Investigation into the Use of Nuclear Magnetic Resonance Spectroscopy for Viability Predictions, in Real Time, on Mouse Preimplantation Embryos

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Abstract **- In Western societies, up to 4% of children born are conceived using assisted reproduction technologies (ARTs), with IVF (in vitro fertilization) now the clinically preferred method of treatment for infertility. It is known that nuclear magnetic resonance (NMR) spectroscopy can be used to identify and quantify metabolite levels in cells and embryos. In these experiments we evaluated embryo metabolism in real time. The development of preimplantation mouse embryos subjected to NMR was compared to embryos subjected only to the manipulations (SHAM), to embryos subjected to deuterium-containing medium (D_2O), and to control embryos. Mouse embryos analyzed with NMR, at the two-cell stage, exhibited lower ($p<0.05$) development to the blastocyst stage than control embryos. SHAM treated and NMR analyzed embryos developed at higher rates than embryos only exposed to D_20 at 37 °C, indicating attenuation of D_2O effects at lower temperatures. To determine why NMR analysis attenuates effects of exposure to D_2O, embryos were cultured to blastocysts after exposure to D_2O at ambient or 37 °C. Results showed that at lower temperatures, exposure to D_2O was less harmful. Finally, transfer of embryos into recipients after NMR resulted in two litters of normal pups. This study suggests that NMR techniques can be used for embryo evaluation.**

Index Terms **- Metabolism, remote sensing, embryo development, NMR, bovine.**

I. Introduction

Human IVF was introduced to treat clinical infertility in 1978. From that starting point, Western societies currently have up to 4% of all children born having been conceived using assisted reproduction technologies (ARTs) [1]. Embryo implantation remains one of the rate-limiting factors in IVF. The ability to identify which embryo has the highest developmental potential from a given cohort would increase the chances of achieving pregnancy while minimizing the number of embryos transferred in each cycle. Morphological assessment is currently the main method used to determine embryo viability during IVF cycles. It includes observation of the developmental pattern of embryos during culture, including the timing and rate of cleavage, cell number and fragmentation. Quantitative techniques have been developed for the non-invasive assessment of embryo metabolism, and their value as predictors of embryo viability is the subject of ongoing investigations [2].

It has been demonstrated that metabolic criteria such as glucose uptake and lactate production can be used to identify viable embryos for transfer [3, 4]. Various applications of ^{1}H-NMR spectroscopy have been successfully used to analyze the metabolome in biological samples such as cell culture models [5] as well as in human blood, urine, and other body fluids [6-8]. Over the past few years, ^{1}H-NMR spectroscopy has been applied to evaluate embryo culture media in order to predict pregnancies with favorable results [9, 10]. The development of the nano-NMR probe (nanoprobe) now allows the analysis of small (<40 microliters) sample volumes with high resolution [11]. The measurement of metabolites in small volumes opens the possibility of using NMR to measure metabolic markers of viability in single embryos. The studies described above all were designed to evaluate embryo viability, however all of these studies use indirect analysis of the embryo. In the present study, our goal was to examine embryo viability directly, if possible, using the NMR to quantify embryo viability in real time.

Before this method can be used for the selection of viable embryos, however, it must be shown that embryos can develop properly following NMR. This is especially important if NMR is to be used in species other than the mouse or on manipulated embryos, since the value of these embryos will be much higher. Although a number of studies have investigated the bio-effects of magnetic resonance imaging (MRI) on living systems, there is no clear consensus on the safety hazards [12-16]. In addition, most studies have focused on long duration (hours to days) NMR exposure. In this study, we examined the effects of short duration (30 - 40 minutes) nano-probe NMR on mouse embryos. During nano-probe NMR analysis, the embryos are subjected to high magnetic fields (11.75 Tesla), high spinning frequencies (2000 Hz), and exposure to deuterium oxide (D_2O). D_2O has been shown in mammalian cells and sea urchin eggs to inhibit mitosis at concentrations about 75% [17, 18]. This inhibition of mitosis could adversely affect the development of embryos in vitro.

