CULTURE OF CELLS FROM MAMMALIAN TISSUE CRYOPRESERVED WITHOUT CRYOPROTECTION

A Thesis

by

LARA NICOLE CHARLES

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2008

Major Subject: Physiology of Reproduction

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Approved by:

Chair of Committee, Duane C. Kraemer
Committee Members, Paul Harms
Charles Long
Head of Department, Gary Acuff

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ABSTRACT

Culture of Cells from Mammalian Tissue Cryopreserved Without Cryoprotection.

(May 2008)

Lara Nicole Charles, B.S., Texas A&M University

Chair of Advisory Committee: Dr. Duane C. Kraemer

Donor cells for nuclear transfer are usually prepared by the culture of fresh tissue. However, animal carcasses are sometimes frozen without cryoprotectants and if it were possible to obtain live cells from carcasses (tissue) preserved in this manner, it could be very beneficial in nuclear transfer cloning of trophy or extinct animals.

This study tested the hypothesis that tissue samples of skin, muscle, and oral mucosa could be cryopreserved without cryoprotection. The tissue samples were taken from euthanized goats and placed into a -20°C freezer for varying lengths of time. The samples were thawed by two different methods. One method was in 37°C water bath and the other was on ice, thawing to room temperature from 1°C to 25°C. The samples were then processed and placed into an incubator to evaluate cell growth.

Skin samples frozen for up to 34 days obtained cell growth to confluency and the cells were then cryopreserved with cryoprotectant. The cells were able to tolerate the potentially lethal effects of ice nucleation and dehydration brought about by ice formation and colligative factors.

Although this method of cryopreservation has been shown to yield growth of cells that might be useful for nuclear transfer cloning, it is not the recommended method to cryopreserve tissues if cryoprotectants are available or if only short term storage is needed. These procedures would be especially useful when a precious animal dies unexpectedly and cryoprotectant is not available and the sample can not be processed before 10 days.

DEDICATION

This is dedicated to my family, who has been my backbone for support. To my parents, who have never told me I could not do something, and who have always told me they love me and support me. I can always count on ya'll for anything, whether you can help or not. Mom and Dad, thank you for the support, guidance, encouragement, reassurance, and everything else you have given me without asking. You have always put my needs above your own. Thank you for all of the financial support over the last nine years, without you, I would not be where I am today. I truly appreciate what you do for me.

To my sisters Celia and LaDonna, thank you for being there for me when I needed to talk or whine or complain. To my niece, Amanda, and nephew, Justin, you have always given me the strength to strive for greatness. I love you very much. To Sabrina, for wanting to be just like me and become an AGGIE! To my grandparents, Poo and Granddaddy, your love and support have driven me through tough times. I would also like to thank God, for walking with me every day of my life.

Lastly, thank you to my fiancé, Shane, for putting up with me during my times of stressing out. I am not sure anyone else would have stayed by my side through all of the tough times we have had. At the end of the day, you are still there to hold me and tell me you love me.

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I would like to thank Dr. Paul Harms for supporting me through this project and throughout my master's career. I am always able to approach you and ask questions or for help. I would like to thank Dr. Charles Long for pushing me to become better. Your support and help has been much appreciated. I would also like to thank my fellow graduate students for being there when I needed to vent. Thanks to Christy Bormann, for showing me how to start my project in culture. Last, but certainly not least, I would like to thank Dr. Duane Kraemer for being so patient with me during the writing process. Without you, this would not have been possible. You have helped me learn how to become a better writer and think outside the box.

NOMENCLATURE

BPE	Bovine Pituitary Extract
DMEM	Dulbecco's Modified Eagle's Medium
Me_2SO	Dimethyl Sulfoxide
EGP	Epidermal Growth Factor
FBS	Fetal Bovine Serum
PBS	Phosphate Buffered Saline
mL	Milliliters

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CHAPTER I

INTRODUCTION

Donor cells for nuclear transfer are usually prepared by the culture of fresh tissue. However, animal carcasses are sometimes frozen without cryoprotectants and if it were possible to obtain live cells from carcasses (tissue) preserved in this manner, it could be very beneficial in nuclear transfer cloning of trophy or extinct animals.

This study has determined a range of days in which a tissue can be placed in a regular freezer without any cryoprotective measures and still grow a culture of cells for cloning. In a pilot study to determine if this option is possible, bovine skin and muscle were collected and frozen for ten days in a regular -20°C freezer. The samples were thawed, washed, and cultured. After about a month there were cells growing from the skin sample, but they had a very short life span. This proposed research carried the pilot study farther and explored the duration of freezing and the effects of different thawing methods. After cell growth had occurred the cells were kept and frozen with cryoprotectants for possible future studies including: using these cells for nuclear transfer cloning.

