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THE CULTURE OF DERMAL REPLACEMENTS

A THESIS

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In the Department of Biology

by

Kristine Peterson

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The Culture of Dermal Replacements

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THE CULTURE OF DERMAL REPLACEMENTS

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Collagen-based wound dressings have been used to prevent excessive fluid loss and infection in individuals with severe skin damage, as well as to promote the re-growth and healing of the patient's own skin. In this study, problems encountered with cryopreservation of collagen-based wound dressings were researched.

A fibroblast cell-line was expanded in culture and seeded onto collagen sponges. The sponges were then cultured for varying lengths of time before freezing them at -80°C. The sponges were analyzed to assess the total number of cells as a function of time in culture.

Toxicity tests were performed using varying concentrations of two cryoprotectants, Dimethyl Sulfoxide (DMSO) and Glycerol. Varying concentrations were tested in an effort to find the concentration of each that gave the highest cell survival and minimal toxic effects.

Over a four hour period, concentrations of up to 10% DMSO did not have an effect on cell viability. The results obtained at 12% DMSO showed that the viability of the cells began to decrease at three hours, and 15% DMSO had a gradual, increasingly toxic effect over time. At glycerol concentrations of 5, 10, and 15%, it was observed that the total cell number was reduced.

Efforts were made to culture sponges to obtain a concentration of 1×10^9 fibroblast cells per sponge in order to provide a detectable signal for analysis by Magnetic Resonance Imaging. Using MRI, the mechanism of cell death due to cryopreservants can be observed and a more efficient

protocol for storing sponges created. However, the highest number of cells cultured per sponge was less than 1.4×10^8. Additional research is needed to improve either the fibroblast concentration on collagen sponges or the resolution obtainable by MRI analysis.

INTRODUCTION

Each year, thousands of people suffer severe skin damage. Of the approximately 130,000 individuals hospitalized annually for injuries resulting from exposure to fire, most require emergency treatment because fire has destroyed a large portion of their skin (20). Collagen-based wound dressings, also known as sponges, may be used to prevent excessive fluid loss and infection in these individuals, as well as to promote the re-growth and healing of the patient's own skin. However, such dermal replacements require weeks of preparation. A collagen sponge must be formed and seeded with fibroblast cells before being placed on the wound site. The fibroblast cells aid in the process of wound repair and, importantly for skin substitutes, appear to be non-immunogenic. A practical method of storage must be found so that these collagen sponges can be ready for use in emergency situations.

This experiment is a preliminary study in a research project using Magnetic Resonance Imaging (MRI) to investigate the problems encountered with storing collagen sponges. Dermal replacements were cultured and analyzed to determine the total cell concentration as a function of time in culture. Cryopreservants (CPAs) are essential in the process of freezing cultured collagen sponges in order to protect the

fibroblast cells from injury due to ice formation. However, too high a concentration of CPAs can have lethal effects during the freezing and thawing process. Toxicity tests were performed using varying concentrations of two CPAs, Dimethyl Sulfoxide (DMSO) and Glycerol, in an effort to find the concentration of each that gave the highest cell survival and minimal toxic effects.

The goal of this research is to determine whether or not the method used to make and culture the sponges is sufficient for fibroblast growth. In order to conduct further studies concerning the transport and viability of engineered dermal replacements using MRI, 1×10^9 cells per sponge are needed to make a detectable signal. The process of cell death in sponges due to CPAs can then be observed and imaged. With such information, an improved method for storing dermal replacements could then be developed.

BACKGROUND

Of the skin substitutes available today, none are without major problems. Autografts, which replace the lost skin with cells harvested from a patient's own skin, are not acceptable solutions for those with major burn injury over a large portion of their body (15, 19). Allografts from a live donor or cadaver are a potential source for disease transmission. They are also in short supply, are often rejected, and eventually need to be replaced (15, 19). Typical xenografts, such as porcine skin grafts, have usually required traumatic removal from the wound (19).

Alternative skin substitutes have been extensively researched over the past few decades, and collagen-based wound dressings, inoculated with non-immunogenic fibroblast cells, have been found to meet many of the requirements for a successful dermal replacement. Collagen fibers are a major component of the basement membrane in skin, provide tensile strength, an ability to anchor, and elasticity (18). As a skin substitute, these fibers are useful because they improve cell differentiation, cell attachment, and growth of fibroblast cells (5).

Collagen gets its tensile strength qualities from the long, uninterrupted triple helical regions of which it is composed (14). This allows for a stable and suturable wound dressing, and works to inhibit wound contracture as well (6, 21). The flexibility of collagen fibers allows the collagen dressing to fit tightly to a rough woundbed to help prevent infection (21). In addition, collagen is a weak antigen, making rejection less likely than with other types of skin

substitutes (6). Furthermore, collagen is biodegradable by collagenase enzymes, making traumatic excision unnecessary. Importantly, this quality also allows a collagen-dressing to be replaced by patient's own tissue (20). Finally, collagen breaks down into a nontoxic material.

A unique characteristic of collagen is its ability to be formed into various shapes and sizes. Such flexibility allows a collagen-based dressing to be made to the width of the skin (21). By freezing and then freeze-drying a collagen dispersion, it can be shaped into a three-dimensional sponge capable of supporting cell growth. Further, ice crystals grow and form channels during the freeze-drying process. Therefore, cells can infiltrate inside the collagen sponge via these channels to aid in re-growth and healing of tissue.

Because small collagenous peptides and collagen alpha-chains are chemotactic, collagen and the products it degrades into may act as attractants for fibroblast cells in wound repair (13). A collagen substrate is also necessary for fibroblast migration (5). Fibroblasts are one of the three types of cells found in the dermis, and they are important for several reasons. The initial healing of cutaneous skin is the result of an invasion into the wound area of inflammatory cells such as neutrophils, granulocytes, macrophages, and platelets which release mediators to attract primarily fibroblasts to the wound (14). Fibroblasts synthesize the connective tissue matrix as well as secrete enzymes needed to break down and remodel the matrix (19). Research indicates that fibroblasts stimulate replication of epidermal cells grown on a collagen sponge (5). Importantly for skin

substitutes, fibroblasts also appear to be non-immunogenic in the allogeneic situation (9).

