

USE OF A NOVEL JOINT-SIMULATING CULTURE SYSTEM TO GROW ORGANIZED EX-VIVO THREE-DIMENSIONAL CARTILAGE-LIKE CONSTRUCTS FROM EMBRYONIC EPIPHYSEAL CELLS

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ABSTRACT

A method for growth and maintenance of vital cartilaginous tissue is necessary for cartilage repair by in-vitro produced biologic implants. A previously tested perfusion system simulating joint activity was used. Whole epiphyses collected from thirty 11-day-old chick embryos were divided into two groups. One group was grown in a tissue culture dish for 10 days. The other group was placed in a perfusion system termed a joint-simulating device (JSD). After a period of 10 days, histology and immunohistochemistry were performed on five epiphyses from each group. Histologically, epiphyses grown in the device coalesced into a homogenous three-dimensional mass. The bridging tissue between individual epiphyses was highly cellular (PCNA staining positive) and was composed of mesenchymal stem cells as shown by expression of FGF receptor 3. No such tissue formed between epiphyses in the tissue culture dish and the epiphyseal cores were shown to be necrotic. The rest of the epiphyses were evaluated for radioactive sulfate incorporation into glycosaminoglycans (GAGs). A tenfold increase in sulfate incorporation occurred in epiphyses grown within the JSD as compared to the traditional culture method. In conclusion, embryonic epiphyses could be a suitable source for the ex-vivo growth of tissue-engineered cartilage constructs that might later be used as an in-vivo cartilage implant. The joint simulating device effectively maintains cartilage viability and bioactivity for as long as 10 days.

INTRODUCTION

Cartilage essentially lacks self-repair capacity. In recent years, single autologous chondrocyte transplantation obtained by arthroscopic biopsy and grown in monolayer cultures has been advocated by several groups among first-line procedures for inducing repair and regeneration of articular cartilage defects.^{1,2}

The major limiting factor of this method, however, is the need for a well-defined defect with surrounding healthy cartilage as a prerequisite. It is therefore not suitable for coverage of large areas of joints denuded of cartilage. These would require the development of other repair techniques.

One alternative which is still experimental on animal models is to transplant the cartilage cells or grown-in culture ectopically into the soft tissues of the recipient and allow them to grow and develop further in a favourable milieu with rich vascularization before their final implantation as articular cartilage substitutes.^{3,5}

Another challenging option presently under investigation is cartilage tissue engineering using three-dimensional (3-D) cartilage constructs grown ex-vivo.⁶⁻¹¹ These constructs may involve scaffolds, adhesives, and cells and/or tissues grown under optimal conditions.⁶⁻¹¹ Previous experience, however, with such 3-D constructs under static culture conditions failed to support cell growth, and viability remained limited to the implant liquid interface and its close vicinity (50 μ m).¹² Proper growth of three-D cartilage ex-vivo, therefore, requires a milieu favoring chondrogenic proliferation and maturation. Various instruments have been devised lately to achieve this purpose. They appear under a variety of names: bioreactors, perfusion chambers, rotating vessels and joint-simulating devices. They all continuously irrigate the culture sample with fresh medium enriched with carbon dioxide and containing nutrients and growth factors in abundance.⁶⁻¹¹ Constant perfusion also avoids the local accumulation of waste products that might be toxic to the chondrocytes. The perfusion flow in itself has a strong influence upon the growing cartilage by mimicking hydrostatic and gravitational forces normally present in the joint, thereby modulating its biomechanical properties.¹³⁻¹⁷ Thus, another advantage of perfusion devices is the possibility to apply hydrostatic loads, whether constant or cyclic, by controlling flow pressures, rates and directions.¹³⁻¹⁷ The present study com-

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compares the fates of embryonic chick epiphyses grown in regular static tissue culture and in a perfusion joint-simulating system.

