COVER SHEET

TITLE: Effects of white yolk concentration on maturation, fertilization, and blastocyst development of bovine embryos produced \textit{in vitro}

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YEAR: 2004

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Dated: 6/1/06
Effects of white yolk concentration on maturation, fertilization, and blastocyst development of bovine embryos produced in vitro

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Abstract
Most in vitro embryo production (IVP) systems use some form of bovine sera in media. The most commonly used sera in maturation of bovine oocytes is fetal calf serum. A potential problem arising from this practice is disease transfer to the embryo as the serum is coming from the same species as the embryo. A potential substitute (white yolk from avian eggs) derived from a different order of animals could ameliorate this problem. This study was done to determine if white yolk plasma is a suitable supplement to use in the maturation of bovine embryos. Eight replications were done: a control (10% fetal calf serum), and three treatments, replacing fetal calf serum with 5%, 10% or 15% white yolk plasma. Data on fertilization, cleavage, and blastocyst development rate and post freezing/thawing survival were collected. There were no significant differences among treatments for fertilization, cleavage, blastocyst development, or post freezing/thawing survival indicating that white yolk is a suitable substitute for fetal calf serum in bovine oocyte maturation systems.

Introduction
The most commonly used medium for in vitro maturation of bovine oocytes employs Tissue Culture Medium 199 (TCM 199) supplemented with 10% fetal calf serum, follicular stimulating hormone, luteinizing hormone, and estradiol-17β (Watson, et al. 2000). This medium supports meiotic development in 90% of bovine oocytes and prepare them for fertilization and development through blastocyst (Watson, et al. 2000). Although this medium provides essential nutrients and perhaps growth factors to the maturing oocyte, fetal calf serum use is a potential problem in that there is probability of disease transfer. Sera or serum containing media can be filtered to remove bacteria, however, viruses, mycoplasma and certain other disease-causing organisms or molecules cannot be removed by filtering. A non-mammalian source of nutrient supplementation should greatly reduce the possibility of disease transfer.

In birds, reptiles, and egg laying mammals, oocytes and early embryos are completely surrounded by white yolk (WY) (Romanoff and Romanoff, 1949). It thus occurred to us that WY might be suitable as a blood serum substitute in the maturation of bovine embryos. Herein we test this hypothesis.

Methods and Materials
The experiment was conducted in 8 replicates during the Fall of 2003. All culture was in 60 mm Petri dishes at 39°C in air with 5% CO2 and high humidity.
Cumulus-oocyte-complex recovery and maturation treatments

Ovaries were obtained from spent cows at Emmepac, Inc. in Milwaukee, Wisconsin. They were transported to the laboratory at 30° C in physiological saline. Antral follicles 2 to 6 mm in diameter were aspirated using an 18-gauge, 1.5-inch needle and vacuum pump. Cumulus-oocyte-complexes (COC) having at least two layers of intact tightly adhered cumulus cells were selected from the aspirant, washed 3X in TL HEPES (Bio Whittaker, Walkersville, MD; modified to contain 3 mg/mL bovine serum albumin, 0.2 mM sodium pyruvate, and 50 µg/mL gentamycin), and randomly assigned to one of four treatments for in-vitro maturation. The control treatment was the normal maturation medium used in our laboratory and was TCM-199 (BioWhittaker), supplemented with 10% v/v heat-treated fetal bovine serum (Gibco BRL, Grand Isle, NY), 0.2 mM sodium pyruvate, 3 µg/mL luteinizing hormone (Sioux Biochemical, Sioux Center, IA), 3 µg/mL follicle stimulating hormone (Sioux Biochemical), and 25 µg/mL gentamycin. The other treatments were formulated as the control except the FCS was replaced with 5, 10, and 15% v/v white yolk plasma.

COCs were cultured in groups of 10 in 50 µL drops of maturation medium under mineral oil (Sigma Chemical Co., St. Louis, MO).