Fibroblasts generally synthesize collagen as 5-10% of their total protein (13). The new collagen they deposit into the wound bed strengthens the healed wound (14). Through the addition of ascorbic acid to a collagen-based wound dressing, cells are capable of maintaining a higher rate of collagen synthesis. Essential for the formation of normal connective tissue, ascorbic acid stimulates the proliferation of cells in culture (13).

Glycosaminoglycans (GAGs), components of proteoglycans in the extracellular matrix, are also important components of skin substitutes. They can be added to the collagen dispersion when forming a sponge. Research has shown that GAG makes the collagen sponge more elastic, creates more open pores, helps control blood clotting, and is a weak antigen which degrades to a nontoxic substance (20).

In particular, hyaluronic acid, a GAG consisting of repeating N-acetylglucosamin-glucuronic acid disaccharides, has been shown to enhance cell mobility by expanding the volume of the hydrated sponge and increasing the space between collagen fibrils (5,12). It may also have a role in cell migration by facilitating adhesion-disadhesion between the cell membrane and matrix (12).

Another common glycosaminoglycan, chondroiton-6-sulfate, stimulates cell replication earlier but does not aid cell infiltration into the sponge (5). The deposition of new extracellular matrix is also more frequently observed with hyaluronic acid than with chondroiton-6-sulfate (5). Research shows hyaluronic acid produces

a highly packed deposition of extracellular matrix and hastens the formation of granulation tissue necessary for the healing process (12).

Glycosaminoglycans can be crosslinked with collagen to decrease the sensitivity of collagen to degradation. Cross linking in a vacuum oven, which forms chemical bonds through an amide condensation reaction, allows the collagen dressing to be degraded at an appropriate rate. Too fast a degradation rate would not allow for wound closure, and too slow a rate would prevent replacement by the patient's own tissue (21). The surface of crosslinked sponges also improves cell to cell contact and attachment and orientation of basal cells (5).

Collagen-based wound dressings have been used in skin injury cases, and the results appear promising. In a study conducted at the University of Miami School of Medicine, a skin substitute consisting of a collagen matrix containing human fibroblasts and a sheet of stratified human epithelium was grafted onto cancer removal sites in human patients. By three months, 11 of the fifteen patients had at least a 75% take of the skin substitute. It was not clinically rejected, was non-toxic, and produced better than expected healing (17).

Further research on collagen-GAG substrates has indicated the long-term closure of chronic wounds is due to the replacement of the allogeneic seeded cells by the patient's own cells. Two of four patients treated in a study at the University of Cincinnati College of Medicine achieved wound closure with a cultured skin substitute (CSS) in a shorter period of time than the duration of their wounds before treatment with the CSS. The wounds remained healed four

months after grafting, and no allogeneic cells were found. Autologous cells grew inward from the wound perimeter and gradually replaced allogeneic cells seeded onto the skin substitute. External factors may have contributed to the unsuccessful wound closures of the two remaining patients. One patient had a very large wound which did not close with conventional or other experimental alternatives. The other patient showed poor compliance with wound care protocol and continued to use tobacco and alcohol. Further research is needed on larger populations of patients to verify the accelerated wound closures indicated by the CSS (3).

Another probable cause for unsuccessful results with skin substitutes is the development of chronic wounds during the three to four week period required to culture the grafts, which makes the healing process more difficult (9). Although collagen-based wound dressings may make a successful alternative to other skin substitutes, advances in storage procedures are needed to ensure their immediate access.

Research is being pursued to solve the storage problems of dermal replacements. Because of the more difficult healing that is involved with chronic wounds, it is vital that dermal replacements be easily accessible for use in emergency situations. Cryobiology is one area of science working to overcome these difficulties. The freezing of cells and cellular systems are studied at temperatures ranging from above zero to -196°C.

Research in this area not only aims to understand preservation, but also deals with killing unwanted tissues such as malignant cells (2). The major problem cryobiologists must contend with is how to best freeze cells to maximize their survival (15). Injuries to cells due

to freezing can result from intracellular ice formation as well as exposure to concentrated medium (2).

The plasma membrane works to block the growth of ice crystals in the cytoplasm of a cell even as the external water begins to freeze. As cells are first frozen, water flows out through the cell membrane because of the lower outside chemical potential and freezes externally. If the cells are frozen too quickly, water does not have sufficient time to penetrate the membrane and leave the cell. The result is the formation of ice crystals within the cell (15).

In contrast, if cells are frozen slowly, the cell is able to dehydrate and intracellular freezing does not occur. However, the loss of water in the interior of the cell causes an increase in the concentration of intracellular solutes, and injuries result from the exposure to concentrated medium (2).

Cryoprotectants have been used successfully to reduce ice formation. Glycerol and DMSO are two types of commonly used cryoprotectants which have low molecular weights and are able to permeate the plasma membrane (2). They do not have special protective properties, but rather are successful because of their effect on concentration.

Different hypotheses may explain how they function. Cells can be found in unfrozen channels between ice crystals. The ice crystals enlarge by drawing water out of these channels, increasing the solute concentration of the channel. The cells then shrink due to osmosis. It is proposed that because the total concentration of solutes is unchanging at a given temperature, the higher the concentration of cryopreservants in the channel, the lower the NaCl concentration will be. It is thought slow freezing may damage cells because of the rise

in electrolyte concentration, and an increase in NaCl concentration is suppressed by the presence of cryopreservants.

Another explanation for the low survival rate of cells during freezing is due to a decrease in the fraction of solution that remains unfrozen. Cryoprotectants increase the amount of unfrozen solution and therefore their viability (15). However, while increases in cryoprotectant concentrations increase cell survival, concentrations that are too high have toxic effects (2).

Problems with thawing can also be found. Generally, cells cooled rapidly must be warmed rapidly to prevent recrystallization (2). Fast cooling causes small crystals to be formed intracellularly, and these crystals fuse together into larger crystals if the warming rate is too low. Often, warming rate of slowly frozen cells has little or no effect on cell viability. However, it is hypothesized that certain slowly cooled cells such as mouse embryos, human red blood cells, and many higher plant cells can undergo osmotic shock if rapidly warmed (15).

Progress in the area of cryoprotectants may be the answer to the problems involved with storing dermal replacements. Ideally, a storage bank for dermal replacements similar to the common blood banks in use today would be created to provide immediate access to skin substitutes.

