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Research Article

A Noninvasive Method for Assessing Oocyte Competency

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ABSTRACT

There is an increasing demand to evaluate oocyte competency and viability for oocyte cryopreservation and use for *in vitro* fertilization. Oocytes suffer decreased survival of cryopreservation as compared to embryos, due to physical characteristics of the oocyte. The objective of this study is to determine if a specific gravity device (SGD) can estimate oocyte viability based on oocyte descent through the buoyancy system. All experiments were performed in a research laboratory with a randomized block design with repeated measures. Three hundred-seventy-six oocytes were collected from seven mice and randomly assigned to one of four treatments: exposure to 60°C for 30 min, acidified media for 1h, ethylene glycol-glycerol cryoprotectant for 1h, or standard culture to serve as control. To further analyze the relationship between oocyte descent time and viability, 98 additional oocytes were passed through SGD before and after treatment. Oocytes were stained with Coomassie Blue to determine membrane permeability and estimate viability based on treatment. Oocytes treated with 60°C heat, acidified media and ethylene glycol/ glycerol cryoprotectant solution demonstrated altered descent times from control and pre-treatment oocytes ($P<0.05$). Oocytes exposed to heat and cryoprotectants descended more rapidly through SGD than control and pre-treatment oocytes ($P<0.05$). Oocytes treated with acidified media descended more slowly through SGD ($P<0.05$). Permeation of stain into oocytes exposed to lethal treatments confirmed changes in membrane integrity post-treatment and further indicates SGD can detect such shifts. This suggests SGD can predict competency between live and dead oocytes. In conclusion, SGD can detect shifts in oocyte density due to altered membrane permeability, which can suggest information about oocyte competency. This information can help differentiate between high- and low-quality fresh oocytes to help select which oocytes to freeze and result in improved oocyte cryopreservation and fertilization.

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Introduction

Oocyte cryopreservation allows women to delay fertility for various medical or personal reasons. In animals, oocyte cryopreservation allows for female genetic storage. Additional advantages include cryobanking of oocytes for donation, preserving genetics of endangered species, and overcoming ethical issues and legal restrictions associated with embryo cryopreservation [1-8]. Despite the advantages associated with oocyte cryopreservation, maintaining oocyte competency throughout

cryopreservation is challenging. Thus, improved methods to select for oocyte competency before freezing is warranted.

Oocyte quality can be indicated by cumulus cell association and adherence to the oocyte [9-12]. However, to improve fertilization, it is common practice to remove cumuli, either mechanically or enzymatically. Denuding the cumulus oophorus does not impact oocyte survival during cryopreservation [13]. Without intact cumulus cells, morphological analysis with light microscopy is the only current noninvasive method to predict the quality of an oocyte. High quality

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oocyte morphology has been indicated by a perfectly spherical shape, regular zona pellucida, and a translucent, homogeneously coloured cytoplasm without inclusions [14]. While oocyte morphology is helpful, it is often insufficient in determining quality as only 30-40% of cryopreserved, high quality bovine oocytes reach the blastocysts stage after fertilization [15]. These demands improvements in both freezing technologies and in preemptive selection techniques, which need to be simple and rapid to perform, inexpensive, reliable, and noninvasive.

The low success rate of cryopreserved oocytes is partially due to intracellular ice crystal formation lysing the cell and affecting its structural integrity [15, 16]. Oocyte cryopreservation can reduce oocyte viability by inducing premature extrusions of the cortical granules which harden the zona pellucida, induce volume changes which affect the structural integrity of the oolemma and disassemble the meiotic spindle, which can result in chromosomal abnormalities [17-20]. To prevent structural damages caused by intracellular ice formation, cryoprotectants (CP) are used to dehydrate the cell. Because the oocyte is one of the largest cells in the body and spherical, its low surface area to volume ratio and inherent concentration gradient of its spherical shape challenge the permeability of CP [1, 9].