MATERIALS AND METHODS

Cell Culture

Femoral and tibial epiphyses were aseptically harvested from thirty 11-day-old chick embryos. Dissection from the surrounding soft tissues was facilitated by pre-digestion with collagenase (1% in phosphate buffered saline, ICN Biomedicals Inc. Costa Mesa, CA). The epiphyses were suspended in culture medium (Dulbecco's minimal essential medium, Biological Industries, Beit Haemek, Israel) to which 10% fetal calf serum (Biological Industries, Beit Haemek, Israel) and antibiotics (Penicillin 10,000 units per ml, streptomycin 10 mg per ml, amphotericin B 0.025 mg per ml) were added.

Epiphyses were then divided into three groups: Two groups (1 and 2) were incubated in regular static culture dishes. A third group (3) was incubated within a closed laminar flow perfusion joint-simulating device (JSD) previously described and successfully used for growth and maintenance of embryonic cartilage of human origin.¹⁸ The present experiment was carried out at a flow rate of 570ml/h, with a peak pressure of 150mmHg and a pressure pulse rate of 150 cycles/min. In previous experiments these values were found to be optimal.¹⁸ All groups were cultured for a period of ten days.

Histology and Immunohistochemistry Assays

Ten epiphyses from each group were taken for histological evaluation. Epiphyses were immersed in formalin solution (4%, pH-7.4) containing 0.5% cetylpyridinium chloride for 24-48 hours, and later transferred into 4% formalin solution for an additional 48 hours. The samples were dehydrated with alcohols, embedded in paraffin, and sectioned by a standard microtome. Blocks were cut into two types of sections: 5 μ -thick sections for routine histological and histochemical examinations on regular glass slides, and 20 μ -sections glued on polylysine-coated glass slides for immunohistochemistry. The paraffin was removed with xylol and samples were rehydrated in serial alcohol. Each sample was stained with Mayer's hematoxylin eosin, Masson's trichrome and Alcian blue (pH1.0. and 2.5) using routine staining techniques.¹⁹

The presence of various specific antigens was assessed in tissue sections by immunohistochemistry: anti-fibroblast growth factor receptor 3 (FGFR3) antibody (1:100 dilution, Santa Cruz, Menlo Park, CA), anti-

proliferating cell nuclear antigen (PCNA, 1:100 dilution, Dako, Glostrup, DK). Incubation with the primary antibody at optimal dilutions was performed in the humidity chamber for 16 hours at 15°C. The slides were later incubated with a secondary antibody (Swine anti-rabbit 1:150 Dako, Glostrup, Denmark) amplified by peroxidase-antiperoxidase complex (1:150 Dako, Glostrup, Denmark). The substrate was diaminobenzidine (Dako, Glostrup, Denmark). Negative controls included second-layer-only and DAB-only preparations.

Cellular Viability Assays

Cellular viability was assessed by counting living cells with intact nuclei versus non-living cells within five square frames (of 50m X 50m each) that were randomly placed over the micro-section under 400 x magnifications. Average percentage of living cells could thus be calculated (Figure 3).

Biochemical Assays

The epiphyses of group 1 were boiled for five minutes so as to obtain non-viable tissue to serve as a control for non-specific sulfate uptake unrelated to GAG synthesis.^{20,21} Five epiphyses from each group were then weighted and incubated for 24 hours with five μ Ci/ml of radioactive sulfate (³⁵SO₄ carrier-free). The reaction was stopped by boiling for five minutes. The samples were solubilized by papain (5% in buffer, ICN Biomedicals Inc., Costa Mesa, CA) digestion at 65°C. The papain buffer contained 0.1M sodium acetate, 0.005M EDTA and 1mg/ml cysteine chloride. Dialysis against distilled water (containing 0.01M of Na₂SO₄ in the initial dialysate) for several days removed proteolytic papain products and non-incorporated sulfate precursor. Tissue remnants were discarded by centrifugation (10,000 rpm for 10 minutes at 4°C). GAG molecules were precipitated by the addition of chondroitin sulfate (1 mg per ml, ICN Biomedicals Inc., Costa Mesa, CA) as a carrier of a concentrated NaCl solution and of cetylpyridinium chloride (ICN Biomedicals Inc., Costa Mesa, CA) during an overnight incubation. The precipitate was collected and re-solubilized in 2M calcium chloride. The latter dissociated the cetylpyridinium chloride molecules from the formed GAGs. The GAGs were isolated by precipitation in ethanol:ether (9:1 vol/vol) solution in the cold by centrifugation. The supernatant was discarded and the precipitate was re-solubilized in distilled water. Scintillation fluid was added (Hydroluma by 'LUMAC', Baanstraat 115-117, 6372AE Landgraaf, The Netherlands) and radioactive counts were measured in aliquots by a liquid scintillation counter (Downer Packard Tri-Carb, model 3380, Packard Grove, IL).^{20,21}



