

Open Pulled Straw (OPS) Vitrification of Mus Musculus Morula and Blastocyst Survival in Two  
Common Cryopreservation Medias

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# **Open Pulled Straw (OPS) Vitrification of *Mus Musculus* Morula and Blastocyst Survival in Two Common Cryopreservation Medias**

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## **ABSTRACT**

The objectives of this study were to: (1) determine an optimal cryoprotectant of mice embryos; and (2) determine whether morula or blastocyst stage is ideal for vitrification in both medias. One experiment was performed using two different medias for vitrification with open pulled straws (OPS) with morulae and blastocysts. In the first protocol, we called V, embryos were exposed to 10% ethylene glycol (EG) for 5 minutes, then 40% ethylene glycol and 0.6 M galactose for about 30 seconds, loaded into an OPS, and plunged into liquid nitrogen. In the second protocol, we called V<sub>G</sub>, embryos were exposed to 7.5% EG + 7.5% dimethyl sulfoxide (DMSO) for 3 minutes, 16.5% EG + 16.5% DMSO and 0.5 M sucrose for about 30 seconds, and loaded and plunged into liquid nitrogen. Cryoprotectants were removed after warming in three steps at 3-minute intervals. All embryos were cultured for 24-48 hours after warming. Survival rates for morulae and blastocysts were similar ( $P > 0.05$ ) in both media. The overall survival of all embryos, regardless of stage of development, was better for embryos vitrified using the V method rather than the V<sub>G</sub> method ( $P < 0.05$ ).

## **INTRODUCTION**

A replacement of freezing, vitrification is a method used that involves very rapid warming and cooling rates, small volumes, high viscosity and high concentrations of cryoprotectant solutions (Campos-Chillon et al., 2009). Vitrification offers many benefits that include timesaving procedures, easy to perform, does not require expensive equipment, and avoids the formation of damaging ice crystals. During cooling, the solutions become supercooled and because of the high concentration of cryoprotectants, they remain liquids but as the temperature drops there is a continuous rise in viscosity until it doesn't flow on a measurable time scale becoming a glass-like state (Telea et al., 2008). However, since vitrification requires high amounts of cryoprotectants, toxicity or osmotic damage to embryos can occur (Cha et al., 2011).

There are a few common cryoprotectants used in the vitrification of embryos. Ethylene glycol (EG) is commonly used, since it is one of the major permeable cryoprotectants; it has a low molecular weight and a low toxicity to mammal oocytes or embryos (Cha et al., 2011). As well as using EG alone as a cryoprotectant, high survival rates of embryos that were vitrified at the blastocyst stage with a combination of EG and DMSO together have been reported (Cha et al., 2011). As DMSO enters into the cell, it accelerates its characteristics of forming a glass-like state and it increases the permeability rate (Cha et al., 2011). Studies have shown that it is common for sugars or other compounds to be added to cryoprotectants because they offer beneficial results in vitrification procedures by preventing ice crystal formation. Sugars raise the total solute concentration of a solution without increasing the toxicity of the media (Shaw and Jones, 2003). Sucrose and galactose are non-penetrating cryoprotectants while EG and DMSO are membrane-penetrating cryoprotectants and can be slightly toxic (Telea et al., 2008; Shaw and Jones 2003). The permeating cryoprotectants lower the freezing point, and replace some of the

bound water molecules in and around intracellular components, therefore preventing damage from ice crystals. The non-penetrating sugars stay outside of the cells and aid in dehydration by exerting an osmotic effect on the cells before and during preservation; they may also help stabilize the membrane by stabilizing the phospholipid heads (Shaw and Jones 2003).

The objectives of this study were to: (1) determine if the DMSO and sucrose protocol or the EG and galactose protocol had a significant difference in survival of embryos when compared; and (2) determine whether stage of early embryo development (morula or blastocyst) had an effect on the survivability in either of the vitrification protocols.

## **MATERIALS AND METHODS**

Unless otherwise stated, all chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) and concentrations of media were prepared by Dr. Campos-Chillon.

