# BRIEF COMMUNICATION: High hydrostatic pressure treatment increases cryo-tolerance of *in vitro* produced bovine embryos

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## Introduction

Cryo-preservation is a critical tool to manage the logistics of implementing assisted reproductive technologies in practice, which depend upon the availability of synchronised recipient cows for embryo transfer. However, the various cryo-preservation methodologies are noted for compromising the integrity of cells within the embryo, hence reducing subsequent viability. The challenge faced by practitioners in this field is to increase the success rate of producing offspring following embryo cryo-preservation.

Recently, an innovative high hydrostatic pressure (HHP) treatment (Pribenszky & Vajta 2011) has been reported to improve the survival of embryos following cryo-preservation. It is hypothesised that sub-lethal environmental stress, induced by high pressure, increases the concentration of specific chaperone proteins in embryos (Pribenszky et al. 2010). Synthesis of these proteins results in an increased tolerance of the cells to subsequent stress induced by treatments such as vitrification, an ultra-rapid cooling method of cryo-preservation, which may increase the efficiency of this biotechnological procedure.

Here we report initial investigations treating Day 7 *in vitro* produced bovine embryos with high hydrostatic pressure before vitrification (HHP+VIT) and evaluating their cryo-survival after subsequent warming *in vitro*, by morphological assessment of blastocyst-stage embryos, and by differential staining of live and necrotic cells within each blastocyst, in comparison to vitrified control (VIT) blastocysts. In addition, we determined the *in vivo* viability of HHP+VIT and VIT embryos, following transfer to recipient heifers, in comparison to non-vitrified fresh control (FC) blastocysts.

## Material and methods

In vitro produced bovine embryos were produced according to standard methods (Thompson at al. 2000). On Day 7 of in vitro culture, blastocyst-stage embryos were graded according to their morphological quality. This included visual assessment of the relative number of cells, trophectoderm to inner cell mass ratio, degree of blastocoel expansion, presence of extruded cells within the perivitelline space and overall appearance, to classify embryo quality as: Grade 1 (Excellent/good); Grade 2 (Fair); or Grade 3 (Poor); which correlates with subsequent embryo survival in vivo (Robertson & Nelson 1998). Embryo evaluations were performed by the same person.

### *High hydrostatic pressure treatment and vitrification of blastocysts*

Approximately equal proportions of Grade 1 and 2 blastocysts were randomly selected on Day 7 for treatment either with or without HHP before vitrification. For each treatment, a maximum of 20 blastocysts were aspirated into 0.25 mL plastic straws in Embryo Hold medium (AgResearch, Hamilton, New Zealand), without air bubbles, and the ends sealed with plastic plugs. The straws were then cooled to room temperature (25–26°C). Those blastocysts treated with HHP where then placed into the pressure chamber of the controlled pressurizing device (HHP 100; Cryo-Innovation Ltd., Budapest, Hungary) using water as the pressure medium. The rate of pressurisation and subsequent decompression to atmospheric pressure was 10 MPa/sec. The magnitude and duration of the pressure had been optimised in previous experiments. HHP treated blastocysts were exposed to 60 MPa for one hour and, after a recovery period of a further one hour, vitrified using the Cryologic vitrification method (Trigal et al. 2012). The VIT control group represented those blastocysts left at room temperature in straws as described above, for the corresponding length of time, before vitrification. Following vitrification, in both HHP+VIT and VIT groups, blastocysts were stored in liquid nitrogen before subsequent warming to 38.5°C for morphological assessment, differential staining or embryo transfer.