The primary experiment used goat carcasses. The experimental endpoint was whether or not there was cell growth in the tissue cultures. Statistical comparisons were made between the percentage of frozen vs. control samples which exhibited cell growth in culture.

This thesis follows the style of Cryobiology.

The methods developed in this project have the potential of: decreasing the total cost of tissue storage by not using cryoprotectants; reducing the amount of time spent freezing, thawing, and cleaning the cells at the time of initial storage; harvesting tissue in the field that could still be viable without the need for cryoprotectants; saving genetics from a frozen dead animal randomly found in the field, and pets whose carcasses have been frozen instead of being stored at non-frozen temperatures before processing. These methods might aid in the preservation of wildlife and endangered species and, although unlikely, could help bring back extinct species that have been frozen in glaciers for centuries.

The hypothesis is that some cells in mammalian tissue survive cryopreservation without cryoprotection.

The objectives were:

1. To determine the effects of freezing duration on the growth of cells from goat tissue frozen without cryoprotectants.
2. To determine the effect of thawing rate on growth of tissue frozen without cryoprotectants.

CHAPTER II

LITERATURE REVIEW

Since the discovery of the benefits of cryoprotectants, there has been only limited research on cells cryopreserved without cryoprotectants. Jinsong Li and Peter Mombaerts [5] from The Rockefeller University have used nuclear transfer and embryonic stem cell techniques on mouse cells that were frozen without using cryoprotectants. Frozen keratinocyte stem cells and cumulus cells were kept in medium without cryoprotectants at -80°C for one day to one year. The frozen keratinocyte and cumulus cells were used to produce blastocysts by nuclear transfer and these blastocysts were used to produce thirteen embryonic stem cells lines. After chromosome analysis, these lines showed to have normal karyotypes. Eight of these cell lines proved to be pluripotent when injected into tetraploid blastocysts and four clonal mice resulted from nuclear transfer of cells from one of the cell lines. The chromosome stability and genetic integrity seemed to be sustained in mouse cells when frozen without the use of cryoprotectants. The authors did not indicate how long the specific cells that resulted in the embryos had been stored.

Narumi Oganuki et. al.[8] from Japan froze mouse carcasses in the -20°C freezer without automatic defrost for fifteen years and were able to produce live, normal offspring by using microinsemination of spermatozoa recovered from the frozen testis. This group also froze epididymides and testes from one week to one year using four different freezing methods. These four methods include plunging the freezer tube

directly into liquid nitrogen, placing tubes directly into the -80°C freezer, placing freezer tubes into a freezing container and then into the -80°C freezer, and finally placing tubes into a freezing container in a -80°C freezer and one day later placing them into liquid nitrogen. All four of these methods were performed without the use of cryoprotectants. The three types of cells used for microinsemination were testicular sperm, elongated spermatids, and round spermatids. The whole carcasses were thawed in a water bath at 25°C for five minutes and the testes and epididymides were thawed in a water bath at 25°C for approximately two minutes. The thawed spermatozoa and spermatids were non-motile and stained positive with propidium iodide indicating significant damage and death of the cells. However, the spermatozoa appeared to maintain their genetic integrity through freezing and thawing because they were able to produce live offspring by microinsemination. Within 24 hours after sperm injection, >80% of ova developed into two-cell embryos.

According to Lovelock's hypothesis, freezing damage is caused by an increased concentration of electrolytes. Slight protection from damage can be attributed to the use of serum in the freezing medium [11]. During freezing, cells undergo changes in volume which can be lethal if beyond their osmotic tolerance limits [4]. Alterations in temperature, water content, state of water and solute concentration affect the intra and extracellular ice crystal formation [6]. Cryoinjuries are induced by osmotic shock resulting from excessive cryodehydration, intracellular ice, and fracturing of the frozen medium upon thawing. These types of damage appear to be non-repairable and lead to cell death [2]. Ice initially nucleates in the extracellular space and causes water to leave

the cell. If the cooling rate is too fast not enough water is lost, resulting in intracellular ice formation and in cell death because of membrane damage. If the cooling rate is too slow the cells may shrink excessively and may be exposed to high solute concentrations for too long [7]. The addition of cryoprotectants modifies water permeability, lowering the hydraulic conductivity and thus limiting volumetric changes and osmotic stress [9].

