Early Experience in Chondrocyte Culture for Autologous Transplantation in University Malaya Medical Centre

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ABSTRACT

An articular cartilage lesion of the knee is a therapeutic problem as the capacity of cartilage to heal is very limited[1,2,5]. Autologous chondrocyte transplantation seems to hold much promise as a popular and accepted mode of treatment. The ability to establish chondrocyte culture remains the key in performing this procedure. We examined our technique of harvesting and culturing chondrocyte in rabbits and found that were able to culture more than $5x10^7$ cells on the first passage. This was more than what is recommended for ACT to be successful[1,5]. Further results were examined and reviewed to ascertain the maximum yield for our cultures. This in turn would help us to determine the best method for chondrocyte culture in preparation for ACT. Initial results in our experimental rabbit model involving transplant of cultured chondrocyte into defective areas were also promising.

Keywords: Chondrocyte culture, autologous transplantation (ACT), tissue engineering

INTRODUCTION

Articular cartilage damage is increasingly common due to increased number of high impact injury to the knee.[13] Treatment regimens were aimed at inducing cartilage repair with various surgical based procedures recommended. Among these include lavage, chondral shaving, spongilization, abrasion arthroplasty, subchondral drilling and microfractures.[3] However, results of these methods of treatment were unconvincing. For instance, subarticular drilling was once suggested as the treatment option of choice. This involves creating small holes under the damaged surface using mechanical drills. In doing so it is thought that subarticular bleeding created by drilling would release precursor or mesenchymal stem cells from the marrow allowing regeneration of chondrocyte. It has been postulated that the effect would result in articular surface regeneration. This was later found to be untrue as in-depth examination showed that this technique produced produce only fibrocartilage and therefore do not offer a long-term cure. Mosaicplasty was later introduced as an alternative to subarticular drilling. This technique involves plugging osteochondral plugs harvested form non-articular surface of the knee to defective articular surfaces. Initial results were encouraging but were later found to cause hardening of the articular surface due to its fibrous healing. Graft matching and contouring to the recipient articular surface is also difficult. Donor sites can be a limiting factor. Furthermore, the fibrocartilaginous interface between the donor and recipient site may contribute to breakdown in the long run. This in turn would cause damage to the adjacent surface contact area resulting in early degenerative change to the knee.

Tissue engineering techniques have brought new hope to this problem.[9] Transplantation regimens were later introduced as treatment options. These include the grafting of autologous perichondrial, synovial, or mesenchymal tissue: the implantation of autologous chondrocytes; and the grafting of autologous or allegeneic osteochondral tissue.[12] As available donor sites were insufficient to replace the damage areas, tissue engineering techniques solves this problem by increasing the number of cells during in-vitro cell proliferation. Autologous chondrocyte transplantation (ACT) was thus introduced in the early half of 1990s as a method of treatment promising better results than previous methods and today has been established as an accepted procedure for articular cartilage damage. This technique involves multiple stages of surgery beginning with harvesting minute amount of articular cartilage from the non weight bearing region of the affected knee arthroscopically.[1,2,5] Concurrently, diagnostic arthroscopy can be performed

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to help diagnose other related knee injuries which would otherwise be difficult to diagnose. Retrieved tissue is then processed in culture suites leading to a controlled cell proliferation in an in-vitro environment. After three weeks of culture and maximal yield is achieved (cell counts of 2.6 X 10^6 to 5 X 10^6), open surgery is performed on the same knee with the cultured cells transplanted into the defective area. This technique was first described by Brittenberg and Petterson and has since been accepted as the standard method for ACT.[1,5] ACT is a biological repair process that shows astonishing results and brings promise to a condition once thought incurable.

As in all new technology, cost has been the main issue. ACT is expensive and may not be viable enough for the mass public. In 2001, there were 3300 individuals employed by 73 companies actively engaged in tissue engineering research.[10] During the past 10 years, an estimated USD$3.5 billion has been invested in this sector, with recent expenditures of USD$610 million per year. This annual rate of spending has been increasing by a compounded 16 percent.[9] In the US, the procedure cost ranges from USD$17,000 to USD$38,000, with an average cost of approximately USD$26,000 per procedure. Genzyme Tissue Repair (a commercial tissue engineering company in the US) charges a fee of USD$10,360 dollars for culturing, growing, and shipping these cells.[8, 11]

In Malaysia, this concept of surgery is new and is not readily available. The key to success in ACT is in the cell culture which in Malaysia is the limiting factor. It is our hope that by intiating a chondrocyte culture programme, we would one day be able to provide ACT as a treatment modality to our local population at a fraction of the cost of what current commercial companies would offer for a similar service.