Another possibility for the potential deleterious effects of NMR analysis is the lack of adequate thermal stability during the procedure. The NMR is normally performed with

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samples at ambient temperature, thereby subjecting the embryos to decreased temperatures, compared with incubation conditions, for extended periods of time. Furthermore, there is a risk of physical damage resulting from the high spinning speeds involved in the NMR process. Finally, there is concern that the high magnetic field (11.75 T) may result in abnormal development following analysis based on experiments of post-implantation embryos exposed *in utero* [19].

II. MATERIAL AND METHODS

A. Reagents and Media

Unless otherwise stated, all reagents were purchased from Sigma-Aldrich (*USA*). M2 medium is Krebs-Ringer solution buffered with HEPES and supplemented with 0.4% bovine serum albumin (BSA). This medium is known as modified Whitten's medium [20]. M16 medium is Krebs-Ringer bicarbonate solution with 0.4 % BSA [21]. D_2O-PBS it is a phosphate buffered saline in deuterium water (D_2O) (Cambridge Isotope Laboratories, Andover, MA) with 0.4 % BSA.

B. Experiment 1

All animal studies were conducted with the approval (IACUC protocol# A7R343) of the Institutional Animal Care and Use Committee at the University of Illinois at Urbana-Champaign. ICR mice were superovulated using 10 IU pregnant equine chorionic gonadotrophin (eCG, Sigma Chemical Co., St. Louis, MO) administered intraperitoneally 92 hours prior to collection of embryos. At 44 hours prior to embryo collection, these females were administered 10 IU human chorionic gonadotrophin (hCG, Sigma Chemical Co., St. Louis, MO) and placed with B6/SJL F1 males. This injection regime allows hybrid embryos to be collected at the 2-cell stage.

At the time of collection, donor females were sacrificed using CO_2 asphyxiation. Oviducts were removed from the donors and placed in 2 mL of M2. A 30-guage needle attached to a 1cc syringe filled with M2 is placed into the infundibulum of the oviduct. By depressing the plunger on the syringe while the needle is within the oviduct, the embryos are flushed into the petri dish containing M2. Embryos were examined, and only morphologically normal embryos were counted and assigned to treatment groups.

Embryos were washed into 2 mL fresh M2 prior to being placed in treatment groups. Control embryos were washed through three 40 µl drops of M16 before being placed into culture in 40 µl M16 under mineral oil at 37 ºC and 5% CO_2. Embryos assigned to the SHAM group were sequentially washed through seven 70 µl drops of D_2O-PBS. This is the medium, which was used to maintain the embryos during the NMR procedure. These embryos were then loaded by mouth pipette into NMR nano-probe analysis tubes. The D_2O group embryos were washed through 70 µl drops of D_2O-PBS and then placed into a 40 µl drop of D_2O-PBS under mineral oil at 37 ºC with 5% CO_2 in air. The NMR group embryos were washed in D_2O-PBS and loaded into an NMR nano-probe analysis tube as in the SHAM group, and then subjected to NMR, as described below.

Following the spectroscopy on the NMR group, all treatment groups were washed through four 70 µl drops of M2 before being placed into 40 µl M16 under paraffin oil at 37 ºC with 5% CO_2. All groups were then cultured for 96 hours. Embryo development was assessed and recorded every day, (22-26 hours). This experiment was replicated three times.

The NMR experiments were performed on a Varian Unity Inova 500 MHz spectrometer (Varian NMR, Palo Alto, CA) using a Varian nano-NMR probe (nanoprobe). The nanoprobe is designed to obtain high resolution NMR spectra of very small sample volumes (<40 microliters) by spinning the sample at the magic angle (54.4 degrees from the vertical axis) [22]. The probe temperature was 21°C and the spin rate was about 2 kHz. Proton one-dimensional spectra were obtained using a pre-saturation pulse sequence (pre-sat - 90° - acquire) to diminish the residual water signal. NMR spectra were averages of 256 scans and were acquired in 28 minutes. The total time the embryos were in the spectrometer was approximately 40 minutes. Each free induction decay (FID) was collected as 256 transients with 20,416 data points, a spectral width of 5.5 kHz, a pulse width of 12 msec, and a relaxation delay of 5 seconds. Prior to Fourier transformation, the FIDs were multiplied by an exponential line broadening factor of 1.0 in order to increase the signal to noise ratio. Chemical shifts are given relative to the residual water peak at 4.80 ppm. Peak assignments were based on comparison to literature values for chemical shifts and to the NMR spectra from known solutions of lactate.