During the freezing process cells undergo many changes, including: volume, osmotic pressure, pH, and changes in morphology. Some of these changes are due to the type of cryoprotectant. It has also been shown that fast cooling rates cause more damage than slower cooling rates. This was confirmed by intracellular ice "flashing" visualized by cryomicroscopy. The slower cooling rates allow time for cryodehydration to progress to a point where the cytosol is vitrified [2].

In addition to preserving cells for cloning, Freshney [3] indicates that cryopreservation can also be useful for managing:

- Genotypic drift due to genetic instability
- Senescence and the resultant extinction of the cell line
- Transformation of growth characteristics and acquisition of malignancy-associated properties
- Phenotypic instability due to selection and dedifferentiation
- Contamination by microorganisms
- Cross-contamination by other cell lines
- Misidentification due to careless handling
- Incubator failure

- Saving time and materials maintaining lines not in immediate use
- Need for distribution to others

Factors such as cell type, rate of cooling, osmotic pressure, change in pH, changes in volume, and changes in morphology influence cell survival during cryopreservation. Therefore it seems logical to hypothesize that in carcasses or tissue blocks, these factors would vary considerably throughout the sample, and that a few of the cells may survive. There are no reports of studies exploring this possibility for somatic cells.

CHAPTER III

MATERIALS AND METHODS

Experimental Design

The experimental design is illustrated in Table 1. One fresh sample of goat tissue per organ was collected as a control. This showed that the tissue is able to grow under normal culture conditions. The other samples were collected and frozen in a -20°C freezer without the use of cryoprotectants. Two samples were removed from the freezer and thawed simultaneously. One sample was thawed in a 37°C water bath and the other in ice water warming to room temperature from 1°C to 25°C. This showed if fast or slow thawing made a difference in the tissue viability.

The data that was recorded and included the date the tissue was collected, how many days the sample was frozen, how many days the sample was in culture before there was cell growth, dates cells were passaged, and cell count when frozen.

Table 1: Experimental Design

Treatment Thaw Method	Goat Skin			
Days Frozen	0	1-10	11-20	21-34
Ice Water (1°C-25°C)		15	15	10
Water Bath (37°C)		15	15	10
Non-Frozen	10			
Total	10	30	30	20

N = Number of Samples in each treatment group

Treatment Thaw Method	Goat Muscle			
Days Frozen	0	1-10	11-20	21-34
Ice Water (1°C-25°C)		15	15	10
Water Bath (37°C)		15	15	10
Non-frozen	10			
Total	10	30	30	20

N = Number of Samples in each treatment group

Treatment Thaw Method	Goat Oral Mucosa			
Days Frozen	0	1-10	11-20	21-34
Ice Water (1°C-25°C)		10	2	0
Water Bath (37°C)		10	2	0
Non-frozen	5			
Total	5	20	4	0

N = Number of Samples in each treatment group

Tissue Preparation

From each animal, samples of muscle, oral mucosa, and skin were collected post mortem. The skin and muscle samples were approximately equal in size. One small sample of each tissue type was placed into DMEM/F-12 medium in a 50mL conical tube while being held for culture. The other samples were placed on crushed ice and later placed into the freezer at -20°C.

All of the following procedures were performed under the hood using sterile technique.

Media Preparation

The medium used was DMEM/F-12 powder mix with 10% fetal bovine serum (FBS) and 0.1% gentamicin. Phosphate Buffered Saline without calcium and magnesium (PBS) was diluted from a 10X concentration to a 1X solution for tissue and cell washing. Trypsin (10%) in PBS was used for passaging cells. The freezing medium for the cultured cells consisted of 45.5% DMEM/F-12 medium and 45.5% (FBS) with 9% Dimethyl Sulphoxide (Me_2SO). One milliliter cryovials were used for storage of the frozen cells.

Tissue Processing

Fresh cultures and frozen cultures were processed the same way except for the thawing process. Two samples from each tissue type were removed from the freezer and placed on crushed ice. This study tested two thawing rates. One rate was in a 37°C water bath and the other was in ice water warming to room temperature from 1°C to

25°C. When both of the samples were thawed they were processed the same way the fresh tissue was cultured.