MATERIALS AND METHODS

Part 1: Cell yield from different depot

This experiment was conducted using the articular cartilage from New Zealand white rabbits aged at 24 weeks as a source of chondrocyte. Open surgery were performed bilaterally on the hips, knees and shoulders of the rabbits allowing articular cartilage to be harvested. Harvested tissue is then digested using collagenase for 24 hours. Cells released from tissue are then collected and cultured on to a mixture of DMEM and HAM-12 culture media. Cultures are stored in a CO_2 incubator at 5% concentration. The amounts of cell growth are calculated using a cell counter performed every 2 days. This observation is continued until confluence is reached after which the culture is trypsinized to release cells for further passage. Again cell count is performed on every other day. At the end of the second passage, characterization using histology techniques and immuno chemistry were performed.

Six New Zealand white rabbits were sacrificed for this experiment. Extractions of knee, hip and shoulder chondrocytes (KC, HC and SC respectively) were performed and underwent the aforementioned process.

Part 2: Rabbit knee chondrocyte transplantion model (Pilot model)

Six New Zealand 24 week old White rabbit was used in this part of the study to determine the feasibility of transplanting cultured autologous chondrocyte following the same culture technique as described in the earlier part of this experiment.[5] Open knee surgery was performed on both hind legs of the animal creating a full thickness articular cartilage defect measuring 5mm diameter on the weight bearing area of the medial femoral condyle. The skin incisions were then closed surgically while the harvested cartilage undergoes cell culture as performed in part 1 of the study. After 3 weeks, the right knee of the same rabbit is operated with the cultured tissue transplanted into the defective site as described by Brittenberg and Petterson[1,5]. This rabbit is then sacrificed at the end of 24 weeks with both knees examined macroscopically and microscopically. Immuno chemistry staining was also performed to prove the presence of collagen type II being express in the extracellular matrix

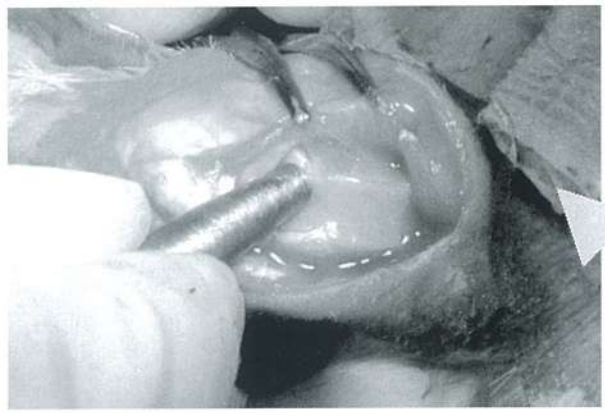

Figure 1. A defect was created on the medial femoral condyle using our custom made cartilage cutter down to the level of the laser mark (note the blue arrow) removing articular cartilage of a depth of less than 5 mm thickness and 5 mm wide.

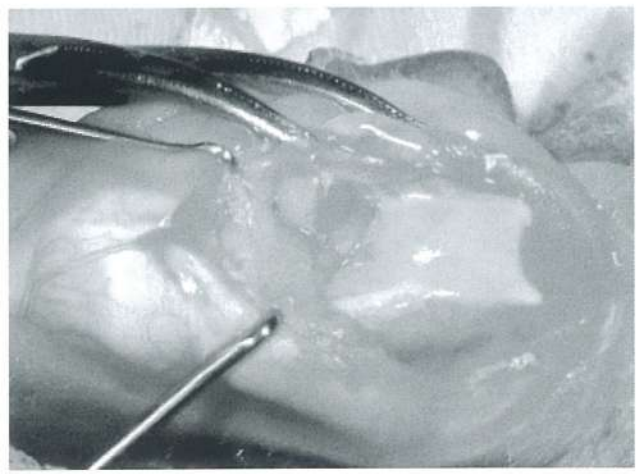

Figure 2. An area of the defect created in the weight bearing area of the femoral condyle.