Figure 1. Epiphyses grown for 10 days in the joint-simulating device coalesced and formed three-dimensional cartilaginous tissue (x 6).

Statistical Analysis

The non-parametric Mann-Whitney Test (CSS, StatSoft Inc., Tulsa, OK) was used to compare the different groups since the variables were not normally distributed.

RESULTS

Comparative histological examination of the epiphyses grown under the two different conditions, regular static culture and joint-simulating device (JSD), yielded the following findings: Epiphyses in the JSD coalesced to form a three-D cartilaginous construct (Figure 1) with bridges of perichondral tissue interconnecting the individual epiphyses.

This newly formed tissue was found to be highly cellular and fibrous in nature (Figure 2, a & b). It was shown to express fibroblast growth factor receptor 3 (FGFR3) uniformly as determined by immunohis-

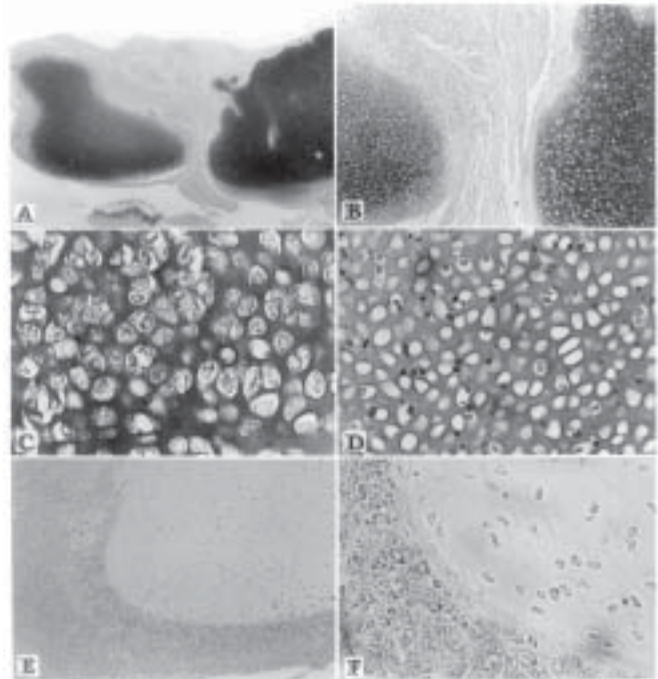


Figure 2. (a) Newly formed tissue bridges the gaps between the epiphyses (alcian blue, x 40). (b) The bridging tissue is highly cellular and has a fibrous-like appearance (alcian blue, x 100). (c) Cartilage grown in the joint simulator—the cells are uniformly viable even in the deepest layers (alcian blue, X 400). (d) Epiphyseal cartilage grown in a regular tissue culture—the peripheral zones appear viable, the cores however contain diffuse areas of cellular necrosis. Empty lacunae and cellular debris are ubiquitous (alcian blue, X 100). (e) The neo-cartilaginous tissue surrounding the epiphyses contains FGFR3 positive cells (anti-FGFR3 1:100, DAB detection, x 100). (f) Same as (e), magnification X 400.