Each tissue sample was placed into a 50mL conical tube with 1mL Chlorhexidine (Nolvasan) and 29mL of PBS. The tissue was placed into the Chlorhexidine solution for approximately two minutes, shaking occasionally. Each instrument was dipped into 95% Alcohol and flamed between samples and each Pasteur pipette used was flamed. The chlorhexidine solution was aspirated and the tissue sample, with sterile forceps, was placed into a Petri dish with medium. All of the fat, hair, and other wastes from the sample were removed at this point. The cleaned piece was transferred into another Petri dish with clean medium and cut into 1mm pieces. The pieces used for culture were obtained from the center of the sample, the outside edges were discarded. The medium was aspirated and replaced with 10mL of clean medium and transferred to a 15mL conical tube. Once the tissue was settled the medium was aspirated and replaced with 10mL of PBS. The PBS and the tissue were pipetted up and down to clean the tissue of foreign matter. The tissue was washed again with PBS and lastly with tissue culture medium. Six milliliters of new medium were added to the tissue and split into two vented T25 flasks. Each flask was labeled and placed into a 5%CO2 incubator.

Tissue Feeding

Until the flask was confluent, the tissue pieces remained in the flask. The medium was aspirated from the flask, being careful not to remove the tissue pieces, and replaced with 3mL of fresh medium. When there was cell growth, 5mL of medium were

added to the flask. If there were excess debris in the flask it was rinsed with warmed PBS, aspirated, and fresh medium was added. Cultures were fed at least twice per week.

Cell Passaging

When the tissue started to form cell colonies the contents of the T25 flask were sub-passaged into a T75 flask. Once the cells in the T75 flask were confluent they were trypsinized, counted using a hemocytometer, and frozen.

Cell Freezing

The *Cell Passaging Process* was followed for cell cryopreservation. Freezing medium was used to resuspend the cell pellet and 1mL of the cell suspension was pipetted into each cryotube with 100µL of Me_2SO. Each tube was labeled with my initials, date, cell type, animal ID, and passage number before being placed into the -80°C freezer. The vials were closed, inverted, and placed in the freezing chamber in 95% isopropanol. After the cells had been in the freezer for at least 24 hours, they were placed into liquid nitrogen.

Statistical Analysis

Data were analyzed by analysis of variance (ANOVA) using JMP IN 5.1 by the SAS Institute. Sources of variation included thaw rate, days frozen, and tissue type. Specific treatment comparisons were made using Chi Square with Pearson's coefficient and the Student T test. Differences of $P < 0.05$ will be considered significant.

CHAPTER IV

RESULTS

Percent that Attained Growth

When comparing the number of days the tissue was frozen the data were divided into four groups. Group 0 is for tissue not frozen, group 1 is for tissue frozen 1-10 days, group 2 is for tissue frozen 11-20 days, and group 3 is for tissue frozen for 21-34 days. Statistical analysis of the data was performed to show the percentage of samples which attained growth for the three groups. A sample was considered to have grown when a monolayer of cells was visible in the culture vessel. Group 0 had over 90% of the samples that attained growth and was significantly different than all other treatments (Table 2). Groups 1 and 2 were not different as showed using the Chi Square test and the Pearson coefficient. Group 3 was statistically different than all other groups. The percent which attained growth was approximately half of the other two frozen groups.

Table 2. Comparison of Percent that Obtained Growth for Time Frozen in Goat Skin, Muscle, and Oral Mucosa

Group	# Days Frozen	% Attained Growth
0	0	91.7^{c}
1	1-10	45.5^{a}
2	11-20	44.7^{a}
3	21-34	17.5^{b}

Values within column with different superscripts are different, $p < 0.001$.Chi Square-Pearson

The data were also analyzed to determine whether there is a difference between the three tissue types in the percentage which attained cell growth when collected fresh.

This comparison includes the frozen tissue as well as the fresh, non-frozen tissue. The difference between muscle (18%) and oral mucosa (28.1%) in the percent that attained growth was not statistically different. Skin samples had a significantly higher percent (80%) that attained growth than muscle and oral mucosa (Table 3).

Table 3. Comparison of Attained Growth for Freshly Collected Goat Skin, Muscle, and Oral Mucosa

Group	% Attained Growth
Muscle	18.0[a]
Oral Mucosa	28.1[a]
Skin	80.0[b]

Values within column with different superscripts are different, p <0. 0001. Chi Square-Pearson

The three tissue types that were frozen, thawed, and cultured are muscle, oral mucosa, and skin. All three tissue types were statistically different from each other for percent that attained growth when frozen and thawed. The percent of tissue samples that attained growth were 6.8%, 21.4%, and 78.4% for muscle, oral mucosa, and skin, respectively (Table 4).