C. Experiment 2

Embryos were collected as in experiment 1, with embryos randomly divided into control, PBS, and D_2O-PBS embryos. The control group was washed through four 40 µl drops of M2 and then placed into 40 µl M16 under paraffin oil at 37 ºC and 5% CO_2. The PBS embryos were treated as any other NMR group embryos, but were washed in standard PBS (salts diluted in ultra-filtered water) and placed into the nano-probe sample vessels with PBS. The D_2O-PBS group was treated as the NMR group in Experiment 1. Both PBS and D_2O-PBS groups were analyzed with NMR as above and cultured in 40 µl M16 for 96 hours following recovery from every 24 hours. This experiment was replicated three times.

D. Experiment 3

Embryos were collected as in experiment 1, with embryos randomly divided into either ROOM, WARMED or control groups. The control group was washed through four 40 µl drops of M2 before being placed into 40 µl M16 under paraffin oil at 37 ºC and 5% CO_2. The treatment groups were washed through seven 70 µl drops of D_2O-PBS and then placed into 40 µl drops of D_2O-PBS under paraffin oil. The ROOM group was placed in a dish, which had been left at room temperature for 1 h, while the WARMED group was placed in a dish, which had been placed on a 37 ºC, slide warmer for 1 hour. The embryos were left in these dishes for 2 h and then washed through four 40 µl drops of M2 before being placed into culture in 40 µL drops of M16 under paraffin oil at 37 ºC and 5% CO_2. The embryos were

cultured for 96 h, with development observed every 24 h. This experiment was replicated four times.

E. Experiment 4

Embryos for this experiment were collected in the manner described above and treated according to the protocol for the NMR group in Experiment 1. Recipient females that had a cervical plug on the day preceding the experiment were used as donors. Morphologically normal embryos were assigned in groups of 20. Following the NMR, embryos were washed through four 70 µl drops of M2 before being placed into 40 µl drops of M2 under mineral oil. These embryos were then transferred into recipients by treatment group. The transfers were accomplished by surgically exposing the oviduct of the recipients and then puncturing the oviduct with a 26-guage needle. A pipette containing the embryos was then introduced through the puncture and the embryos were expelled into the lumen of the oviduct.

F. Statistical Analysis

Categorical data were analyzed using chi-square analysis. An alpha of 0.05 was selected prior to analysis. The data are summarized in the frequency tables.

III. RESULTS

A. Experiment 1

The Control group embryos developed to blastocyst stage at a higher rate (44/53, 83%) than the other groups ($p<0.05$) (Table I). There was no difference between the NMR (24/44, 54.5%) and SHAM groups (19/40, 47.5%), though both groups had higher ($p<0.05$) development than the D_2O group (11/40, 27.5%). There were significant time effects seen in the model for blastocyst development. The day of culture at which the embryos reached a given stage of development were determined and then compared. The mean day at which embryos reached eight cells differed ($p<0.05$) only between the control (1.38d, n=50) and NMR (1.67d, n=37) groups (Table I). Development to morula was faster ($p<0.05$) for control (1.98d, n=48), NMR (1.97d, n=29), and SHAM (1.83d, n=24) groups compared to the D_2O (2.46d, n=13) group. Blastocyst development was also significantly faster in control (3.02d, n=44), NMR (3.00d, n=24) and SHAM (3.00d, n=19) versus the D_2O (3.18d, n=11).

