of choice for protecting against slow freezing injury in the rat embryo (13,14). Neither of these investigators reported any detrimental effect of Me_2SO on viability post-thaw.

Both addition and dilution of Me_2SO was carried out at room temperature. Many investigators add the cryoprotectant at 0°C (13,29,70) and few at room temperature (14). Schnieder suggests that exposure to the cryoprotectant is more efficient at room temperature since the permeation of a solute into the blastomeres is dependent not only on the permeability coefficient of the cell membrane but also on the temperature coefficient of that permeability (78). Addition at 0°C may not allow sufficient permeation to afford protection during slow cooling.

Both in vitro and in vivo tests of viability were used to assess the effects of freezing and thawing on the BB rat embryo. The Blastocyst stage survived best whether embryos were frozen slowly to -40°C or -75°C before rapid cooling to -196°C in all assays of viability.

Fluorescein diacetate provides a rapid initial method of assessing viability. Using this assay the viability of both 8-cell and blastocyst embryos was significantly reduced after cryopreservation. There was however, no difference between the freezing protocols as measured by this assay. Blastocysts survived slow cooling to -40°C significantly better than did 8-cell embryos.

In vitro culture is a more stringent test of viability. Development in culture was only considered positive if 8-cell embryos developed into morula or blastocysts, and blastocysts or morula expanded within 24 hours. In some experiments blastocysts which had developed in

vitro from 8-cell were held in culture for an additional 18 to 24 hours to observe expansion and occasionally, loss of the zona pellucida.

Fresh control embryos developed significantly better than did any of the other experimental groups. The addition and dilution of Me_2SO had a marked effect on survival of BB rat 8-cell embryos. There was no difference between the two freezing protocols for the 8-cell embryos as measured by In vitro embryo culture.

Survival of 8-cell BB rat embryos was significantly reduced (31%), regardless of the freeze thaw protocol. This may be attributed to the apparent adverse effects of 2 Me_2SO, as fresh 8-cell embryos did not develop well in culture after exposure to this concentration of Me_2SO. Whittingham reported 61% of 8-cell rat embryos developed into blastocysts after slow cooling to -80°C and slow thawing (13).

Blastocysts survived both freeze-thaw protocols significantly better than 8-cell embryos. When frozen slowly to -40°C prior to transfer to -196°C, and subsequent rapid thawing, blastocysts developed as well as (74%) either control groups in culture. Survival of blastocysts frozen slowly to -75°C was lower than those exposed to Me_2SO alone and then assessed in culture.

In vivo transfer of the BB rat embryo after freezing and thawing gave very low survival rates regardless of the protocol followed. Again, the best results (13.5%) were obtained when blastocysts were cooled slowly to -40°C prior to transfer to -196°C and then transferred to pseudopregnant recipients after rapid warming. The large number of embryos included in this group may have biased these results. No blastocysts frozen slowly to -75°C prior to rapid freezing to -196°C and

subsequent slow thawing survived after embryo transfer, however, only a few embryos were included in this group.

The low success of embryo transfer after cryopreservation was attributed to the lack of success of the transfer technique and not the freeze-thaw protocols as very poor results were obtained in control transfers as well.

One other study considered the same two freeze-thaw protocols in 1980 (14). These investigators used 1.5 M Me_2SO as the cryoprotective additive and a faster warming rate for embryos cooled slowly to -40°C. Wood and Whittingham only looked at in vivo transfer as a measure of viability. Their results were slightly better when slow cooling was stopped at -80°C (21%). They also considered different slow cooling rates but made no conclusive remarks. These investigators only considered the rat blastocyst stage for cryopreservation.

Viability as measured by in vitro assays showed no difference between the two freezing protocols employed in this study. Since slow cooling to -40°C prior to transfer to liquid nitrogen for storage and subsequent rapid thawing is less time consuming, this protocol may be the better of the two for the BB rat embryo. The blastocyst is the more stable stage of development for cryopreservation in the BB rat as measured by these in vitro assays.