tochemical techniques (Figure 2, e & f) while epiphyses grown in tissue culture dishes did not express FGFR3. Additional parameters indicated high rates of cellular proliferation as assessed by PCNA staining. Areas of active proliferation, staining positive with PCNA, were limited to the newly formed inter-epiphyseal connecting tissues. The epiphyses grown in the JSD appeared uniformly viable both superficially and in the depth of the three-D construct. The cells were found to be intact, seated within typical lacunae with a viable nucleus, abundant cytoplasm and surrounded by rich characteristic extra-cellular matrix heavily stained with alcian blue (Figure 2c). Sections of the cartilaginous tissue grown in the regular static culture dishes, on the other hand, showed large areas of central necrosis with empty lacunae, pyknotic nuclei and cellular debris scattered all over (Figure 2d). The extra-cellular matrix stained relatively poorly with alcian blue indicating depletion of GAG macromolecules during the culturing period (Figure 2d).

Cellular viability in terms of percentage of viable cells was found to be clearly in favor of the JSD as compared

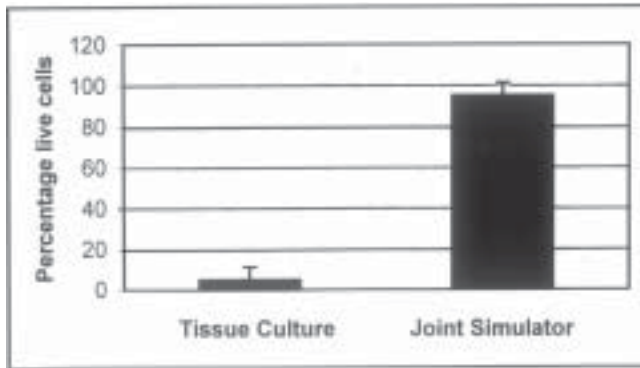


Figure 3. Cellular viability in the joint-simulating device is clearly superior to viability in tissue culture.

to the static tissue culture (Figure 3). A comparative biochemical study was run in parallel on other epiphyseal samples, comparing synthetic activity by ^{35}S incorporation into GAGs for an additional 24 hours after termination of the culturing period. The radioactivity figures obtained from ^{35}S -isolated-GAG molecules showed an elevated rate of sulfate incorporation in the epiphyses grown in the JSD of an order of magnitude tenfold higher than the rate measured in an equal amount of wet epiphyseal tissue grown in traditional static cultures (Table 1). The data revealed a significant higher level of sulfate incorporation with the JSD device (non-parametric Mann-Whitney test, $p < 0.008$).

DISCUSSION

Since the first steps of growing cells in cultures, most findings and landmark achievements were obtained by employing cells in monolayers. Until recent years, culturing technologies failed to support growth and survival of three-dimensional cartilage chunks. Innovative approaches have yielded new perfusion systems that allow for cells and tissues to be maintained ex-vivo for relatively long periods of time,^{13,18,22} even up to 90 days.²³

This new field of tissue engineering is rapidly evolving, and present attempts focus on formation of composite cartilage tissue constructs with features that would make them suitable for in-vivo implantation. They should be inherently stable, big enough to cover up large defects and stiff enough to be cut and shaped as needed. They should not evoke any immunogenic response in the recipient nor transmit any acquired or genetic disease. To achieve that goal, cells are being cultured in conjunction with biomaterials that would act as scaffolds and with a variety of growth factors.^{5,24}

The ideal scaffold has to be both biocompatible and biodegradable as it is gradually being replaced by the cells that synthesize their own inter-cellular milieu or matrix over time.²⁵ Many biomaterials are presently under investigation as potential scaffolds.²²

TABLE 1

Radioactive sulfate ($^{35}\text{SO}_4$) incorporation into isolated GAG molecules by epiphyses grown in a joint-simulating device compared to static tissue culture (CPM/mg wet weight, $n=5$, Mean \pm S.D.).

