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Optimization of culture media replenishment regimens for cartilage tissue engineering

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OPTIMIZATION OF CULTURE MEDIA REPLENISHMENT REGIMENS FOR CARTILAGE TISSUE ENGINEERING

by

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No pain, no gain.

How many roads must a man walk down, before you can call him a man.
Bob Dylan (from *Blowin' in the wind*)

Life was like a box of chocolates. You never know what you're gonna get.
from *Forrest Gump*

Dedicated to my loved family and my great country.

Acknowledgments

I am now on this page after one and a half year's research journey in Albro Lab. It's a difficult and a very special journey, especially because of the COVID-19. So, at the very beginning, I really appreciate all the assistance and support from the Boston University community. Notably, the BU Healthway. It's your hard work in the back that guarantees everything goes on stably this year. Thank you for keeping everyone healthy and safe.

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OPTIMIZATION OF CULTURE MEDIA REPLENISHMENT REGIMENS FOR CARTILAGE TISSUE ENGINEERING

YANLI LYU

ABSTRACT

Cartilage tissue engineering (TE) is a promising osteoarthritis therapy whereby cell-seeded constructs are generated *in vitro* for use in restoring degenerated cartilage in patients. While cartilage TE technology has exhibited growing clinical success, it continues to be encumbered by the utilization of high cost and laborious protocols, such as the need for frequent replenishment of culture media (every other day) during the duration of standard *in vitro* cultivation phases (2-8 weeks). This constitutes a significant time/cost burden for researchers and clinical technicians. Interestingly, the adoption of this convention is based on traditional cell culture protocols, rather than on a fundamental understanding of the stability of culture media constituents in current cartilage TE culture systems, leading one to consider that current TE replenishment protocols may be far from optimized. In the current study, we hypothesize that larger media volumes can be used to: 1) mitigate the depletion of constituents and accumulation of waste products in tissue constructs over time and accordingly, 2) reduce the media replenishment frequency required to generate engineered cartilage with functional mechanical properties and composition.

Bovine chondrocyte-seeded agarose constructs (Ø4mm×2mm) were cultivated for 7 weeks in chondrogenic media of increasing cumulative media volumes (3mL, 6mL, 9mL, 18mL, and 54mL) and replenishment frequencies, including the conventionally utilized thrice-weekly and lowered frequencies of weekly, biweekly, and replenishment-free. The stability of influential media constituents (glucose, ascorbic acid, insulin), waste product accumulation (assessed via pH), and the properties of constructs were monitored over time.

Results demonstrated that concentrations of growth-promoting media constituents and pH decreased over culture duration but this decrease can be mitigated by the use of larger replenished media volumes. For all replenishment frequencies, tissue construct mechanical properties and sulfate glycosaminoglycan (sGAG) content generally increased with replenished media volumes. For weekly, biweekly, and replenishment-free frequencies, the generation of constructs with native properties required the higher replenished media volumes per replenishment but did not require the use of higher cumulative media volumes. These results suggest that functional engineered cartilage can be generated with lower media replenishment frequencies or replenishment-free conditions. These protocols may be adopted in clinical and research-grade TE platforms to reduce labor costs and contamination risk.

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List of Abbreviations

AAP	.....	Ascorbic Acid
CM	.....	Chondrogenic Media
DMEM	.....	Dulbecco's Modified Eagle Media
DMMB	.....	1,9-Dimethylmethylene Blue
ECM	.....	Extracellular Matrix
ELISA	.....	Enzyme-Linked Immunosorbent Assay
FBS	.....	Fetal Bovine Serum
GAG	.....	Glycosaminoglycan
GLU	.....	Glucose
IGF	.....	Insulin Growth Factor
INS	.....	Insulin
ITS	.....	Insulin-Transferrin-Selenite
MOPS	.....	Missouri Osteochondral Preservation System
MSC	.....	Mesenchymal Stem Cell
OA	.....	Osteoarthritis
PBS	.....	Phosphate-Bufffered Saline
PG	.....	Proteoglycan
RPMI	.....	Roswell Park Memorial Institute
TE	.....	Tissue Engineering
TGF-β	.....	Transforming Growth Factor-beta

Chapter 1

Introduction

Osteoarthritis (OA) is a degenerative joint disorder associated with the degeneration of articular cartilage, leading to pain and debilitation. OA predominantly afflicts the weight-bearing joints (e.g., knees, hips, and spine). OA affects over 32.5 million adult patients in the US and incidence rates are increasing. Thus, therapies to treat OA are urgently needed.

1.1 Articular Cartilage and OA Therapy - Cartilage Tissue Engineering (TE)

Articular cartilage is a type of hyaline cartilage that can bear high mechanical loads. Articular cartilage predominantly consists of chondrocytes and extracellular matrix (ECM). The ECM is majorly constituted by water, proteoglycans (PGs), and collagens [1, 2, 3]. The predominant PG type in articular cartilage is aggrecan[1], which consists of a core protein which is covalently tethered to a large number of negatively charged sulfate glycosaminoglycans (sGAGs) side chains. Charge-to-charge repulsion and the domestic swelling pressure induced by GAG provide cartilage with its high capacity to support compressive loads [1]. In addition, collagens, a superfamily of fibrous proteins, abundantly exist in the cartilage ECM [4, 5]. Collagen fibrils enable cartilage to bear tensile loads and maintain the tissue structure [1, 4].

Cartilage tissue engineering (TE) is an emerging OA treatment technology whereby cells are encapsulated in polymer scaffolds to generate new cartilage replacement tissues. The cartilage TE process involves four integral steps: cell isolation, tissue construct fab-

rication, *in vitro* tissue cultivation, and surgical implantation (Fig. 1·1)[6]. Autologous chondrocytes can be isolated from the residual healthy regions of a patient's articular cartilage[7]. Further, mesenchymal stem cells (MSCs) can be isolated and primed for chondrogenic differentiation[8, 9]. Isolated cells are then mixed with a polymeric scaffold, forming the engineered cartilage tissue construct. Cartilage tissue constructs are cultivated *in vitro* in an anabolic media formulation for a two to eight weeks maturation stage. The goal of this stage is to initiate the generation of a functional ECM that can support physiologic mechanical loads. Lastly, the mature cartilage tissue constructs will be surgically implanted into the patient to replace the damaged cartilage tissue, which will ultimately repair the OA. The work of this thesis focuses on the development of protocols for *in vitro* maturation of engineered cartilage tissues.

