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Strategies to Improve the Developmental Competence of Water Buffalo Oocytes *In Vitro*

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Mammalian oocytes undergo spontaneous maturation when isolated from the ovarian follicles and cultured *in vitro*. Use of these oocytes in the production of embryos for eventual transfer to recipients to produce youngs have been widespread, but only to a limited extent especially in bubaline species. Apparently, understanding of the mechanisms governing the bubaline oocytes acquisition of developmental competence *in vitro* remained a challenge, especially on the role of various supplemental compounds/substances being incorporated in the maturation medium and the role of ovarian follicular environment surrounding the oocyte. This review focuses on the dynamics of these compounds/substances with particular emphasis on how it interact with the metabolic needs of the oocytes and the nature and diversity of compounds that transfer between cumulus cells and the oocyte and its impact on the process of developmental acquisition. In one particular case, we have demonstrated that IVM supplementation with cysteamine improved the developmental potential of swamp buffalo oocytes to developed up to the blastocyst stage following fertilization *in vitro*.

Keywords: Oocyte maturation, developmental competence, embryo production, *in vitro*

Introduction

In most developing countries, the major concern is on how to meet the increasing demand for more food production (both crops and livestock) by its rapidlygrowing population. In the livestock sector, genetic improvement of local breeds through assisted reproductive technologies (ARTs) to help improve and increase its production in a much faster rate have been adopted. Recent progress in the application of some ARTs, including the *in vitro* maturation/fertilization/culture (IVM/IVF/IVC) and intracytoplasmic sperm injection (ICSI) systems have made it possible to produce large number of embryos for offspring production with the desired genetictraits. However, the efficiency of there application is dependent on a number of factors, including the source and quality of oocytes, culture and activation conditions, quality of resulting embryos and synchrony between the stage of

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embryos and the recipients. Of these, oocyte quality and its achievement of complete maturation *in vitro* remained the most critical factor for determining the developmental competence of resulting embryos to term. Hence the induction of oocyte maturation *in vitro* need to be taken seriously because of its potential to utilize the vast supply of oocytes within the ovary and transform this into embryos for offspring production. The critical challenge here is to understand what constitutes oocyte developmental competence and the mechanisms governing it , especially between the oocyte and somatic cell interactions. Apparently, the somatic cells of the follicle particularly the cumulus cells play a key role in the acquisition of oocyte developmental competence *in vivo*. However, our understanding of the nature and diversity of compounds that transfer between the cumulus cells and the oocyte via gap junctions during the final phase of follicular development is limited (Albertini et al., 2001; Gilchrist et al., 2004). The reason why studying in detail the interaction between the oocytes and its surrounding somatic cells during oocyte growth and maturation is important.

The *In vitro* maturation of oocytes differs to*in vivo* maturation in two fundamentally important ways. Firstly, oocytes collected from small to mid-sized antral follicles have not yet completed “oocyte capacitation”, thus do not possess full molecular and cellular machinery required to support early embryogenesis (Hyttel et al., 1997; Sirard et al., 2006). Secondly, mechanically removing the oocyte from the follicle results in loss of natural meiotic inhibiting environment, that results to “spontaneous” meiotic maturation of the oocytes *in vitro*. The reinitiating of M-phase in spontaneously maturing oocytes occurs via different intracellular molecular cascades, from the “induced” meiotic maturation that occurs *in vivo* in response to gonadotropin cascade. Hence, embryos and fetuses produced from IVM have some potential for impaired development than those generated from *in vivo* matured oocytes (Thompson et al., 1995; Rizos et al.,2002).Therefore,varyingapproaches in the development of an IVM systems that would rescue and assist the oocytes to gain developmental competence instead of undergoing natural demise *in vivo* by subordination and atresiamust be undertaken. The purpose of this paper is to review available informations from different experimentations in *in vitro*maturation of mammalian oocytes with potential application in water buffalo.And, to identify which of them could be applied in themass production of embryos and offsprings particularly in developing countries where buffalo remained there most important livestock resource, providing milk, meat and work power.

