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## Viability of bovine opu-derived oocytes to honeybee as cryoprotectant

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**Abstract.** This study designs to determine the effectiveness of Honeybee (HB) as cryoprotectants (CP) on viability vitrified-thawed bovine oocytes derived from OPU using Trehalose as control. Cattles were subjected to superstimulation protocol, per session conducted five days where three days both cattle were administrated 100 mg follicle stimulation hormone (FSH) within 24 h once and two days of “resting period” totalling two sessions. The “coasting period” (FSH starvation) between sessions was four days (96 h). Oocytes collection via OPU were performed at fifth day (120 h). The ovarian growth was observed via ultrasonographic before OPU. Prior to vitrifying oocytes with treatment Trehalose (T1) and HB (T2) followed by warming protocol, oocytes subjected to *in vitro* maturation (IVM). Oocytes viability were evaluated by fluorescein diacetate staining. Results showed ovarian growth for first session was larger size follicles than the second session for both cattle. Total number of oocytes obtained were 60. Oocytes viability treatment T2 was significantly higher (90.9%) than T1 (70.4%). This study concludes that HB as CP in vitrification protocol was able to achieved high oocytes viability with oocytes derived via OPU suggesting Honeybee as an alternative CP for oocytes vitrification.

### 1. Introduction

Many studies attempted to devise better cryopreservation protocol that aim to optimize the outcome of freezing and thawing [1]. The choice for a suitable sugar cryoprotectant agent (CPA) for vitrification were limitless, which common sugars such as glucose, fructose, sorbitol, sucrose, raffinose, and Trehalose were used with the hopes of finding the best sugar CPA resulting superior vitrification performance [2]. Honeybee (HB) has been used for semen cryopreservation in bull and results were comparable to natural mating indicating HB could be a potential choice for sugar CPA oocytes cryopreservation [3].

Ovum pick up (OPU) that utilises ultrasound guided follicular puncture improves *in vitro* production (IVP) for better embryo transfer (ET) with high popularity in Brazil [4]. OPU gain more popularity with various donor cow breed. Commonality above documentation were cow donor



subjected to *in vitro* fertilisation (IVF) via OPU (OPU/IVF). Progression in OPU/IVF was integration of hormonal follicle stimulation hormone (FSH) administration to promote follicular response and oocytes recovery [5] and improvement on embryo production [6] that creates OPU/IVF as a potential alternative for multiple ovulation embryo transfer (MOET) [7].

Superior sugar CPA that able to vitrify OPU retrieved bovine oocytes might improve OPU/IVF system and HB could be a potential choice of sugar CPA because of HB well documentation in semen extenders with various animal species including bull [8]. However, report on HB for bovine oocytes cryopreservation were inconsistent [9]. Thus, this study aims to determine effectiveness of Honeybee (HB) as cryoprotectants (CP) on viability vitrified-thawed bovine oocytes derived from OPU.

## 2. Materials and Method

All the chemicals and media used in this experiment were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and all the apparatus were brought from Thermo Fisher Scientific Company Ltd. (Bangkok, Thailand) unless stated otherwise.

### 2.1. Experimental animals

This study was conducted at Faculty of Agriculture, Princess of Naradhiwas University, Narathiwat Province, Thailand (Latitude 6.4557° N, Longitude 101.7898°E). A total of 2 Thai native cows aged 15 months used were normal cyclic cattle and healthy. The cattle were kept with pasture with concentrate feed supplementation feed

#### 2.1.1. Experimental design and superstimulation protocol

Superstimulation protocol conducted followed previous study with some alteration [10]. Cattle cycle did not synchronize and dominant follicle did not remove due to time and resources limitation. Animals were subjected to superstimulation protocol which per session lasted for five days totalling two sessions. Per session conducted with three days with single administration of 100 µL follicle stimulating hormone (FSH) (Folltropin®, Vétoquinol USA, Inc., Texas, USA) within 24h interval followed by two days of “resting period” totalling five days. The “coasting period” (FSH starvation) between first session and second session were four days (96 h) prior to second session.