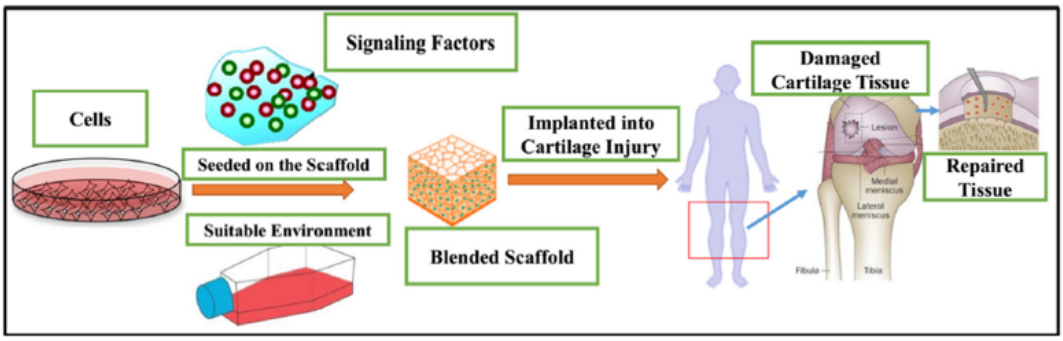


Figure 1·1: Four steps of cartilage tissue engineering. Cells are isolated from a source tissue and embedded into the scaffold. After *in vitro* culture in a suitable environment, the mature constructs will be implanted into lesion sites for tissue repairment. Reprinted by permission from: [Springer] [Bio-Design and Manufacturing] [Ansari, M., Eshghanmalek, M. Biomaterials for repair and regeneration of the cartilage tissue. Bio-des. Manuf. 2, 41–49 (2019). https://doi.org/10.1007/s42242-018-0031-0 (Biomaterials for repair and regeneration of the cartilage tissue, Mojtaba Ansari, Mahdi Eshghanmalek), [COPYRIGHT] (2018)

1.2 *In vitro* Cultivation in Cartilage Tissue Engineering

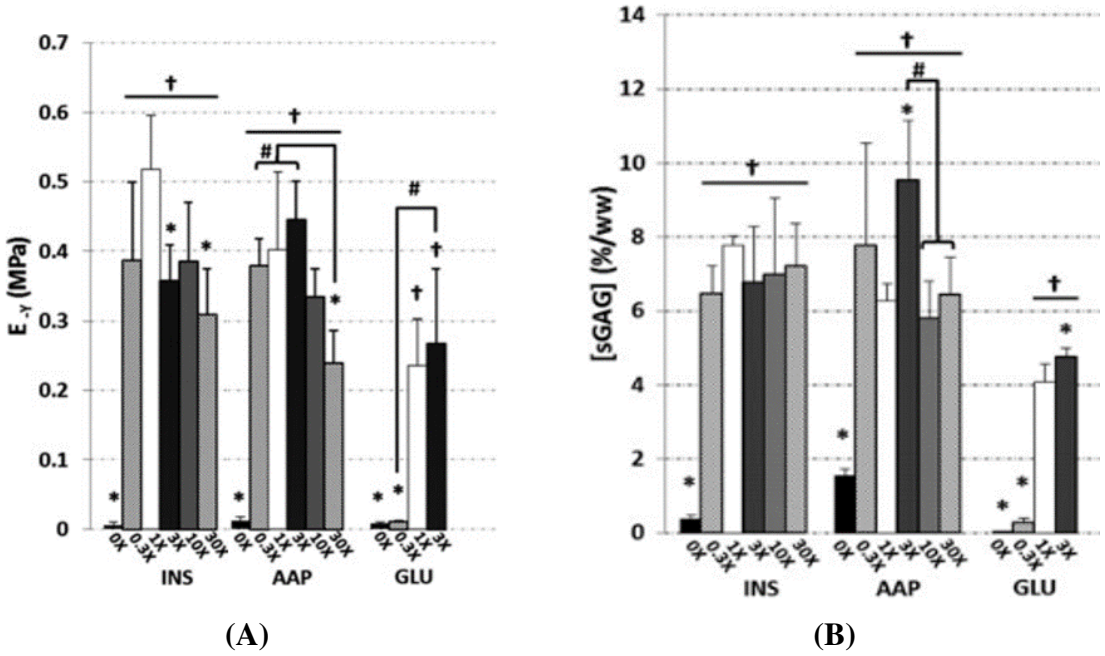
1.2.1 Chondrogenic Media (CM) in Cartilage Tissue Engineering

Tissue culture is a process by which cellularized tissues are cultivated *in vitro* under conditions that attempt to recapitulate the native environment. The composition of culture media is one of the critical factors that influence this process. Since Sydney Ringer[10] first developed a media to successfully perform *in vitro* culture in 1882, significant efforts have been undertaken to improve media formulations (a detailed outline of this timeline can be found in Appendix 1). Today, there are numerous standardized media formulations, including Dulbecco's Modified Eagle Media (DMEM)[11], Ham media[12], Roswell Park Memorial Institute (RPMI) media [13], and others [10]. Each standardized media consists of supplies of metabolic sources (e.g., glucose, pyruvate), vitamins (e.g., ascorbic acid (vitamin-C), vitamin-B), amino acids, and hormones (e.g., insulin) to support cellular processes [14].

The culture media in cartilage TE has been developed accordingly. Traditionally, media for cartilage TE was supplemented with fetal bovine serum (FBS)[15] in order to provide an extensive array of biomolecules needed to promote tissue growth. However, serum-based formulations have encountered limited success in cartilage TE, as marked by the generation of tissues with sub-native mechanical properties and biochemical composition. A major advance in the past decade has been the development of chondrogenic media (CM) formulations that improve chondrogenesis and cartilaginous ECM biosynthesis. In 2005, Kisiday *et al.*[16] reported that a supplement consisting of insulin-transferrin-selenite (ITS) could serve as a suitable substitute for serum, promoting the progress of the development of the serum-free media in cartilage TE. Since then, the supplementation of ITS, combined with other growth mediating constituents (ascorbic acid, dexamethasone, and TGF-β)[17] has served as the foundation for a serum-free, chemically-defined CM formulation to be applied in cartilage TE protocols. Importantly, in 2008, Byers *et al.*[18] demonstrated that with short-term, transient exposure to TGF-β3 in a serum-free, chemically-defined CM,

constructs can achieve mechanical and biochemical properties that match native cartilage tissues in only 2 months of culture.

Since then, the serum-free, chemically-defined CM has been applied extensively to cartilage TE investigations. A complete list of constituents in CM can be found in Appendix B and Appendix C.



(A) **(B)**

Figure 1·2: (A) Young's modulus (E_Y) and (B) sulfate GAG (sGAG) content of engineered tissue constructs after 42 days of culture in response to varying concentrations of insulin (INS), ascorbic acid (AAP), and glucose (GLU). Concentrations are relative to conventional doses (1×) present in standard CM formulation.

The successful generation of engineered cartilage with functional properties is dependent on the presence of sufficient levels of an array of key constituents in the CM. Cigan *et al.*[19] established the influential role of several key CM constituents towards tissue growth. His works established that the presence of insulin and ascorbic acid was essential, but their concentrations could be administered at levels as low as 30% of conventional doses without detriment to construct development. Moreover, the presence of glucose was similarly es-

sential and reduction of glucose to 30% of the conventional dose was detrimental to tissue growth (Fig. 1·2 A B). The work of Cigan *et al.*[19] established that while several CM constituents are critical for engineered cartilage development, they can potentially undergo partial depletion during culture without detriment to tissue formation.

