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MATURATION AND CULTURING OF BUFFALO EMBRYOS WITH PYRIDOXINE IMPROVED THEIR DEVELOPMENT POTENTIAL

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ABSTRACT

Several trials were done to enhance the systems of in culture and vitro maturation (IVM) for production of good quality and competent buffalo embryos. But till now the blastocyst rate of yield and calving following in vitro transfer of produced buffalo embryos is unfortunately still low. Cathepsin B inactivation leads to enhance potential of development of buffalo embryos cattle. L-trans-Epoxysuccinyl-Leucylamido-(4-guanidino) Butane (E-64) is widely used as an inhibitor of CTSB. However, E-64 also inhibits the activity of other proteases as papain, trypsin, cathepsin L(CTSL) and calpain,. In addition, pyridoxine plays an essential role in several cellular activities. Pyridoxine supplementation improved the developmental competence in cattle but in the present study, we investigated the influence of pyridoxine during IVM on the developmental competence of buffalo oocytes. Significant improvement of the developmental competence of oocytes of bovine by increasing both the blastocyst resulted by pyridoxine supplementation to the maturation, fertilization or culture medium. The results indicated that pyridoxine is an essential tool in improvement of buffalo oocytes developmental competence and for subsequent embryo quality.

Key words: buffalo oocytes, cathepsin B, pyridoxine, vitamin B6 coenzyme.

INTRODUCTION

Buffaloes are important vital livestock in many Mediterranean, Asian, European, and Latin American countries; that's because they are important milk and meat source **(Azawi et al. 2009 and, Perera 2011).** Several studies have been applied for improvement of the competence of development of oocytes and embryos of bovine **(Song et al., 2009; Balboula et al., 2010; Yamanaka et al., 2010; Song et al., 2012)**. Until now we cannot compare the quality of the embryos *in vitro* produced (IVP) embryos with others produced *in vivo* **(Lim et al. 2007)**. The reproductive performance of buffaloes is low due to delayed onset of puberty, silent estrus, and poor conception rates, low numbers of grafian follicles, low response to hormonal stimulation, low efficacy of superovulation and embryo transfer programs **(Mahesh et al. 2017)**. Although relatively high rates of oocyte maturation, fertilization and cleavage had been achieved in buffalo, the blastocyst yield and calving rates following transfer of IVP buffalo embryos is still unfortunately low.

Vitamin B_6 and coenzymes mainly present in food as pyridoxine, pyridoxal and pyridoxamine and are essential for metabolism of amino acids and nucleic acids synthesis

(Katunuma et al., 2000). Many roles in antioxidant activity have been found for vitamin B_6 **(Endo et al., 2007)**. The characteristic structure of pyridoxine provides details of the mechanism of inhibition on CTSB activity in some non-germinal cells like helper T lymphocyte type-2 **(Katunuma et al. 2000)**. The natural origin of pyridoxine as an in activator of CTSB from starchy vegetables, whole-grain (including cereals), liver, fish, and organ meats give it an advantage **(Lindberg et al. 1983, Stover and Field 2015)**. Relation between pyridoxine and cathepsin b activity has been established in bovine oocytes before, as addition of Pyridoxine (250 µM) during in vitro maturation significantly inhibited the cathepsin B activity **(Aboelenain et al. 2017)**. Moreover, addition of pyridoxine during maturation time markedly enhanced the subsequent developmental competence of the treated oocytes **(Aboelenain et al. 2017)**. The present study was to investigate the effect of pyridoxine supplementation during maturation time or culture days on improvement of the developmental competence of oocytes of buffalo and the subsequent embryos quality.

MATERIAL AND METHODS

The chemicals used were obtained from Sigma Chemical Co (St. Louis, MO, USA).