The prolonged exposure to CP necessary to dehydrate the cell is often the cause of decreased oocyte viability, as CP can alter the intracellular pH, induce oxidative stress, cause chemical toxicity and osmotic shock [18, 20-22]. Oocytes are also more prone to chilling sensitivity due to their high cytoplasmic lipid content [18, 23]. These factors decrease oocyte viability after cryopreservation and cannot always be identified by morphological analysis. Therefore, new methods to detect oocyte viability before freezing are necessary for improved success of oocyte cryopreservation.

Previously, our lab created a method to detect embryo viability with a noninvasive embryo assessment technique (NEAT) using a specific gravity device (SGD) [24, 25]. Applying this technique to oocytes would allow further indication of oocyte viability, thus reducing time and energy spent freezing and thawing incompetent oocytes. The objective of the present study was to determine if the SGD can predict oocyte competency prior to cryopreservation. By studying oocyte buoyancy before and after exposure to lethal treatments, SGD efficacy in predicting oocyte viability was evaluated.

Materials and Methods

All experiments were approved by Texas Tech University Animal Care and Use Committee. Oocytes ($n=278$) were collected from twenty-two, 6-8-week-old mice, stimulated using standard protocol [25]. After micro-dissection from the oviduct and ovary, oocytes were randomly assigned to one of four treatment groups: Control, pH adjusted media, prolonged exposure to CP or treatment at 60°C (Table 1). All oocytes passed were assessed through SGD before treatment to establish initial descent time. After initial pass through SGD, the first group of oocytes were placed into standard culture conditions (36.9°C, 5.8% CO₂, balance room air) to serve as control. Oocytes in other treatment groups were then either immersed in Modified Ham's F-10 Media which has been adjusted to a pH of 6.0 from 7.4 with Hydrochloric Acid for 1 h, the F3 solution of the Global Blastocysts Fast Freeze kit for 1h or exposed to a

temperature of 60°C for a period of 30 min. Oocytes were then returned to normal culture conditions and passed back through SGD for final descent rate estimations. After all data were collected, comparisons were made between initial and post treatment descent times to establish if there were any changes in oocyte buoyancy over the course of treatment.

To further examine the relationship between oocyte viability and buoyancy, ninety-eight additional oocytes were passed through SGD. After initial descent times were measured, oocytes were randomly assigned to one of four treatment groups (Table 1). Treatments were performed as previously described and oocytes were dropped through SGD post-treatment. After final measurements through SGD, all oocytes were immersed in Coomassie blue post treatment for 1 minute and then flushed with PBS to determine oocyte membrane integrity and estimate viability. Comparisons were made based on the uptake of stain into oocyte and treatment group.

Table 1: Number of oocytes per treatment group. Oocytes from each mouse were divided amongst different treatment groups to reduce variation due to maternal influence. Unequal oocytes per treatment group are due to unequal numbers of oocytes harvested per mouse on a given day when experiment was performed.

	Number of Oocytes Per Treatment Group	Number of Stained Oocytes Per Treatment Group
Control	69	24
pH	80	26
CP	80	24
Heat	49	24
Total:	278	98

Statistical Analysis

All data were analysed using the Statistical Package for the Social Sciences (SPSS ver. 12; Chicago, IL). The basic analysis was a two-way analysis of variance of treatment by time using a P -value of 0.05 for significance. In case of significance by the original analysis, the differences within time or treatment were reanalysed with either Student's t -test or one-way analysis of variance with Tukey's means separation. A Chi Square analysis was used when appropriate.

Results

There was no significant difference between oocytes treated with F3 media, pH adjusted media and controls before treatment (Figure 1; $P > 0.05$). Oocytes treated with acidified media had slower descent times than before treatment (Figure 2; $P < 0.001$). When embryos were stained to verify effectiveness of kill treatment, 71% of control oocytes and 92% of acid treated oocytes appeared to have intact membranes, because the stain was not absorbed into the cell (Figure 3).