Another consideration for engineered cartilage growth outcomes is the presence of cell generated metabolism waste products in culture media. Cell secreted waste products can impact cell viability and biological processes. Waste products include CO_2 and lactate. Lactate, is a product of glucose metabolism; specifically, it is the end product of anaerobic glycolysis [20]. Increased lactate accumulation will lead to a lower pH, which can be harmful to engineered cartilage growth. While CO_2 exhibits a degree of diffusion out of conditioned media, lactic acid will accumulate in the media and needs to be periodically removed. Therefore, the waste accumulation-induced problems in the culture media in *in vitro* cultivation is another crucial and fundamental issue that needs to be considered.

1.2.2 Media Replenishment Method in Cartilage Tissue Engineering

While a significant amount of work had been conducted on the development of growth-promoting media formulations, far fewer efforts have been undertaken towards the optimization of media replenishment protocols. The need for the periodic replenishment of culture media is fundamental to cell/tissue culture applications, serving to restore cell-depleted nutrients (e.g., glucose, vitamins, amino acids), restore degraded signaling molecules (e.g., TGF-β, insulin), and clear cell-secreted waste products (e.g., lactic acid). In cartilage TE, the conventional paradigm to meet this need is to subject tissue constructs to relatively low volumes of media but replenish the media frequently—often daily or every other day. This convention has been adopted by both research-based and clinical TE platforms. Huang *et al.* [21] describes the culture media replenishment methodology for clinical tissue-engineered cartilage products (Table 1.1). Frequent media replenishment protocols constitute a significant time/cost burden for researchers and clinical technicians and may be

associated with an increased tissue contamination risk.

Table 1.1: Culture media replenishment methodology of clinical cartilage tissue engineering products. Culture duration is the total days spend on *in vitro* cultivation.

Product name	*In vitro* culture duration	Media replenishment protocol
CaReS®	10-13 days	Every three days[22]
Hyalograft®	2 weeks	Twice a week[21]
NeoCart®	3 -5 weeks	Culture apparatus (Robotics)[23]
RevaFlex™	More than 40 days	Every 3 – 4 days [24]

Interestingly, the adoption of this convention is based on traditional cell culture protocols, rather than on a fundamental understanding of the intrinsic stability and cell consumption rates of culture media constituents in TE tissue engineering systems. As such, it is worth considering that the current TE replenishment protocols may be far from optimized and that it may be possible to generate functional engineered cartilage with larger media volumes and less frequent media replenishment or even with replenishment-free culture systems. In particular, the use of serum-free and chemically-defined CM formulations serves as a unique opportunity to optimize media replenishment protocols based on the dosage needs of the tissue constructs and stability of specific molecular constituents in the media formulation.

Chapter 2

Study

2.1 Introduction

Over the past several decades, significant advances in cartilage tissue engineering(TE) have been made, including the progress of the serum-free and chemically-defined chondrogenic media (CM), mentioned in the previous chapter. Before the development of the serum-free and chemically-defined CM, because the supplemented serum contains many unknown constituents, it is difficult to entirely research on the exact development of every specific constituent in the culture media during the cultivation. Therefore, the requirement for the periodic media replenishment of the traditional serum-contained culture media is fundamental to cultivation, serving to restore cell-depleted nutrients (e.g., glucose, vitamins, amino acids), restore degraded signaling molecules (e.g., TGF-β, insulin), and clear cell-secreted waste products (e.g., lactic acid). This frequent and periodic media replenishment protocol continues to be encumbered by the utilization of high cost and laborious protocols. This constitutes a significant time/cost burden for researchers and clinical technicians. However, in current cartilage TE, the progression of the serum-free and chemically-defined CM provides the unique opportunities to investigate and monitor every constituent in the CM during the cultivation and over time. Thus, generally, the objective of the current study is to optimize media replenishment regimens for cartilage tissue engineering to generate the engineered cartilage with native level of functional properties with the serum-free, chemically defined chondrogenic media. We hypothesize that larger replenished media volumes can be used to: 1) mitigate the depletion of constituents and accumulation of waste prod-

ucts in tissue constructs over time, and accordingly. 2) reduce the media replenishment frequency required to generate engineered cartilage with functional mechanical properties and composition. Specifically, this thesis assessed the influence of different cumulative media volumes (3mL, 6mL, 9mL, 18mL, and 54mL) and various media replenishment frequencies (thrice-weekly (TW), weekly (W), biweekly (BW), replenishment-free (RF)) in total six weeks cultivation, and eventually find out an optimized media replenishment regimen.

2.2 Materials and Methods

Constructs were cultivated in CM with varying replenishment regimens for six weeks and assessed for mechanical properties, biochemical contents (sulfate GAG (sGAG), DNA contents), and cell viability. Constituents in the culture media, including glucose, insulin, ascorbic acid, and pH, were measured over time for different groups with and without tissue.

Cell Isolation and Tissue Constructs Fabrication

Chondrocytes were isolated from immature bovine cartilage (3-6 months). In brief, cartilage was harvested from bovine carpometacarpal joints and digested via 1000 U/ml collagenase (Type IV) at 37°C overnight. The chondrocyte suspension was filtered, centrifuged at 1000 g for 15 minutes, and resuspended in CM. Chondrocytes were seeded in 2mm thick slabs in 2% agarose at a density of 30×10^6 cells/mL. Cylindrical tissue constructs were generated via a biopsy punch at Ø4mm× 2mm.

Tissue Cultivation

The tissue constructs were cultivated at 37°C in CM, consisting of high glucose DMEM supplemented with 50 μg/mL L-proline, 1% PS/AM antibiotic-antimycotic, 100nM dexamethasone, 100 μg/mL sodium pyruvate, 50 μg/ml ascorbic acid 2-phosphate and 1% ITS premix (6.25 μg/mL insulin, 6.25 μg/ml transferrin, 6.25 ng/mL selenite acid, 1.25

mg/mL bovine serum albumin, 5.35 mg/ mL linoleic acid). The tissue constructs were exposed to 10 ng/mL active TGF-β3 for the first week of culture and maintained in TGF-β3 free media for the following six weeks. This transient TGF-β administration induces high ECM biosynthesis rates, as shown previously [17]. During the post-TGF-β administration 6-week culture phase, constructs were cultivated in culture media that was replenished thrice-weekly (conventional frequency), weekly, biweekly, or un-replenished (replenishment-free). For each replenishment frequency, constructs were exposed to either 3mL, 6mL, 9mL, 18mL, or 54mL of cumulative media volume administered over the entire 6-week culture duration. The volume of media administered for each group at each replenishment point is indicated in Table 2.1. The relationship between the cumulative media volume and replenished media volume is shown in Equation 2.1.

$$\text{Cumulative media volume} = \text{Replenished media volume} \times \text{Numbers of media replenishment} \tag{2.1}$$

Table 2.1: Engineered cartilage construct media supplementation protocols vary with respect to media replenishment frequency (thrice weekly [conventional], weekly, biweekly, and replenishment-free) and the cumulative media volume administered per construct over the entire 6-week cultivation period (3mL, 6mL, 9mL, 18mL [conventional], and 54mL). Values in brackets indicates replenished media volume administered per construct at each media replenishment point. Green highlight represents media supplementation regimen that is conventionally used in most TE protocols.