Oocytes recovery and IVM:

Collection of buffalo ovaries were done from a nearby slaughterhouse. After washing of ovaries several times in a normal saline solution, IVM was performed according to**Takahashi et al. (1996) and Sharma et al. (2010)**. In brief, collection and aspiration of cumulus oocyte complexes (COCs) were done from medium sized follicles using an 18-gauge needle. Quality of the selected COCs was evaluated as described before **(Leibfried and First 1979 , Pawshe et al. 1994, Chauhan et al. 1998)**. Each 10-15 COCs are assigned to 100 µl drop of maturation medium, tissue culture medium (TCM)-199 supplemented with 10% heat inactivated fetal calf serum, 10 µg/ml LH, 5 µg/ml FSH and 1 µg/ml estradiol-17β. Drops are covered by mineral oil and incubated for 20-22 h at 38.5 °C in a humidified atmosphere of 5% CO_2 in air.

***In vitro* fertilization (IVF):**

IVF and *in vitro* culture (IVC) was conducted according to a procedure described previously **(Samake et al. 2000, Kang et al. 2014)**. Separation of motile spermatozoa by swim up for one hour in the medium of fertilization, modified Tyrode's Albumin-Lactate-Pyruvate (TALP) with 6 mg/ml of bovine serum albumin (BSA) **(Parrish et al. 1988)**. COCs were co-incubated with motile sperm for 18 h at 38.5°C under a humidified atmosphere of 5 % CO_2 in air under sterile mineral oil.

***In vitro* culture (IVC):**

Denudation of the putative zygotes from the surrounding cumulus cells was done mechanically by repeated pipetting **(Aono et al. 2013)**. Presumptive zygotes (10–15 zygote/50 µl droplets) were cultured in Charles Rosenkranz containing amino acids (CR1aa) medium **(Sagirkaya et al. 2006)** at 38.5 °C under a humidified atmosphere of 5% CO_2, and 90% N_2 and covered with mineral oil for 7 days **(Badr 2009)**. The cleavage and blastocyst rates were calculated **(Totey et al. 1992)**.

Experimental Design:

II. 1. Effect of inhibition of cathepsin B at the time of maturation on the buffalo oocytes maturation rate:

To evaluate the relation between apoptosis and oocytes maturation, the effect of inhibition of cathepsin B on the maturation rate was assessed. Different concentrations of Pyridoxine, Sigma-Aldrich) were supplemented to the TCM-199 (0, 50, 125, 250, and 500 µM). After 22 h of maturation, nuclear maturation of oocytes was evaluated by assessing the extrusion of the first polar bodies in the previtelline space after denuding by repeated pipetting the COCs as reported previously **(Nandi et al. 2002)**.

II.2. Effect of inhibition of cathepsin B at the time of maturation on the buffalo embryos developmental competence

Developmental competence of oocytes was evaluated after addition of PN (0, 50, 125, 250, and 500 µM) to the IVM medium. COCs were then incubated with sperm for fertilization and then cultured after 22 h of maturation. Assessment of the competence of development was performed by observing both cleavage and blastocyst rates on day 2 on days 8 respectively.

II. 3. Effect of inhibition of cathepsin B at the time of fertilization and culture on the buffalo embryos developmental competence

To confirm if CTSB inhibition is required during oocytes maturation only or also required for early embryos development. COCs were then incubated with sperm for fertilization and then cultured after 22 h of maturation. Then, addition of PN (0, 50, 125, 250, and 500 µM) to the fertilization medium and also the culture medium in the first 3 days of IVC was done. Assessment of the competence of development was performed by observing both cleavage and blastocyst rates on day 2 on days 8 respectively.

Statistical analysis:

Each experiment was performed at least 3 times in each analysis and the data were expressed as the means ± SEM. The statistical significance of differences was analyzed by Student's t-tests for independent samples. Data for experiments containing more than two groups were analyzed by one-way ANOVA with Fisher protected least significant difference using the SPSS software version 16.0 (SPSS Inc., Chicago, IL, USA); $P < 0.05$ was considered statistically significant.

