BioProcessing JOURNAL
TRENDS & DEVELOPMENTS IN BIOPROCESS TECHNOLOGY

Robust and Scalable GMP Manufacturing of Chondrocyte Cell Therapy for Cartilage Regeneration

By Vinayak Virupaksh Kedage, Rajesh Pratap Singh, Parvathi Chandran, Vidya Jadhav, Sujata Shinde, and Satyen Sanghavi

ABSTRACT

Cell therapy has emerged as a promising technology that involves implanting live cells to replace/repair and restore normal function of damaged tissue. Autologous chondrocyte implantation (ACI) has been proven effective for the regeneration of articular cartilage in defective cartilage tissue. The process starts with the collection of healthy tissue from an eligible patient, then isolation and expansion of desired cells *in vitro* under good manufacturing practice (GMP) conditions, qualification before release of the final cell product, and finally, implantation into the patient. The promise to deliver autologous cell therapies has its own challenges in robust and reproducible manufacturing. To commercialize a cell therapy, it is imperative that a robust and scalable manufacturing process is set up that is consistent, in terms of quality and quantity, in order to deliver the intended therapeutic effect.

We analysed the manufacturing parameters of over 100 cartilage samples that were used to deliver our proprietary, commercialized autologous cell therapy. The paper addresses the most cited challenges in the manufacturing of autologous cell therapies and describes a robust process of *in vitro* human chondrocyte cell culture. Also included are key factors in manufacturing for attaining a high-quantity and quality product for articular cartilage regeneration.

INTRODUCTION

Cell Therapy

Cell therapy involves injecting or implanting live cells to replace damaged tissue and restore lost function.[1] Autologous cell therapies make use of the patient's own cells to manufacture the therapeutic product so that there is no risk of rejection or graft-versus-host-disease.[2] The process involves extraction of healthy tissue from the patient, isolation, culture, and expansion of extracted cells *ex vivo* (in a laboratory setting), and implantation of the final cell therapy product into the patient.[3] Autologous cell therapies have been researched for many years, but very few are currently available commercially in the global market.[4]

Cartilage Defects

Cartilage is a connective tissue located in various areas of the body including joints between bones, knees, and ankles, ends of the ribs, between the vertebrae in the spine, and in the ears and nose. Cartilage is categorized into three types: hyaline cartilage, fibrocartilage, and elastic cartilage.[5] A type of hyaline cartilage, articular cartilage, is found between the articulations of synovial joints and acts as a shock absorber, minimizing friction. It provides the mechanical strength in weight-bearing joints but has only a limited ability to regenerate after an injury.

Cartilage defects, specifically osteochondral defects, involve damage in a focal area of the articular cartilage along with the underlying bone. As articular cartilage lines the ends of the bones, treatment of defects in these areas is very challenging. Conservative treatments such as oral medication and pain management are thoroughly tested before considering minimally invasive surgeries.[6] Interventions such as microfracture, drilling, mosaicplasty, and allograft transplantation have been used, but results are variable, depending on lesion size and extent of articular cartilage damage.[7] Regenerative

medicine and tissue engineering techniques have been researched and used as tools for cartilage repair over several decades.[8-11]

Autologous Chondrocyte Implantation for Articular Cartilage Defects

Autologous chondrocyte implantation (ACI) is a cell therapy technique used for the regeneration of articular cartilage.[12-14] For ACI in the knee joint, healthy chondrocytes (cartilage cells) are isolated from cartilage tissue harvested from a nonweight-bearing area of the knee joint. Chondrocytes are isolated, cultured, and expanded *ex vivo* to a defined number of cells (therapeutic dose), which forms a part of the final cell therapy product to be implanted in the defect site.[15]

ACI for deep cartilage defects was first performed in 1987 by Mats Brittberg, and the study results were published in 1994.[16] Several studies have since reported the long-term clinical benefits of ACI for the repair of articular cartilage. One study reported that for large, symptomatic, full-thickness lesions of the distal femur, ACI can result in early improvements that are sustained through follow-up (up to ten years).[17,18] Another study reported significant clinical improvements of ACI and satisfactory implant survival rates over a 20-year monitoring period.[19]

The challenge for a seemingly established, autologous cell therapy technique lies in its scalability and robust, reproducible manufacturing manner that can enable administration to many patients.