Cumulative Media Volume Administered Over Entire Cultivation / Media Replenishment Frequency	3mL	6mL	9mL	18mL	54mL
Thrice-Weekly (TW)	TW-3mL [0.17mL]	TW-6mL [0.33mL]	TW-9mL [0.5mL]	TW-18mL [1mL]	-
Weekly (W)	W-3mL [0.5mL]	W-6mL [1mL]	W-9mL [1.5mL]	W-18mL [3mL]	W-54mL [9mL]
Biweekly (BW)	BW-3mL [1mL]	BW-6mL [2mL]	BW-9mL [3mL]	BW-18mL [6mL]	BW-54mL [18mL]
Replenishment-Free (RF)	RF-3mL [3mL]	RF-6mL [6mL]	RF-9mL [9mL]	RF-18mL [18mL]	RF-54mL [54mL]

Media Constituents Measurements

The stability of insulin, glucose, and ascorbic acid in CM over time in culture was assessed. Assessments were performed in conditioned media for all culture groups and for media in the absence of constructs. Aliquots from conditioned media were collected once a week. Insulin was measured by a human insulin enzyme-linked immunosorbent assay (ELISA) (R&D Systems). Glucose was measured by the Amplex Red Glucose/Glucose Oxidase Assay Kit (Invitrogen). Ascorbic acid was dephosphorylated by phosphatase (Sigma) and then measured via a fluorometric assay, as described by Vislisel *et al.*[25]. Lastly, pH was monitored over time (Mettler Toledo) as an assessment of lactic acid waste product generation.

Mechanical Testing

The constructs' Young's modulus (E_Y) was measured via a customized mechanical testing system. In brief, constructs were maintained in phosphate-buffered saline (PBS) and subjected to unconfined compression with a nonporous platen, consisting of a 10% platen-to-platen strain at a rate of 1 μm/s. After 15 minutes of stress relaxation, Young's Modulus was calculated using the equilibrium reaction load and the cross-sectional disk area.

Biochemical Analysis

After the mechanical testing, constructs were weighed and digested with 0.5 mg/ml proteinase-K at 56°C overnight for biochemical analysis. Sulfate GAG (sGAG), and DNA content were assessed by 1,9-dimethylmethylene blue (DMMB) dye-binding assay and PicoGreen dsDNA quantitation, respectively.

Confocal Cell Live/Dead Quantification

Tissue constructs were diametrically sectioned (around 100 μm thick) and stained by cell viability (LIVE/DEAD Viability/Cytotoxicity Kit, Invitrogen) and imaged with confocal microscopy (Olympus FV3000) to reflect the distribution of viable chondrocytes. CellProfiler (Broad Institute of MIT and Harvard) was used to quantify the cell viability

in the periphery. The quantification area is 1 mm × 1 mm at the corner of the construct. Quantification was performed for the thrice weekly and replenishment-free groups.

2.3 Results

Media Constituents Measurements

In the absence of cartilage tissues, glucose and ascorbic acid (Fig. 2·1 A C) were relatively stable in the chondrogenic media (CM) over 42 days. In contrast, the insulin concentration decayed to 60% of initial levels after 28 days (Fig. 2·1 B), indicating a limited intrinsic stability of the insulin protein. For low media volumes in the presence of constructs (3mL per construct), glucose levels exhibited a pronounced reduction, decreasing to 38% of initial levels after 28 days — this decay can be attributed to cellular consumption of glucose. pH levels also decayed from 7.6 to 6.9 over this 28-day period, attributed to the cell - mediated secretion of lactic acid (Fig. 2·1 D). Insulin decrease was more pronounced with constructs for low media volumes, decaying to 20% of initial levels after 28 days (Fig. 2·1 B), indicating insulin loss in media can be partially attributed to cell activity in addition to its intrinsic instability. Higher media volumes mitigated glucose, insulin, and pH decreases — glucose decreased to only 80% of initial levels, insulin decreased to only 58% of initial levels, and pH only decreased to 7.4 after 28 days for 18mL per construct media volumes (Fig. 2·1 A B C D). Ascorbic acid exhibited a pronounced decrease at these higher media volumes, however, we did not analyze samples at lower volumes for comparison.

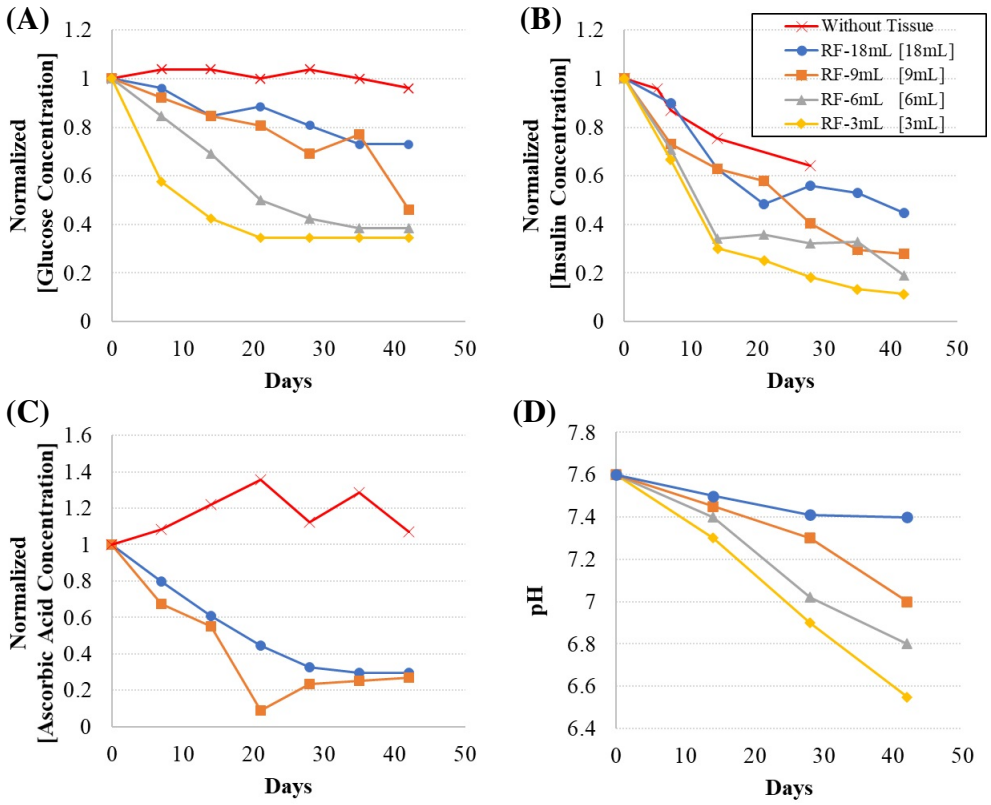


Figure 2·1: Media constituent stability. Concentration of (A) glucose, (B) insulin, (C) ascorbic acid, and (D) pH in conditioned media over time in the presence of constructs with varying media supplementation levels and without constructs. For all groups, constituents' stability is monitored under replenishment-free conditions for the entire 42 days culture duration.