BB rat embryos were stored in liquid nitrogen for up to 4 years. Blastocysts stored at -196°C for 4 years survived well on thawing (71.43%) as measured by in vitro culture. Mouse embryos have been stored for up to 11 years without detrimental effects (12). The only biological reaction which takes place at -196°C, background radiation, is not a significant problem (85). There are however, a few practical

problems which could affect the viability of the stored embryos. Low levels of liquid nitrogen or total failure of a storage container are realistic possibilities (12), and there is the remote possibility of cross contamination between materials in the liquid nitrogen tank especially if vials are not sealed properly (11). In mouse embryo cryobanking, mutant strains tend to survive less well than do hybrid strains (12). The health status of the thawed embryos will depend on that of the foster mother and the colony where they will be introduced (11).

A very recent technique, vitrification, has been developed as a new method of cryopreserving mouse embryos (110). Vitrification involves exposure of the embryos to very high concentrations of a mixture of cryoprotectants. The vitrifying solution (VSI), contains Me_2SO, acetamide and propylene glycol. Toxicity of these cryoprotectants is reduced by decreasing the time and temperature of exposure to the embryos. Mouse embryos exposed to 87.5% VSI survive very well (94.7%) when they are held for 20 min at 4°C in this solution. The embryos dehydrate to the degree that no ice forms when they are subsequently cooled at very rapid rates (200°C/min) to -196°C. They also survive rapid dilution of the cryoprotectants after rapid thawing (300°C/min). When slow cooling of mouse embryos are observed under the cryomicroscope no intracellular freezing occurs if slow cooling is stopped at -40°C and the embryos are cooled rapidly to -196°C (18). The cytoplasm is thought to form a glassy state or vitrify in this procedure as well.

Viability Assays

A. Fluorescein Diacetate Assay

The use of fluorescein diacetate as an initial test of viability of frozen thawed embryos has not been widely used. In this study, embryos appearing morphologically normal on thawing always fluoresced. This test did not reveal the loss of viability of 8-cell embryos exposed to the cryoprotectant Me_2SO. Although a high percentage of 8-cell BB rat embryos fluoresced (65%), the ability of 8-cell BB rat embryos to develop in culture after exposure to Me_2SO and freezing was significantly reduced (31%). The ability of blastocysts to fluoresce and develop in culture was similar in all protocols considered.

Since this test is only a measure of the integrity of the plasma membrane and eterase enzyme activity (92), it is possible that 8-cell embryos lose their viability after exposure to Me_2SO or post-thaw as time progresses or that other cell mechanisms are damaged beyond repair during the freezing or thawing process. Incubation of the embryos with FDA after *in vitro* manipulation or cryopreservation may give a better indication of viability.

Mohr and Trounsen (92) studied the effect of FDA and UV light on 2-cell mouse embryos. Neither of these factors affected the ability of the mouse embryo to develop in culture or to develop *in vivo* after transfer to a foster mother. This technique could be used as an initial nonintrusive measure of viability after freezing and thawing before subsequent transfer to a pseudopregnant recipient.

B. Embryo Culture

In vitro culture of mammalian embryos is an established test of viability. Most studies employ Brinsters technique (15) of culturing embryos in microdroplets of medium under paraffin oil (13,14,29). A few studies in the 1950's cultured mouse embryos in serological tubes in a small amount of medium (26,41,42). Both techniques were attempted in this study as initial results with Brinsters method gave very low survival rates for control groups. The test tube or batch method was very successful and technically much easier. Fresh 8-cell and blastocyst embryos developed significantly better with the batch method of culture. Brinster suggests that the main disadvantage of the batch method is, loss of embryos on recovery from the test tubes (111). This was never a problem in this study. The culture chamber atmosphere was saturated with water vapor at 95% humidity and was precisely controlled. There was no problem with evaporation and subsequent increased osmotic pressure of the medium which would be fatal to the embryos (24).

The main advantages of the microdrop technique are that small amounts of medium are used and embryos can be quickly identified in the same culture dish. The oil also prevents evaporation (15). There are a few reasons why the microdrop culture method may have failed. The quality of paraffin oil is important. Some paraffin oils contain an impurity which results in the formation of a thin, viscous film between the culture medium and the mineral oil and development of embryos is retarded when this film is present (37). Some components of the culture medium, such as pyruvate, are soluble in mineral oil. These components

will equilibrate with the oil and this may interfere with development of the embryos (37).