Table 4. Comparison of Attained Growth for Goat Skin, Muscle, and Oral Mucosa for Frozen Samples

Group	% Attained Growth
Muscle	6.8[a]
Oral Mucosa	21.4[b]
Skin	78.4[c]

Values within column with different superscripts are different, p <0. 0001. Chi Square-Pearson

Mean Days to First Cell Growth

The difference between the number of days to first cells for the three tissue types is shown in Table 5. This includes frozen and non-frozen samples. Muscle averaged 13.3 ± 2.7 days to cell growth, oral mucosa averaged 3.9 ± 3.83 days to cell growth, and skin averaged 10.8 ± 1.3 days to cell growth. The muscle and oral mucosa groups were statistically different, but skin was not statistically different from either muscle or oral mucosa.

Table 5. Comparison of Days to First Cells by Goat Tissue Type

Group	# in Each Group	Mean ± SEM
Muscle	18	13.3 ± 2.7^{a}
Oral Mucosa	9	3.9 ± 3.83^{b}
Skin	78	10.8 ± 1.3^{ab}

Values within columns with different superscripts are different, $p < 0.05$. Wilcoxon test. Errors are expressed as standard errors of the mean.

The means plus standard deviations of the number of days for the goat skin to grow cells are shown in Table 6. Also shown is the number of samples in each group of days frozen. The days to first cell growth of the muscle and oral mucosa varied from 2 to 40 and 2 to 7, respectively, but the numbers of samples were too small for statistical comparisons. The non-frozen skin group 0 averaged 1.9 ± 2.74, group1 (1-10 days frozen) averaged 5.4 ± 1.66, group 2 (11-20 days frozen) averaged 16.9 ± 1.56, and group 3 (21-34 days frozen) averaged 28.3 ± 5.25 days to cell growth. Groups 2 and 3 were not statistically different from each other but were statistically different from groups 0 and 1. These data indicate that the longer the skin samples are maintained in the freezer, the longer it takes for the cells to grow.

Table 6. Comparison of Goat Skin for Days to First Cells by the Number of Days Frozen

Days Frozen	# in Each Group	Mean ± SEM
0	11	1.9 ± 2.74^{a}
1-10	30	5.4 ± 1.66^{b}
11-20	34	16.9 ± 1.56^{c}
21-34	3	28.3 ± 5.25^{c}

Values within column with different superscripts are different, $p < 0.001$. Wilcoxon Errors are expressed as standard errors of mean.

Thawing Method

The frozen tissues were thawed by two different methods. One method is in the 37ºC water bath and the other is ice water warming to room temperature. When these two thawing methods were compared the percent that attained growth was not statistically different (Table 7). The percentages that attained growth were 44.1 and 35.3 for the ice water method and the water bath method, respectively. The Chi-square test and Pearson's coefficient were used for these analyses.

Table 7. Comparison of Goat Cell Growth by Thaw Method for Skin, Muscle, and Oral Mucosa

Thaw Method	% Attained Growth
Ice Water (1 ºC-25 ºC)	44.1
Water Bath (37ºC)	35.3

Chi Square-Pearson $p = 0.20$

The mean number of days to cell growth was not statistically different for the two thawing methods. Ice water thawing to room temperature averaged 11.64 ± 1.96 days to grow cells and the warm water bath method averaged 13.03 ± 2.11 days to grow cells (Table 8).

Table 8. Comparison of Days to First Cells by Thaw Method for Goat Skin

Thaw Method	# in Each Group	Mean ± SEM
Ice Water (1 ºC-25 ºC)	36	11.64 ± 1.96
Water Bath (37°C)	31	13.03 ± 2.11

ANOVA p = 0.63
Errors are expressed as standard errors of mean.

The muscle, oral mucosa, and skin samples that were thawed using the ice water method had a 9.09, 35.71, and 81.82 percent that attained growth, respectively. The muscle, oral mucosa, and skin samples that were thawed using the warm water bath method had a 4.55, 7.14, and 75 percent that attained growth, respectively (Table 9). Both thawing methods showed muscle to have a 40% probability to have cell growth, oral mucosa had a 7% probability to have cell growth, and skin had a 44% probability to have cell growth (Table 9). Muscle and skin were not statistically different between thawing methods. Oral mucosa seems to grow best when thawed using ice water warming to room temperature.