PROCEDURE

Six frozen vials of fibroblast cells at a concentration of 1 X 10^6 cells/ml were thawed and placed into separate tubes. A total of 14 ml of medium (500 ml Dulbecco's Modified Eagle's Medium, 6.5 ml antibiotic/antimycotic, and 55 ml Fetal Bovine Serum) was divided among the tubes, followed by vortexing. The solutions in each of the tubes were transferred to separate flasks, and each flask was brought to a total volume of 14 ml with growth medium. The fibroblast cells were cultured and expanded for 16 days prior to seeding on a collagen-based sponge. They were maintained in a 37°C incubator at 5% CO_2.

A dispersion consisting of 0.5 g collagen, 0.026 g hyaluronic acid (HA) and 100 ml .01N HCl at pH 3 was blended for one minute to make the collagen sponges. The 1% weight dispersion was poured 3-4 mm thick into twelve 100 x 15 mm petri dishes and frozen in a freeze dryer at -45°C overnight. The following day, the samples were freeze-dried with a condenser temperature of -94°C and a chamber pressure of ~14mtorr. The temperature was slowly brought to room temperature at increments of 5-10°C per hour to prevent collapsing of the sponges. After the freeze-drying was

completed, the sponges were cross linked in a vacuum oven at a temperature of 110°C for 5 days.

The seeding of fibroblast cells on the sponges was carried out by the following procedure. Fibroblast cells were removed from a culture flask and centrifuged. Then, 1.5 ml of DMEM was added to the pellet, followed by vortexing. The cells were counted using a hemacytometer to obtain the initial concentration of cells seeded on each sponge. The pan-side of a hydrated collagen sponge was seeded and placed in a CO_2 incubator. After 1 hour, growth medium with 1.2% ascorbic acid was used to cover the sponges.

Sponges were washed with 1 x Phosphate Buffer Solution (PBS) and frozen on days 1, 4, 8, 12, 16, 20, and 24 after seeding so that the amount of cells cultured on the sponges as a function of time could be assessed by DNA analysis. On days 8 and 16, an additional sponge was frozen for verifying the data on those days. These sponges were subjected to Protease K Digestion and incubated overnight at 37°C. This step digests the collagen sponge and cell membranes, leaving only the DNA of the cells. Initially, one ml of Protease K at a concentration of 1 mg/ml was used per sponge. The solution made consisted of 1 mg Proteinase K, 16 ml H_2O, 2 ml NaH_2PO_4 (1M) and 2 ml EDTA (500 mM). The following day when

the sponges were still undigested, 100 ul of Protease K at a concentration of 10 mg/ml was put on the sponges two separate times. The sponges were also sonicated for 3 seconds each to disrupt them.

A fluorometric assay of the DNA was then done using a standard and comparing the concentration of DNA from the sponges to the standard. A stock solution of calf thymus DNA was mixed with 1x PBS buffer to make a 10 ug/ml concentration of DNA for the standard. This was pipetted into wells in different amounts and brought to the final volume of 500 ul per well with 1 x PBS. The final concentration of DNA per well in ug/ml was 0, 0.25, 0.5, 1, 1.25, 1.5, 2, 2.5, 5, and 10. From each of the digested sponges, 5 and 10 ul samples of DNA were also placed into wells, to which 495 and 490 ul/ml respectively of 1 x PBS were added. Next, an amount of 10 ul Hoechst 33258 dye was added to 50 ml of 1x PBS, and 500 ul of this solution was added to each well. The DNA concentration was then measured using a fluorometer with an excitation of 360, emission of 460, and a gain of 60. The number of cells per sponge was found by dividing the mass of DNA by 1 x 10^{-11} g, the average mass of DNA in fibroblast cells.

A sponge on day 24 was also frozen for histological sectioning in order to examine the extent of cell infiltration into the interior of the sponge. Two sections of the sponge were fixed in 12% Formaldehyde in 1x PBS. Several days later the samples were placed between two foam pieces and put in a basket in formalin (Formaldehyde 3.7% in 1x PBS). They were then taken to a lab for paraffin embedding. Tissue sections of 5 um were made and set in a warm water bath. Each ribbon was put on a slide, dried, and placed on a hot plate for 2 hours. They were then stained with hematoxylin-eosin dye and examined.

In order to perform toxicity tests using the cryopreservants Dimethyl Sulfoxide (DMSO) and Glycerol, solutions of interest were made containing 0, 5, 8, 10, 12, and 15% DMSO and 0, 5, 10, and 15% Glycerol. Fibroblast cells were removed from a flask and centrifuged. They were diluted by resuspending the cells in 10 ml of growth medium, and 2 ml aliquots were introduced into separate test tubes. The tubes were brought to a total volume of 5 ml with growth medium to wash the cells, followed by centrifugation. Next, one ml of the cryopreservant was added to the pellet. Equivalent amounts of this solution and AO/PI dye (1.0 ml of 1mM acridine orange stock solution, 2.0 ml of 0.5 mg/ml propidium iodide stock

solution, and 47 ml Dulbecco's PBS) were combined in order to count the cells using a hemacytometer. Live cells appeared green in color and dead cells were orange.

RESULTS

After 3 hours in 12% DMSO, the viability of the cells began to decrease. Concentrations of 15% DMSO had an increasingly toxic effect on fibroblast cells over the 4 hour period tested (figure 1.1). The results of the Anova statistical test showed a 0.021 probability that the null hypothesis is correct (table 1.1). Concentrations of 0-15% glycerol did not have an effect on cell viability (figure 1.2). The results of the Anova test showed a 0.791 probability that the null hypothesis is correct (table 1.1). The total cell number was reduced, however, in the concentrations of Glycerol tested (figure 1.3). The Anova test showed a 0.015 probability that the null hypothesis is correct (table 1.2).

Figure 1.4 indicates the highest fibroblast cell concentration obtained with the procedure used resulted in approximately 1.4×10^8 cells per sponge. Because each sponge was seeded with fibroblast cells from a separate flask, the initial number of cells varied (table 1.3). Thus, figure 1.5 shows the normalized fibroblast cell population per sponge to account for this difference. Figure 1.6 shows the logarithm of the normalized cell population per sponge.