RESULTS

In part 1 of this study, no significant differences ($p<0.05$) in cell yield were noted in chondrocytes derived from 3 different depots, although mean yield for KCs tended to be highest (Figure 3).

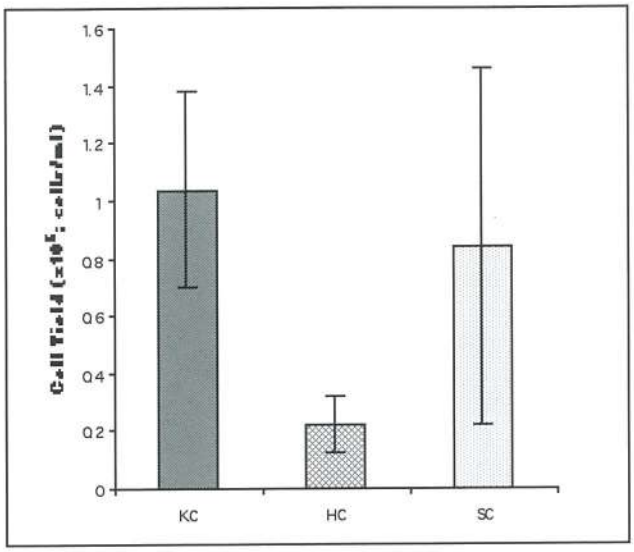

Figure 3. Comparison of chondrocyte cell yield (cells/ml) from 3 different depots; knee, hip and shoulder

We also noted that the highest yield in chondrocyte harvesting was from the knee. Despite good viability following the first passage, P0 (>93%) from all different depots, there is a decreasing trend of viability on passage 2, P1 (Figure 4). However, further testing revealed no statistical significant reduction in cell viability.

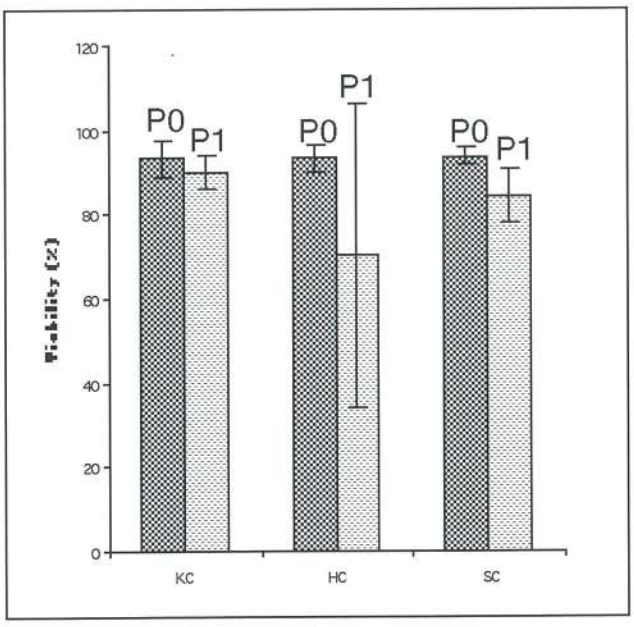

Figure 4. Comparison of chondrocyte viability (%) after serial passaging (P0: after extraction, P1: first passage)

Chondrocyte morphology exhibited much heterogeneity, both within and between cultures, but could be classified largely into 3 types, based on cell shape and polarity. In such monolayer cultures, irrespective of joint source, chondrocytes grew either as confluent sheets, scattered non-confluent cells or aggregated into isolated colonies. In few cultures, chondrocytes formed confluent sheets comprising spindle-shaped/ fibroblast-like, linearly aligned cells with flattened nuclei. In addition, chondrocytes also existed concurrently as diffusely scattered bipolar/ multipolar cells, often exhibiting nuclear degeneration (Figure 5a). However, in several instances, chondrocytes clustered into recognizable isogeneous colonies, varying between 10–35 cells in diameter. In such colonies, component cells were observed to be mainly rounded/ ovoid with prominent rounded nuclei and maintained intimate intercellular contact with their neighbours (Figure 5b). Interestingly, Safranin-O staining was limited mainly to such chondrocyte colonies and not seen in confluent sheets or scattered isolated cells. Intracellular cytoplamic staining was concentrated largely within centres of early colonies (Figure 5c), and Safranin-O positivity was clearly evident in the immediate extracellular space of well-established colonies (Figure 5d). Native articular cartilage controls exhibited Safranin-0 staining in matrix surrounding chondroblasts and was further enhanced in territorial matrix areas related to isogenous chondrocyte groups.