Manufacturing Challenges in the Production of Chondrocytes

Several factors contribute to manufacturing a standardized cell therapy product. Some of the challenges that can severely affect the manufacturing process and thereby clinical efficacy have been elucidated below.[20-23]

Variability in Quantity of Starting Material

Starting material represents the most variable part in the manufacturing process of an autologous pipeline and can have an enormous impact in the subsequent downstream steps. Quantity of the 'healthy' cartilage available for tissue harvest can be very minimal and insufficient in patients who have undergone previous cartilage repair surgeries. This means that the initial amount of tissue and consequently the number of cells that can be extracted will be limited. It can be difficult to obtain a high number of chondrocytes from a small portion of cartilage tissue without sacrificing their chondrogenic potential for further expansion in culture.

Variability in Quality of Starting Material

Factors such as age, previous cartilage injuries like anterior cruciate ligament tears, and prior surgical interventions can influence the quality of tissue available for extraction. Mechanical properties of articular cartilage tissue change with age, impacting the functionality. It can be a challenge to manufacture therapeutic cell product from compromised starting material.

Efficiency of Cell Culture Process

The manufacture of chondrocytes for cell therapy consists of several steps in the production line, such as cell isolation from the harvested cartilage tissue, cell expansion under appropriate growth media, cell purification, and final product formulation. Each of these steps are normally standardized and followed in a step-by-step protocol. In case of variable patient-derived cells, the ideal cell manufacturing process must be perfected to achieve the predefined quality target cell profile required for the final cell therapy product.

Complexity of the Supply Chain

Manufacturing processes of cell therapies are quite different from the batch production protocols normally used in the pharmaceutical industry. The final product in autologous cell therapies is manufactured from the patient's own cells, which serves as the starting material. Hence, no two autologous cell therapy products are alike.

The administration of autologous cell therapy products involves a chain of events from extraction of the patient's cells to cultured chondrocyte implantation. The harvested cartilage tissue is usually sent from the hospital to the cell processing facility through a temperature-controlled transport kit. Any variation in the temperature or protocol timelines of the transport kit may affect the quality of the chondrocytes. Sample collection, shipping, and logistics require a high level of synchronicity among various stakeholders. To preserve sample integrity, back-up plans need to be ready in case of delays during any of these steps.

Delivery of the Final Cell Therapy Product

It is possible that the implantation date will be changed due to unforeseen circumstances such as medical staffing availabilities, ineligibility of the patient to undergo a scheduled procedure, or even a pandemic. The final product must be stored under optimum storage conditions so that the cellular therapeutic properties remain intact.

This paper focuses on a proprietary cell therapy product developed by Regrow Biosciences Pvt. Ltd. using ACI for the treatment of cartilage injuries. Autologous adult live cultured chondrocytes, CARTIGROW®, for the treatment

of articular cartilage injuries has been approved for manufacture and commercial sale by the Drug Controller General of India, Central Drugs Standards Control Organization (regulatory body for pharmaceuticals and medical devices) under the Ministry of Family, Health and Welfare, Government of India. A robust and scalable chondrocyte cell culture technique was designed and established to achieve a final cell therapy product at a predefined dose of over 48 million cells (for defect sizes up to 20 cm^2) that can deliver the intended therapeutic effect. This paper describes the cell culture process and supply chain for large-scale manufacturing of autologous chondrocytes.

MATERIALS AND METHODS

Patient Selection

Candidates included otherwise healthy patients in the 18–62 year age group diagnosed with osteochondritis dissecans or osteochondral lesions in the knee, with defect sizes from 2–19 cm^2 in ICRS (International Cartilage Regeneration and Joint Preservation Society) grade III to IV. To further determine cohort eligibility for ACI, blood samples were collected from each patient and then tested for hepatitis B and C viruses, HIV, and syphilis in-house at Regrow Biosciences. Validated rapid test kits were used following manufacturer's protocols.

All 115 patients undergoing treatment provided the required informed consent. This study was approved by the Internal Ethics Committee of Regrow Biosciences Private Limited.

Collection of Cartilage Tissue

Healthy articular cartilage tissues were harvested arthroscopically by 59 orthopedic surgeons across 48 hospitals from 115 eligible patients with cartilage defects (*e.g.*, due to sports injuries, trauma, or osteochondritis dissecans) from June 2017 through February 2020. Tissue consisted of cartilage with subchondral bone from non-weight-bearing regions of the knee. In each procedure, tissue was transferred to a collection vial containing proprietary collection media aseptically.

