 8% 1.2 mg/ml L-carnitine 8% Fresh control 19%	

Abbreviations: CB, cytochalasin B; CLV, cryoloop vitrification; DMSO, dimethyl sulfoxide; EG, ethylene glycol; LN2, liquid nitrogen; MII, metaphase II stage; PROH, propylene glycol (1, 2-propanediol); SSV, solid surface vitrification; VA, vitrification A solution; VB, vitrification B solutions.

5 | SCNT

5.1 | Cloned buffalo embryo and offspring production

Cloned swamp buffalo embryos were first successfully produced by SCNT using fetal fibroblasts as the donor cells in 1999 (Parnpai et al., 1999). In 2004, three recipients were pregnant after transferring cloned swamp buffalo derived from fetal fibroblasts; however, no recipient could carry to term (Saikhun et al., 2004). Simon et al. (2006) also transferred cloned river buffalo embryos derived from fetal fibroblasts to the recipients but no pregnancy was found. In 2007, the first SCNT swamp buffalo was successfully produced using granulosa cells (Shi et al., 2007). After that, several cloned swamp and river buffalo calves were successfully produced by the conventional SCNT (Wilmut et al., 1997) and the handmade cloning (HMC) methods (Vajta, 2007) by different workers using various donor cell types (Table 7).

5.2 | Epigenetic modification to improve buffalo cloning efficiency

The overall efficiency of cloned animal production is still relatively low (Zhang et al., 2021). Several abnormalities have been found in SCNT embryos and offspring (Keefer, 2015; Niemann et al., 2002; Ogura et al., 2013), which may be caused by incomplete epigenetic reprogramming of the donor cell during SCNT (Tian et al., 2003; Yang et al., 2007). Epigenetic modifications such as DNA methylation and histone modifications play an important role in embryonic development (Niemann, 2016; Sproul et al., 2005). Aberrant epigenetic modifications such as DNA methylation and histone acetylation, and also abnormal gene expression patterns for example insulin-like growth factors (IGF-1 and IGF-2) have been found in cloned buffalo embryos when compared with those of IVF embryos (Jyotsana et al., 2016; Luo et al., 2013; Mohapatra et al., 2015; Pandey et al., 2009; Saini et al., 2016, 2017; Srirattana et al., 2014; Sun et al., 2015; Suteevun, Parnpai, et al., 2006; Suteevun, Smith, et al., 2006).

Trichostatin A (TSA) is a hydroxamic acid inhibitor (Marks et al., 2001) and is one of the most used histone deacetylase inhibitors (HDACi) to improve cloning efficiency in many mammalian species such as mice (Kishigami et al., 2006), rhesus monkeys (Sparman et al., 2010), pigs (Zhang et al., 2007), rabbits (Shi et al., 2008), cattle (Akagi et al., 2011), and cynomolgus monkeys (Liu et al., 2018). To facilitate nuclear reprogramming, the donor cells were treated with 0.3-nM TSA for 48 h prior to SCNT. TSA treatment in donor cells increased the cleavage and blastocyst rates and increased the histone H4 lysine 8 acetylation (H4K8ac) level of SCNT swamp buffalo embryos to a level equivalent to those of IVF counterparts (Luo et al., 2013). Significant improvement in mouse cloning was found when TSA was treated on reconstructed oocytes/embryos (Kishigami et al., 2007). In SCNT swamp buffalo, treatment of TSA at 25 nM for 10 h on reconstructed oocytes could enhance embryo development, but no beneficial effect on the DNA methylation level was observed (Srirattana et al., 2014). When HMC river buffalo embryos treated with 75-nM TSA for 10 h, the global level of histone H4 lysine 5 acetylation (H4K5ac) in blastocysts was increased and level of histone H3 lysine 27 trimethylation (H3K27me3) were decreased, however, the global level of histone H3 lysine 18 acetylation (H3K18ac) was not affected (Selokar et al., 2015). And it was concluded that TSA treatment could not improve embryo development and offspring rate.