Figure 1.7 shows a histological section of a collagen and hyaluronic acid sponge. The seeded cells have infiltrated throughout the sponge.

	0-15% DMSO	0-12% DMSO	0-10% DMSO	0-15% Glycerol
Probability of null hypothesis	0.021	0.044	0.181	0.791

Table 1.1. Anova statistical test comparing the percent viability of cells in different concentrations of DMSO and glycerol.

	DMSO: Total number of cells	Glycerol: Total number of cells
Probability of null hypothesis	0.383	0.015

Table 1.2. Anova statistical test comparing the total number of viable cells in different concentrations of DMSO and glycerol.

Days in Culture	Initial concentration of cells per sponge	Final concentration of cells per sponge (average of 3 DNA counts)
1	478.5 x 10^4	1.98 x 10^7
4	595.5 x 10^4	4.72 x 10^7
8	780 x 10^4	6.06 x 10^7
8	387 x 10^4	7.28 x 10^7
12	648 x 10^4	7.93 x 10^7
16	127.5 x 10^4	11.6 x 10^7
16	834 x 10^4	8.23 x 10^7
20	598.5 x 10^4	12.9 x 10^7
24	430.5 x 10^4	9.94 x 10^7

Table 1.3. Initial and final concentration of cells per sponge and the number of days in culture.

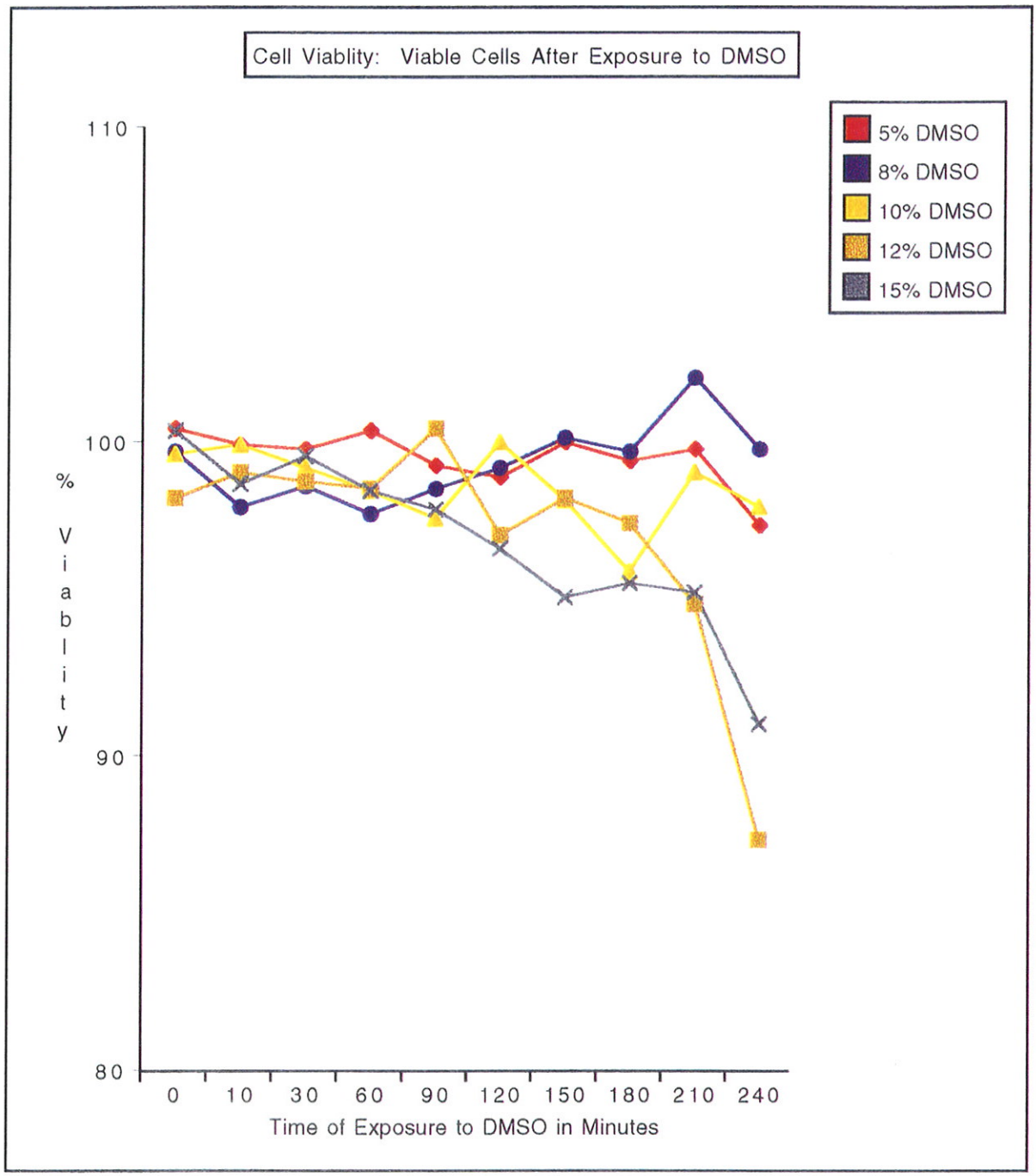

Figure 1.1. Percent of viable cells as a function of time in varying concentrations of DMSO.