Group 1 Control (boiled)	Group 2 Tissue culture	Group 3 Joint simulator
23 \pm 9	1201 \pm 561	11299 \pm 2107

Chondrocytes from multiple sites in the body^{26,27} and from various species were tested, including porcine, murine, bovine,²⁸ avian as well as human,^{14,23} and reported results are basically similar: Matrix is gradually being deposited around the scaffold, and the constructs so formed have a macroscopic cartilaginous appearance although they are small in size. The inter-cellular matrix has a high content of both collagen II and glycosaminoglycans.^{13,16,18}

Perfusion systems have been shown to yield constructs that are more cellular and that contain more inter-cellular matrix than those grown by ordinary tissue culture methods. Moreover, they have been found to have better mechanical properties. A major role in explaining these differences is being attributed to the flow of liquids. The application of shear stress in monolayer cultures resulted in overgrowth due to chondrocyte proliferation.²⁹ Modifications of perfusion flow rates and patterns as well as hydrostatic pressures had a major impact on both cellularity and sulfate incorporation by the matrix.^{13-17,30} Vunjak-Novakovic and colleagues compared static culture to turbulent flow and to dynamic laminar flow in rotating vessels simulating microgravity. Constructs subjected to the latter conditions were the largest, contained the highest fractions of GAGs and collagen II in their matrices and had the best mechanical properties.³⁰

Many technical questions still remain unanswered. We need to clarify how to modulate and integrate different physical parameters such as flow rates, flow pulses, reciprocal stream directions, mechanical hydrostatic pressures and shear stresses so that the impact upon the tissue will be optimal, thereby yielding a cartilaginous tissue of better quality.

Most researchers use mature chondrocytes of allogeneic or isogeneic origin and attempt to manipulate them into de-differentiation to obtain a state of rapid growth and abundant matrix formation.

It is our belief that embryonic cartilage is a better cell source for tissue engineering, as it already possesses these qualities inherently.⁵ It may also be harvested in abundance from human embryos originating from miscarriages and planned pregnancy arrests.

In the current study, a self-manufactured perfusion system termed a joint-simulating device (JSD) was uti-

lized. We had previously tested the performance of the JSD with human cartilage of embryonic origin. It has been shown to be effective in keeping cartilage alive and metabolically active for a culture period of 10 days and was found to be highly superior to standard static cultures. The radioactive sulphate incorporation into GAG molecules was elevated tenfold as compared to the static culture.¹⁸ In view of these promising findings, the present study was designed and conducted.

The results presented herein indicate that the JSD provides a better environment for in-vitro growth of chick embryo epiphyseal cartilage. The epiphyses show improved viability. The cells appear metabolically active as demonstrated by GAG production. These biochemical changes are further substantiated by the microscopic findings. The constant perfusion likely prevents central necrosis of the epiphyses.

An interesting observation relates to the coalescence of individual epiphyses into a 3-D macroscopically homogenous cartilage construct. Adherence of epiphyses to one another was apparently the result of specific stimulation of mesenchymal stem cells, which produced de novo tissue in the perfusion chamber of the JSD. These pre-cartilaginous mesenchymal cells originating from the perichondrium region have been previously shown to express fibroblast growth factor receptor 3 (FGFR3)³¹ and have also been demonstrated to play similar roles in bunion formation and exostosis growth.³² It seems that, very much like the situation in the developing embryo, no artificial scaffold is necessary when whole epiphyses are used, provided that satisfactory conditions for growth are available. Not using a scaffold would offer an obvious advantage. An artificial scaffold can be either stable or biodegradable. The former is not desirable, as it would permanently affect the biomechanical properties of the tissues. The latter is potentially toxic when it biodegrades and may evoke a foreign body reaction.

In conclusion, the results reported herein seem to imply that culturing embryonic epiphyses in perfusion chambers encourages formation of neo-cartilage in vitro. This tissue might later be used as implant material for joint reconstruction.³³ It is possible that the future use of epiphyses harvested from embryos would abolish the need for artificial scaffolds and would thus simplify the growing process of 3-D cartilaginous constructs.

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