Scriptaid, 6-(1,3-Dioxo-1H, 3H-benzo[de]isoquinolin-2-yl)-hexanoic acid hydroxyamide is a drug that acts as a Histone deacetylase inhibitor (HDACi). Scriptaid has proven less toxic than TSA in cloned mice (Van Thuan et al., 2009), cattle (Wang et al., 2011), rabbit (Chen et al., 2013), and pigs (Xu et al., 2013). Adding 500 and 1000 nM of Scriptaid for 10 h into embryo culture media increased the blastocyst formation rate of HMC river buffalo embryos and increased cell number in blastocysts (Panda et al., 2012). Similarly, treatment of SCNT buffalo embryos with 500-nM Scriptaid for 24 h increased blastocyst formation rate and also resulted in higher levels of H3K18ac and lower methylation levels of global DNA at the blastocyst stage, which was similar to fertilized counterparts (Sun et al., 2015).

When donor cells were treated with TSA or 5-aza-2′-deoxycytidine (5-aza-dC), a DNA methyltransferase inhibitor (DNMTi) prior to cloning, acetylation levels of these donor cells were increased and methylation levels were decreased (Saini et al., 2016). Moreover, treatment of 50-nM TSA and 7.5-nM 5-aza-dC in donor cells and/or reconstructed oocytes increased blastocyst rates and decreased apoptosis rate of HMC river buffalo embryos (Saini et al., 2017). However, valproic acid (VPA), another HDACi, treatment in donor cells increased histone acetylation of the cells but could not increase the blastocyst rate of HMC river buffalo embryos (Selokar et al., 2017). Moreover, treatment of donor cells with another DNMTi, RG108

TABLE 7 Summary of the cloned buffalo offspring

Buffalo type	Cloning method	Donor cell	Blastocyst rate (%)	No. of recipient	Pregnancy rate (%)	Calving rate (%)	Reference
Swamp	SCNT	Fetal fibroblast	21.3	16	3 (18.8)	2 (12.5) one calf died	Shi et al., 2007
		Granulosa cell	22.2	5	1 (20.0)	1 (20.0) died after 14 days	
River	HMC	Fetal fibroblast	24.0	5	1 (20.0)	1 (20.0) died after 5 days	Shah et al., 2009
		Newborn fibroblast	33.0	8	2 (25.0)	1 (12.5)	
River	HMC	Embryonic stem cell	27.3	6	2 (33.3)	1 (16.7)	George et al., 2011
Swamp	SCNT	Fetal fibroblast	18.6	16	3 (18.8)	2 (12.5)	Lu et al., 2011
River	HMC	Fetal fibroblast	30.7	4	1 (25.0)	1 (25.0) died after 4 h	Panda et al., 2011
River	HMC	Newborn fibroblast	41.7	9	1(11.1)	1 (11.1) died shortly after birth	Saha et al., 2013
		Fetal fibroblast	39.1	4	1 (25.0)	1 (25.0)	
River	HMC	Fresh semen derived epithelial cell	48.8	12	1 (8.3)	1 (8.3)	Selokar et al., 2014
		Frozen semen derived epithelial cell	51.4	10	2 (20.0)	1 (10.0) died after 12 h	
Swamp	SCNT	Skin fibroblast	25.0	12	10 (83.3)	1 (8.3)	Tasripoo et al., 2014
River	HMC	Skin fibroblast	28.8	3	1 (33.3)	1 (33.3)	Jyotsana et al., 2015
River	HMC	Urine-derived epithelial cell	50.4	5	1 (20.0)	1 (20.0)	Madheshiya et al., 2015
River	HMC	Skin fibroblast	50.0	4	1 (25.0)	1 (25.0) died after 21 days	Saini et al., 2016
Swamp	HMC	Fetal fibroblast	27.9	7	2 (28.6)	2 (28.6)	Liu et al., 2018
River	HMC	Frozen thawed semen derived epithelial cell	One demicytoplast 12.7	8	1 (12.5)	1 (12.5) died after 12 days	Raja et al., 2019
			Two demicytoplast 47.6	8	1(12.5)	1 (12.5)	
River	HMC	Skin fibroblast	40.4	8	2 (25.0)	1 (12.5)	Selokar et al., 2019
River	HMC	Skin fibroblast	42.6	13	4 (30.8)	2 (15.4)	Shyam et al., 2020