Table 9. Comparison of Growth for Thaw Method by Tissue Type

Tissue Type	Thaw Method	% Attained Growth	P
Muscle	Ice Water	9.09	0.40
Muscle	Water Bath	4.55	
Oral Mucosa	Ice Water	35.71	0.07
Oral Mucosa	Water Bath	7.14	
Skin	Ice Water	81.82	0.44
Skin	Water Bath	75.00	

Chi Square-Pearson

CHAPTER V

DISCUSSION

In this study it was found that some tissue samples taken from a carcass, placed into a 50mL conical tube, frozen up to 34 days without using cryoprotectant in a -20ºC freezer, thawed and then cultured were able to produce live viable cells. Not all cells from the tissue sample were alive, but many tissue pieces had more than one cell colony grow. Even though the tissue was damaged by cryopreservation without cryoprotectant, it is clear that a cell line can be produced and the cells may be useful for nuclear transfer cloning. Collecting living skin tissue from a frozen animal carcass would allow the animal to be rescued genetically. Therefore, successful cryopreservation without cryoprotectants could be useful for short term storage of tissues in emergency situations.

Although this method of cryopreservation has been shown to be successful, it is not recommended over the use of cryoprotectants if they are available. If cryoprotectants are not available, the tissue can be placed into the refrigerator for up to 14 days and still obtain cell growth, although at 10 days the percentage of cell growth decreased rapidly [10]. If over 21 day storage is needed and there are no cryoprotectants available, then freezing could be used and result in a cell line from a skin sample about 18% of the time.

During cryopreservation the organism, or cell, goes through many osmotic changes that can cause numerous reactions within the cell. Cryoprotectants cause the cell to become toxic due to elevated salt concentrations and stresses of the osmotic

balance [2]. The cells are also exposed to many volume excursions between the membranes [4]. All of these can be fatal to the cell viability. The use of cryoprotection has been used to save many gametes and cell lines that would otherwise not be available.

This project utilized a very simple method to freeze the tissue without cryoprotectant. It consisted of placing the tissue into a 50mL conical tube and placing it into a -20ºC freezer without an automatic defrost setting. Neither slow cooling nor any type of cryoprotectant was used. The cells are able to tolerate the potentially lethal effects of ice nucleation and dehydration brought about by ice formation and colligative factors [2]. This procedure can be performed in any laboratory or in most homes. The thawing method was also simple, needing only a water bath and a beaker of crushed ice. The tissue culture process used a commonly available culture medium and can also be performed in most tissue culture laboratories. These procedures would be especially useful when a precious animal dies unexpectedly and cryoprotectant is not available and the sample can not be processed before 10 days.

The medium used was Dulbecco's Modified Eagle's Medium/F-12 for cell maintenance and support. Fetal bovine serum was added to the medium to increase cell growth, sodium bicarbonate to maintain pH, and gentamicin as an antibiotic. The cells that grew from the tissue samples appeared to be fibroblast and epithelial cells. Figure 1 shows the goat skin cells first starting to grow in culture. Figure 2 shows goat cells nearing confluency after being in culture. Figure 3 shows goat skin cells growing after being frozen for 34 days. This was the longest time period from which growing cells were obtained.

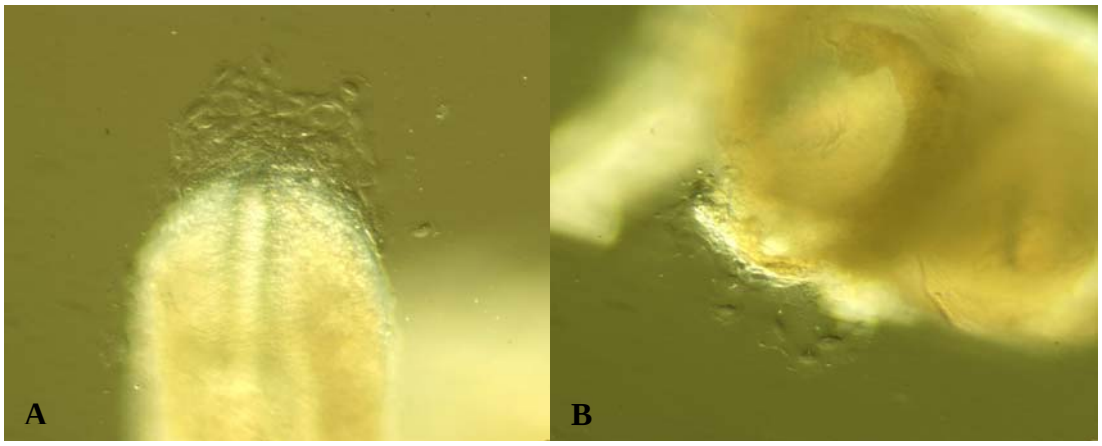

Figure 1. Goat Skin Showing First Cell Growth. A) Goat skin frozen for one day and thawed using the ice water warming to room temperature method (1°C-25°C). B) Goat skin frozen for one day and thawed using the warm water bath method at 37°C.