Transport of Collected Tissue

Cartilage tissue samples were transported under temperature-controlled conditions (2–8°C) from collection sites to the manufacturing facility located in Pune, Maharashtra, India. Care was taken to expedite sample processing within a period of 72 hours from the time of tissue collection. The weight of the harvested tissue and the temperature of the collection kit was recorded upon receipt at the manufacturing facility.

Starting Material Integrity

The samples are kept in quarantine incubators for up to five days, until tested negative for contamination, and then transferred to regular incubators. Cell culturing is performed using media devoid of phenol red due to photo-toxicity. Sterility and mycoplasma testing are carried out at crucial cell culturing stages, especially passage 1 subculture and then an intermediate step before final cell processing. Only those testing negative are further processed.

Manufacturing Process

The manufacturing process was designed according to good manufacturing practices (GMP) regulatory guidelines (Government of India).[24] Manufacturing steps include the assessment of initial samples for culture, cell isolation, cell expansion, cell harvesting for the final cell product, and release of the final cell therapy product based on release criteria specifications. Manufacturing parameters such as sterility, endotoxin, cell viability, cell purity, doubling time, number of days in cell culture, karyotypic analysis of cultured cells with patient's blood were performed for each of the 115 samples. Sterility was tested automatically using the BACTEC™ FX blood culturing instrument (Becton Dickinson). PCR (Veriti, Thermo Fisher)-based mycoplasma testing was performed using Venor® GeM mycoplasma detection kit (Minerva Biolabs). Rapid qualitative endotoxin analysis was performed using Endosafe®-PTS™ (Charles River) kinetic chromatography. Cell purity (for non-viable impurities) was tested using QuantiChrom™ BCG albumin assay kit (BioAssay Systems). Manufacturer protocols were followed for all kits and instrumentation.

In Vitro Culture of Chondrocytes

Bioprocessing and cell culture were performed in a biosafety level-2 (BSL-2), Grade B cleanroom environment. All tissue samples were processed within 72 hours of collection for cell isolation using a standardized procedure. Cartilage tissues were washed with fresh, proprietary buffer 2–3 times and minced before washing 2–3 more times. The minced cartilage tissue was kept overnight at ambient temperature for enzymatic digestion using a proprietary combination of enzymes. After digestion, the isolated chondrocyte cells were filtered with a 40 µm cell strainer (Becton Dickinson) to obtain single-cell suspension.

Initial cell counts were taken by a Vi-CELL automated cell counter (Beckman Coulter) and then cells were seeded in T-25 flasks (Corning) containing proprietary media for chrondrocyte cell culture. The chondrocytes were

passaged, and media was changed until 70–80% cell confluency was achieved within two weeks. Cells were enzymatically harvested using recombinant trypsin (TrypSOL, BioGenomics) and seeded in larger T-150 flasks (Corning) for further expansion in a CO_2 incubator. Media was changed every 48 hours until 70–80% cell confluency was achieved in two weeks or less. Additional cells were grown in treated 30 mm culture dishes (Corning) at ambient temperature. After confluency was reached, cells were stained (1% alcian blue in 3% acetic acid) for immunohistochemistry analysis and observed under 100× magnification as a confirmation of chondrogenic identification. Chondrocytes were enzymatically harvested using a proprietary combination of enzymes, in the same manner as before, and then run at 1300 rpm in a benchtop centrifuge (Thermo Fisher Scientific) for ten minutes. Cell counts and cell viability were measured by an automated cell Coulter Counter (Beckman Coulter) and hemocytometer, respectively.

A chondrocyte suspension of 1×10^5/100 µL was used for cell characterization. Both fluorescein isothiocyanate (FITC)-labeled $CD44^+$ and phycoerythrin (PE)-labeled $CD151^+$ (mouse monoclonal antibodies, Thermo Fisher Scientific) were used for staining cells prior to a 20-minute incubation. Then cells were washed with PBS to remove unbound antibodies and resuspended in fresh PBS. The samples were acquired on a FACSCanto™ II flow cytometry system (BD Biosciences) using a 488 nm blue laser and analyzed with FACSDiva™ software (BD Biosciences).