RESULTS

1. Effect of Vitamin B_6 coenzyme addition to IVM on the maturation rates of buffalo oocytes.

After addition of different concentrations of PN to the TCM-199 (0, 50, 125, 250, and 500 µM) for 22 h of, the nuclear maturation rates were significantly improved (Table 1). Highest maturation rates were observed in the concentrations of 125 and 250 µm where they are significantly higher than control and 500 µm groups.

Table 1: Effect of Vitamin B_6 during IVM on the maturation rates of buffalo oocytes

Treatment	Maturation rate %
Control	54.46 ± 2.24 [a]
PN 50 µm	57.53 ± 4.14 [a, b]
PN 125 µm	62.59 ± 2.06 [a, b]
PN 250 µm	68.28 ± 4.34 [b]
PN 500 µm	48.33 ± 3.70 [a, c]

Effect of CTSB inhibition on the maturation rate was assessed. Different concentrations of Vitamin B_6 coenzyme were supplemented to the TCM-199 (0, 50, 125, 250, and 500 µM). Total number of examined oocytes is 227. Different superscript letters across the treatments indicates a significant difference ($P < 0.05$).

2. Effect of Vitamin B_6 coenzyme addition during IVF on the developmental competence of buffalo zygotes:

Different concentrations of PN were supplemented to the IVF medium (0, 50, 125, 250, and 500 µM). Penetration and fertilization rates were improved more than control group especially 250 µM PN as shown in Table 2.

Table 2: Effect of Vitamin B_6 during IVF on the penetration and fertilization rates of buffalo zygotes

Treatment	Penetration rate (%)	Fertilization rate (%)
Control	61.69 ± 2.00	39.03 ± 6.43
PN 50µm	65.97 ± 5.42	40.41± 5.41
PN 125µm	65.04 ± 4.68	44.44 ± 5.55
PN 250 µm	66.11 ± 8.32	56.41 ± 6.72
PN 500 µm	65 ± 12.58	36.94 ± 9.62

Effect of CTSB inhibition on the developmental ability of the putative zygotes was assessed. Different concentrations of Vitamin B_6 coenzyme were supplemented to the IVF medium (0, 50, 125, 250, and 500 µM). Total number of examined zygotes is 245. The highest rate was observed in 250 µM concentration.

3. Effect of Vitamin B_6 coenzyme addition during IVC on the developmental competence of buffalo embryos:

Different concentrations of PN were supplemented to the IVC medium (0, 50, 125, 250, and 500 µM). Cleavage, Morula and blastocyst rates were improved significantly more than control group especially 250 µM PN as shown in Table 3.

Table 3: Effect of Vitamin B_6 coenzyme addition during IVC on the developmental competence of buffalo embryos:

Treatment	Cleavage rate (%)	Morula rate (%)	Blastocyst rate (%)
Control	29.22 ± 3.58	4.42 ± 0.71 [a]	3.18 ± 1.59 [a]
PN 50µm	32.80 ± 5.53	10.31 ± 2.44 [a]	6.84± 1.12 [a]
PN 125µm	35.25 ± 3.52	16.82 ± 4.77 [a, b]	7.64 ± 1.04 [a]
PN 250 µm	49.01 ± 0.98	29.43 ± 5.45 [b]	18.28 ± 2.10 [b]
PN 500 µm	30.97 ± 6.80	6.14 ± 3.40 [a]	1.96 ± 1.96 [a]

Effect of CTSB inhibition on the developmental ability of the putative zygotes was assessed. Different concentrations of Vitamin B_6 coenzyme were supplemented to the IVC medium (0, 50, 125, 250, and 500 µM). Total number of examined zygotes is 282. The highest developmental rates were observed in 250 µM concentration

DISCUSSION

CTSB inhibition has been established as a new method for getting a better result concerning the competence of oocytes and embryos of bovine **(Balboula et al. 2010, Balboula et al. 2010, and Min et al. 2014)**. The results showed that pyridoxine supplementation to the medium of IVM resulted in a significant enhancement of rates of blastocyst rates in buffaloes. Addition of vitamin B_6 to the IVF medium increased the fertilization number and penetration rates. It was reported that activity of CTSB was inversely correlated with bovine embryos quality and, hence, it could be used as an indicator of embryo quality **(Balboula et al., 2010; Aardema et al., 2013;Aboelenain et al., 2017)**. The present results showed that pyridoxine supplementation to the IVC medium significantly increased morula, cleavage and blastocyst rates. Generally, our results indicated that pyridoxine is a critical additive led to enhance the developmental potential and quality of buffalo oocytes and their blastocysts.