#### 2.2. Follicular aspiration and bovine oocytes collection

Follicular aspiration and bovine oocytes collection followed protocol from previous study [11]. Cattle were restrained and administered 0.1 mg/kg body weight (BW) Xylazine (X-lazine, L.B.S. Laboratory Ltd., Part, Bangkok, Thailand) and cattle become less aggressive after 10 minutes, followed by 5 mL of 2% Lidocaine Hydrochloride (Locana, L.B.S. Laboratory Ltd., Part, Bangkok, Thailand) for cattle immobilisation. Follicles later were aspirated with a portable ultrasound device with intravaginal 7.5-MHz transducer (Honda®HS-2100, Honda Electronics Co. Ltd, Japan) using 17 G x 490 mm cow ova vacuum needle (COVA Needle; Misawa Medical, Tokyo, Japan). An aspiration pump (Welch Model No.2515C-75, Gardner Denver Welch Vacuum Technology, Inc. Sheboygan, Wisconsin, USA) aspirated follicle by vaginal wall punctured using COVA needle with ultrasound guided probe. Vacuum was set to 120 mmHg and 22 mL/min aspiration rate via the COVA needle. Aspirated follicles were collected to a 50 mL tube containing small volume of mDPBS follicular fluid which act as a saline and washing solution for aspirated follicles.

Aspirated oocytes classified based on oocytes morphology [12]. Prior to *in vitro* maturation (IVM), recovered cumulus cells were classified as (1) Grade A, > four layers of cumulus cells; (2) Grade B, three or four layers of cumulus cells; (3) Grade C, one or two layers of cumulus cells; (4) Grade D, denuded oocytes; (5) Grade E, oocytes that are expanded with the cumulus cells. Thus, only oocytes from Grade D and E were not used and continue to maturation, whilst the rest proceeded to IVM. Thus, the result obtained with the highest yielding was Grade A (n=35, 58.3%) followed by Grade B (n=12, 20.0%), Grade C (n=10, 16.7%), Grade D (n=3, 5.0%) and none for Grade E.

### 2.3. *In vitro* maturation (IVM) of bovine oocytes

Recovered oocytes from cattle followed procedure with some minor modification [13]. Oocytes were washed for last time with 35 mm dish containing mDPBS supplemented with FBS, P-S stock + 5% polyvinylpyrrolidone (PVP). Oocytes were then transferred to 35 mm dish containing four droplets of 100  $\mu$ L with immediate separation accordance to oocytes grade. Droplets containing oocytes were covered with paraffin oil and dishes were placed into CO<sub>2</sub> incubator at 38.5°C, 5% CO<sub>2</sub> in air at maximum humidity for 24 hours.

### 2.4. *Vitrification of matured bovine oocytes*

Vitrification protocols adopted with some alteration from previous study [14]. Prior to the vitrification procedures, oocytes were separated into two different groups: T1 (27 oocytes) and T2 (33 oocytes). Vitrification procedure started with T1 followed by T2. The cumulus cells of oocytes from both groups were removed gently with pipetting in rising solution (RS) and all washing procedure were done in a 4 well dish. Oocytes from T1 were washed once with RS (TCM-199 + 20% newborn calf serum (NBCS) + 0.1% P-S stock) and continued washed three times in an equilibration solution (ES) (TCM-199 + 20% NBCS + 1% P-S stock + 2% ethylene glycol (EG) + 2% dimethyl sulfoxide (DMSO)) and 14 minutes suspension before exposing oocytes to vitrification solution (VS) (17.5% EG + 17.5% DMSO + 50 mg/mL PVP + 0.3 M Trehalose in TCM-199 with 20% NBCS + 1% P-S stock) for three times washing within a minute totalling 15 minutes for vitrification procedure. They were then dropped immediately as droplets for 1-2  $\mu$ L containing 5 oocytes per drop (Solid surface vitrification method-SSV). Droplets were directly dropped onto liquid nitrogen (LN<sub>2</sub>) cooled folded aluminium foil. Using nitrogen-cooled forceps to move vitrified droplets into 15 mL tube where 15 mL tube also nitrogen-cooled beforehand and tubes were later stored in LN<sub>2</sub> tanks for ten days. Procedure above remained same for T2 except T2 uses different VS (17.5% EG + 17.5% DMSO + 50 mg/mL PVP + 1 M HB (21.74% v/v) in TCM-199 containing 20% NBCS + 1% P-S stock) with Honeybee (HB)[15].

### 2.5. *Thawing of matured vitrified bovine oocytes*

Thawing procedure followed [3]. Prior to thawing oocytes, warming solutions (WS) were prepared, where T1 uses WS 1 (0.3 M Trehalose + 20% NBCS + 0.1% P-S stock in TCM-199), while T2 uses WS 2 (1 M HB (21.74% v/v) + 20% NBCS + 0.1% P-S stock) respectively. Vitrified droplets containing oocytes in the 15 mL tubes were moved from the LN<sub>2</sub> tank using nitrogen-cooled forceps to a 4 well dish containing TCM-199 supplemented with 0.15 M, 0.075 M and 0.0375 M Trehalose of WS 1 for one minute each. Thereafter, the oocytes were washed for the last time with TCM-199 until the following procedures. The procedures were also similar for T2, except vitrified droplets containing oocytes were transferred to a 4 well dish consisting of TCM-199 supplemented with 0.5 M and 0.125 M HB of WS 2 for one minute each.