### *In vitro and in vivo development of vitrified blastocysts*

All vitrified blastocysts, in both HHP+VIT and VIT groups, were warmed to 38.5°C and incubated for at least two hours before evaluating re-expansion of the blastocoel cavity and assessing morphological quality *in vitro*. Representative Grade 1 and 2 embryos were transferred to recipient heifers to assess *in vivo* development up to Day 65 of gestation. Two transferable quality blastocysts from either the HHP+VIT or VIT treatment groups, in addition to FC blastocysts, were transferred to the uterine horn ipsilateral to the *corpus luteum* of each synchronised recipient female. A total of 136 embryos were

**Table 1** *In vitro* morphological assessment of Day 7 bovine IVP embryos treated either with (HHP+VIT) or without (VIT) high hydrostatic pressure before vitrification.

| Treatment | Embryo grade before treatment | Number of embryos | Number and percentage of embryos per grade, after warming | | |
|---|---|---|---|---|---|
| | | | 1 | 2 | 3 |
| HHP+VIT | 1 | 26 | 14 (54%) | 10 (38%) | 2 (8%) |
| VIT | 1 | 31 | 14 (45%) | 15 (48%) | 2 (6%) |
| HHP+VIT | 2 | 103 | 3 (3%) | 67 (65%)[a] | 33 (32%)[a] |
| VIT | 2 | 90 | 3 (3%) | 29 (32%)[b] | 58 (64%)[b] |

**Table 2** *In vivo* embryo survival on Day 35 and Day 65 of gestation. HHP+VIT = High hydrostatic pressure treatment before vitrification; VIT = Vitrification control; FC = Non-vitrified fresh control embryos.

| Treatment | Total number of transferred embryos | Number of transferred embryos and grades | | Embryo survival on Day 35 of gestation | Embryo survival on Day 65 of gestation |
|---|---|---|---|---|---|
| | | Grade 1 | Grade 2 | | |
| HHP+VIT | 48 | 14 (29%) | 34 (71%) | 23 (48%) | 18 (38%) |
| VIT | 40 | 14 (35%) | 26 (65%) | 22 (55%) | 22 (55%) |
| FC | 48 | 30 (62%) | 18 (38%) | 27 (56%) | 19 (40%) |

transferred. Embryo survival was monitored by ultrasonography at ten day intervals between Day 35 to Day 65 of gestation to detect fetal heartbeats and determine the percentage of transferred embryos that resulted in potentially viable fetuses; after which pregnancies were terminated. All animal manipulations were approved by the Ruakura Animal Ethics Committee.

### *Differential staining of live and necrotic cells in blastocysts*

Only blastocysts assessed as being of Grade 2 quality at the start of the experiment were used for differential staining. After warming and two hours of re-expansion at 38.5°C, HHP treated and Control vitrified blastocysts were washed in phosphate buffered saline with 1% polyvinyl alcohol (PBS/PVA; Sigma-Aldrich, Auckland, New Zealand) and placed for 10 minutes in PBS/PVA that contained 0.04 µg/mL propidium iodide (Life Technologies, Auckland, New Zealand) and 4 µg/mL Hoechst 33342 (Life Technologies, Auckland, New Zealand). After washing in PBS/PVA, the blastocysts were mounted on glass slides and examined using a fluorescent microscope (Leica DMI6000B, Leica Microsystems, Wetzlar, Germany). Necrotic and live cells were identified by red and blue coloration, respectively, and cell numbers were quantified using the public domain Image-J software, version 1.36b (National Institutes of Health, Bethesda, Maryland, USA).

### *Statistical analyses*

Data for the morphological assessment of blastocysts were analysed with Fisher's exact test. *In vivo* embryo survival for the three treatments was compared with a chi-square test. The percentage of live embryonic cells in blastocysts were analysed with a t-test.