Analysis

Relative gene expression was detected in chondrocytes at different stages: cell isolation P1 (passage 1), and subculture P2 (passage 2) for type II collagen and aggrecan genes. To confirm that the expanded chondrocytes were true to type without any dedifferentiation, three random samples were selected with RNA extracted at each stage, and subjected to RT-qPCR analysis. The expression of collagen-II and aggrecan genes were analysed. Gene expression levels of chondrocytes cultured at different stages normalized to their respective glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene expression levels (internal control) was measured.

Process Controls

If any of the quality-control or the in-process quality control tests fail, a root cause analysis can be initiated, and corrective and preventive action are performed, if necessary, from the back-up cells.

Transport of Final Cell Therapy Product

Following qualification by release testing criteria, the final cell therapy product was packed in a validated, pre-cooled delivery kit (incorporating biologics data-logging) and transported at 2–8°C within 72 hours of manufacture.

Statistical Analysis

Continuous and quantitative variables were summarized using descriptive statistics and compared using the Student's *t*-test or nonparametric test, as applicable. *p*-values <0.05 were considered significant. Statistical analysis was performed using Prism software version 9 (GraphPad).

RESULTS

In this current study, 115 autologous cartilage tissue samples were harvested from 115 select patients to manufacture cell therapy products for each of their ACI procedures. The challenges faced during the manufacturing process through product delivery were overcome using the techniques described in the following sections.

Effect of Variability in the Starting Material

The mean weight of 115 harvested cartilage tissues was 214.83 ± 143 mg. The lightest and heaviest tissue weights were around 100 mg and 626 mg, respectively. **Figures 1 A–C** show harvested cartilage tissue taken before processing.

Logistics and Supply Chain

It was imperative that a very stringent supply chain process was in place to avoid any mishaps. For this study, an effective supply chain meant stringent coordination and open communication between the collection sites,

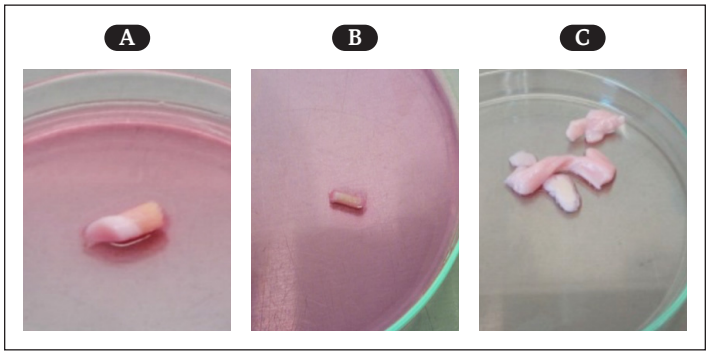


FIGURE 1.
(A) Optimum weight of harvested tissue (250 mg);
(B) the lowest weight harvested tissue (100 mg); and
(C) loose bodies (582 mg).

in-house operations, logistics groups, and cell processing teams. **Figure 2** outlines the needle-to-needle process flow.

Process Verifications

Primary cell culture and passage 1 sub-culture processes involved cell count and cell viability checks to ensure optimum numbers for seeding density of 3000–5000 cells per/cm² of flask area of live cells. Cell characterization, karyotyping analysis, and immunohistochemistry analyses using alcian blue staining were performed to confirm that the final cell therapy product contained highly specialized chondrocyte cells necessary for therapeutic efficacy.

The alcian blue dye was used to stain the proteoglycan components of the extracellular matrix, acidic polysaccharides (*e.g.*, glycosaminoglycans in cartilage cells) to aid in chondrocyte identification (**Figure 3**).[25] Cell line characterization and marker expression analysis was performed to verify purity of the cell population and the presence of the desired phenotype over two passages. Irrespective of the weight of harvested cartilage tissue or initial cell yield, the final cell count, cell viability, and cell characterization analyzed via cell surface marker expression of CD44⁺/CD151⁺ (shown in **Figure 4**) was optimum.