Construct Properties in Response to Varying Replenishment Regimens

In an initial study, construct cell numbers and mechanical properties were measured at day 42 after culture with conventional low replenished media volumes at each supplementation point (1mL and 3mL) with varying media replenishment frequencies to highlight the importance of media replenishment for these replenished media volumes. It is important to note that the cumulative media volume administered to constructs over the 42-day culture duration increases with replenishment frequency (e.g., 18mL for Thrice-Weekly versus 6mL for Weekly versus 3mL for Biweekly). Construct DNA and mechanical properties exhibited a trend of decreasing with decreasing replenishment frequency, as anticipated. (Fig.

2·2 A B).

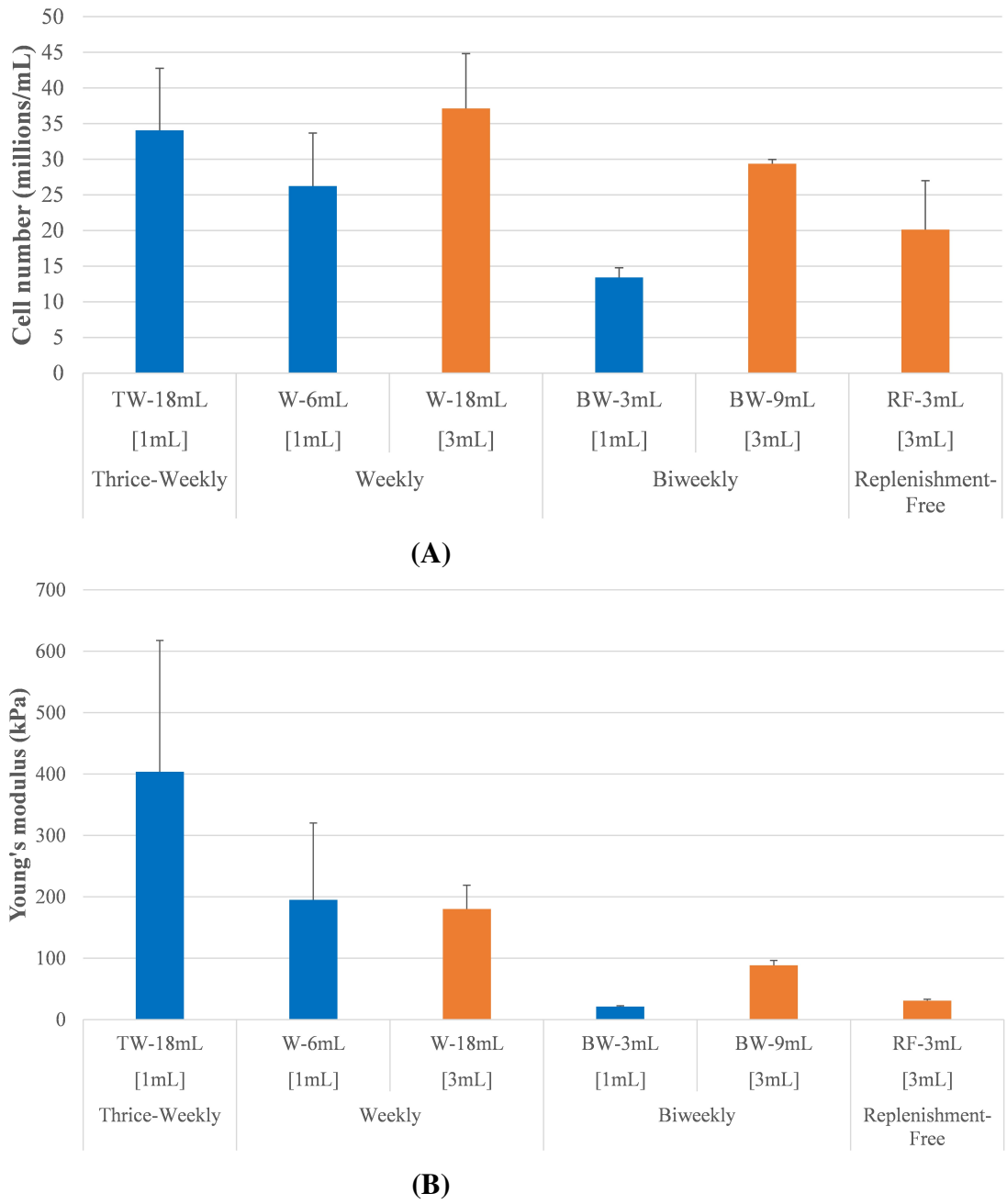


Figure 2·2: (A) Cell number and (B) Young's modulus (E_Y) of constructs after 42 days of culture with low replenished media volumes (1mL and 3mL) to tissue constructs and varying replenishment frequencies. Values in brackets indicates replenished media volume administered per construct at each replenishment point.

In the next study, construct outcomes were assessed in response to varying media replenishment frequencies but with the larger replenished media volumes, designed to match or exceed the cumulative media volume over the culture duration that is administered during the conventional replenishment regimen (18mL per construct over 42 days).

Here, we observed that for the conventional replenishment frequency (TW), the administration of conventional cumulative media volumes achieved constructs that approached native E_Y and sGAG content after 42 days. With reduced cumulative media volumes, TW replenishment induced a reduction in tissue properties. For less frequent replenishment groups (W, BW, RF), construct E_Y, sGAG, and DNA (cell numbers) generally increased with cumulative media volume administration. Constructs reached native E_Y properties for groups of W-54mL, BW-18mL, BW-54mL, RF-18mL, RF-54mL. Importantly, these results demonstrate that engineered cartilage with native properties can be generated using lower replenishment frequencies, provided that one administers the same level of total volume of media over the culture duration. Using this methodology, one can generate native-property-matched cartilage even under replenishment-free culture conditions.