The survival of fresh 8-cell embryos using the batch method of culture was similar (72.5%) to those reported for 8-cell rat embryos cultured under oil (79.0%) by Mayer and Fritz (27). These investigators used Biggers, Whitten and Whittingham medium substituting BSA with FCS. Folstad, Bennet and Dorfman reported a higher percentage (90%) of 8-cell rat embryos developing to morula or blastocysts after 24 hours in culture using Brinsters medium (BMOC-3) (28). Wood and Whittingham tested various culture media using 8-cell rat embryos and obtained best results (44%) with medium based on Tyrodes solution (14). Better results were achieved using Brinsters medium (BMOC-3) in this study than in Wood and Whittingham's study.

In vitro culture of the BB rat blastocyst was a good measure of viability. Seventy-four per cent of blastocysts cooled slowly to -40°C and 59% cooled slowly to -75°C before transfer to -196°C expanded in culture in 24 hours on thawing. Hatching from the zona pellucida was observed in 33% of rat blastocysts cultured from the morula or blastocyst stage in 20 to 25 hours. Hatching occurred in control and experimental groups. Mayer and Fritz suggest that the substitution of FCS for BSA may initiate hatching (27), however, the medium used in this study only contained BSA. Wood and Whittingham did not observe hatching from the zona in their study (14).

The effect of hydrogen ion concentration on embryo development has not been extensively studied. Brinster points out that when considering the effect of pH, the concentration of energy source and CO_2 content of the medium are also taken into account (24). The availability of energy

sources such as pyruvate and lactate to the developing embryo is related to the pH of the medium. The lower the pH, the lower the concentration of substrate necessary for optimal development. The CO_2 concentration also directly affects the pH of the medium since a bicarbonate buffer is necessary to support mammalian embryo development.

Although an in depth study of the effects of pH on BB rat embryo development was not undertaken in this study, several observations were made. The concentration of CO_2 (5%) was electronically controlled by the culture chamber. Even with this control, a constant monitoring of the pH of the culture medium was necessary. A fyrite gas analyzer was also used as a second control in maintaining an optimum pH range for development of the BB rat embryo in vitro.

The BB rat embryo is very sensitive to changes in pH. Fresh 8-cell embryos developed best at a pH of 7.27 to 7.32. A more basic pH was detrimental to development of the 8-cell embryo. Blastocysts developed well at a pH of 7.36 where 8-cell embryos did not. This factor was the most critical in determining the success of in vitro cultivation of the BB rat embryo. For viability assays, only embryos cultured at a pH ranging from 7.27 to 7.32 were considered in the final data.

In Vivo Embryo Transfer

In vivo transfer of frozen-thawed embryos is the ultimate test of survival. Transfer of rat embryos has not been as successful as for the mouse embryo. Many factors play equally important roles in determining the success of this technique. The induction of pseudopregnancy, normality of donor embryos, synchrony between the developmental stage of

donor embryos and stage of pregnancy of the recipient uterus and the technical aspects of performing a successful transfer must all be perfect for success (32-34).

In this study, survival of the BB rat embryo in vivo was very low. Only 4% of fresh 8-cell and 7% of fresh blastocysts developed into normal fetuses. For these reasons this test for viability on frozen-thawed BB rat embryos did not depict the effects of the different cryopreservation protocols but rather the effects of the embryo transfer technique itself.

The BB rat 8-cell and blastocyst embryo can be successfully cooled slowly to -40°C and then stored at -196°C before rapid warming, as 2 out of 80, 8-cell embryos and 26 of 193 blastocysts did develop into live fetuses. Twelve rats were born live and developed normally after being frozen for 15 days to 4 months. Photographic plate 4 shows a frozen-thawed BB rat blastocyst. Photographic plate 5 shows 21-day old BB rats previously frozen for 2 weeks. Only two papers report the successful transfer of rat embryos after cryopreservation. Whittingham transferred 8-cell rat embryos after slow cooling to -80°C and subsequent storage in liquid nitrogen, and slow warming. Nine per cent of these developed in vivo after slow thawing (13). Wood and Whittingham studied two different freeze-thaw protocols and reported slightly better survival of rat blastocysts transferred to recipients on thawing using the same freeze-thaw protocol as Whittingham (14). Twenty-one per cent of the blastocysts frozen with this protocol developed in vivo where only 14 % of blastocysts cooled slowly to -40°C before storage in liquid nitrogen developed in vivo when thawed rapidly.