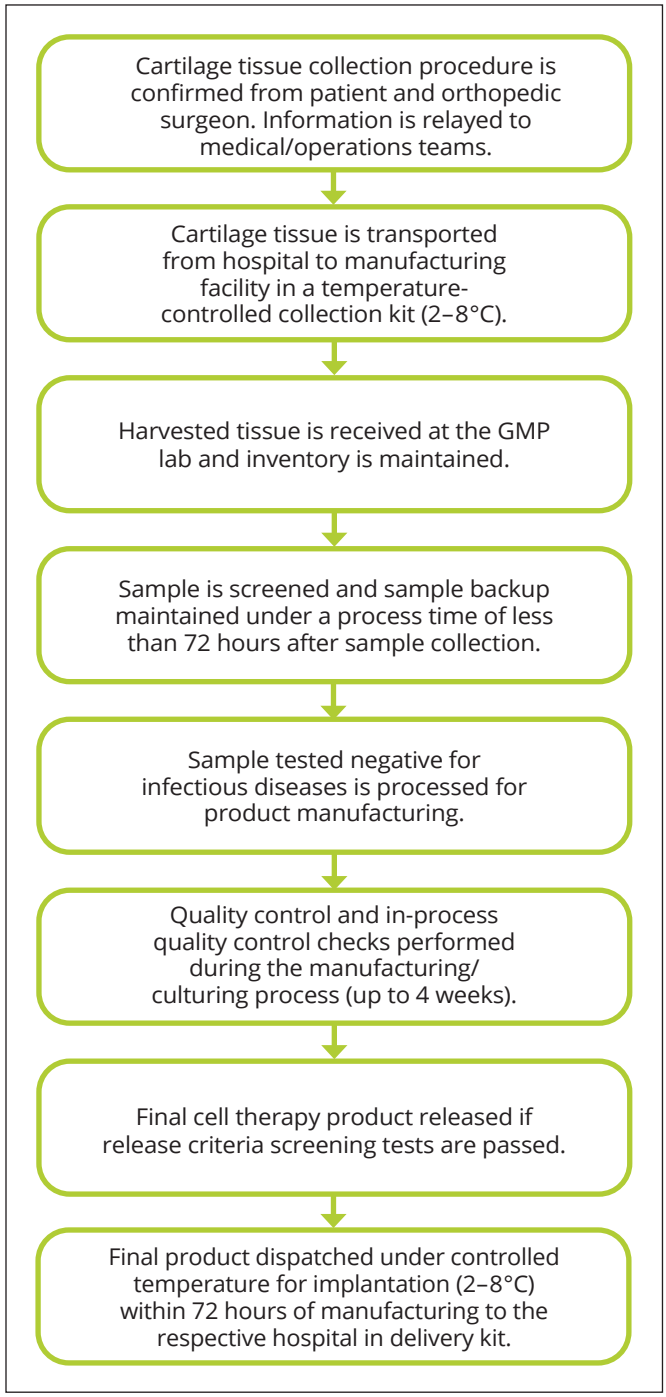


FIGURE 2. The sequence of steps involved in the logistics and supply chain, from collection of cartilage tissue to implantation procedure.

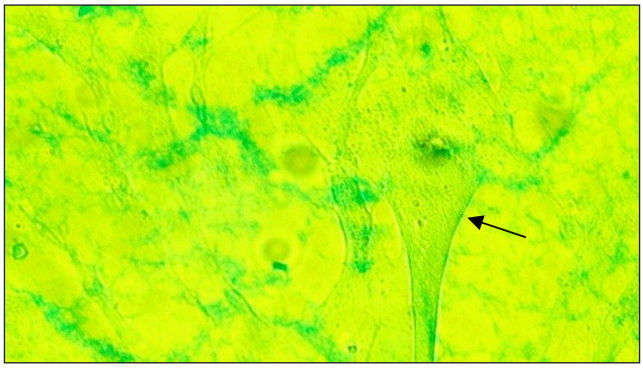
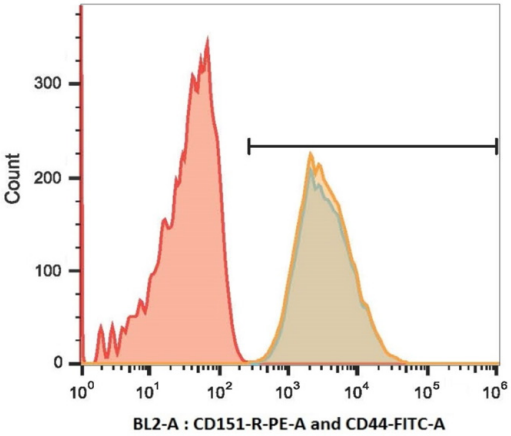


FIGURE 3. Alcian staining of chondrocyte cells for identification. The arrow shows stained proteoglycan as an extracellular matrix. (100x magnification)



Sample Name	Subset Name	Count	Frequency of Total	Frequency of Parent
STAIN 1.fcs	CD151-R-PE-A	8,768	87.7%	99.9%
STAIN 1.fcs	CD44-FITC-A	7,969	79.7%	90.8%
UNSTAIN 1.fcs	Lymphocytes	20,585	86.8%	86.8%

FIGURE 4. Cell surface marker expression of CD44⁺ and CD151⁺.