Fertilization

After 22 to 24 h of maturation, COCs were washed in TL HEPES and transferred in groups of 10 to 44 µL drops of fertilization medium under mineral oil. The fertilization medium contained IVF-TL (Specialty Media, Lavallette, NJ) supplemented with 0.2 mM sodium pyruvate, and 25 µg/mL gentamycin. A Percoll gradient method was used to isolate a population of motile sperm (Parrish, et al. 1986). A sperm suspension was added to the drops to obtain a final concentration of 1 x 10⁶ sperm per mL. Each drop was supplemented with heparin (2 µg/mL), 2.5 nM epinephrine, 0.01 nM hypothalmine, and 0.08 nM penicillin. Sperm and COCs were co-incubated for 18-22 hours.

Embryo Culture

Cumulus cells were removed from the COCs by vortexing for 3 min. Presumptive zygotes were washed 3X in TL HEPES, approximately 20 presumptive zygotes from each replicate/treatment combination were reserved for microscopic evaluation of fertilization. Remaining presumptive zygotes were transferred in groups of 25 into 50 µL drops of culture medium. Embryos in runs 1-6 were cultured in synthetic oviductal fluid (SOF, Specialty Media, Walkers, MD) supplemented with 8 mg/mL of fatty acid free BSA (Sigma). Embryos in runs 7 and 8 were cultured in the media described above, supplemented also with 3% white yolk. Embryo culture is considered Day 1 of development.

White Yolk Collection

White yolk was obtained from chicken eggs from the University of Wisconsin flock. The white yolk containing latebra occurs at the center of the more familiar yellow yolk (Figure 1).
Endpoints Examined

During culture, these physiological stages were examined. On day of culture, 20 presumptive zygotes from each treatment were mounted on slides, with 10 ova per slide. Slides were placed in a fixative having 3 parts ethanol and 1 part glacial acetic acid. After the slides had been in fixative for at least 24 hrs, they were stained with acetocarmine and viewed under a high power inverted microscope (100X). Each presumptive zygote was determined to be normally fertilized, polyspermic, or unfertilized. Normal fertilization was defined as having 2 pronuclei. Polyspermy was defined by more than 2 pronuclei or 2 pronuclei and a devandensed sperm head. In unfertilized oocytes, full maturation has not occurred and often a metaphase plate is visible.

On day 2 of culture, cleavage data was collected by microscopic examination. This is an indirect measurement of fertilization rate, although it is usually slightly higher than directly determining fertilization rate as embryos can divide parthenogenically or if they are polyspermic. Cleavage rate was calculated as the number cleaved divided by the total number of presumptive zygotes in culture for that treatment (approximately 80 for each replication).

On days 7 and 8 of culture, the number developing to the blastocyst stage was recorded. At day 7, blastocyst rate was calculated by dividing the number of blastocysts by the number of embryos in culture for a given treatment. Day 7 is the normal day that blastocysts would be transferred into recipient cows or frozen for later use. The total blastocyst rate also includes blastocysts formed on day 8.

Figure 1. Schematic of a hen's egg.

Our collection procedure made use of the differences in physical chemistry of white and yellow yolk. Yellow yolk upon cooling irreversibly phase changes to a gel; white yolk forms an ice. To collect white yolk, eggs were kept at -20°C for several days. Eggs were removed and warmed in a water bath to facilitate removal of shell and albumin. Yolks were bisected with a wire and the halves were allowed to warm. Upon warming a pool of liquid white yolk formed at the latebra (Figure 2), which was then aspirated with a micropipette and placed in a 1 mL vial. White yolk was frozen at -20°C until used for maturation medium. Prior to use the white yolk was centrifuged and the plasma filtered through a 0.2 μm syringe filter.

Figure 2. Picture of egg yolk that has been frozen and thawed.
To further evaluate embryo fitness, all blastocysts were cryopreserved using 10% glycerol in phosphate buffered saline with 0.4% BSA. Embryos were warmed and scored as blastocyst, hatched blastocyst or dead 24 hrs post-warming.