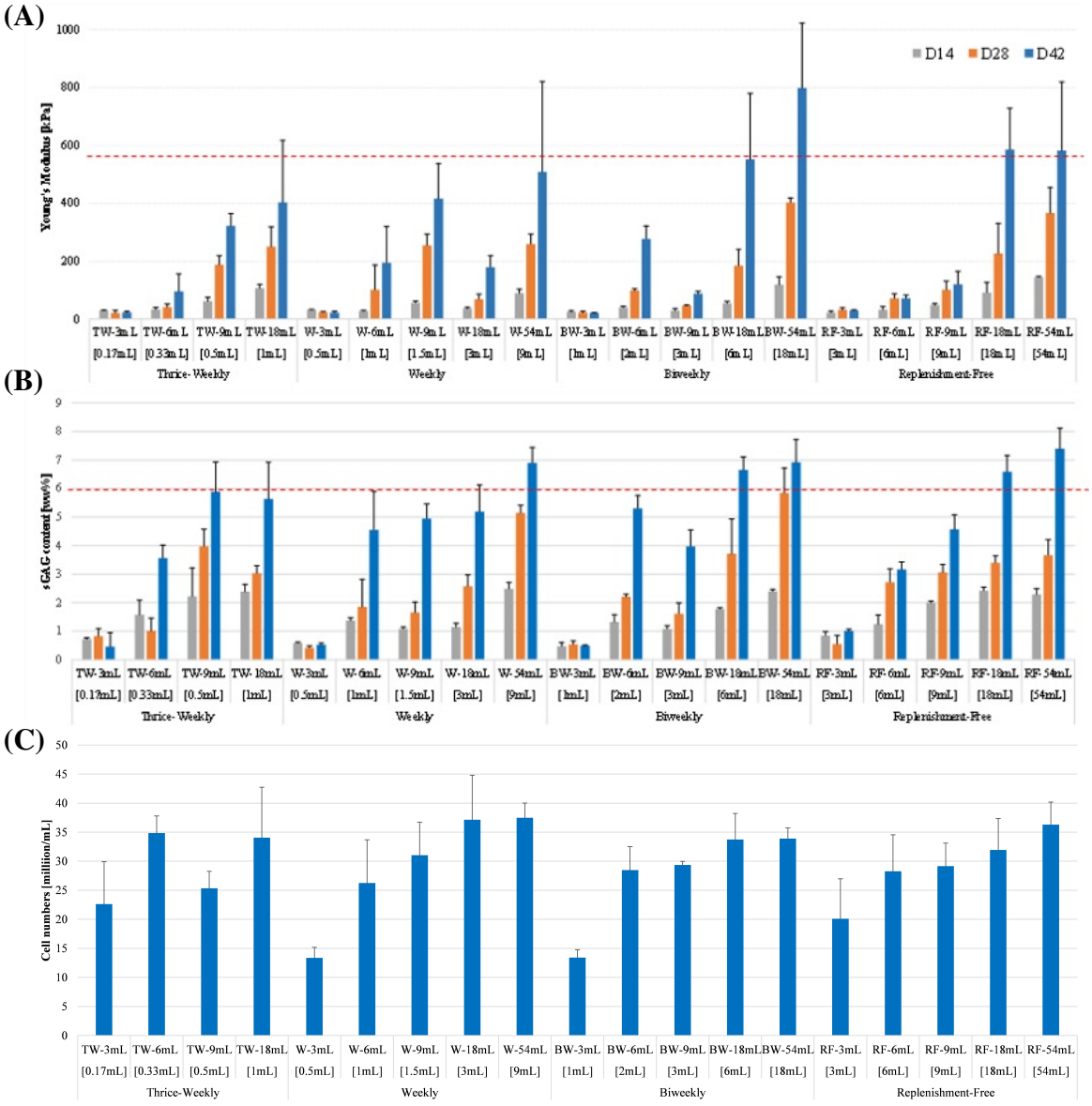


Figure 2·3: Mechanical and biochemical properties of constructs. (A) Compressive Young's modulus (E_Y) and (B) sGAG content of constructs at day 14, day 28, and day 42 for thrice-weekly (TW), weekly (W), biweekly (BW), and replenishment-free (RF) media replenishment groups. Value in group name indicates cumulative media volume administered per construct over the entire culture duration. Value in brackets indicates replenished media volume administered per construct at each replenishment point. Red horizontal dashed line shows the mechanical property and sGAG content of the native articular cartilage. (C) Cell number of constructs at day 42.

Cell Live/Dead Quantification

The periphery cell viability showed significant cell death in the periphery both in thrice-weekly media replenishment (TW-3mL) and replenishment-free (RF-3mL) groups (Fig. 2·4 A B), which were two groups with the lowest cumulative media volume. However, when the administered cumulative media volumes increase to 6mL or higher, no cell viability loss is observed (Fig. 2·4 A B). The cell viability is consistent with the DNA content measures (Fig. 2·2 C). Cell viability loss was most prominent in the cell peripheral regions (Fig. 2·3 C E). As such, the corner area is adopted for cell viability quantifications.