Embryos from the control group seemed to be of a higher quality at the end of culture than those from the treatment groups, as evidenced by less fragmentation and heterogeneity of the cytoplasm. Although embryos were not graded for this experiment, the quality of the embryos in the treatment groups was generally lower than the controls. An embryo was considered to be a blastocyst if a clear blastocoel was present. In many of the treatment blastocysts, however, there was a large, single blastomere, which was present even when an inner cell mass and trophoblast were distinguishable. Furthermore, embryos from the treatment groups often had irregular membranes, fragmented cells, or smaller blastomeres. All of these signs seem to indicate less healthy embryos than those in the control groups.

B. Experiment 2

The development of embryos to the blastocyst stage following NMR in PBS (12/15, 80%) was higher ($p<0.05$) than that of embryos having undergone NMR in D_2O-PBS (7/15, 46.7%) (Table II). Both treatment groups have less ($p<0.05$) blastocyst development than for control (14/15, 93.3%). The rate of development varied between groups, with development to morula faster in controls (1.92d, n=15) compared to PBS (2.12d, n=14) and D2O (2.23d, n=10) groups. Blastocyst development was also faster ($p<0.05$) in controls (3.00d, n=14) versus PBS (3.18d, n=12) and D_2O (3.32d, n=7) groups (Table II).

C. Experiment 3

The development of embryos to blastocyst was higher ($p<0.05$) in the control (49/60, 81.67%) group compared to the ROOM (36/60, 60%) group. Both control and ROOM groups had higher blastocyst development compared to the WARMED (19/60, 31.67%) group. Development to morula was also higher ($p<.05$) in the control group (60/60, 100%) when compared to the ROOM (40/60, 66.67%) group. Both still had higher development to morula than the WARMED (29/60, 48.33%) group. The ROOM (60/60, 100%) and control (59/60, 98.33%) groups had greater development to the eight-cell stage than the WARMED (38/60, 63.33%) group (Table III). The rate at which embryos developed did not differ ($p<0.05$) except at the morula stage, where embryos in the control (1.93d, n=15) group were faster than embryos in the ROOM (2.3d, n=10) and WARMED (2.43d, n=7) groups (Table III).

D. Experiment 4

Three groups of 20 embryos were sent for analysis in the NMR. One of the groups was lost during recovery from the NMR sample tube, leaving only 2 groups available for transfer. Two recipients were used, with one recipient receiving 20 embryos, the other 21. Although only 20 embryos were assigned to each group, group 2 had 21 embryos transferred. The reason for this number is that one embryo recovered after NMR had been removed from the zona and the blastomeres were separated. Both of these demi-embryos were transferred to the recipient, and each was counted as an embryo. The first recipient gestated for 20 days following transfer while the second required 21 days. The litters contained 8/20 (40%) and 6/21 (28.6%) for a total of 14/41 (34.2%) (Figure I). At weaning, the weight and head to rump length of the NMR pups were compared to those of B6/SJL F1 pups of the same age from a breeding colony. The mean weight of the NMR pups, 12.2 g, did not differ ($p<0.05$) from that of the breeding colony pups, 13.1 g. The head to rump length of the NMR pups, 3.3 cm, also did not differ ($p<0.05$) from the control pups, 3.4 cm. The NMR pups show no outward signs of abnormality. Random matings of F1 offspring from NMR pups have resulted in litters of 9-16 pups in 5 of 6 matings.

Table I: Embryo developmental percentages at different stages for Experiment 1

Treatment	8 Cell	Time to 8-cell	Morula	Time to Morula	Blastocyst	Time to Blastocyst
Control	50/53, 94%[a]	1.38d [a]	48/53, 91%[a]	1.98d[a]	44/53, 83%[a]	3.02d[a]
NMR	37/44, 84%[a]	1.67d [b]	29/44, 66%[b]	1.97d[a]	24/44, 54.5%[b]	3.00d[a]
SHAM	29/40, 72.5%[a]	1.44d [a,b]	24/40, 60%[b]	1.83d[a]	19/40, 47.5%[b]	3.00d[a]
D_2O	19/40, 47.5%[b]	1.46d [a,b]	13/40, 32.5%[c]	2.46d[b]	11/40, 27.5%[b]	3.18d[b]

[abcd]Values within columns with different superscripts differ ($p<0.05$).