### **Embryo Production**

Mice provided by California Polytechnic State University's (CPSU) mouse colony were super ovulated and bred. All mice were kept in a light and temperature controlled environment. Each female received a 0.1 mL (100 microliter) intraperitoneal (IP) injection from 80 international units (IU)/mL concentration of pregnant mare's serum (PMS) at a pre-determined time. Exactly 47 hours later, each female received a 0.1 mL (100 microliters) of human chorionic gonadotropin (hCG) and were placed with a male for breeding to occur. The ratio of female to male ratio was 1:1 and each pair was kept in a separate cage to ensure breeding could occur. The injections were given using 25-gauge needle and a 1 mL syringe. Exactly 3 or 4 days later the females were harvested for morulae or blastocysts, respectively. The mice were harvested by cervical separation. The belly area was wet with ethanol to prevent hair from entering the sterile body cavity. The tools used (small scissors and fine-tipped forceps) were sterilized with ethanol prior to usage. Uteruses from the super ovulated and fertilized mice were then dissected out as close to the oviducts and cervix as possible and flushed in tissue culture medium-199 (TCM-199) with 25 gauge needles and 1 mL syringes under a dissection microscope. About 0.3 to 0.5 mL of TCM-199 was used to flush each side of the uterine horn and out of the uterine body for each uterus.

### **OPS and Vitrification**

Pulled French mini-straws (250 microliters) were heat-softened over a hot plate, and pulled manually until the inner diameter decreased from 1.7 mm to approximately 0.8 mm and the wall thickness of the central part decreased from 0.15 mm to approximately 0.07 mm. They were cooled in air, then cut at the narrowest point with a razor blade (Vajta et al., 1997).

After making a 1 microliter drop with embryos in it, the capillary effect was used to load the narrow end of the straw. Directly after the embryos were loaded into the straw, it was submerged into liquid nitrogen where the liquid column would turn into a glass-like state immediately, keeping all contents intact (Vajta et al., 1998). Warming occurred when the straw was placed directly into the holding medium. The holding medium entered the straw and within a couple seconds the vitrified medium became liquid. The embryos floated out of the straw into the holding medium due to sedimentation and capillary action (Vajta et al., 1998; Gabor Vajta). If the embryos did not come out of the straw when warmed, up to 10 microliters of air maximum was used to gently push out the liquid into the holding medium.

### **Vitrification and warming of embryos in “V” media:**

Embryos harvested that were either in the morula or blastocyst stage had been vitrified using the OPS method. The embryos were placed in a holding medium consisting of modified phosphate-buffered saline (mPBS) plus 20% fetal calf serum. A warming plate was used to keep the embryos and all media warm while working, and was kept at 39 degrees Celsius. A vitrification dish with 4 wells was set up; the first well consisted of 1000 microliters of holding medium, the second well ( $V_1$ ) had 800 microliters of 10% EG dissolved in holding medium, and a 20 microliter drop of 40% EG dissolved in holding medium and 0.6 M galactose ( $V_2$ ) was made in an unused portion of the dish for each group of embryos per straw.

Embryos were moved from the holding medium with a pipet set at 1 microliter, placed into  $V_1$  and a timer was set for 5 minutes. In the last 30 seconds, the embryos were found and collected in the pipet. Once the 5 minutes was up, the embryos were placed into the 20-microliter drop of  $V_2$  and mixed about 5 times. After mixing the embryos in the  $V_2$  medium, they were placed in their own 1 microliter drop in another portion of the dish and were loaded in the OPS and plunged into the liquid nitrogen. As soon as the 5 minutes was up in the  $V_1$  well, one additional minute was allowed to move the embryos into the  $V_2$  and mix, place into the OPS and plunge into the liquid nitrogen.