Media formulation for in vitro maturation

The IVM system is intended to yield oocytes that have completed the 1st meiotic division (nuclear maturation), can be fertilized and support male

pronucleus formation of penetrating spermatozoon (cytoplasmic maturation) which can result in embryos capable of full- term development. At present however, the most commonly used media (eg., TCM-199, MEM, Ham's F-10) for IVM of mammalian oocytes were designed for the culture of somatic cells and tissues to address the metabolic needs, particularly the longer-term needs of cell lines and not for the complex and dynamic needs of maturing cumulus-oocyte complexes (COCs) (Thompson, 2006). This is clearly a deficiency in IVM technology that could have contributed to fertilization abnormalities such as polyspermy,parthenogenesis and asynchronous development of male and female pronuclei (Crozet, 1991;Ocampo et al., 1993). Unlike in embryo culture media where significant improvements were made by basing media formulations on the major cation and anion concentrations and metabolic substrates of reproductive tract fluids, as well as on metabolic needs of growing preimplantation embryos (Gilchrist and Thompson, 2007). It is obvious that the follicular fluid compositions varies greatly from commonly used IVM media (Sutton et al.,2003: 2005), particularly glucose which is the major energy substrate for the COCs.

A practical approach then is on designing a chemically defined IVM medium based on the composition of the follicular fluid to ensure the complete maturation of the oocytes when cultured *in vitro* replacing the commonly used biological media. Chemically defined media are reproducible at different times and in different laboratories and can be varied in a controlled manner. Whereas, biological media may contain biological fluids of unknown chemical composition, such as serum, which could contribute to variability in responses being studied. There are only two steps in the design of a chemically defined medium, the choice of compounds to include, and the concentrations of selected compounds (Biggers, 1998).A good example is the development of synthetic oviductal fluid (SOF) based on the biochemical analysis of ovine oviductal fluid (Tervit et al., 1972) used for the culture of cleaving embryos of sheep and cattle which upon modifications were also used as a single medium to support the maturation , fertilization and culture of bovine and bubaline embryos (Gandhi et al., 1999).SOF has subsequently been modified by the addition of amino acids, citrate (Keskintepe et al., 1995), the removal of glucose (Takahashi and First, 1992) and the inclusion of EDTA for the initial 72 hrs of the culture period (Gardner et al., 1997).

Improving oocyte glutathione (GSH) synthesis

During the development and maturation of the oocytes in the ovary, the GSH content increases as the oocyte approaches the time of ovulation. GSH accumulation in the ooplasm is necessary, as the oocyte prepares for fertilization and it is an important cytoplasmic factor for sperm nuclear decondensation and hence for male pronucleus formation after fertilization(De Matos et al., 1996). GSH play a critical role in reducing the

disulfide-rich-protamines in the sperm nucleus (Perreault et al, 1988; Yoshida *et al.*, 1993) which must be unpacked in the oocyte during fertilization in order for sperm DNA to participate in embryonic development (Zirkin *et al.*, 1989: Perreault, 1990).The supplementation of IVM medium with cysteamine has been demonstrated to enhance cysteine-mediated GSH synthesis (De Matos *et al.,* 1995; Ocampo *et al.*, 2000).Cysteine and cystine are present in IVM media (eg., TCM-199, Hams F-10, MEM) commonly used in mammalian oocytes but under *in vitro* culture conditions, cysteine is readily oxidized to cystine making it unavailable for synthesis(Bannai, 1984; Issels *et al.*, 1988; De Matos *et al.*, 1997). Cysteamine reduces cystine to cysteine and promote its uptake through γ-glutamyl cycle, making its synthesis dependent on the presence of cysteine in the medium (Meister, 1983; Meier and Issels, 1995). Another option is the supplementation of IVM medium with β-mercaptoethanol, cysteine (Abeydeera *et al.*, 1998; De Matos and Furnus, 2000) or L-carnitine (You *et al.*, 2012; Chankitisakul *et al.*, 2013) which has been demonstrated to stimulate the synthesis of intracellular GSH.GSH has been shown to be synthesized throughout IVM of pig, hamster and buffalo oocytes resulting to improved sperm chromatin decondensation and hence MPN formation of penetrating spermatozoa (Perreault *et al.*, 1988; Yoshida *et al.*, 1993; Ocampo *et al.*, 2000). Depletion of GSH during oocyte maturation reversibly block the decondensation of MPN and pronuclear apposition during fertilization (Sutovsky and Schatten, 1997). The antioxidant activity of GSH protects the cells from the damaging effects (oxidative stress) of free oxygen radicals generated during metabolism causing developmental arrest at the earliest period of embryonic gene expression.