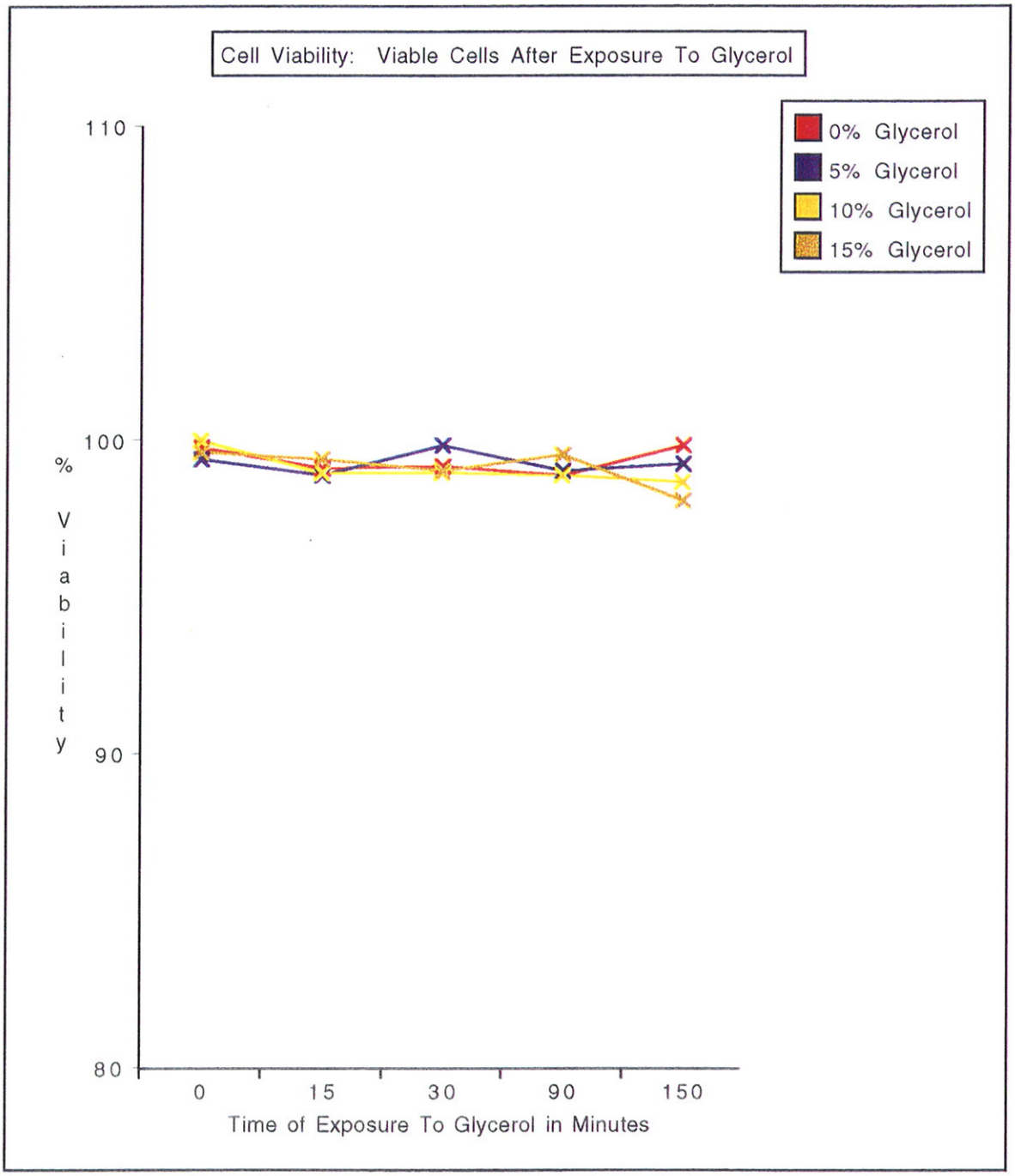

Figure 1.2. Percent of viable cells as a function of time in varying concentrations of Glycerol.

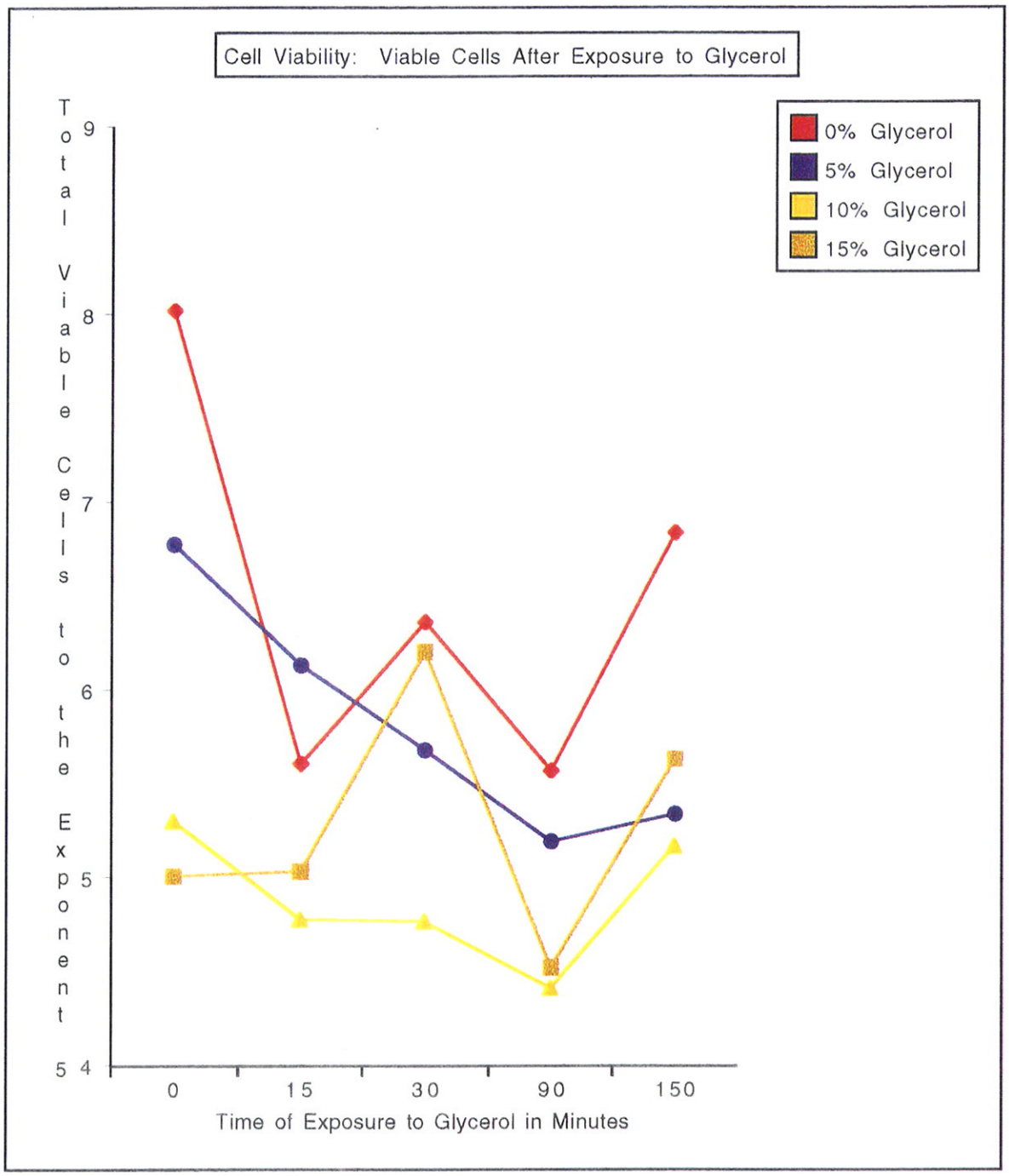

Figure 1.3. Total number of viable cells after exposure to varying concentrations of Glycerol.