The straws had been left in a liquid nitrogen tank between 1-7 days then were warmed. Warming was done in a 4-well plate with media that had been warmed by the warming plate previously mentioned. The first well contained 800 microliters of 1 M galactose prepared in holding medium ( $V_{w1}$ ), the second well contained 800 microliters of 0.5 M galactose prepared in holding medium ( $V_{w2}$ ), the third well contained 800 microliters of 0.25 M galactose prepared in holding medium and the fourth well contained 800 microliters of holding medium. A culture plate was made for incubation after the warming process, which consisted of three 30-microliter drops of embryo culture medium ( $G_2$ ), covered by mineral oil to prevent evaporation of the growth medium during incubation. One straw at a time was taken out of the liquid nitrogen and placed into the bottom of the first well. Capillary action and the tip of the finger on top of the straws or less than 10 microliters of air if capillary action did not work, was pushed through the straw to expel the embryos from the straws. Once the embryos were in  $V_{w1}$ , a timer was set for 3 minutes. Before time was up, embryos were located and picked up with a pipet set to 10 microliters and moved into  $V_{w2}$  for 3 minutes. Once the 3 minutes was up in  $V_{w2}$ , embryos were moved into  $V_{w3}$  for 3 more minutes. After the final 3 minutes is up in  $V_{w3}$ , embryos were placed into the holding medium in well #4. Once all embryos had completed the warming process and were in the final holding medium, they were moved into the  $G_2$  plates, “washed” as they were moved through each of the 3 drops, then placed in the incubator. The embryos were incubated overnight and checked on approximately 24 hours later to determine survival of each trial. A few circumstances, further incubation was needed and embryos were checked again within 48 hours after first starting incubation.

The incubator in CPSU’s embryology lab is shared between various laboratory classes and research projects. The settings should be at 39 degrees Celsius, 6%  $CO_2$ , and 90% humidity. Due to constant opening from sharing the incubator, a few times the settings have been noticed to be a little low or off with the temperature no lower than 37 degrees Celsius,  $CO_2$  stayed pretty constant but could have changes around +/- 0.1% and humidity at some times was down into percentages in the 70s.

### **Vitrification and warming of embryos in “VG” media:**

Embryos harvested that were either in the morula or blastocyst stage had been vitrified using the OPS method. The embryos were placed in a dish of holding medium. A warming plate was used to keep the embryos and all media warm. A vitrification dish with 4 wells was set up; the first well consisted of 1000 microliters of holding medium, the second well had 800 microliters of 7.5% EG + 7.5% DMSO dissolved in holding medium ( $VG_1$ ), and a 20 microliter drop of 16.5% EG + 16.5% DMSO dissolved in holding medium and 0.5 M sucrose ( $VG_2$ ) was placed in an unused portion of the dish for each group of embryos per straw.

Embryos were moved from the holding medium with a 1-microliter pipet, placed into  $VG_1$  and a timer was set for 3 minutes. Once the 3 minutes was up, the embryos were placed into the 20-microliter drop of  $VG_2$  and mixed about 5 times. After mixing the embryos were placed in their own 1-microliter drop and were loaded in the OPS by capillary action and placed into the liquid nitrogen. One additional minute was allowed to move the embryos into  $VG_2$  and mix, place into the straws and plunge into the liquid nitrogen.

The straws had been left in a liquid nitrogen tank between 1-7 days then were warmed. Warming was done in a warmed 4-well plate. The first well contained 800 microliters of 0.3 M sucrose prepared in holding medium ( $VG_{w1}$ ), the second well contained 800 microliters of 0.2 M sucrose prepared in holding medium ( $VG_{w2}$ ), and the third and fourth well contained 800 microliters each of holding medium. One straw at a time was taken out of the liquid nitrogen and placed into the bottom of the first well. Capillary action and the tip of the finger on top of the straws, or less than 10 microliters of air if capillary action did not work, was pushed through the straw to expel the embryos from the straws. Once the embryos were in  $VG_{w1}$ , a timer was set for 3 minutes. Before the time was up, embryos were located and picked up with a pipet set to 10 microliters and moved into  $VG_{w2}$  for 3 minutes. After the 3 minutes was up in  $VG_{w2}$ , embryos were moved into the first holding well to be mixed a couple times. After the embryos had been mixed with the holding, they were placed into the final holding medium in well #4. Once all embryos had completed the warming process and were in the final holding medium well, they were moved into the  $G_2$  plates, “washed” three times through each drop, then placed in the incubator. The embryos were incubated just the same as they were in the first protocol.