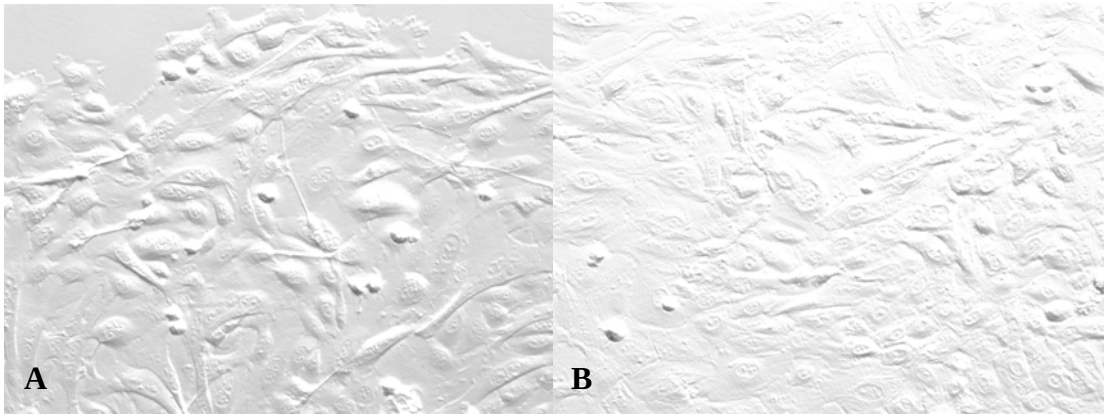

Figure 2. Goat Skin Cells at Confluency. A) shows cells obtained from goat skin frozen for seventeen days and thawed using the ice water warming to room temperature method (1°C-25°C). B) shows cells obtained from goat skin frozen for seventeen days and thawed using the warm water bath method at 37°C.

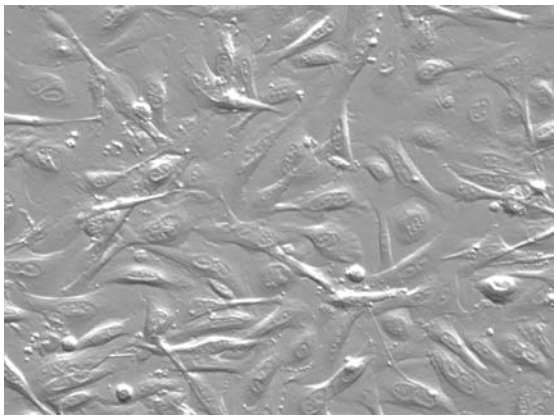

Figure 3. Goat Skin Cells Frozen for 34 Days. Goat skin cells in culture after having been frozen without cryoprotectant for 34 days. The sample was thawed using the warm water bath method at 37°C.

The analysis consisted of three tissue types, (skin, muscle, and oral mucosa), and two thawing methods, (warm water bath (37°C and ice water (1°C-25°C)), and the results revealed that skin is the most likely of the three tissue types to produce cell growth from tissue frozen without cryoprotectants. Cell growth was obtained in almost 80% of the attempts. The reason for this is probably that skin has relatively large numbers of stem cells within the dermis and is continually growing new skin. This could also be due to the fact that skin is a barrier to the body and is able to withstand temperature, osmolarity, and other environmental changes better that other tissue types.

Oral mucosa tissue samples grew more quickly than skin and muscle (3.9 days $\pm$ 2.7) after being frozen, thawed, and cultured. Skin and muscle required longer for cell growth (10.8 days $\pm$ 1.3 and 13.3 days $\pm$ 2.7, respectively) and were not significantly different from each other in the number of days to first cells growth. The number of days a tissue sample was placed into the freezer appeared to be directly correlated to the number of days it took the sample to have cell growth while being cultured. When the

tissue sample was frozen for a longer period of time of up to 34 days, it could take up to 66 days to show cell growth within the culture flask. After a sample was in culture for about three months it was discarded if there was no cell growth.