Abbreviations: HMC, handmade cloning; SCNT, conventional somatic cell nuclear transfer.

could decrease DNA methylation level in buffalo donor cells and could increase the blastocyst formation rate of SCNT buffalo embryos (Sun et al., 2016).

There are a number of reports that nonchemical and biological agents were used for improving buffalo embryo production efficiency. Transfection of 50-nM *DNMT1* small interfering RNA (siRNA) to 1-cell stage HMC buffalo embryos decreased levels of *DNMT1* mRNA and DNMT1 protein and increased blastocyst formation rate but did not alter the DNA methylation level (Selokar et al., 2015). Treatment of buffalo donor cells with cell-free extracts from buffalo matured oocyte (BOE) decreased expression levels of *HDAC1*, and increased H3K9ac level as well as *OCT4* and *NANOG* pluripotency-related gene expression levels in the donor cells. Moreover, HMC river buffalo embryos produced from BOE-treated donor cells had similar *OCT4*, *NANOG*, and *SOX2* expression levels to those in IVEP blastocysts (Sadeesh et al., 2017). microRNAs (miRNAs) are single-stranded non-coding RNA molecule (about 22 nucleotides) that are involved in oocyte maturation and fertilization (Gilchrist et al., 2016), embryo development (Hossain et al., 2012), maternal-to-zygotic transition (Mondou et al., 2012), and epigenetic reprogramming and pluripotency (Onder & Daley, 2011). miRNA-145 is involved in early embryonic development (Tesfaye et al., 2009) and differentiation of stem cells (Xu et al., 2009) and was found to be a higher expression in cloned embryos than that in IVEP embryos. Treatment with an inhibitor of microRNA-145 (80 nM) for 1 h after electrofusion could decrease the apoptotic index and increase the blastocyst rate of HMC

river buffalo embryos (Sah et al., 2020). Other miRNAs play on embryonic development, miRNA-21 is involved in the regulation of apoptosis and miRNA-29b plays an important role in controlling DNA methylation in cells. Treatment of miRNA-21 (40 nM for 1 h) and miRNA-29b (40 nM for 1 h) mimics improved blastocyst quality, reduced apoptosis, and altered gene expression but did not increase the blastocyst rate of HMC buffalo embryos (Rashmi et al., 2019).

Canonical WNT (wingless-related mouse mammary tumor viruses) signaling pathway has been reported to inhibit embryonic development (Tepekoy et al., 2015). Dickkopf-1 (DKK1) is a secretory inhibitor of the canonical WNT signaling pathway, which could increase blastocyst formation, conception, and birth rates of HMC river buffalo embryos (Shyam et al., 2020). DKK1 can also increase the preimplantation development of bovine IVF embryos (Denicol et al., 2014).

Histone methylation plays an important role during embryonic development and is regulated by histone methyltransferases and histone demethylases (Jambhekar et al., 2019). Aberrant epigenetic reprogramming of histone 3 lysine 9 trimethylation H3K9me3 was found in cloned bovine (Pichugin et al., 2010; Santos et al., 2003), mouse (Ribeiro-Mason et al., 2012), and rabbit (Yang et al., 2013) embryos. Histone demethylase Kdm4d could regulate the level of H3K9me3. In SCNT buffalo embryos, the expression level of Kdm4d was significantly lower while the level of H3K9me3 was significantly higher when compared with the IVEP buffalo embryos. Microinjection of Kdm4d mRNA could correct the H3K9me3 level, increase the expression level of ZGA (*ZSCAN5B*, *SNAI1*, *eIF-3a*, and *TRC*) and pluripotency-related genes (*POU5F1*, *SOX2*, and *NANOG*) and promote the developmental ability of buffalo SCNT embryos (Feng et al., 2021). Events of epigenetic modification such as histone acetylation, DNA methylation, and histone methylation should be deeper studied during buffalo embryonic development. The effects of epigenetic modulators on the full-term development of cloned buffalo embryos are needed to be determined.