### **Statistical Analysis**

Survival rates were analyzed for statistical significance with a two-proportion z-test. The software used for the tests was Minitab. P-values < 0.05 were considered statistically significant.

## **RESULTS**

### **Survival of embryos in “V” media:**

Total embryos vitrified were 71 and broken down into the morula or blastocyst stage. 25 morulae were vitrified, 23 were recovered after warming and out of those warmed, 10 survived and showed growth. 46 total blastocysts were vitrified, 38 were recovered after warming and 20 of them survived. There was an overall survivability of 49% between both morulae and blastocysts in the first protocol. Blastocysts had 53% survivability and morulae had 43% survival rates (Fig. 1 and 2). The sample size was large enough to run a two-proportion test. There was no significant difference (P-value= 0.486) between the survival rates regarding the stage of embryo development (morula or blastocyst).

### Survival of embryos in “VG” media:

The amount of embryos we vitrified was 67 divided between morula and blastocyst stage. 45 total blastocysts were vitrified, 35 were recovered after warming and only 6 survived and showed growth. There were 22 total morulae, 9 were recovered after warming and 4 survived and showed growth. Morulae and blastocysts together had a 23% overall survival rate in the VG protocol. Blastocysts had a 17% survival rate and morulae had a 44% survival rate in the second protocol. There was a concern that the population of morulae may have been too small but after running a two-proportion test, the population was shown to be large enough and the Fisher’s exact test gave a P-value= 0.175; the conclusion was that there was no significant difference in survival between the two stages of development (Fig. 1 and 2).

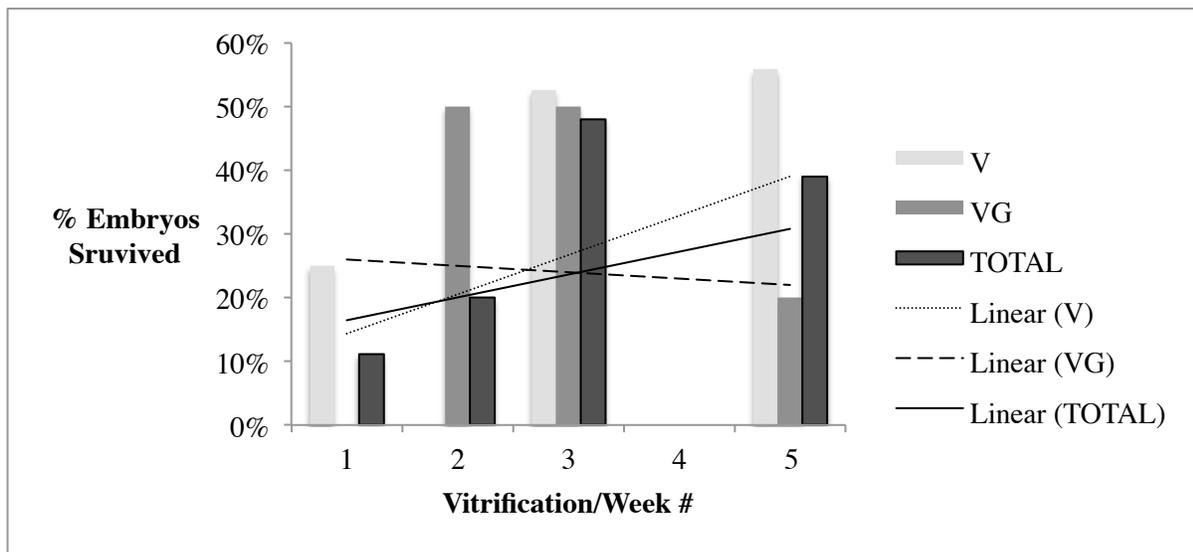


Fig. 1. Percentage of surviving embryos in each protocol each vitrification trial/week. Overall survival (solid line) increased as well as embryos in the V protocol; embryos in the VG protocol had a slight decrease in survival rates over the course of this study.