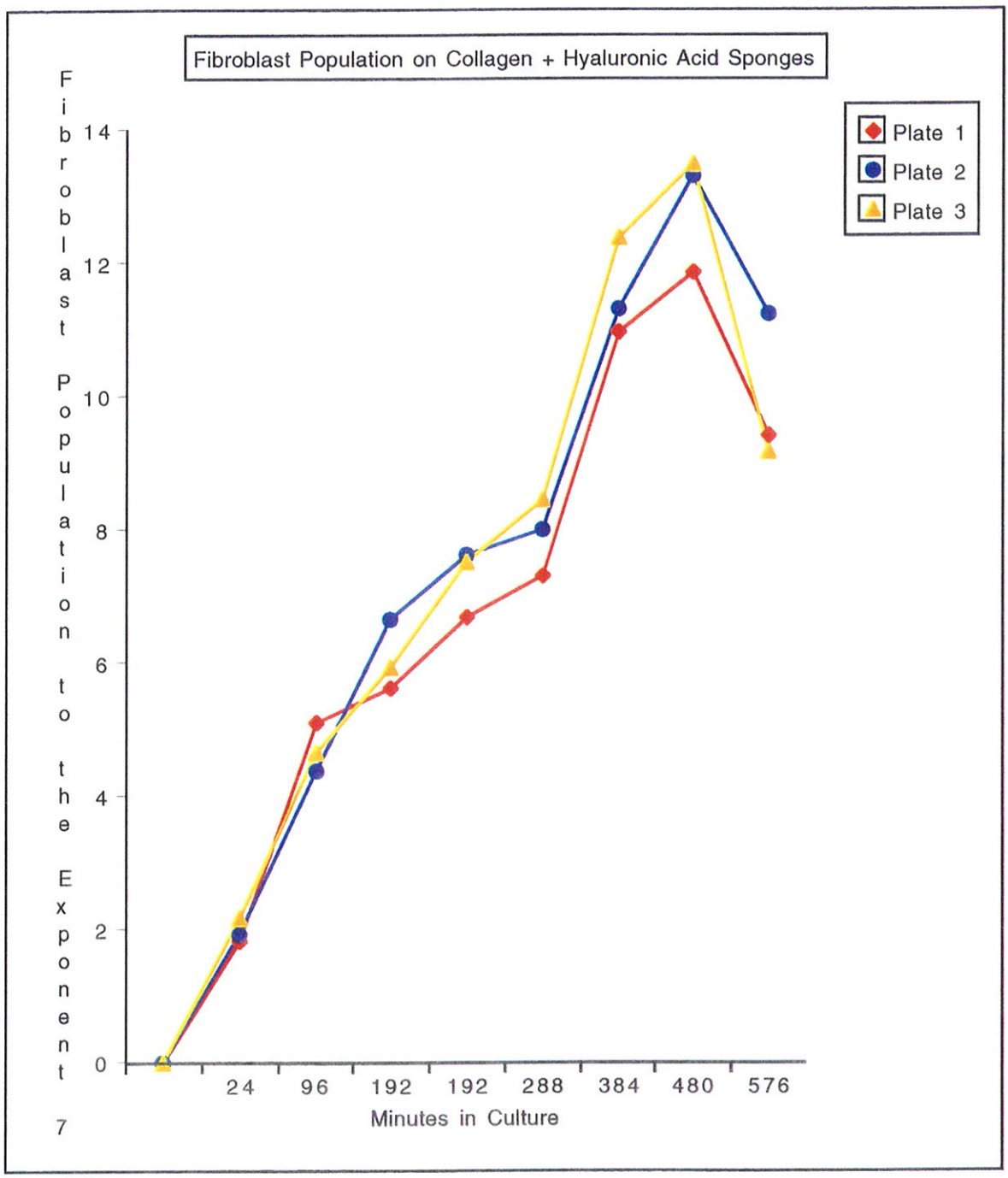

Figure 1.4. Total fibroblast Population on collagen and hyaluronic acid sponges.