The improvement of the developmental competence of oocytes due to pyridoxine supplementation can be explained by disrupting and inhibiting the pathway of CTSB-induced apoptosis. **Balboula et al. (2013)** reported that apoptosis could be stimulated in the presence of some proteases as CTSB that originated and secreted from the partly improper lysosomes as a result of moderate stresses. CTSB release could initiate apoptosis by activation of executioner caspases and initiator caspases **(Vancompernolle et al., 1998)**. The rates of *CTSB* transcript abundance and TUNEL-positive cells were significantly decreased in blastocysts produced from oocytes treated by pyridoxine that suggested pyridoxine presence in the medium improved the potentiality of developmental for bovine oocytes by antagonizing CTSB, which is considered the first stimulus of the apoptotic pathway. Additional research is essential to evaluate there is another path of vitamin B_6 has to enhance the potentiality of development of buffalo oocytes.

Pyridoxine has been used to treat many diseases including adult-onset clinical conditions **(Stover and Field, 2015)** and carpal

tunnel syndrome **(Aufiero et al., 2004)**. Maternal dietary supplementation of pyridoxine was used to improve the gene expression patterns of *in vivo* derived blastocysts of porcine **(Dalto et al., 2015)**. Furthermore, pyridoxine could be used in women during pregnancy for treatment of vomiting and nausea **(Wibowo et al., 2012; Stover and Field, 2015)**. Additionally, pyridoxine has nontoxic and natural properties so acts as a natural component to improve the developmental competence and the quality of embryos by inhibiting CTSB *in vivo*.

In conclusion, this study reveals that supplementation of IVM or IVF or IVC media with Pyridoxine improves fertilization, morula, cleavage and blastocyst rates.

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الملخص العربي

انضاج وزراعة أجنة الجاموس فى وجود البيرديوكسين أدى إلى زيادة قدرتها على النمو

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اجريت العديد من المحاولات لتطوير انظمة الانضاج والنمو المعملى لانتاج اجنة ذات جودة عالية وقدرة على النمو. ولكن للاسف حتى الان مازالت معدلات البلاستوست والولادات الناتجة من الاجنة المحضرة معمليا منخفضة. تثبيط الكازيبسين ب ادى الى زيادة فى قدرة اجنة الجاموس على النمو. تعتبر مادة اى ٦٤ من اشهر المواد المستخدمه فى تثبيط الكازيبسين ب ولكن مع ذلك تلك المادة تقوم بتشبيط بعض المواد الاخرى فى الخلية مثل الكازيبسين لام والبابين والكالبين والتريبسين. بالاضافة الى فيتامين ب ٦ (البيريدوكسين) يلعب دور مهم فى العديد من العمليات الحيوية داخل الخلية. اضافة البيريدوكسين الى ميديا الانضاج المعملى ادى الى تحسين قدرة بويضات الابقار على النمو. لكن فى هذة الدراسة تم تقييم تاثير اضافة البيريدوكسن الى ميديا الانضاج والاخصاب والنمو المعملى لبويضات واجنة الجاموس. اضافة البيريدوكسين حسنت من القدرة على النمو وجودة الاجنة ومعدل البلاستوست.هذة النتائج اثبتت ان البيريدوكسين اصبحت مادة واعدة لزيادة معدل وجودة اجنة الجاموس المنتجة معمليا.

الكلمات المفتاحية: بويضات الجاموس، الكازيبسين ب، البيريدوكسين، فيتامين ب٦