Improving oocyte DNA methylation

Oocyte quality is closely related to developmental competence, and the acquisition of oocyte competence accompanies spatiotemporal changes in histone acetylation and DNA methylation (Bui *et al.*, 2007; Endo *et al.*, 2005; Mason *et al.*, 2012). The methylation level of DNA increases with oocyte growth as has been found in bovine oocytes in which oocytes derived from large antral follicles have higher DNA methylation than those derived from small antral follicles (Lodde *et al.*, 2009; Fagundes *et al.*, 2011). DNA methylation has been suggested to continue up to the metaphase 2 stage (Imamura *et al.*,2005) and decreases as oocyte age (Kim *et al.*,2009).The supplementation of IVM medium with folic acid is responsible for global DNA hypermethylation in oocytes and high level of histone acetylation in early developmental stage embryos (Sato *et al.*, 2013). Folic acid is a member of vitamin B family and serve as a methyl donor (Boxmeer *et al.*, 2008). Also, oocytes cultured in maturation medium with

folic acid show increased GSH content and improved developmental ability (Kim et al., 2009).

Reduction of oocyte exposure to reactive oxygen species (ROS)

Handling or culturing of oocytes or embryos in a high oxygen atmosphere, glucose concentration or during artificial treatments (eg., electric stimulus for cell fusion or during activation of somatic cell nuclear transferred oocytes) result to an increased ROS levels (Fujitani et., 1997; Thompson *et al*., 2000; Koo *et al*., 2008), causing cell membrane lipid peroxidation (Nash-Esfahani *et al*., 1990; Noda *et al*., 1991), DNA fragmentation and influences RNA transcription and protein synthesis (Takahashi *et al*., 2000) which lead to *in vitro* developmental blocks and early embryonic death (Noda *et al*., 1991; Goto *et al*., 1993). Thus, modifications of IVM culture system that would reduce ROS activity is necessary. IVM medium supplementation with antioxidants has been demonstrated to stimulate the synthesis of GSH and decreased the ROS levels in oocytes (Luberda, 2005; Wu *et al*., 2011).Also, a cysteine-containing medium might prevent cell death triggered by oxidative stress or ROS (Cetica *et al*., 2001). N-acetyl-cysteine is a potent antioxidant that has been proven to reduce oxidative stress in oocytes and improve embryo development (Whitaker *et al*., 2012). L-carnitine (β-hydroxy-γ-trimethylammonium-butyric acid), an antioxidative agent, protects cell membranes and DNA from damage induced by oxygen free radicals (Zhou *et al*., 2007). Significant improvement in the integrity of microtubule, chromosome structural integrity and decreased level of apoptosis in mouse M-II oocytes and 8- cell embryos were observed when incubated in medium with 0.6 mg/ml L-carnitine (Mansour *et al*., 2009). Also, use of 0.3 mg/ml L-carnitine improved blastocyst formation in mice by reducing the blocking effects of actinomycin-D, hydrogen peroxide and tumor necrosis factor-α on embryonic development and decreasing levels of DNA damage (Abdelrazik *et al*., 2009). In bovine, L-carnitine addition improved embryonic development in vitro by exhibiting an extensive relocation of active mitochondria to the inner oocyte cytoplasm (Yamada *et al*., 2006).