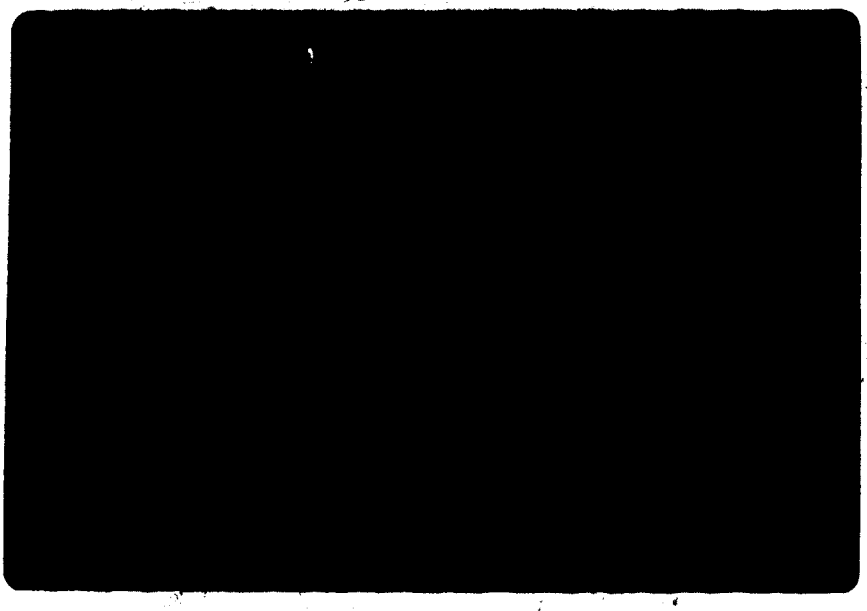

Plate 4: BB rat blastocyst after freezing and thawing.

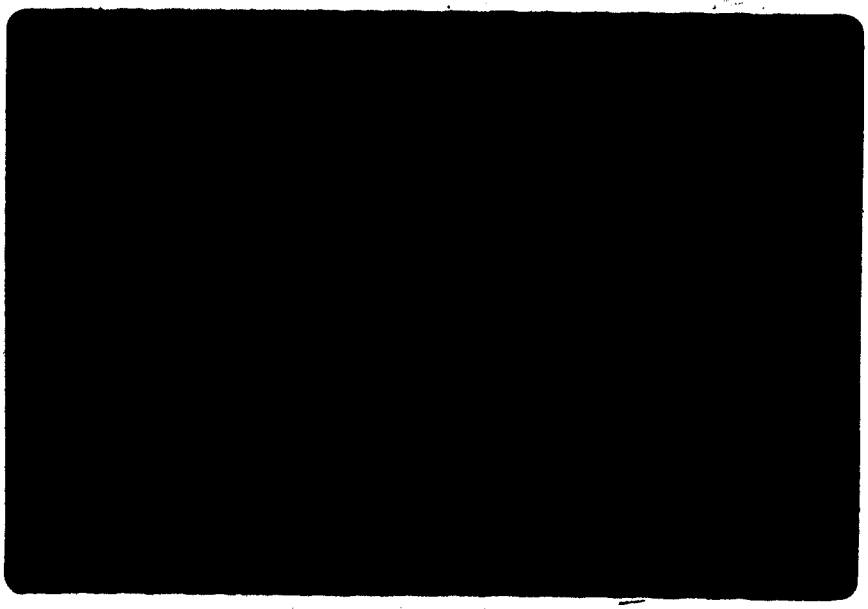

Plate 5: 21-day old BB rats previously stored in liquid nitrogen at the blastocyst stage for 2 weeks.

An interesting observation in their study was that more embryos warmed rapidly appeared morphologically normal (58%) than embryos warmed slowly (49%) (14). In the present study, a much larger number (75-80%) of embryos appeared normal on thawing.