This experiment showed that the thawing method did not seem to make a difference for the skin and muscle samples. Skin attained cell growth on about 82% of the samples for slow thawing (1°C-25°C) and 75% of the samples for fast thawing (37°C). Muscle obtained cell growth on about 9% of the samples for slow thawing (1°C-25°C) and 4.5% of the samples for fast thawing (37°C). On the other hand, oral mucosa produced cell growth in 35% of the attempts when thawed using ice water gradually warming to room temperature (1°C-25°C) and produced cell growth in 7% of the attempts when thawed using the 37ºC water bath. This was not expected due to the fact that the normal way to thaw semen and embryos is rapidly thawing in a 37ºC water bath for a short period of time.

Ogonuki et. al. reported that mouse spermatocytes in epididymides and whole mouse bodies were able to be frozen for up to fifteen years and produce apparently normal live offspring when used for microinsemination. It was shown that the sperm were still genomically intact when used in microsurgical sperm injection [8]. The main difference between this and the current study is that Ogonuki was testing nuclear function as opposed to cellular function.

It has been reported that keratinocyte stem cells and cumulus cells frozen without cryoprotection were able to function when used in nuclear transfer experiments. No cryoprotection was used, and the cells were stored in a simple medium from 1 day to 1

year [5]. The main difference between this study and the current study is that the stem and cumulus cells were frozen individually instead of within a piece of tissue.

Our data show that some cells are still viable when frozen without cryoprotectants and may be useful for nuclear transfer cloning. It appears there is no difference in cell survival in samples that were frozen from 1 to 20 days. Once the sample reached the twenty day mark of being frozen, the percent of samples that attained growth dropped by approximately half.

This study shows that some cells survive freezing without cryoprotection and maintain the ability to proliferate. It could be possible that even though the cells did not proliferate, the nuclei might still be viable. Previously, it has been shown that donor cells treated to 55 or 75°C can be reprogrammed and can sometimes develop into viable cloned lambs, even though they did not grow when cultured [1].

A decrease in storage temperature is able to extend the time interval between animal death and culture. When decreasing the storage temperature, the cooler the temperature became, the more it minimized pathological changes such as neutrophil infiltration, thrombosis of small vessels and necrosis of epidermal and dermal tissue. In an experiment performed in Spain, cell growth was obtained up to 14 days post mortem if the tissue sample was maintained at 4°C [10]. The main difference with Silvestre's work and this experiment is the temperature at which the tissue sample was stored. Silvestre was able to store skin samples at 4°C and obtain cell growth up to 14 days post mortem and this experiment stored skin samples at -20°C and obtained cell growth up to 34 days post mortem. Thus, freezing doubled the storage time.

There has also been a long term interest in the wooly mammoth that has been frozen in the permafrost for millions of years. This experiment could shed some light on the subject of bringing back the wooly mammoth, but there are a lot more variances to consider. The tissue samples used in this study were collected fresh from the carcass and placed into a freezer that maintained a constant temperature and was not exposed to different environmental factors such as rain, wind, temperature changes, and light. The wooly mammoth has been exposed to all of these environmental factors, if not more, and was not maintained at a constant temperature in the permafrost. When the environmental temperature is higher, cell death takes place more quickly and microbial contamination is greater [10]. Another major difference between this experiment and the wooly mammoth is the amount of time frozen. The wooly mammoth has been frozen for centuries and the tissue samples in the present study showed cell growth after being frozen for only 34 days.

Future studies might include determining if the nucleus is still viable after being stored at freezing temperatures for various periods of time. It may be found that nuclei cryopreserved without cryoprotectants could be useful even if there is no cell growth in culture. If this is possible, then nuclei could be transferred into a donor cell and result in a cloned animal.

The culture medium used in the present study was a relatively simple medium. It would be of interest to know whether bovine pituitary extract (BPE) or epidermal growth factor (EGP) and other growth factors would aid in the salvage of cells from carcasses that have been frozen for extended periods of time.

CHAPTER VI

CONCLUSIONS

The data from this experiment support the hypothesis that some cells in mammalian tissues survive cryopreservation without cryoprotection. The storage time under these conditions appeared to be limited to 34 days. This experiment determined that there was no difference in skin cell growth in culture between the two thawing methods tested. Although this method of cryopreserving has been shown to yield cells that might be useful for nuclear transfer cloning, it is not the recommended method to cryopreserve tissues if cryoprotectants are available or if only short term storage is needed.

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VITA

Name:	Lara Nicole Charles
Address:	Reproductive Sciences Laboratory Texas A&M University 500 University Drive West College Station, Texas 77845-446
Email Address:	lara.charles@gmail.com
Education:	B.S., Animal Science, Texas A&M University, 2004. M.S., Physiology of Reproduction, Texas A&M University, 2008.