Table II: Embryo Development percentages and rates for Experiment 2.

Treatment	8-cell	Days to Eight-Cell	Morula	Days to Morula	Blastocyst	Days to Blast.
Control	15/15, 100%[a]	1.32d[a]	15/15, 100%[a]	1.92d[a]	14/15, 93.3%[a]	3.00d
PBS	15/15, 100%[a]	1.55d[b]	14/15, 93.3%[a]	2.12d[b]	12/15, 80%[b]	3.18d
D_2O	12/15, 80%[a]	1.67d[b]	10/15, 66.7%[b]	2.23d[b]	7/15, 46.7%[c]	3.32d

[abcd]Values within columns with different superscripts differ ($p<0.05$).

Table III: Embryo Development rates and percentages for Experiment 3

Treatment	8-cell	Days to Eight-Cell	Morula	Days to Morula	Blastocyst	Days to Blast.
Control	60/60, 100%a	1.32d	60/60, 100%a	1.93d[a]	49/60, 81.7%[a]	3.00[d]
Warm	59/60, 98.3%a	1.44d	40/60, 66.7%[a]	2.43d[b]	36/60, 60%[b]	3.00[d]
Room Temp	38/60, 63.3%b	1.46d	29/60, 48.3%c	2.3d[b]	19/60, 31.7%[c]	3.00[d]

[abcd]Values within columns with different superscripts differ ($p<0.05$).

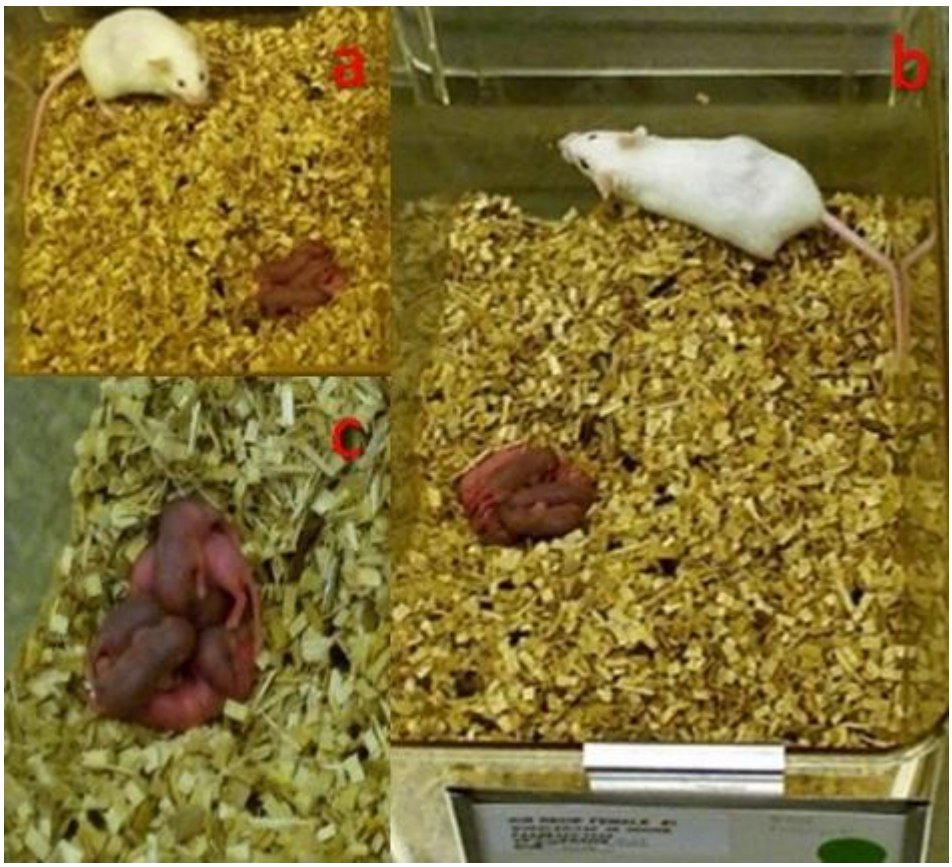

Figure I: Photographs of pups born following transfer of NMR analysis a) and b) the two recipient with their pups; c) magnification of one group of pups in this picture allows observation that there are no negative effects on development.