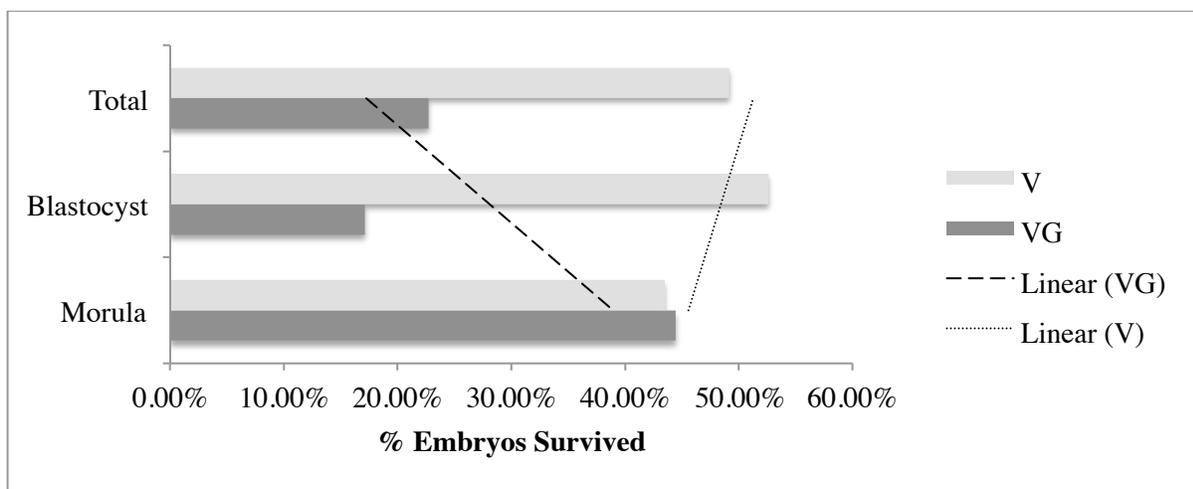


Fig. 2. Early embryo survival for each vitrification protocol. Significant differences were seen between each protocol but not between stages of development (two-proportion test).

## DISCUSSION

In this study, two cryopreservation medias were evaluated using morula and blastocyst stages. Our objective was to determine the best stage of early embryo development for vitrification and the best media protocol for vitrification. The stages of development in either of the two protocols used were determined to show no significance in the survival rates of embryos after using a two-proportion test. After running another two-proportion test, it was determined that there was a significant difference in overall survival rates of embryos amongst the “V” and “VG” media. A P-value= 0.003 was given which shows that there is a significant difference in the survival depending which media protocol was used. Survival rates were better overall in the V media than in VG.

Sucrose and galactose are both beneficial to include in vitrification by preventing ice crystals, however the two sugars differ in their molecular weight and viscosity since one is a monosaccharide and the other is a disaccharide. Galactose is a monosaccharide, giving it a lower molecular weight than the disaccharide sucrose. Galactose is able to facilitate dilution of cryoprotectants better than sucrose because of the differences in molecular weight and viscosity (Campos-Chillon et al., 2009). The sugars also act as an osmotic buffer, preventing ‘osmotic shock’ following dilution of the cryoprotectant after warming (Alcolak et al., 2011). The presence of these macromolecules could decrease toxicity of the cryoprotectants, and change the physical properties of the solution (Wang et al., 2011). From this information, galactose seems like it would be the better sugar of the two to add to a vitrification cryoprotectant. Our data showed that the protocol that included galactose was superior.

Wang et al. (2011) states that EG and DMSO together has a better glass-formation characteristic and higher permeability than EG alone, which improves survival rates. It also states that bovine oocytes have a higher permeability to DMSO than EG as well. Another study stated that DMSO containing vitrification solutions did not lead to cell membrane damage and death as quickly as the DMSO free vitrification solutions (Alcolak et al., 2011). With all the positive effects reported by including DMSO in vitrification procedures, the expectation in our study would show decent survival rates in the VG protocol. However, our survival rates were not as high as we should have seen with only 22.72% of total embryos surviving in that protocol. Some broken zona pellucidias were observed after warming which shows our warming technique has not been perfected yet.