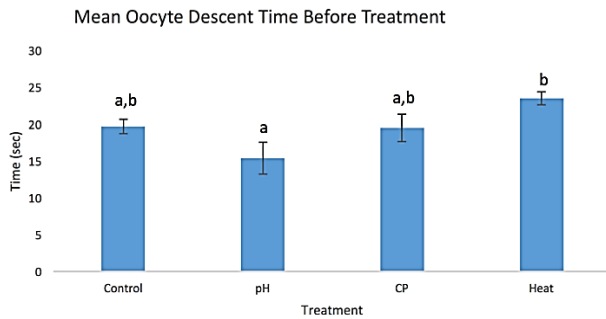


Figure 1: Mean oocyte descent time before treatment. Error bars represent standard error. Bars with different letters a–b represent differences between mean descent time ($P < 0.05$).

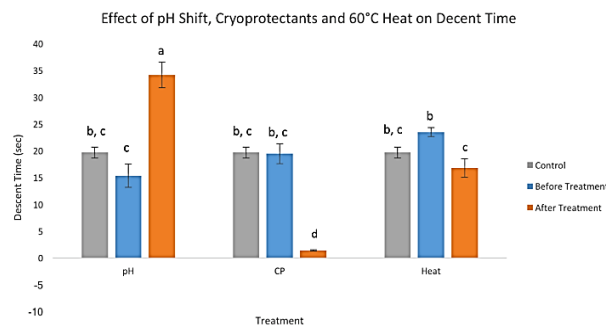


Figure 2: Effect of pH Shift, Cryoprotectants and 60°C Heat on descent time. Comparison of oocyte descent time between control, pre-treatment and post-treatment in oocytes treated with exposure to acidified media, F3 solution and 60°C heat. Error bars represent standard error. Bars with different letters a–c represent differences in descent time ($P > 0.05$).

Conversely, oocytes treated with CP and heat had more rapid descent times through SGD than before treatment (Figure 2; $P < 0.001$). Oocytes exposed to CP for 1h experienced drastic shifts in descent time; faster than both control and pre-treatment oocytes (Figure 2; $P < 0.001$). 100% of desiccated oocytes with CP absorbed stain and were deemed to be non-viable. Before treatment, there was no significant difference between heat treated oocytes and control group (Figure 1; $P > 0.05$). After heat treatment, oocytes displayed faster descent times than before treatment, but were not statistically significant from control oocyte descent times (Figure 2). Only 20.8% of heat treated oocytes appeared to maintain cell membrane integrity by not absorbing stain, suggesting the majority of oocytes were damaged in the treatment process (Figure 3).

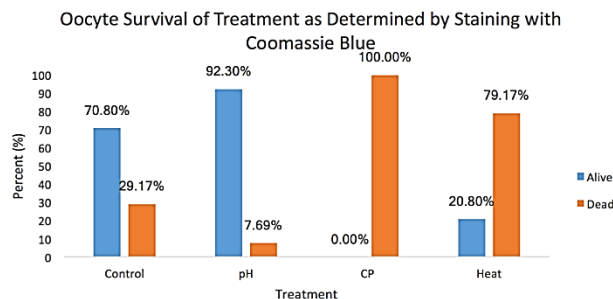


Figure 3: Oocyte survival of treatment as determined by staining with Coomassie blue. Percent of oocytes in each treatment group (control, pH, CP and heat) which appeared to be live by not absorbing stain or those which appear to be dead because the cell did uptake stain.

Discussion

SGD was effective in detecting differences between pre-treatment and post-treatment descent times in each group. When oocytes were treated with pH adjusted culture media, oocytes demonstrated longer descent times than before treatment (Figure 2). While stain was not absorbed in the majority of oocytes cultured in acidic conditions, SGD demonstrated the ability to detect differences between viable and non-viable oocyte descent times. Denuded oocytes lack the ability to regulate cellular pH [26–28]. This can be contributed by lack of tight junctions between cells (as an oocyte is a single cell) and inactive regulatory mechanisms in oocytes [26, 29, 30, 31]. In oocytes, pH is known to affect cytoskeletal elements and mitochondrial localization, which are both correlated to developmental incompetence [32–34]. Decreased pH can denature proteins, which limits the effect of the membrane and the cell cannot regulate bidirectional leakage. For these reasons, it is likely oocytes exposed to acidified culture media lost the ability to regulate bidirectional leakage. Therefore, SGD can allow early estimation of oocyte viability because, water, being a smaller molecule than Coomassie Blue, can permeate and alter cellular density before the stain could permeate into the cell. This demonstrates the sensitivity of SGD to detect oocytes suffering from shifts in pH from those in healthy culture conditions based on oocyte density.