Production Results

Table 1 shows the cell culture parameter values obtained in the isolation and manufacturing phases using different amounts of collected cartilage tissue. The initial subculture cell counts was dependent upon the amount of starting material. The average initial cell count was 1143 ± 466 cells/mg. The smallest amount of starting material (cartilage tissue) yielded an initial cell count of 2.92×10^5, and the largest cartilage harvest yielded a relatively higher initial cell count of 4.78×10^5. As mentioned earlier, further cell processing and final cell product manufacturing was dependent on the initial cell count following cell expansion. There were instances where the quantity of starting material was minimal, so additional growth factors were added to the medium and critical quality control process parameters were monitored.

Whenever the quality of harvested cartilage tissue was compromised and contained fibrous cartilage or loose bodies (fragments of cartilage that float freely in the knee joint, as seen in **Figure 1C**), modified growth media was used to eliminate further dedifferentiation and in-process quality control parameters were also monitored carefully.

The mean age of the autologous donor patients was 34 years, and the youngest and oldest patients ranged from 18 to 62 years, respectively. With such an age variance, one would have expected to see differences in the quality of the isolated cell population affecting the final cell therapy product. However, our cell culture process proved to be robust, with no drastic variations in cell parameters. Cartilage tissue from the youngest patient yielded an initial cell count of 3×10^5, while the oldest patient yielded a comparable cell count of 4.1×10^5 (**Table 2**).

Analysis

Figure 5 shows the RT-qPCR results of relative gene expression with type II collagen and aggrecan at different culture stages. This validation test used three random samples. There was minimal variation in the gene expression and chondrocytes at different stages such as isolation, and P1 and P2 subcultures. Thus, the developed GMP manufacturing process yielded true-to-type of chondrocytes with no dedifferentiation of the cells during *in vitro* expansion.

These parameters formed necessary specifications for

TABLE 1. Cell culture parameters of chondrocytes cultured from different amounts of harvested cartilage tissue.

ISOLATION PHASE			MANUFACTURING PHASE						
Size of Collected Cartilage Tissue	Initial Cell Count ($\times 10^5$)	Final Cell Count ($\times 10^6$)	Doubling Time (hours)	Number of Days Cultured	Cell Characterization (%)	Cell Purity (g/dL)	Cell Viability (%)	Endotoxin (EU/mL)	Karyotyping
100 mg*	2.92	52.18	90.20	25	95.2	0.135	96.13	0.75	No chromosomal abnormalities
626 mg**	4.78	72.02	86.98	30	94.6	0.239	97.38		

*The smallest amount of tissue harvested **The largest amount of tissue harvested

TABLE 2. Cell culture parameters of chondrocytes cultured from cartilage tissue samples of young and old patients.

ISOLATION PHASE			MANUFACTURING PHASE						
Age of Patient (years)	Initial Cell Count ($\times 10^5$)	Final Cell Count ($\times 10^6$)	Doubling Time (hours)	Number of Days Cultured	Cell Characterization (%)	Cell Purity (g/dL)	Cell Viability (%)	Endotoxin (EU/mL)	Karyotyping
18*	3.04	94.86	85.44	27	99.3	0.327	97.26	0.75	No chromosomal abnormalities
62**	4.10	98.89	89.38	25	98.2	0.327	96.96		