Supplementation with recombinant activin-A

During mammalian development**,** some cytokines, including activin-A play a functional role in the process of cellular proliferation, differentiation and morphogenesis. Activin is an important member of the transforming growth factor β superfamily, and are homodimers or heterodimers of the βA or βB subunits of inhibin linked to one another by a single disulfide bond (Hata *et al*., 1988).Dimerization of these subunits gives rise activin-A (βAβA), activin- AB (βAβB) and activin-B (βBβB) (Ying, 1988). The expression of protein and mRNA for activin-A and activin receptors in the

ovary has been localized in both oocyte and granulosa cells of follicles of various developmental stages (Izadyar *et al*., 1998; Sidis *et al*., 1998; Van der Hurk and Van de Pavert, 2001;Thomas *et al*., 2003; Silva *et al*., 2004). Aside from being a local regulator of folliculogenesis, activin-A also directly stimulate FSH synthesis and secretion, promote release of GnRH (Childs and Unabia, 1997), stimulate increase of FSH and LH receptors in granulosa cells and play a role in progesterone production (Alak *et al*., 1996; Tsuchiya *et al*., 1999). Therefore, the granulosa cells are likely to be the main source of paracrine factors crucial for oocyte maturation. Supplementation of IVC medium with activin-A was shown to enhance embryo development of prepubertal goat oocytes (Hammami *et al*., 2014) (Table 1).

Table 1. Effect of IVM supplementation on embryo production

IVM supplement	Species	Blastocyst development	Remarks
Cysteamine	Bovine Buffalo Porcine Murine Ovine	Improved Improved Improved Improved Improved	Increased intracellular GSH level, reduced oxidative stress
ß-Mercapto-ethanol	Bovine Porcine Ovine	Improved Improved Unchanged	Increased intracellular GSH level,reduced oxidative stress
Cysteine or Cystine	Bovine Porcine	Improved Improved	Increased intracellular GSH level, reduced oxidative stress by reducing levels of reactive oxygen species in the oocytes
L-Carnitine	Porcine Murine Bovine	Improved Improved Improved	Increased GSH and decreased ROS level, energy source via β-oxidation, improved cell activity, facilitate extensive relocation of active mitochondria to the inner oocyte cytoplasm
Folic Acid	Porcine	Improved	Influences DNA methylation of oocytes, has antioxidant property and improved GSH content
Activin-A	Caprine Bovine Porcine	Improved Unchanged Unchanged	Stimulate FSH synthesis and secretion, promote release of GnRH and plays a role in progesterone production

Attenuating spontaneous oocyte maturation in vitro

In vivo, the oocytes gradually and sequentially acquire meiotic and developmental competence during folliculogenesis as regulated by a complex interaction of stimulators and inhibitors in the hypothalamus-pituitary-ovary system. The oocytes during early follicle growth actively synthesized RNA as characterized by dispersed chromatin configurations and non-compacted nucleoli (De Smedt et al.,1994; Fair *et al*., 1995) before gradually reduces its synthetic activityuntil the oocyte reaches a quiescent

state (oocyte capacitation), where it acquires the cytoplasmic machinery needed to support embryo development (Hyttel *et al.*, 1997; Brevini-Gandolfi, 2001).The oocyte remains arrested at the dictyate stage (late prophase of 1st meiosis) until the occurrence of gonadotrophin surge (Tsafriri, 1978) overriding the natural meiotic inhibiting environment of the follicle as mediated by specific epidermal growth factor (EGF)-like peptides, secreted by mural granulose cells in response to the LH surge (Park et al., 2004). In vitro, once the oocytes in the follicles are released for IVM, the process of oocyte capacitation got interrupted and meiotic process resumes, leading to spontaneous germinal vesicle breakdown down to 1st polar body extrusion (Pincus and Enzmann, 1935) in the absence of certain crucial oocyte cytoplasmic events and components required for complete developmental competence of the oocyte. Furthermore, meiotic resumption *in vitro* occurs very rapidly, regardless of the degree of developmental competence acquired,and may have causes an abrupt and premature breakdown of oocyte-cumulus cells gap junctions (Thomas *et al.*, 2004), leading to loss of important cumulus cell metabolites, such as ions, nucleotides and amino acids.