Oocytes treated with F3 solution for 1h had significantly faster descent times than fresh oocytes and controls (Figure 2; $P < 0.001$). The cause for this is likely two-fold. First, the glycerol and ethylene glycol solution dehydrated the cell and reduced its radius, thus reducing resistance as the oocyte descended through the fluid filled chamber. Second, while water was removed from the cell, CP likely entered the cell, increasing cellular density. These phenomena can be supported by experiments performed by Paynter *et al.*, in which shrink-swell experiments demonstrate oocytes initial non-spherical shrinkage after exposure to hypertonic glycerol CP solutions resulting in increased surface area [35]. Glycerol ethylene glycol have high molecular weights and can permeate into the membrane with long time exposure. This should contribute to the faster descent times observed in the CP treated oocytes [35, 36]. This suggests the SGD was effective in detecting the change in oocyte buoyancy after exposure to CP. Rapid descent times of CP treated oocytes through SGD likely represent decreased resistance and increased cellular density after CP exposure. Stain data supports this theory as 100% of CP treated oocytes absorbed Coomassie Blue. It is well documented CP alters both physical parameters of the oocyte (e.g. volume and surface area) and intracellular osmolality [37]. SGD was able to detect these changes by means of oocyte buoyancy and could differentiate between healthy and osmotically damaged oocytes.

With respect to heat treated oocytes, there was no significant difference between post-treatment and control descent times (Figure 2; $P > 0.05$). However, post-treatment descent times were more rapid than pre-treatment, indicating SGD can detect changes in buoyancy in oocytes damaged with high temperatures (Figure 2; $P < 0.05$). While heat treated oocytes did not demonstrate as dramatic shifts in buoyancy as compared to other groups (mean descent time before treatment was 23.5s as compared to 16.82s after treatment), this observation resembles previous

data in zygotes in which dramatic shifts after heat treatment were not observed until 24h [25].

This study suggest SGD is a non-invasive and quantitative measurement of oocyte competency, which can be used to supplement a morphological analysis to select only the highest quality oocytes to cryopreserve. Damaged cells undergo changes which are not always easily visually apparent. These osmotic changes can be detected by SGD and oocyte descent time can be used to select healthy oocytes. When selecting which oocytes are of highest quality, it appears that more average oocyte descent times within a maternal cohort should be considered competent over fast and slow counterparts (Figure 2). Measuring buoyancy of all oocytes in the cohort prior to freezing would allow specification of healthy oocytes.

As assisted reproductive technologies continue to grow, there will be an increased demand to freeze oocytes. Currently, all non-fertilized oocytes retrieved will be frozen whether or not they demonstrate high competency. Embryologists' time, space in cryotanks and resources are spent freezing non-viable oocytes, which will never regain viability. If SGD can be used to select higher quality oocytes to freeze, thus reducing the number of non-viable oocytes preserved, time and resources can be conserved.

Conclusion

SGD detected differences in oocyte descent time before and after exposure to lethal treatments. This can be used as a further means to predict oocyte quality prior to cryopreservation. Descent times of control and pre-treatment oocytes appear to represent healthy oocytes and display more average buoyancy compared to post-treatment oocytes. This indicates oocytes outside at least one standard deviation from the mean are likely non-viable and further efforts should not be invested on them. Freezing fewer non-viable oocytes will save time and resources during freezing, thawing, fertilization and culture, and will increase fertilization rates of *in vitro* produced embryos.

Author Contributions

All four authors were significant contributors to the work and manuscript preparation on this project. SP developed the original concept and initial design of SGD. CW and LP refined the design prior to embryo testing. CW, MO, SP and LP designed experiment. CW performed testing with mouse embryos. While CW developed the original manuscript, all four were involved in editing for final content.

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Conflicts of Interest

Cara Wessels has financial interest in Embryotics, LLC which has licensed this technology from Texas Tech University. All other authors have no conflict of interest.

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