5.3 | Generation of transgenic buffalos

The production of transgenic animals has numerous potential applications in establishing human genetic disease models, producing pharmaceutical proteins, and improving the growth performance and disease resistance of farm animals (Laible et al., 2015). In 2018, Lu et al. transfected enhanced green fluorescent protein (eGFP) into male swamp buffalo fetal fibroblasts using the electroporation technique. A total of 72 blastocysts produced from transfected donor cells were transferred to 36 recipients and six recipients became pregnant. At the end of gestation, the pregnant recipients delivered six healthy transgenic calves and one stillborn transgenic calf (Lu et al., 2018). Producing offspring with the desired sex is a significant goal in livestock production. The combination of CRISPR/Cas9-mediated gene editing and SCNT techniques could apply to sexing preimplantation embryos. The eGFP gene was integrated into the Y chromosome of swamp buffalo fetal fibroblasts. When these cells were used as the donor cells, the results showed that eGFP reporter is suitable for the visualization of the sex of embryos (Zhao et al., 2020). Moreover, the blastocyst rate of transgenic SCNT embryos was similar when compared with that of the nontransgenic group (Zhao et al., 2020). This report showed that the transgenic donor cells had no negative effect on buffalo embryonic development. On the other hand, when human insulin gene was transfected into buffalo fetal fibroblasts using nucleofection and these transgenic cells were used as the donor cells, the blastocyst rate of SCNT was lower than that of of nontransgenic donor cells (Mehta et al., 2018). Moreover, when Venus construct (derivative of the enhanced yellow fluorescent protein) was transfected into river buffalo fetal fibroblasts using electroporation technique, the morula and blastocysts rates of HMC river buffalo embryos produced by Venus transgenic cells were found lower than that of nontransgenic cells (Kumar et al., 2018).

5.4 | Interspecies SCNT (iSCNT) in buffalo

iSCNT, where the recipient cytoplasm and donor nucleus are derived from different species, provides an alternative tool for the preservation of endangered species using oocytes and recipients from related domestic species (Beyhan et al., 2007). iSCNT buffalo blastocysts were successfully produced using bovine oocytes as the recipient cytoplast (Kitiyanant et al., 2001; Lu et al., 2005; Saikhun et al., 2004). However, the mixing of two populations of mitochondrial DNA from the buffalo donor cell and bovine recipient oocyte has been found in iSCNT buffalo embryos (Srirattana et al., 2011). Incompatibility between the nuclear and mitochondrial genomes is thought to be one of the major causes of developmental arrest among iSCNT embryos (Ogura et al., 2013). To improve iSCNT efficiency, treatment of buffalo–bovine iSCNT reconstructed oocytes with 20-μM zebularine (DMNTi) and 2-μM BIX-01294 (HDACi) could decrease the respective levels of 5-methylcytosine and histone 3 lysine 9 dimethylation (H3K9me2). The quality of iSCNT blastocysts was improved due to the significant expression of *OCT4* and *CDX2* in BIX-01294 and *CDX2* in zebularine treatments. However, treatment with zebularine and BIX-01294 did not enhance developmental competence of iSCNT embryos (Alsalim et al., 2018). For iSCNT, river buffalo donor cells were transferred to swamp buffalo enucleated oocytes (Yang et al., 2010). The result showed that the blastocyst rate of river-swamp embryos was not different from the swamp–swamp embryos. A total of 30 river-swamp blastocysts were transferred to 13 recipients, four recipients established pregnancy. While three of them were aborted, one live river-swamp buffalo calf was born. These results indicate that swamp-river buffalo embryos can develop to full term (Yang et al., 2010). Moreover, buffalo oocytes also have the potential to reprogram somatic cells from bovine and goat up to the blastocyst stage (Selokar et al., 2011). In addition, wild buffalo iSCNT embryos were successfully produced through HMC using recipient oocytes from river buffalo (*Bubalus bubalis*) and skin fibroblasts from wild buffalo (*Bubalus arnee*) (Priya et al., 2014). The blastocyst rate of wild buffalo iSCNT embryos was about of 38–50% (Priya et al., 2014; Saini et al., 2015). iSCNT could be used as an alternative approach in