## Results

The results presented in Table 1 showed that HHP treatment significantly increased the percentage of Grade 2 cattle blastocysts that remained of transferable quality following vitrification and warming (67/103 = 65%) compared to vitrified controls (29/90 = 32%) ($P < 0.001$). Conversely, a significantly higher proportion of Grade 2 embryos at the start of the experiment had deteriorated to non-transferrable Grade 3 (Poor) quality after warming in the VIT group compared to those receiving HHP treatment (Table 1). For those blastocysts assessed as Grade 2 at the start of treatment, in both the HHP+VIT and VIT groups there were three embryos classified as Grade 1 after warming. This may have been the result of mis-classification of blastocyst quality either before or after vitrification and warming (Table 1: 3/103 HHP+VIT and 3/90 VIT). In contrast, with Grade 1 blastocysts at the start of the experiment, a similarly high percentage remained of transferable quality following vitrification and warming either with (24/26 = 92%) or without HHP treatment (29/31 = 94%).

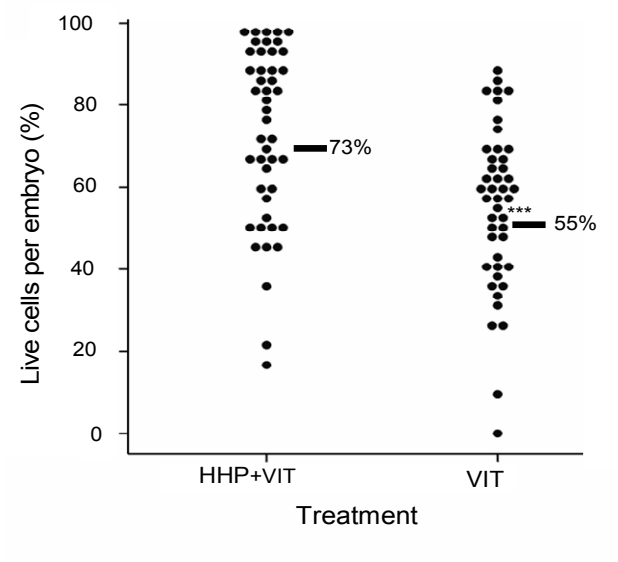
The results of differential staining showed that on average HHP+VIT treated Grade 2 blastocysts had 18% (Standard error of difference = 4.3%) more live cells after warming ($P < 0.001$) than blastocysts which were only vitrified (Figure 1).

In terms of *in vivo* development, there was no significant difference in embryo survival to Day 65 of gestation between HHP treated and vitrified (18/48 = 38%), vitrified (22/40 = 55%) or FC Grade 1 and 2 embryos (19/48 = 40%) ($P = 0.3$) (Table 2).

## Discussion

In this study, the defined HHP treatment significantly improved the cryo-survival of Grade 2 blastocysts after vitrification and warming, with a higher proportion of treated blastocysts remaining of transferable quality. In addition to morphological assessment, this improvement in embryo quality was also evidenced by the HHP treated blastocysts having

**Figure 1** Percentage of live cells in individual vitrified bovine IVP blastocysts after warming, either with (HHP+VIT) or without (VIT) prior high hydrostatic pressure treatment. Bars represent the respective mean values. *** = P <0.001.




a significantly higher percentage of live cells after vitrification and warming. Together, this suggests that the HHP pre-treatment conferred a degree of cryo-tolerance and reduced the degree of cellular damage, especially for more marginal Grade 2 blastocysts. Grade 1 blastocysts did not appear to suffer the same degree of cryo-injury following vitrification and HHP treatment provided no additional benefit to these embryos. Importantly, the *in vivo* developmental potential of vitrified blastocysts was very similar to FC blastocysts, up to Day 65 of gestation at least. Related studies have shown that sub-lethal HHP treatment prior to vitrification improved embryo survival, promoted *in vitro* development and increased hatching rates of bovine blastocysts from their surrounding *zonae pellucidae* after warming (Pribenszky & Vajta 2011). Trigal et al. (2012) reported that after vitrification and warming, HHP treated bovine IVP blastocysts that subsequently hatched in culture, had more cells in the inner cell mass, which is highly correlated to the quality of the embryo.

Further studies are needed to reveal the molecular mechanisms and/or biological implications involved in stress induced tolerance of *in vitro* produced bovine embryos following HHP treatment.

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