*The youngest patient **The oldest patient

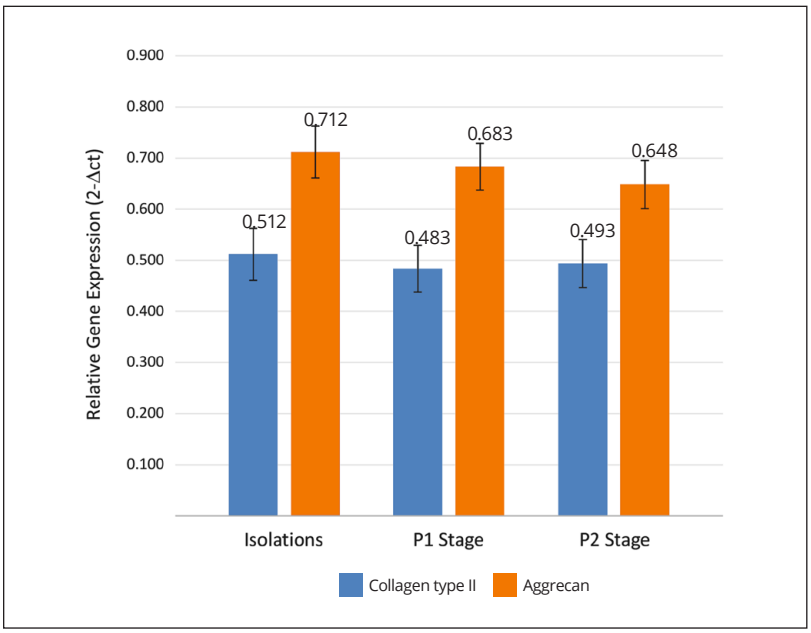


FIGURE 5. Relative RT-qPCR analysis of chondrogenic gene expression in different stages of chondrocyte cell culture.

the release criteria that was met before release of the final product (**Table 3**). Of note, reference and sample backups of cells were maintained at cell isolation and final harvest stages, and as an extra measure, at the P1 sub-culture stage.

Efficacy of the Cell Culture Process

The average final cell count of 115 samples cultured through our optimized cell culture process was determined to be $72.47 \pm 15.41 \times 10^6$ chondrocytes. The growth kinetics of all the chondrocyte samples were studied to determine cell doubling times. Of those 115 samples, the mean *ex vivo* doubling time was 83.25 ± 5.8 hours (mean ± SD), comparable to the doubling time of chondrocytes cited in literature for monolayer cultures, which was 1.7–3.5 days.[25]

TABLE 3. Release criteria for the final autologous chondrocyte cell therapy product.

PARAMETER	SPECIFICATION
Cell Number	Over 12×10^6 cells per vial
Cell Viability	≥80% dye-excluding cells
Microbial Sterility	Negative/no growth
Endotoxin	<3 EU/mL
Mycoplasma	Negative
Cell Purity Test	<1 g/dL serum albumin
Cell Characterization	≥80% $CD44^+/CD151^+$ expression (FACS)
Karyotypic Analysis	No chromosomal abnormalities

Delivery of Final Cell Therapy Product

Because unforeseen circumstances can potentially delay the implantation procedure, our proprietary cryopreservation medium allows the maintenance of cell viability and stability for as much as two years. Depending on seeding densities ranging from 0.5–4 million cells, the chondrocyte cells are cryopreserved at two stages, P1 and in the final cell processing phase. **Figures 6A** and **B** show a pre-labeled borosilicate glass vial (with patient ID and hospital details) containing the final cell product. The stainless steel enclosure (**Figure 6C**) ensures the vial's integrity during handling and transportation at 2–8°C.

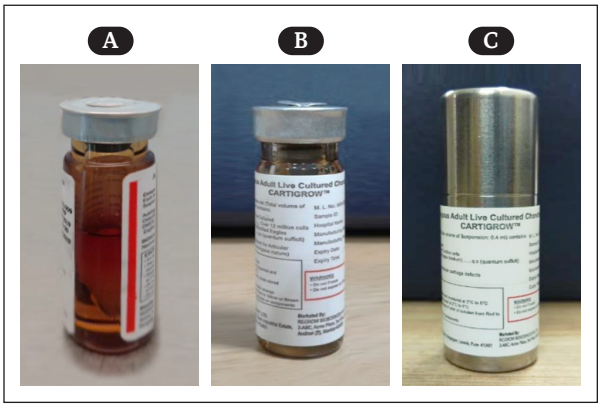


FIGURE 6. (A) V-vial showing the suspended CARTIGROW® product (autologous adult, live-cultured chondrocytes); **(B)** front view of the labeled V-vial; and **(C)** protective V-vial canister shield for transport purposes.