There are several possible reasons for such low success in this technique. The Sprague-Dawley female recipients may not have been in a state of pseudopregnancy. These females were mated with vasectomized hooded males when vaginal smears showed proestrus. Repeat vaginal smears were done on 2 days following mating to note the presence of a continuous state of diestrus which indicates pregnancy or pseudopregnancy (59). In some cases, smears were checked again on day 4, as cycling females would again be in proestrus. If females showed any other stage of the estrus cycle they were not used as recipients. Many of these rejected females did not begin cycling regularly for up to 3 weeks. Since rodent require physical stimulation to induce pseudopregnancy (31,32), the physical trauma incurred during the embryo transfer may upset this state of pseudopregnancy. One way to determine whether the recipient is still in the state of pseudopregnancy would be to monitor serum progesterone levels before and after the transfer procedure. The cycling of this hormone would not continue if pseudopregnancy persisted.

In all transfer experiments, control or experimental, only morphologically normal BB rat embryos were transferred. On thawing abnormal embryos were included in the data, but not transferred.

Synchrony of the state of pseudopregnancy and the developmental age of the embryo was varied in control and experimental groups. Eight-cell embryos were transferred to day 3 and 4 pseudopregnant females. Dickman

and Noyes suggest transfers on day 3 of pseudopregnancy are never successful (34). In this study, although all survival rates were low, asynchronous transfers appeared to be better for 8-cell embryos (4 live fetuses and 2 implant sites) than synchronous transfers (1 live fetus). Blastocysts were transferred on day 4 or 5 of pseudopregnancy and survived equally as well. Transfers done late on day 5 are usually less productive (34), therefore, day 5 transfers were always performed before 2 pm in this study.

Asynchronous embryo transfers may be advantageous if embryos have suffered developmental delay because of in vitro manipulations such as cryopreservation (31). Freezing and thawing may delay resumption of normal metabolic processes. Frozen-thawed rabbit embryos survive better when transfers are asynchronous (112). Some investigators believe that post-thaw culture allows cryopreserved embryos time for recovery prior to transfer (12). This technique was tried in this study with a small group of embryos (data not shown). The effect of culture on the survival of frozen thawed rat embryos and subsequent transfer was also considered in this study. Fresh 8-cell embryos were cultured for 24 hours and the resulting blastocysts frozen slowly to -40°C, stored in liquid nitrogen and rapidly warmed before transfer. Four out of 7 of these embryos developed into live fetuses. Allowing blastocysts to recover from the freeze-thaw procedure by culturing for 18 hours gave poor results. No live fetuses resulted after synchronous transfer, but degenerating implant sites were present on day 14 post-transfer. Resumption of development may begin in culture after thawing and may result in a less desirable candidate for implantation. Late mouse

blastocysts do not survive embryo transfer as well as early blastocysts (34).

A major cause of failure may have been the actual transfer technique. Experience is definitely important and for this reason some of the early attempts at transfer may have failed because of lack of experience. Trauma to the uterus is unacceptable and usually causes loss of the entire transfer (32). Injection of air may also have been a cause of failure (113). In this study, embryos were transferred in a minimal amount of medium trapped in the very tip of a drawn siliconized, sterile pipet between two tiny air bubbles. Without these air bubbles there was less control over the amount of medium entering the uterus. Mouse embryos survive best when minimal amounts of medium are transferred with the embryos (58).

After transfer of the embryos the pipet was held in place for 5 to 10 seconds to allow the embryos to settle. This was done to reduce the possibility of the embryos being drawn back through the puncture hole immediately after removal of the pipet.

Lack of sterility may also have been a problem in this study. Precautions were taken to ensure sterility, such as, sterilization of all media, glassware and surgical instruments used, however, the early luteal phase uterus is particularly susceptible to infection and in the rabbit this is the principle cause of failure (32).

It is obvious that further investigation into the area of <u>in vivo</u> transfer of the rat embryo is necessary. There are very few studies on the effects of <u>in vitro</u> manipulation followed by embryo transfer in the rat. Since the establishment of an embryo cryobank for the BB rat

depends on the success of *in vivo* transfer, further research must be focused on this area.

CONCLUSIONS AND RECOMMENDATIONS

Conclusions

(1) Diabetic BB rat embryos can be successfully frozen and thawed. Normal young develop after transfer of thawed 8-cell embryos and blastocysts into pseudopregnant recipient females.

(2) The ability of the 8-cell BB rat embryo to develop in culture was significantly reduced when exposed to 2 M Me_2SO, the cryoprotective agent used in this study.