An alternative approach to improve the developmental competence of oocyte *in vitro* is by delaying or temporarily preventing spontaneous nuclear maturation while promoting development of the ooplasm. This could be achieved by culturing COCs with known peptides, eg., β-endorphin (O, 1990), vasoactive intestinal peptide (Sato, 1990), atrial natriuretic peptide (Tornell *et al.*, 1990), U-D-4 (Sakakibara *et al.*, 1993) or nonpeptidesubstances, eg., dibutyryl cAMP (Cho *et al.*, 1974), hypoxanthine (Eppig and Downs, 1987), hyaluronic acid-like substance (Sato *et al.*, 1990) with meiotic inhibiting activity. Others have used invasive adenylate cyclase activators such as FSH or forskolin (Luciano *et al.*, 1999) and/or phosphodiesterase (PDE) inhibitors, such as the non-specific inhibitor PDE type 4-specific inhibitor Rolipram or the PDE type-3 specific inhibitor Milrinone (Thomas *et al.*, 2002; 2004a). These PDE inhibitor delay germinal vesicle breakdown and simultaneously extend the extent and duration of oocyte-CC gap-junctional communication during meiotic resumption phase. After which the inhibitor is removed and the maturation allowed to continue before IVF. The approach will allow a prolonged oocyte-cumulus cell gap-junctional communication for continued mRNA and protein accumulation within the ooplasm facilitating exchange of positive regulatory molecules and metabolites for improving oocyte quality and its developmental competence. Also, by preventing immediate meiotic resumption *in vitro*, the oocytes undergo a more controlled prophase-1 to metaphase transition (from a dispersed to compacted chromatin configuration and from a non-compacted to compacted nucleoli configuration) indicating a transcriptionally active to inactive oocyte which incidentally occurs during the final phase of acquisition of developmental competence in vivo just before the LH surge (Mattson, 1990; Hyttel *et al.*,

1997; Sirard *et al*., 2006). Therefore, inclusion of PDE inhibitors and/or related cAMP modulating agent agent in IVM media is more likely to improve buffalo oocyte developmental potential (Table 2).

Table 2. Effect of PDE inhibitors and cAMP modulating agents during IVM on embryo production.

IVM Treatments	Species	Embryo Development	Reference
Milrinone	Bovine	Improved	Thomas et al., 2004
Rolipram	Bovine	Improved	Thomas et al., 2004
cAMP analogues	Porcine	Improved	Funahashi et al., 1997
Invasive adenylate	Bovine	Unchanged	Aktas et al., 1995
Cyclise	Bovine	Improved	Luciano et al., 1999
PDE inhibitors	Porcine	Unchanged	Grupen et al., 2006
	Murine	Improved	Nogueira et al., 2003
	Human	Unchanged	Nogueira et al., 2006