ACI Treatment Outcomes

Improvements in functional activity and pain reduction were assessed for all patients at pre- and post-operative consultations. Patients were evaluated using the Lysholm knee scoring and visual analog scales for acute and chronic pain post-treatment.

Based on pre- and post-operative data, the 115 manufactured cartilage cell therapy products that were implanted in patients with knee defects showed significant treatment efficacy. Pain reduction or elimination is a good indicator of *in vivo* cartilage regeneration (data not shown).

DISCUSSION

Autologous cell therapies are the new wave of medicine and are taking center-stage as the mode of treatment for many diseases. For these

therapies to deliver their full transformative potential, it is necessary to develop large-scale manufacturing capabilities. ACI for cartilage defects has been around since the 1980s. However, there is huge variability among studies with respect to selection of biopsy, chondrocyte culture, and corresponding therapeutic outcomes. There are many manufacturing challenges that are generally considered to impede commercial viability. Factors such as donor-to-donor variability, cell culture process parameters, logistics, and supply chain have been tackled to make CARTIGROW® production a robust and scalable process. Our process has been successfully used for 115 patients to facilitate cartilage regeneration in the knee.

The quantity and quality of the starting material is absolutely critical to the production of the final cell product. However, our culture process, along with the addition of proprietary growth factors, has been proven to support the expansion of variable starting materials, without any compromise in the final product's therapeutic potential. A key factor we have focused on is minimizing the number of chondrocyte passages in the manufacturing process, which ensures that the chondrogenic potential of the cells is not compromised. Longer cell culture times and multiple passages may fail to provide therapeutic efficacy, due to the loss of chondrogenic potential and increased probability to dedifferentiate.[26, 27] Important quality control and in-process quality control tests ensure that the quality of the cells being processed is maintained through all crucial stages of manufacturing. Cell growth and characterization parameters are highly important for homogeneous suspension in the final cell product. Strictly regulated and critically monitored release criteria, at the end of manufacturing and at the time of product release, ensures that a high-quality cell product that is safe and effective is implanted. The study shows favorable treatment efficacy after implantation of 115 manufactured chondrocyte products in patients with knee cartilage defects.

The production and delivery of personalized cell therapies involves a complex supply chain and logistics plan, from extraction of the cells from the patient to the implantation of the cells. It is possible to have delays in the process due to multiple stakeholders and process steps. We were able to deliver the final product (live-cultured chondrocytes) in a sterile container formulated in a medium that facilitates and imparts a decent shelf-life of 72 hours, from the end of the manufacturing process to actual patient implantation. Anticipating a worst-case scenario, it is very important to keep necessary sample back-ups at crucial stages during isolation and subculture stages so that the patient does not have to undergo a second procedure of cartilage extraction.

Cell therapies are proven to transform patient's lives and will be the mode of curative therapy for a variety of diseases in the near future. Our manufacturing process sets a standard for commercial capability, given the therapeutic success of the patients involved in this study.

CONCLUSION

We have designed a robust manufacturing process for the CARTIGROW® cell therapy product using autologous chondrocytes specifically for ACI. It is scalable for commercial purposes and has been proven successful in regrowing cartilage for over 100 patients with cartilage defects of the knee.

DISCLOSURE

Satyen Sanghavi is one of the promoters of the company, whereas Dr. Vinayak Virupaksh Kedage, Rajesh Pratap Singh, Parvathi Chandran, and Vidya Jadhav are employed by the company.

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ABOUT THE AUTHORS

Vinayak Virupaksh Kedage[1] is Director of Laboratory; Rajesh Pratap Singh[1] is Senior Manager of Cell Therapy; **Parvathi Chandran**[2]* is Manager of Research, Strategy and Communication; Vidya Jadhav[1] is Assistant Manager of Cell Therapy; Sujata Shinde[1] is Assistant Manager, Quality Control; and Satyen Sanghavi[2] is Chief Scientific Officer.

1. Regrow Biosciences Pvt Ltd, 22 Shah Industrial Estate, Nangargaon, Lonavala – 410401, Maharashtra, India
2. Regrow Biosciences Pvt Ltd, 2 ABC ACME Plaza, Andheri Kurla Road, Andheri (East), Mumbai – 400059, Maharashtra, India

Corresponding Author:
Email: parvathi.c@regrow.in | Phone: +91 -7045938934