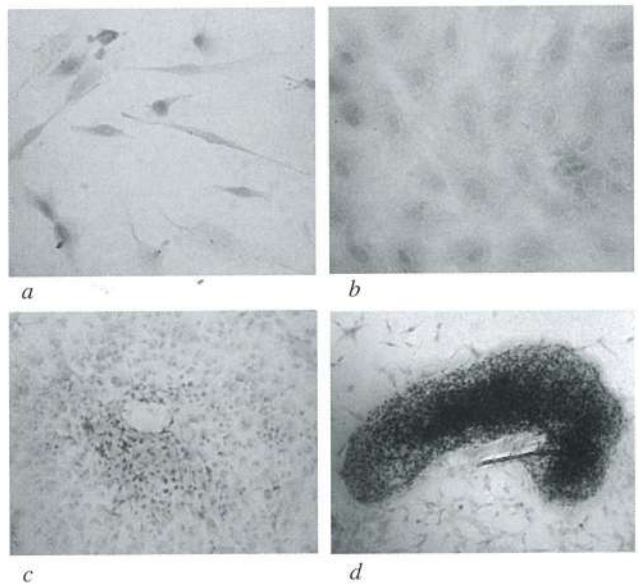

Figure 5. Heterogeneity of chondrocyte morphology and Safranin-O staining demonstrated in monolayer cultures; (a) scattered isolated bipolar (arrow) and multipolar (asterisk) cells ; (b) highly congruent ovoid cells with rounded nuclei / perinuclear 'haloing'(indicating active Golgi apparatus); (c) early colony showing Safranin-O staining restricted to central core (approximately 12 cell diameter area; arrows); (d) established colony strongly expressing Safranin-O into extracellular space (arrows) (note: adjacent negatively stained bipolar/multipolar cells) [Final magnification: x400 (a, b); x100 (c,d)]

In part 2 of this study, 6 rabbits were sacrificed at 24 weeks after the reimplantation of the cultured chondrocytes into the artificially created partial thickness cartilage damage of the right knee while the left knee remained untreated. Histological sections made using H&E and Safranin-O staining techniques on both operated knees showed good repair outcomes in the transplanted side. It is worth noting though that the although the chondrocyte dispersion in the transplanted model did not show as good

a morphology as the native cartilage, the heavy staining of Safranin-O at the extra cellular matrices of the transplanted site showed good cellular expression of articular cartilage related proteoglycans and glycosaminoglycans. On further examination we also noted that the transplanted area had also lesser articular thickness as compared to the native cartilage as evident in Figure 6. Despite this observation, the functional state of the articular cartilage was not determined although they exhibit the same morphology as what we expect to have in native cartilage

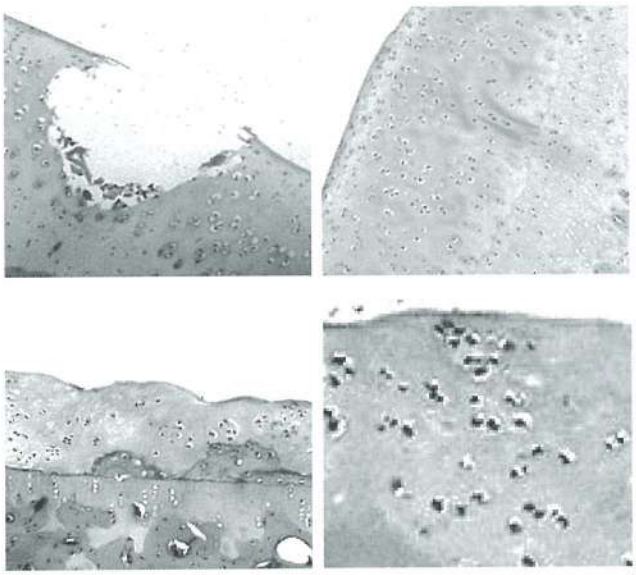

Figure 6. Histological findings of untreated and transplanted knees in one of the rabbits. Note the defect still remains uncovered and no filling of the defect is noted (Above, left). When compared to cartilage engineered with articular chondrocytes (below, left) to native rabbit articular cartilage (above, right), the chondrocytes of the newly formed tissue demonstrated morphological characteristics almost similar to those of native cartilage, with cells located within typical chondrocyte lacunae and surrounded by cartilaginous matrix. (below and right) There was also no significant morphological difference in histological staining of the matrix among the experimental groups (Safranin-O; magnification, _40).