Use of oocyte-secreted growth factors

The oocyte plays an active role in the regulation of ovarian somatic cell function (Eppig, 2001; Gilchrist *et al.*, 2004) by secreting soluble growth factors, oocyte-secreted factors (OSFs), which modulate a broad range of granulosa cell (GC) and cumulus cell (CC) functions associated with growth and differentiation, including promotion of cellular growth (Vanderhyden et al., 1992; Gilchrist et al., 2006) and prevention of death (Hussein et al., 2005), modulation of steroidogenesis (Vanderhyden *et al.*, 1993; Li *et al.*, 2000), regulation of cumulus cell expansion and metabolism (Buccione *et al.*,1990; Salustri *et al.*, 1990; Sutton *et al.*, 2003a; Sugiura, 2005) and kit-ligand expression (Joyce et al.,2000). The two growth factors unique to gametes are growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15). OSFs promote CC and GC proliferation using combination of superfamily receptors, namely BMP receptor-II and activin-receptor like-kinases 4/5, to activate SMAD2/3 intracellular signaling molecules (Gilchrist *et al.*, 2006). Conversely, OSF GDF9 is less important in the anti-apoptotic effects of oocytes whereas BMP15 and BMP6 play a major role (Hussein *et al.*, 2005). Also, it appears that some combination of OSF GDF9 and BMP15 (but not BMP6) is required for CC expansion (Dragovic *et al.*, 2005; Yoshino *et al.*, 2006), suggesting that the oocyte secretes these paracrine growth factors to regulate its own microenvironment directing the lineage decision of its neighboring cells. It has been assumed that COC microenvironment is necessary for appropriate "oocyte capacitation" or oocyte cytoplasmic development prior to maturation of which the wider follicular environment adversely influenced the oocyte in many cases (Gilchrist *et al.*, 2004).

In using bovine oocyte as a model, IVM supplementation with recombinant GDF9 or BMP15 has been shown to improved blastocyst formation to as high as 60% with subsequent improvement in the quality as

evidenced by increased total cell count (Table 3). Also in mouse, addition of GDF9 during oocyte maturation increases total cell count and almost doubles fetal survival post transfer (Yeo *et al*., 2006). Such improvement in the embryo quality is tantamount to improved embryo freezability and post-implantation developmental potential to term. Thus, addition of OSFs in the IVM media has become necessary for buffalo oocyte maturation if we are to improve its embryo production *in vitro*. The results presented provided evidence that secretion of growth factors by the oocytes together with appropriate regulation of CC function, is an important function the oocyte must undertake. The ability of the oocyte to regulate its own microenvironment via OSFs constitute an important component of oocyte developmental competence. Furthermore, the results support the possibility of developing diagnostic markers of oocyte developmental potential based on specific CC functions under the control of OSFs. And, the increase in embryo production efficiency, up to 60% in the absence of serum during IVM, as demonstrated in cattle, clearly has significant clinical and commercial applications for buffaloreproduction.

Table 3. Effect of maturing oocytes with native or recombinant OSFs on embryo production

IVM treatment	No. of oocytes	Blastocysts (%)
Native OSFs		
COC	182	39[a]
COC + DO (0-24 hr)	176	51[b]
COC + DO (9-24 hr)	158	61[c]
Recombinant OSFs		
Control	205	41[a]
GDF9	191	50[b]
BMP15	189	58[b]

[a,b,c] Values differ significantly (Hussein et al., 2006)

Conclusion

Production of buffalo embryos *in vitro* using oocytes from slaughtered female offers a lot of advantages in terms of basic information gatherings for developmental biology research. Also, use of these embryos for breeding will greatly accelerate genetic gain by shortening genetic interval. The main limiting factor in the efficient utilization of these oocytes is the “spontaneous maturation” that occur, by simply removing the COCs from the meiotic inhibiting effects of the follicle and then cultured *in vitro*.Nevertheless, the volume of informations that are steadily being gathered from the researches on other mammalian species on the factors regulating the acquisition of developmental competence of the oocytes has progressively translating into efficient development of culture conditions for buffalo oocytes. The supplementation of IVM medium with substance(s) that would help increase the methylation level of DNA during oocyte

growth, convert the endogenous lipid components in the cytoplasm to energy source via β-oxidation, increase the GSH level,reduce ROS activity and stimulate the secretion of paracrine growth factors necessary in the regulation of oocytes own mcroenvironment aresome of the developments that benefited the oocytes in acquiring the capacity for competence *in vitro*.The use of such supplement(s) either singly or in combination in the IVM appear to bethe most likely approach in improving the developmental potential of buffalo oocytes *in vitro*.

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