In an experiment by Shirazi et al. (2010), two vitrification methods were performed and embryo survival progressively increased as the developmental stage progressed. It has also been reported by Cuello et al. (2004) that the survival rate of blastocysts had a significant difference than in the morula stage, which had the poorest survival and hatching rates after warming. A majority of other studies and experiments have shown higher survival rates in the blastocyst stage after vitrification and warming. In our experiment, we would anticipate better survivability in the blastocyst stage, however we discovered there was no significant difference between the stages. This was probably influenced by conditions and techniques we used.

Further research is needed to confirm our results. We may have gotten inaccurate data due to a few conditions. Sterility of media was a concern during a couple of the last trials due to a high amount of contamination seen in the embryo culture media. The contamination could have come from the culture media, pipet tips, dishes, or straws we used. Everything used is shared amongst other students using the embryology lab so it is possible sterile techniques may not have been used prior to our usage causing our materials to become contaminated. We tried to keep our materials labeled and separate to avoid contamination, however some items could

have been easily accessed and used without our knowledge. The culture media in the last two trials showed the most contamination which could have come from using any of the materials that we assumed were sterile but could have been contaminated prior to our procedures. Sterile media that was left over from previous trials got transferred over each week for another trial. It is possible that we could have accidentally contaminated some of our media this way without realizing if sterile technique was not proper. Pipet tips were another possible concern of contamination since supplies in the embryology lab began to run low towards the end of our experiment. Many pipet tips, which were assumed sterile, were shared amongst various students and classes, including us, subsequently that may have been a source of our contamination in the last two trials. The incubator and liquid nitrogen tanks were another cause of concern in our experiment. Since they are shared as well, proper environmental conditions may not have been maintained, causing lower survival rates.

Another possible problem we encountered was the mice provided might not have been reproductively sound or mature. We started to see a decrease in the amount of embryos produced as we got further into the experiment, even though our procedures did not change. Due to this realization, we increased the amount of mice for harvesting each week in order to get an adequate amount of data.

This was also a learning experience for Lisa and I so some of the timing of moving embryos between each media was not exact and could have altered the outcomes of our data on survival rates (Fig. 3).