(3) There was no significant difference in survival as measured by *in vitro* assays, of the BB rat 8-cell or blastocyst stage when slow cooling was stopped at either -40°C or -75°C before rapid cooling to -196°C and subsequent rapid or slow warming respectively. Since slow cooling to -40°C and rapid thawing is less time consuming and is equally as effective in cryopreserving BB rat embryos, the author feels this is the better freeze-thaw protocol.

(4) The BB rat blastocyst survived both freezing the thawing protocols significantly better than the 8-cell stage when tested with either *in vitro* assays or *in vivo* embryo transfer. The blastocyst appears to be the better preimplantation

developmental stage for *in vitro* manipulation, cryopreservation and embryo transfer in the BB rat.

(5) Superovulation with Pregnant Mare Serum Gonadotrophin followed by Human Chorionic Gonadotrophin is not a reliable method for obtaining large numbers of normal embryos in the BB rat.

(6) The ability of BB rat blastocysts to fluoresce in the presence of the dye, fluorescein diacetate, was a good indication of viability and agreed well with *in vitro* culture results.

(7) The *in vitro* culture technique, microdrops under oil, used by most investigators for mammalian embryo culture gave very poor results in this study. Embryos cultured in serological tubes (batch culture), in a small amount of Brinsters' medium (BMOC-3), survived significantly better than those cultured using the conventional method.

(8) Eight-cell-BB rat embryos appeared more sensitive to changes in pH than the blastocyst stage. The optimal range of pH for the successful culture of these stages of the BB rat embryo was 7.27 to 7.32.

(9) *In vivo* transfer of the BB rat embryo gave very low survival rates in all groups, control and experimental, in this study. The blastocysts frozen slowly to -40°C, stored in liquid nitrogen and thawed rapidly survived significantly better

after embryo transfer than any other protocol. Despite several changes to the original method of embryo transfer, survival remained low. It was concluded that the low survival of BB rat embryos transferred to recipient females was not a failure of the freeze-thaw technique but of the method of in vivo transfer itself.

Recommendations

(1) Development of BB rat 8-cell embryos in culture is markedly reduced after exposure to 2M Me_2SO. This concentration may be toxic to the 8-cell BB rat embryo or the method of addition and/or dilution used in this study may be detrimental to the viability of this stage of development. Rat embryos survive exposure to 1.5 M Me_2SO (13,14). Further investigation into the effects of varying concentrations of Me_2SO and method and temperature of addition and dilution on the survival of the 8-cell BB rat embryo is necessary to alleviate this problem. Both glycerol and ethylene glycol have been used successfully as the cryoprotective additive for the mouse embryo (74), and ethylene glycol for the rat (116). Perhaps the 8-cell stage will tolerate the presence of a cryoprotective solute such as glycerol or ethylene glycol better than Me_2SO.

(2) The FDA assay provided a rapid non-intrusive means of assessing initial viability of frozen thawed BB rat blastocysts. This assay is less time consuming than the *in vitro* culture test and has no detrimental effect on further development (92). FDA may be a more efficient means of initial *in vitro* assessment prior to the use of further treatments such as *in vivo* embryo transfer.

(3) The method of batch culture for the BB rat embryo gave significantly better results than the conventional method for

cultivating mammalian embryos in microdrops under oil. The author found this technique to be less time consuming and recovery of cultured embryos from the serological tubes was never a problem. This technique may provide more satisfactory results for rat embryo culture.

(4) In vivo embryo transfer of the BB rat embryo gave very low survival rates. The rat embryo does not survive as well as the mouse in embryo transfer experiments. There is a need for further investigation into the method of inducing pseudopregnancy in recipient females. The fertility of the recipient varies from strain to strain and the most suitable strain for a particular experiment needs to be assessed to improve the success of this technique. Uniparous recipients or genetic marker recipients that are already pregnant may provide more suitable recipients for rat embryo transfers. The technique of embryo transfer may be at fault. It is possible that the state of psuedopregnancy is disturbed in some way during the surgical transfer itself. Different transfer techniques may improve results.

(5) Since the embryo transfer technique has been adopted for the rat from methods developed in the mouse model, it may be necessary to attempt this technique using the mouse model before using the rat model. This would ensure that failure of the proceedure is not due to the actual transfer technique

itself. Once established in the mouse model, the method could then be carried over into the rat model.

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