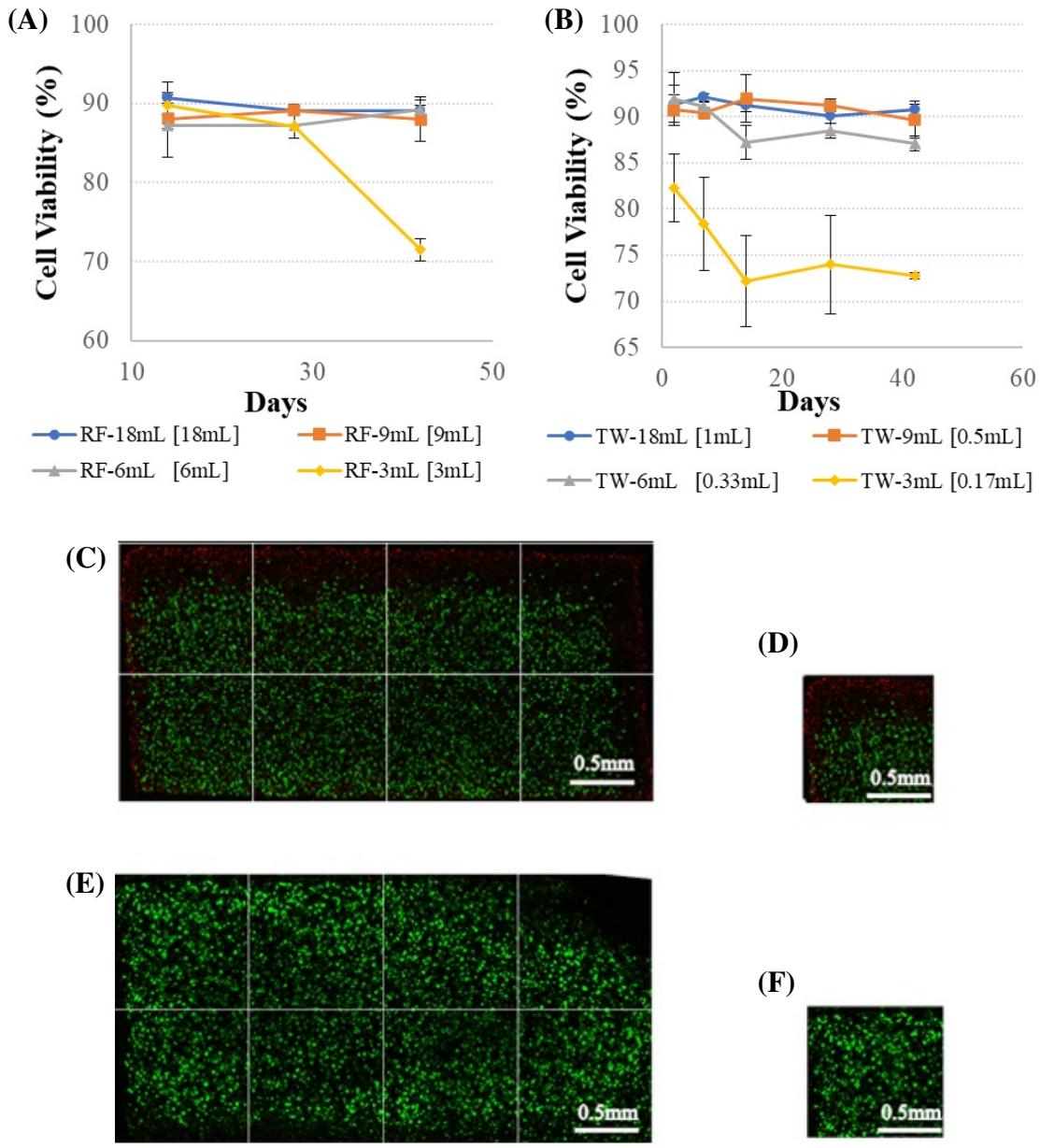


Figure 2·4: Cell viability quantification. (A) Periphery cell viability quantification for thrice-weekly media replenishment groups with different cumulative media volumes (n = 3). (B) Periphery cell viability quantification for replenishment-free groups with different cumulative media volumes (n = 3). (C) Cell live/dead image for a whole construct (with cell death). (E) Cell live/dead image for a whole construct (without significant cell death). (D & F) The quantification area is the top left square of figures C and E (size is 1mm×1mm). Red dots indicate the dead cells and green dots represent the live cells.

2.4 Discussion

While the field of cartilage tissue engineering (TE) has progressed considerably over the past 25 years, few efforts have been undertaken to optimize media replenishment protocols. The results of this work support that the generation of functional cartilage tissue specimens can be achieved using media replenishment frequencies that are far lower than those conventionally adopted, provided that sufficient volumes of media are administered.

Prior work and the initial experimental characterization of this study emphasize the importance of media supply for cartilage TE outcomes. Here it is confirmed that for the administration of conventional replenished volume of media (1mL) and low replenished volume (3mL) to constructs, thrice weekly media replenishment protocols are indeed necessary to generate engineered cartilage with functional properties. For these low replenished volumes, a reduction to weekly replenishment frequency leads to a 50% reduction in construct E_Y (Fig. 2·3 B). The need for frequent media replenishment for conventional low replenished media volumes (1mL) is unsurprising, and consistent with prior investigations[18].

In consideration of this established constraint, the principal hypothesis of the current project was that the required frequency of media replenishment can be reduced through the administration of larger replenished volumes of media to tissue constructs. Through the parametric investigation of the influence of variations to media volumes and replenishment frequencies on the material properties and composition of engineering cartilage, this hypothesis has been validated. Here is it shown that by increasing the replenished media volume administered at each replenishment point, a native cartilage E_Y can be reached in constructs for weekly or bi-weekly replenishment frequencies or for entire replenishment-free culture regiment (Fig. 2·3 A). Importantly, while a reduction in replenishment frequency requires the administration of higher volumes of media at each replenishment point, the total volume of media administered over the course of the culture duration does not increase.

Essentially, a cultivation protocol of "front-loading" the media administration at the start of culture performs equally well to the conventional use of frequent media replenishments.

The emergence of serum-free, chemically-defined chondrogenic media formulations for cartilage TE provides a unique opportunity to develop optimizations for culture protocols. Historically, the need for frequent replenishment protocols (daily or every-other-day) was established for cell culture protocols using serum, a biologic supplement that consists of thousands of potentially influential nutrients and signaling molecules. The identification of the numerous key growth-promoting molecules in serum and the characterization of their stability in TE applications would constitute a significant challenge. In contrast, chemically-defined chondrogenic media is composed of only a few dozen nutrients and signaling molecules—as such, the identification of their intrinsic stability and consumption rates for media replenishment optimization becomes a far more feasible endeavor. To this end, in the current study, the stability of several chondrogenic media constituents is monitored. The monitoring of insulin, ascorbic acid, and glucose were selected based on their established importance for engineered cartilage growth[19]. Interestingly, the stability of these mediators is dependent on their mechanism of degradation in tissue culture systems. While glucose exhibits long-term stability in cell-free culture media at 37°C, it can undergo accelerated depletion as a result of cell consumption (Fig. 2·1 A). In contrast, insulin levels undergo depletion even in the absence of live cells, likely due to the characteristic intrinsic instability of the protein at 37°C (Fig. 2·1 B). The supply of consumption-limited mediators, such as glucose, can be enhanced by simply increasing the replenished media volume (Fig. 2·1 A B C). However, as results demonstrate, increasing the replenished media volume has a limited effect on mitigating the depletion of mediators with intrinsic stability limitations, such as insulin (Fig. 2·1 B). The successful use of replenishment-free culture platforms in this TE system suggests that although insulin exhibits an exponential decay during culture, levels remain sufficiently high over time to promote high ECM deposition

rates. In the future, if required, the stability of proteins with intrinsic instability limitations can potentially be enhanced via biomaterial delivery platforms[26].

The development of replenishment-reduced or replenishment-free cultivation protocols can potentially be transformative for the field of cartilage tissue engineering. As currently constituted, thrice-weekly replenishment protocols represent a significant labor burden for lab-based researchers and clinical technicians. It further adds a contamination risk, which inherently exists in any sterile tissue manipulation protocol. In recent years, tissue banks have made efforts to move away from replenishment-based cultivation strategies. The Missouri Osteochondral Preservation System (MOPS)[27, 28] represents an example of replenishment-free live tissue maintenance for osteochondral allograft preservation. However, similar platforms have yet to be designed for cartilage TE cultivation platforms. The results of this study support the feasibility of such replenishment-free cultivation approaches. In the future, replenishment protocols will need to be optimized for specific TE systems, which will vary with respect to cell source, cell density, and media composition.

Appendix A

Development of Cell Culture Media

Table A.1: Development of cell culture media

Period/start time	Period name	Representative events
1882-1907	Dawn of the cultivation experiments	Sydney Ringer invented a balanced salt solution with a composition close to that of bodily fluids to keep a frog's heart beating after removal from the body. [29]
1907	Use of natural media	Ross G. Harrison successfully monitored an apparent outgrowth of frog nerve fibers for several weeks in lymph fluid that had been freshly drawn from the lymph sacs of an adult frog.

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Table A.1 – continued from previous page

Period/Start time	Period Name	Representative Events
1911	Endeavors toward synthetic media	Margaret R. Lewis and Warren H. Lewis (1911) demonstrated that the Locke-Lewis solution- which is a modified Locke's solution with added amino acids, bouillon, and glucose (or maltose) – is more effective for chick embryo cell cultivation than simple, balanced salt solutions. [30]
1940	Birth of established cell lines	Earle *et al.*[31] successfully create immortal mouse fibroblasts (L cells), revealing that proliferation from a single cell is possible.
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Table A.1 – continued from previous page

Period/Start time	Period Name	Representative Events
1946	stablishment of basal media and research into protein-free media	To avoid the risk of unknown components in the Baker's media (especially unknown proteins), two main strategies were undertaken. The first strategy uses the media with unknown components, while the second uses defined media. The defined media is comparably efficient in the cultivation of cells relative to the media containing natural ingredients.