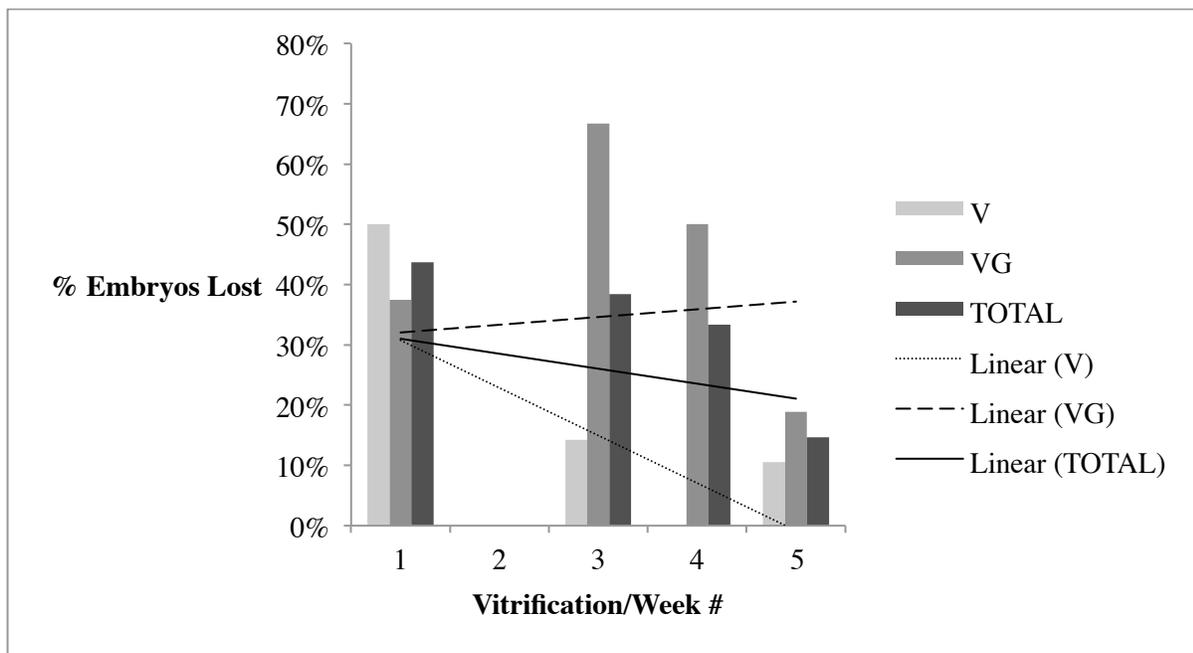


Fig. 3. Percentage of embryos lost. Each bar indicates the percentage of embryos lost each vitrification trial. The solid line indicates the overall embryos lost (learning curve), the dotted line indicates percentage of embryos lost in V protocol and the dashed line indicates percentage of embryos lost in VG protocol.

In conclusion, we found one of the common cryoprotectant protocols used in vitrification to show better survival rates of mice embryos; this was the protocol that used ethylene glycol and

galactose in the vitrification procedures. We did not see any significant differences in the different stages of development in either protocol.

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## REFERENCES

- Alcolak E., Marar E. A., Mytas S. C., Chalvatzas N., Palapelas V., Schöpfer B., Diedrich K., and Al-Hasani S. 2011. Comparison of two different media for vitrification and rewarming of human zygotes: Propective randomized study. *Middle East Fertility Society Journal*. 16:189-193.
- Campos-Chillon L. F., Suh T. K., Barcelo-Fimbres M., Seidel Jr. G. E., and Carnevale E. M. 2009. Vitrification of early-stage bovine and equine embryos. *Theriogenology*. 71:349-354.
- Checura C. M. and Seidel Jr. G. E. 2007. Effect of macromolecules in solutions for vitrification of mature bovine oocytes. *Theriogenology*. 67:919-930.
- Cha S. K., Kim B. Y., Kim M. K., Kim Y. S., Lee W. S., Yoon T. K., and Lee D. R. 2011. Effects of various combinations of cryoprotectants and cooling speed on the survival and further development of mouse oocytes after vitrification. *Clin. Exp. Reprod. Med*. 38(1):24-30.
- Cuello C., Gil M. A., Parrilla I., Tornel J., Vazquez J. M., Roca J., Berthelot F., Martinat-Botte F., and Martinez E. A. 2004. Vitrification of porcine embryos at various developmental stages using different ultra-rapid cooling procedures. *Theriogenology*. 62:353-361.
- Gabor Vajta. 1998. The Open Pulled Straw system. <http://www.gaborvajta.com/the-open-pulled-straw-system/> Accessed Apr. 12, 2012.
- Shaw J. M. and Jones G. M. 2003. Terminology associated with vitrification and other cryopreservation procedures for oocytes and embryos. *Hum. Reprod. Update*. 9:583-605.
- Shirazi A., Soleimani M., Karimi M., Nazari H., Ahmadi E., and Heidari B. 2010. Vitrification of in vitro produced ovine embryos at various developmental stages using two methods. *Cryobiology*. 60(2):204-210.
- Telea A., Pacala N., Ivan A., Caraba V., and Korbuly B. 2008. Studies regarding the crioprotective properties of the vitrification media, with DMSO, sucrose, Ficoll 70 and galactose used in embryo cryopreservation. *Lucrări științifice Zootehnie și Biotehnologii (Scientific Works and Animal Biotechnology)*. 41(1):162-168.
- Vajta G., Booth P. J., Holm P., Greve T., and Callesen H. 1997. Successful vitrification of early stage bovine in vitro produced embryos with the open pulled straw (OPS) method. *Cryo-Letters*. 18:191-195.
- Vajta G., Holm P., Kuwayama M., Booth P. J., Jacobsen H., Greve T., and Callesen H. 1998. Open pulled straw (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos. *Mol. Reprod. Dev*. 51:53-58.