DISCUSSION

In part one of our study, we found that there were slightly higher cell yield from knee (KC) depot as compared to the shoulder (SC) or hips (HC). Optimal viability was noted immediatedly post-extraction from native cartilage and tended to be reduced on passaging. However, it must be stressed in both these studies, sample size was small. Further work would have to be carried out in order to indicate whether allogeneic (such as SCs/HCs) or autologous chondrocytes (KCs) would be most suitable in terms of yield / viability for grafting knee articular cartilage defects in a rabbit model. Other researchers have shown that implanted autologous chondrocytes contributed to better cellular repair in cartilage defects[15, 16].

The heterogeneity of chondrocyte morphology in monolayer culture highlighted the importance of a conducive *in vitro* environment to enable optimal expression of differentiation. Chondrocytes aggregating in colonies, with increased intercellular contact, exhibited a mature phenotype similar to that seen in isogenous chondrocyte groups found in native hyaline cartilage. As colonies comprise dense aggregates of chondrocytes, it is highly likely that homotypic cell-cell interaction/signaling is enhanced, possibly via calcium-dependent adhesion molecules[17]. In addition, many cultures also demonstrated scattered isolated cells of poor morphology, indicating dedifferentiation in a probably sub-optimal environment[17]. Thus, the monolayer culture system allows expression of mature chondrocyte differentiation, albeit limited largely to colonies.

Significantly, synthesis and secretion of Safranin-0 positive extracellular matrix glycosaminoglycans and proteoglycans were strongly correlated with good chondrocyte morphology[18] and were associated largely with established colonies. Staining of chondrocyte matrix with Safranin-0 indicated high glycosaminoglycan and proteoglycan content, key components of cartilage ground substance which contribute to its biomechanical properties of water-retention and flexibility. Hence, this study has demonstrated that chondrocyte function and presumed biomechanical character is largely dependent on attainment of superior chondrocyte morphology associated with colony formation.

In part two of the study, we found that in all our transplanted knees, articular cartilage repair were evident as compared to the untreated knees. The heavy staining of Safranin-O in all transplanted sites signifies the cellular expression of proteoglycan and glycosaminoglycans as exhibited by the native cartilage. Morphologically, these expressions together with cellular characteristics denote the viability of passaged cells and the proof that our cultured autologous chondrocyte transplantion techniques works. It would have been interesting however, if the tissue were functionally tested in its biomechanical properties as to see if the repair site will exhibit the same properties as that of normal cartilage[16] . It has been suggested that biomechanical testing using indentometers would have been the ideal way of providing this information.[5, 16] This would further provide supporting evidence that the repair would also give similar functional properties as seen in the unaffected cartilage. Furthermore, evidence of cellular proliferation and expressions may not be accurate based on mere histological examination.[4] Electron microscopy would have shown a dissimilar result as reported by Mitchell 1980[4] and cellular proliferation would have been better demonstrated using more advanced technique such as proteomics analysis.[6] In view of all these issues, newer research areas are currently undertaken with the additional analysis afore mentioned performed, in hope that further evidence of chondrocyte characteristics and expression following better transplant techniques can be established producing better tissue repair results.

CONCLUSION

Full-thickness articular surface defects are a challenging orthopedic problem. The latest research shows that autologous chondrocyte transplantation is a viable

treatment option yielding acceptable clinical results since the past 9 years.[7] Although clinical application for ACT have been widely available in the west for more than a decade, this treatment option is not readily available in south east Asian countries due to the high cost involved in performing this procedure.[1, 2, 5, 10, 11,12] It has been recognised that the high cost as a result of the number of procedures involved, the specific facility required to culture harvested chondrocytes and rehabilitation involved in patients following surgery has been the leading factor in the slow progress of this treatment this region. It is the hope of our department to further develop this technology which can be used to provide this treatment option to the public at an affordable price thus solving the disparity of treatment option of cartilage damage previously only available in advanced countries. It is therefore prudent that continued, intensive research leading to cheaper production techniques and increased availability must be done to provide valuable information of which clinicians can use in optimizing the treatment for this difficult problem.

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