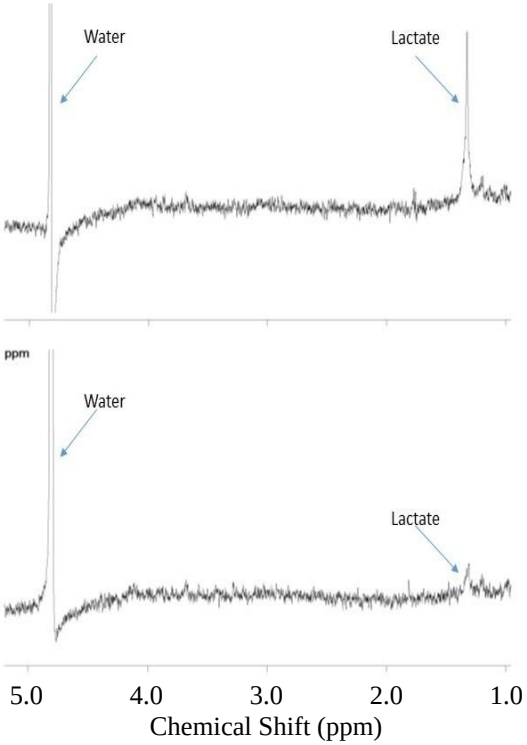

Figure II: Examples of two different run of ^{1}H-NMR spectrum.

IV. DISCUSSION

The aim of our study was to verify if it was possible to perform NMR spectrometry without altering embryo viability. To our knowledge, NMR analysis of preimplantation mouse embryos has not been previously demonstrated. In this study we examined the possibility of using nano-probe NMR analysis to identify embryo viability. The ability of embryos to develop to the blastocyst stage from two-cells following NMR is a promising result for the future use of NMR to detect markers of viability in embryos. The most surprising result in this experiment is the fact that the NMR and SHAM groups had higher development to the blastocyst stage than the D_2O group. There is a perceived additional stress on the NMR and SHAM groups, given that they are at a lower temperature than the D_2O group, required additional handling, and were subjected to a high magnetic field and high frequency rotation (120,000 rpm). One possible explanation for the increased ability of the NMR and SHAM groups to develop in culture after treatment versus the D_2O group is the lack of thermal control during the exposure to the D_2O based medium. The NMR and SHAM groups were not maintained at physiological temperatures. Rather, these groups were allowed to cool to ambient temperature during the experimental period of the NMR (approximately 18 – 22 ºC). This decrease in temperature from the incubator conditions (37 ºC) may be the factor that accounts for the difference in the blastocyst development. The decreased temperature favors destabilization of tubulin, and therefore may decrease the effects of the D_2O on the abnormal formation of mitotic spindles. Furthermore, decreasing temperatures impedes most biologically relevant enzymatic processes, and so any effects of D_2O on enzymatic events would also be decreased. Given this, the question remained whether the NMR itself was injurious to the embryos (apart from the occasional physical damage noted), or if it was only the medium, which was detrimental.

Experiment 2 was performed to try to examine the question of whether the embryos would be unaffected by NMR if the analysis was performed in a water based medium. This experiment showed that the water medium NMR did allow for increased development over the D_2O based medium, but control embryos still fared better in culture. This indicates that although D_2O is a significant factor causing poor performance in vitro, the NMR procedure itself is still slightly harmful to the embryos. The water-based medium, unfortunately, is impractical for NMR analysis, however, because the significant signal returned from the water blocks recognition of other molecules in the sample. The spectrum returned from the water-based group revealed only a large water peak and significant spinning artifacts. Water based media can be used in samples where the molecules of interest are of a significant concentration, but analysis of gametes in water media will require extremely sensitive tools.