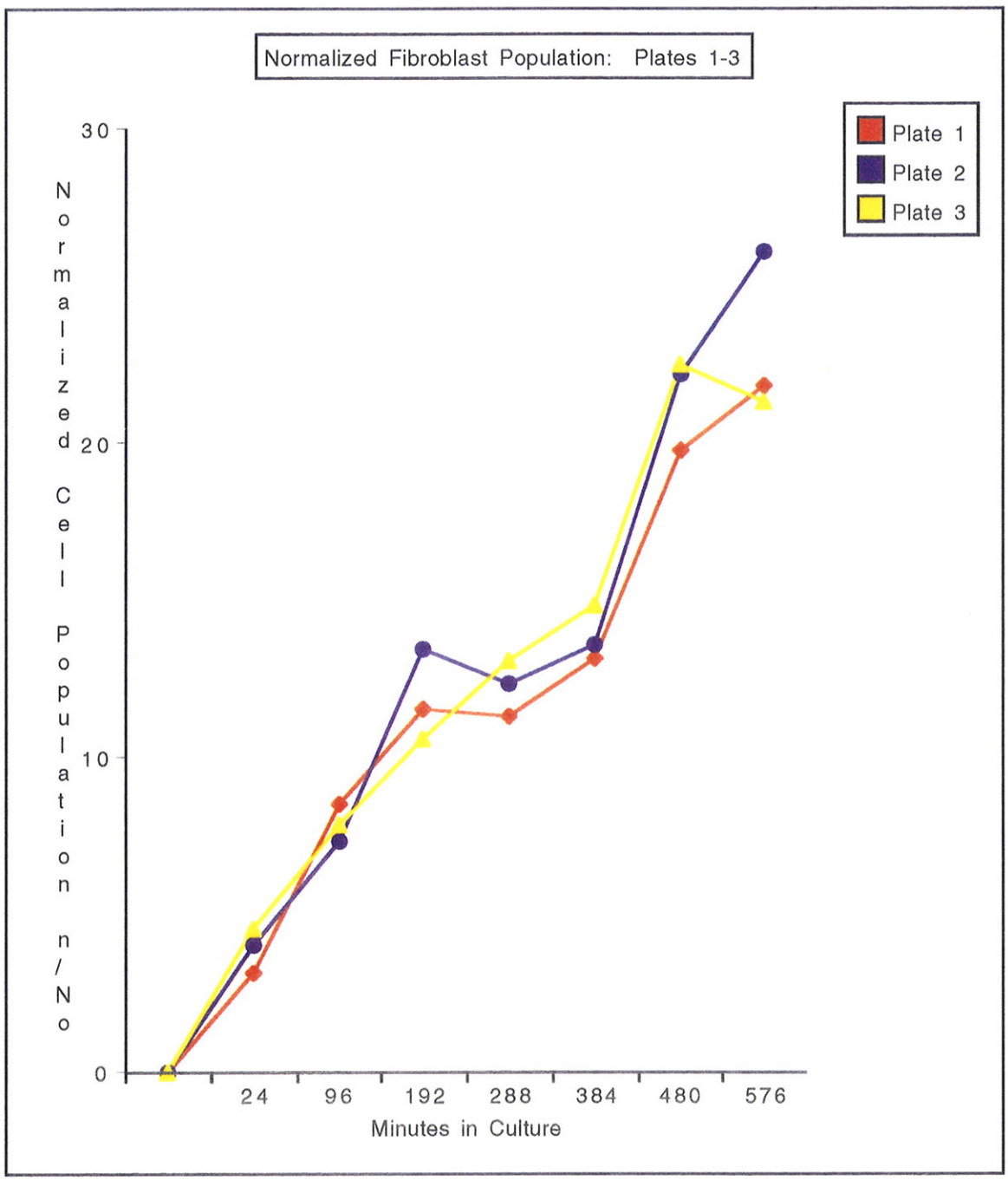

Figure 1.5. Normalized fibroblast population (n/No) on collagen and hyaluronic acid sponges.

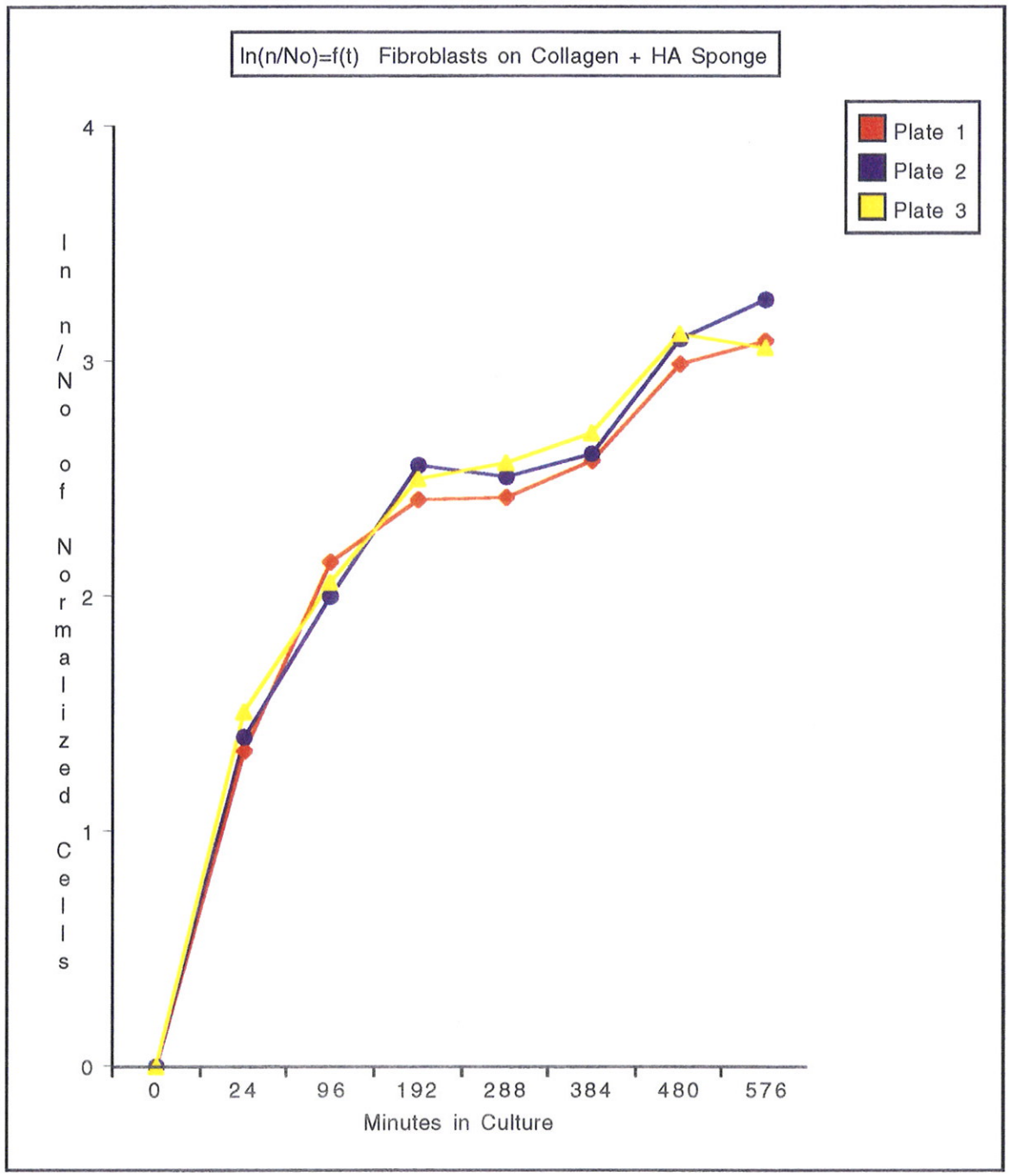

Figure 1.6. Logarithm ln(n/No)=f(t) of the normalized fibroblast population per collagen sponge.