**Statistical Analysis**

Since there were no statistical differences between replicates 1-6 (SOF culture) and 7-8 (SOF + 3 mg/mL WY) the data were pooled for analysis. A two-way Analysis of Variance test of the untransformed percentages was done. A critical value of 0.05 was used to reject the null hypothesis.

**Results**

Fertilization rates did not differ between treatments (Figure 3). Control fertilization rate averaged 70.9%. Average fertilization rates were 71.3, 65.4, 79.5% for 5, 10, and 15% WY treatments, respectively.

![Figure 3. Mean fertilization rates. Fertilization rates were recorded on the day of culture.](image)

Average cleavage rate for control was 74.4%, and that for 5, 10, 15% WY treatments averaged 76.7, 71.6, and 74.9%, respectively (Figure 4). Average cleavage rate was slightly higher than average fertilization rate as expected.

![Figure 4. Mean cleavage rates. Cleavage rates were recorded on day 2 of culture.](image)

**Blastocyst development rates on day 7** did not statistically differ (Figure 5). Blastocyst development data for replicates 3 and 5 were removed from analysis due to embryonic death post-cleavage. Average control blastocyst development for day 7 was 18.6%. For 5, 10, and 15% WY treatments, averages were 15.1, 11.0, and 14.9%, respectively.

![Figure 5. Mean day 7 blastocyst development rates. These were taken on day 7 of culture.](image)

Blastocyst development was again recorded on day 8 for a final rate. These were higher than day 7 development rates as expected as the embryos have had more time to grow. Total blastocyst development (recorded on day 8) for the control treatment averaged 32.1%. 5, 16, and 15% WY
treatments had blastocyst development rates of 30.0, 27.1, and 29.2%, respectively (Figure 6).

![Total Blastocyst Rate](image)

**Figure 6.** Total blastocyst development was recorded on day 8.

There was no statistically significant difference in blastocyst development post-freeze thaw (Figure 7). Blastocyst survival for the control treatment averaged 31.9%, 5, 10, and 15% WY treatments averaged 22.0, 28.5, and 17.1%, respectively. Data for hatching post-thaw was very low and variable between replicates (data not shown). No embryos hatched in many replicates. There was no statistical difference.

![Total Blastocysts Developed Post-Thaw](image)

**Figure 7.** Percent of blastocyst that survived after the freeze/thawing process.

**Discussion**

Since there were no significant differences in fertilization, cleavage and blastocyst rates or fitness post freeze-thaw between the control and three white yolk treatments, this supports our hypothesis that white yolk is a suitable supplement for the maturation medium of bovine embryos produced in an *in vitro* production system.

There did not appear to be any dose response of the increasing white yolk concentrations. The three concentrations appeared to be equal in their ability to sustain embryo maturation. Also no difference was seen in development for embryos cultured in medium supplemented with 3% WY (replicates 7-8, data not shown separate). White yolk appears to be able to not only sustain maturation but also subsequent development of the embryo.

There has been little research on the use of white yolk and allied substances in cell culture and preservation. In 1939, Phillips found that yolk (sum of white and yellow yolk) is an effective extender of bull semen. Huenp used egg yolk to culture bacteria (Romanoff and Romanoff, 1949). Elhussan, Kaefer and Westin (1999) used whole yolk, whole yolk plasma, and whole yolk granules in culture media for bovine embryos. Best results were received with a simple salt solution supplemented with 5% whole yolk plasma and semi-calcium lactate.

Studies completed in our lab have found white yolk to be a suitable supplement also for the culture media. Future studies should be completed using white yolk in every step of the *in vitro* process, maturation, fertilization, and culture.

**Acknowledgements**

I would like to thank the Cargill/Benevenga fund for Undergraduate Research and the Babcock Institute for International Dairy Research and Development for their
support and funding of this project. Also, thank to the entire Rutledge lab who helped make this project a success: Ryan Becker, Casey Jenzi, Kaela Retallick, Amy Fisher-Brown, and Amy

Reene. Thank you for all of your help and knowledge.

References