Experiment 3 was designed to test the possibility that the lower temperature was indeed the reason for better development in the NMR and SHAM groups compared to the D_2O group. This experiment showed that the temperature did indeed affect the impact of the D_2O on the embryos. Cell temperature is under very tight homeostatic control. Cells at normal body temperature, ~36.9 ºC in the case of the mouse (http://www.informatics.jax.org), undergo normal metabolic processes including respiration, cell division and differentiation. It is known that the exposure to D_20 reduces the oxidation of glucose in mammalian cells. This phenomena has been demonstrated in both mouse and rat cells [23]. However, the inhibitory effects of D_20 may be reduced by the cooler temperature conditions of the NMR and SHAM groups. Further, it has been shown that two different small sets of proteins can be induced or down-regulated, respectively, when cells are exposed to cooling [24]. When the cells are cooled their metabolism decreases and therefore, the amount of D_20 taken up by the cells may be decreased leading to less of a deleterious effect of D_20 than is seen at 37 ºC. Taken together these observations provide a plausible explanation for the reduced deleterious effects of D_20 on embryo development in the NMR and SHAM groups. In addition, the manner in which temperature altered the effects of exposure to D_2O may involve the action of tubulin. The decrease in temperature should cause a trend towards depolymerization of microtubules. This may be favorable during exposure to D_2O, since one cause of the mitotic block is an abnormal lengthening of the mitotic spindle in sea urchins (Takahashi and Sato, 1983). Since the effects of D_2O on the kinetics of mitosis in the sea urchin is similar to those in mammalian cells, the depolymerization of tubulin at lower temperatures is likely to play an important role.

It is interesting that while there is a difference in the timing of development of embryos between the control group and the other experimental groups, those slower developing groups are still in line with the developmental

parameters given by Hogan et al. [25]. In addition when we evaluated the presence of abnormality in embryo, fetal and pup development we did not find any differences between the control group and the NMR group, this result is in agreement with Murakami et al., that evaluated the effect of static magnetic field on fetal development in mice and that experiment, no anomalies were found, as well [26]. Our preliminary NMR spectra (Figure II) show that a lactate signal can be detected in the embryos. We would expect to measure a lactate signal, since lactate has many important roles in the embryo. The early embryo relies on lactate as one its primary energy sources, high lactate production is seen at all stages of development, and lactate plays a role in regulating the internal pH of the embryo [16, 27]. Since no lactate was present in the NMR analysis fluid (D_20-PBS), the measured signal must be from lactate stores or production within the embryos. The difference in the height of the lactate signal between the cultured and fresh embryos suggests that the lactate signal may be an indicator of embryo health. In fact, the measurement of lactate production has been used as an indicator of embryo viability [28]. Further studies are needed to determine whether changes in the NMR lactate signal will correlate with embryo viability.

V. CONCLUSIONS

Based on this study, NMR analysis does subject embryos to conditions that adversely affect development to the blastocyst stage in vitro. The effects of the current method, however, are primarily due to the analysis medium and not the NMR procedure itself. Alteration of the analysis medium may allow for better developmental rates. However, these alterations do not compromise the ability to obtain useable spectra from the analysis. It may also be possible to further decrease the temperature of the NMR chamber to attenuate the effects of the D_2O. Embryos can develop fully into offspring following NMR on the embryos, indicating that the i*n vitro* development of the embryos is representative of a capacity to develop fully. New technological advances, such as micro coil NMR, have higher signal to noise ratios and offer a better possibility of single embryo analysis [29]. In summary, the use of NMR to analyze the chemical composition of embryos in vitro is plausible, provided some important modifications can be made to decrease the detrimental effects of the analysis.

VI. DECLARATION OF INTERESTS

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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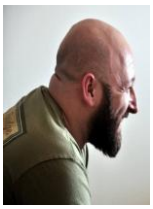

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