buffalo cloning. However, nuclear and mitochondrial genomes incompatibility, mtDNA heteroplasmy, embryonic genome activation of the donor nucleus by the recipient oocyte, and availability of suitable foster mothers for iSCNT embryos are needed to be determined and solved.

6 | CONCLUSION AND FUTURE PERSPECTIVE OF ART IN BUFFALO

ART in buffaloes has achieved considerable success as shown by the birth of live healthy riverine calves ($2n = 50$) out of in vitro produced-vitrified embryos in both the riverine and swamp ($2n = 48$) buffalo recipients, MOET produced embryos, embryos from OPU-derived oocytes, sex-sorted sperm cells, and SCNT.

ES and fixed-time artificial insemination partly solved distinct problems or the extrinsic factors affecting buffalo reproduction. The window on estrus occurrence and time of ovulation can be synchronized using Ovsynch protocols during breeding season and with P_4-based protocols in combination with gonadotropin, estradiol, equine chorionic gonadotropin, human chorionic gonadotropin, and PG during the nonbreeding season. Enhancing PG-based ES protocol with gonadotropin (GnRH or hCG) had a beneficial effect on improving the pregnancy rate. A deep understanding and wide knowledge of follicular dynamics in buffalo are necessary for developing new innovative approaches and improving the currently used regimens for controlled breeding. There is a need to focus on the correlation between ovarian structures and endocrine milieu at various times points during hormonal treatment and the size of follicles at the time of insemination.

IVEP is a potential alternative in the production of desired animals. The quality of the oocytes, the components of the IVC medium, the culture condition, and the quality of sperm cells are important considerations in the success rate. Resultant embryos can withstand cryopreservation and develop to term once given the appropriate condition. With the poor ovulation response of buffalo, IVEP is a good alternative in the production of genetically superior animals. With the advancements in ICSI, reproduction among genetically superior bulls with sperm motility and fertility problems and enhancing fertilization using sex-sorted embryos become possible though the activation factor needs further improvement for a higher success rate.

Cryopreservation of oocytes has been found critically important in the progress and practical application of reproductive biotechnologies in buffaloes. However, overall efficiency obtained with frozen/thawed gametes and embryos remained low. Further research must focus on the biochemical evaluation of various CPAs and careful selection of the most effective CPAs along with efficient carrier methods. The development of procedures for decreasing the detrimental effects of vitrification on buffalo oocytes is needed to increase oocyte availability for reproductive biotechnologies. In addition, the transfer of vitrified buffalo embryos from vitrified/warmed oocytes into recipient animals to produce healthy calves must be seriously pursued in order to prove the full developmental potential of the vitrified/warmed buffalo oocytes. Moreover, variability of protocols with varying efficiencies exists, thus there is a need for standardization of protocols. The reduction of technical variations and mindfulness of quality control of the vitrification system will enhance procedural consistency, repeatability, and efficiency among laboratories. Therefore, future research undertakings should be directed not only on improving the efficiency of the vitrification system but also to narrowing down the variability of the manual system to achieve standardized operation to increase the overall efficiency of oocyte and embryo vitrification, especially in buffaloes.

Buffalo SCNT is a powerful tool for elite animal production, conservation of endangered species, and generating transgenic animals to improve human health and animal production. However, the molecular mechanisms and full-term development of the cloned buffalo embryos are still needing further investigation in order to improve buffalo cloning efficiency.

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CONFLICT OF INTEREST

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