### 2.6. *Viability Assessment of survived vitrified-thawed bovine oocytes*

The vitrified-thawed oocytes were stained with 2.5  $\mu$ g/mL fluorescence diacetate (FDA) containing in mDPBS that supplemented with 5 mg/mL bovine serum albumin (BSA) at 38.5°C for 2 minutes in a dark room without any light sources. Stained oocytes were then washed three times with mDPBS in 5 mg/mL of BSA. The viability of those oocytes was observed under a fluorescence microscope (IX-71; Olympus, Tokyo, Japan) with wavelength excitation of 460-495 nm and emission at 510 nm been used for better observation. Stained oocytes that projected with bright green fluorescence light regarded as live while oocytes that showed otherwise regarded as dead.

### 2.7. *Statistical analysis*

Statistical Analysis System software (SAS 9.1) was used for assessing the data of oocytes viability. Student's T-test were used to allow proper analysis of data addresses the relationship between viability of oocytes and treatment of Trehalose and Honeybee (HB). All obtained results were expressed as mean  $\pm$  standard error (SE) with  $p \geq 0.05$  considered as non-significant.

### 3. Results and Discussion

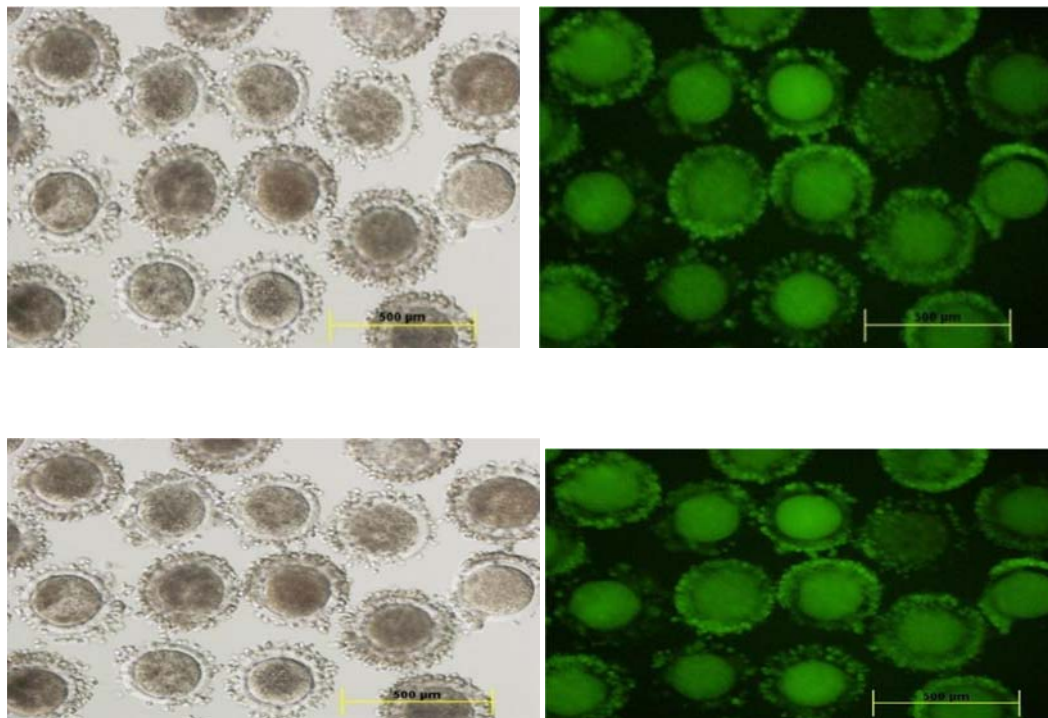
Viability of vitrified matured bovine oocytes after fluorescein diacetate (FDA) staining with Honeybee (HB) (1.0 M) (T2) were significantly higher ( $p$ -value is 0.021) compared to Trehalose (0.3 M) (Table 1) (Fig. 1) which suggested HB superiority over to Trehalose as cryoprotectant (CP) sugar media in this study. HB provides similar dehydration and volumetric alteration status as sucrose to bovine oocytes [15] that might enhance cell osmotic gradient across cell membrane [15] which prevents intracellular ice crystals formation [16]. Preventive ice crystal formation might due to HB chemical properties existed as mixture of 25 sugars (mainly glucose and fructose) with about 95% dry matter [17].