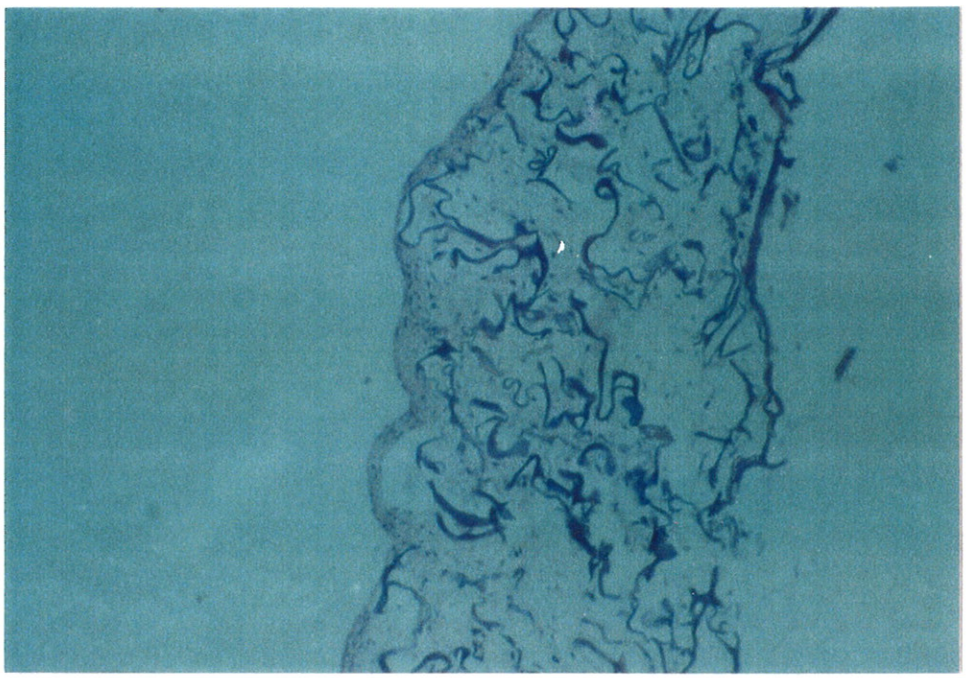

Figure 1.7. Histological section of fibroblasts seeded onto the pan-side of a collagen and hyaluronic acid sponge.

DISCUSSION

Varying concentrations of two cryoprotectants, DMSO and glycerol, were tested in an effort to find the concentration of each that gave the highest cell survival and minimal toxic effects. The toxicity tests conducted were intended to provide a comparison for future tests by MRI. CPAs have not been studied extensively by this technique, and similar tests by light microscopy, a commonly used procedure, would provide a basis for comparison of data obtained by MRI.

The results indicate that the mechanism of cell death may be different in glycerol than with DMSO at the concentrations tested. As shown in figure 1.1, 12% DMSO resulted in cell death due to toxicity. Results of the Anova statistical test indicate that the null hypothesis (the percent of viable cells in 0-15% DMSO is the same) can be rejected (table 1.1). In contrast, concentrations of up to 15% glycerol did not have an effect on cell viability (figure 1.2). The Anova statistical test showed that the null hypothesis (the percent of viable cells in 0-15% glycerol is the same) can be accepted (table 1.1).

A difference in total cell number was apparent in concentrations of glycerol (figure 1.2) that was not observed in concentrations of DMSO table (1.2). The results of the Anova test indicate that there is a significant difference in the total number of cells in 0-15% glycerol (table 1.2). Being that glycerol is a higher molecular weight molecule than DMSO, a possible explanation for these results may be that cells suffer immediate lysis due to osmotic shock as glycerol enters the cell.

However, large variations in total cell number were observed in measurements obtained by light microscopy (figure 1.3). A more precise method of measuring total cell populations is needed to obtain reliable results of cell death due to complete cell lysis. In future toxicity tests, a Coulter Counter should be used to obtain total cell counts.

To produce a detectable signal with MRI analysis, a concentration of 1 x 10^9 cells per sponge is currently needed. Sponges containing less than1 x 10^8 cells, mainly at the periphery of the sponge, were prepared in a past experiment conducted at the University of Minnesota using a similar protocol. A change in the seeding of the sponges was made in this experiment with the goal of achieving the needed concentration of 1 x 10^9 cells per sponge. In the prior experiment, sponges were seeded on the air-side of the

sponge, opposite the bottom of the petri dish. In this experiment, the sponges were seeded on the pan-side of the sponge. The decision was based on research that shows a reaction occurs on the air-side of the sponge, collapsing the pores and preventing infiltration of fibroblasts to the interior of the sponge (6).

Although the cell density obtained (1.4 x 10^8 cells/sponge) is still inadequate for MRI analysis at this point, the results of the histological sections appear promising. As seen in figure 1.7, the protocol used to culture the dermal replacements did allow fibroblast cells to fully infiltrate throughout the entire width of the sponge. These results indicate that collagen sponges can function as good substrates for cell growth, at least in the *in vitro* situation. If the resolution obtainable by MRI can be increased, it is still possible that images of the cultured collagen sponges can be made.

Several possible changes may also be made in an effort to increase the cell concentration on collagen sponges. The initial concentration of cells seeded onto the sponges and the length of time in culture can be increased. An obvious difficulty with this proposal is that the cell concentration increases at a slower pace as time progresses (figure 1.6), and the length of time needed to culture the sponges may not be feasible.

A lower weight percent collagen dispersion, such as 0.5% rather than 1%, may be used to decrease the density of the initial, uncultured sponge and increase its pore size to aid cell infiltration. Maintaining the sponges at -30°C for an extended period of time while freeze-drying may create more open pores as well.

Another possible solution would be to seed two thinner sponges separately and stack them on one another. Since it is easier to achieve cell growth on the surface of sponges rather than on the inside, this may increase cell density.

Finally, the collagen sponge is a very simplified replication of the human skin. Additional components may be needed within the sponge to increase fibroblast growth. It is probable that a combination of these ideas may need to be implemented to increase the concentration of fibroblast cells per sponge.

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