**Table 1.** The percentage of viability and number of vitrified oocytes after FDA staining.

Treatment	Number of vitrified oocytes*	Viability $n$ (%)
T1-Trehalose (0.3 M)	27	19/27 (70.4) <sup>a</sup>
T2-Honeybee (1.0 M)	33	30/33 (90.9) <sup>b</sup>

<sup>a,b</sup> indicated significant differences using student t-test ( $p \geq 0.05$ )

\* Total number of oocytes



**Figure 1.** Fluorescein diacetate (FDA) staining of matured vitrified-thawed bovine oocytes in treatment 1 (T1) Trehalose (1.0 M) (A: Bright field and B: FDA) and treatment (T2) Honeybee (HB) (C: Bright field and D: FDA).

HB extenders are well documented and successful in spermatozoa cryopreservation [18]. Sugar with various saccharides combination in HB [17] provides spermatozoa energy that increases survivability and motility [8]. Sugar combination in HB also provides superior antioxidant effects rather than single sugar element (Trehalose) [19] which protects cell organelle from oxidative stress,

damage [20] and anti-inflammatory effects [17]. Despite the sugar combination provide positive influences to spermatozoa, types of sugar and different animal species spermatozoa can influence extenders performance [21]. High mortality rate with ram spermatozoa from monosaccharides (fructose and glucose) extenders compared to disaccharides extenders [22] while both monosaccharides and disaccharides extenders are superior than trisaccharide extenders [23] indicating inconsistent previous results based on animal species spermatozoa. Despite both HB and Trehalose were common choices as sugar media vitrification for spermatozoa studies, but were never directly compared for oocytes preservation. Thus, both were chosen with hopes of finding superior combination performance despite only relying on bovine oocytes viability to gauge the sugar CP performance in current study.

The first session has larger ovarian follicular size and higher oocytes recovery (High FSH responses) than the second session for both sides in current study. The result from this study agrees that follicle stimulating hormone (FSH) administration: increases follicular response after three days [5], produces highest number of oocytes than single FSH injection [24] and new follicles emergences [25].

However, second session in comparison with first session presented smaller ovarian follicular size and lower oocytes recovery despite both sessions follows the same FSH administrations superstimulation. These results contradict multiple previous studies. Limited resources during this experiment could explained opposite results in second session. Lesser FSH administrated in second session does influences follicular growths and sizes [26]. The limited resources also prevented cattle synchronisation in both sessions but it should be performed as a standard FSH superstimulation protocol as shown in previous studies [10,26]. The aim of synchronisation was to achieved new initiation follicular wave with removal of dominant follicles prior to FSH superstimulation [10,26]. Both non-synchronise Cattle 1 and 2 could have benefited from FSH administration protocol with natural uninterrupted estrous cycle for both cattle which might resulting in high follicular size and recovery in first session.

After OPU first session end, OPU complication could negatively affected the ovarian cattle that lead to the none oocytes recovery in second session. Punctures from the needle could led to ovarian lesion [27], hardening of ovarian consistency during aspiration with the needle [28], and ovarian stroma alteration from aspiration [29] despite follicular puncture does not affect ovarian functionality [28]. The commonality of all previous documented OPU procedure were random number of subjected cattle. Despite the random cattle number subjects, all numbers were high with 80 cattle [26] and 27 cattle [30] which were sufficient enough without using the exact same cattle which unlike this current study due to limited resources. Using the exact same cattle (1 and 2) in current study causes both cattle with follicle reduction because of relative short inter-aspiration interval that prevented healing from previous OPU procedures (first session) [27] which were no such occurrence with other documented OPU procedures.

The intrinsic and extrinsic factors could responsible for unexplainable phenomenon that occurred in second session with small follicular growth in this study. Cattle in present study were subjected to short-term hormonal treatment during hot weather that might cause impaired follicles and poor oocytes qualities [31]. More effective hormonal treatment is necessary for improving the oocytes quality during autumn because poor performance presented in the rate of blastocyst formation despite bovine were administrated with bovine somatotrophin (bST) and FSH [32].

#### **4. Conclusion**

In conclusion, Honeybee as CP able to achieved higher viability compared to Trehalose as control where oocytes were derived OPU in which oocytes subjected to vitrification protocol. However, treated oocytes should subjected in vitro fertilization (IVF) for embryonic development as future studies.

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