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Table A.1 – continued from previous page

Period/Start time	Period Name	Representative Events
1970	Identification of serum-free substitutes and the development of serum-free tailored to a cell type	Larry J. Guilbert and Iscove showed that, besides selenite, a combination of transferrin and albumin is a good serum substitute. [32] Izumi Hayashi and Gordon H. Sato discovered that a combination of several hormones and growth factors is an effective serum substitute. [15]
1970	Improvements to basal media	DMEM/F-12 medium, in which Ham's F12 and DMEM are combined in a 1:1 ratio, occasionally shows better performance when used for certain types of cells.

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Table A.1 – continued from previous page

Period/Start time	Period Name	Representative Events
1978	Medical and industrial applications of animal-cell culture technology	The application of human recombinant insulin in 1982 inspired the world to use culture media for the production of pharmaceuticals.

Appendix B

Constituents in Chondrogenic Media and Their Functions

Table B.1: Constituents in chondrogenic media and their functions

Constituents	**Functions in cell culture media**	**Functions in cartilage tissue engineering**
Glucose	First time isolated from raisins in 1747 by German chemist Andreas Marggraf[33]. As one of the most vital molecules in biochemistry, glucose is the primary fuel source in cell culture.	Glucose is a source of energy and a structural precursor for extracellular matrix production in bone and cartilage. Also, it is an important signaling molecule in articular cartilage and bone[34].

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Constituents	Functions in Cell Culture Media	Functions in Cartilage Tissue Engineering
Hormones (insulin)	Act as a substitution of the undefined component, the serum, in the culture media, a group of hormones including insulin has been determined as a replacement of the serum whose primary role is to provide the hormones[10].	Kisiday *et al.*[16] indicated that insulin (with transferrin and selenium) is suitable as a partial or full substitute for a serum for stimulation of bovine calf chondrocyte division and biosynthesis in 3-D hydrogel culture. Also, there is a relatively more homogeneous accumulation of cartilage-like ECM in insulin (with transferrin and selenium) when compared with the one in FBS (Fetal Bovine Serum) cultured media.

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Constituents	Functions in Cell Culture Media	Functions in Cartilage Tissue Engineering
Amino acids (L-proline, L-glutamine)	Amino acids are the building blocks of proteins and thus are obligatory ingredients of all known cell culture media[14]. The importance of amino acids is further pronounced in chemically defined mammalian cell culture media, making the consideration of their biological and chemical properties necessary. Amino acids are the building blocks of proteins and thus are obligatory ingredients of all known cell culture media[14].	Tao Zhou *et al.*[35] indicated that glutamine is a critical regulator in bone homeostasis via supporting energy as a substitute carbon source through the TCA cycle and providing precursors for protein and nucleic acid synthesis. At the cellular level, glutamine metabolism mediates the bioenergy of bone cells. Paz-Lugo *et al.*[36] stated that increasing glycine in the diet might well be a strategy for helping cartilage regeneration by enhancing collagen synthesis, which could contribute to the treatment and prevention of osteoarthritis.

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Constituents	Functions in Cell Culture Media	Functions in Cartilage Tissue Engineering
Vitamin (Ascorbic acid)	Many vitamins are essential for the growth and proliferation of cells. Vitamins cannot be synthesized in sufficient quantities by cells. Vitamins are most commonly added for growth stimulation.	S. Omata *et al.*[37] elucidated that by increasing Vitamin C (Ascorbic acid) concentration in the culture medium, the modulus of the cultured constructs was significantly increased.
Inorganic salts	Inorganic Salt in the media helps to retain the osmotic balance and helps in regulating membrane potential by providing sodium, potassium, and calcium ions[14].	Similar for all the cell lines.

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Constituents	Functions in Cell Culture Media	Functions in Cartilage Tissue Engineering
Growth factors (TGF-β)	Transforming growth factor-beta (TGF-β) is a superfamily of powerful mediators that can modulate the proliferation differentiation of cells, and maintain the growth and degradation of tissues[14].	Act in small quantities on cells; for example, to induce proliferation, differentiation, migration, secretion, or import. Many cells require supplementation of the medium with growth factors under serum-free conditions[17].
Carrier proteins (BSA, Transferrin)	Bovine Serum Albumin (BSA) is the main protein in blood acting to bind water, salts, free fatty acids, hormones, and vitamins, and transport them between tissues and cells.	–

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Table B.1 – continued from previous page

Constituents	Functions in Cell Culture Media	Functions in Cartilage Tissue Engineering
Trace element (selenium)	Trace elements are often supplemented in serum-free media to replace those normally found in serum. Trace elements like copper, zinc, selenium, and tricarboxylic acid intermediates are chemical elements that are needed in minute amounts for proper cell growth[14]. These micronutrients are essential for many biological processes, such as the maintenance of the functionality of enzymes.	Selenium is always supplemented in the culture media together with insulin and transferrin. Kang *et al.*[38] indicated that selenium is essentially required to induce the proliferation and chondrogenic differentiation of mesenchymal stem cells.

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Table B.1 – continued from previous page

Constituents	Functions in Cell Culture Media	Functions in Cartilage Tissue Engineering
Dexamethasone	McCulloch *et al.*[39]. indicated that dexamethasone is an important regulator of cellular proliferation and differentiation, but paradoxical effects have been noted in a variety of culture systems.	–

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Table B.1 – continued from previous page

Constituents	Functions in Cell Culture Media	Functions in Cartilage Tissue Engineering
Antibiotics	Although not required for cell growth, antibiotics are often used to control the growth of bacterial and fungal contaminants[40]. Routine use of antibiotics for cell culture is not recommended since antibiotics can mask contamination by mycoplasma and resistant bacteria[41]. Antibiotics can also interfere with the metabolism of sensitive cells.	No special functions in cartilage TE. Antibiotics serve as anti-contamination agents.

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Table B.1 – continued from previous page

Constituents	Functions in Cell Culture Media	Functions in Cartilage Tissue Engineering
Fatty acid (Linoleic Acid)	Fatty acid are particularly important in serum-free media as they are generally present in serum.	No literature described the specific function of linoleic acid[14]. Always supplemented, along with insulin, transferrin, and selenite acid.

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Table B.1 – continued from previous page

Constituents	Functions in Cell Culture Media	Functions in Cartilage Tissue Engineering
Buffering system (CO_2/HCO_3 system, HEPES, Phenol red)	Chemical buffering using a zwitterion, HEPES, has a superior buffering capacity in the pH range 7.2-7.4 and does not require a controlled gaseous atmosphere[42]. In a natural buffering system, gaseous CO_2 balances with the CO_3/HCO_3 content of the culture medium. Cultures with a natural buffering system need to be maintained in an air atmosphere with 5-10% CO_2, usually maintained by a CO_2 incubator.	No special functions in cartilage TE, but the high concentration of HEPES might have negative effects on certain types of cells, such as chick embryo epiphyseal chondrocytes and ES cells.

Appendix C

Supplementary of the Constituents in Chondrogenic Media

Table C.1: Supplementary of the constituents in chondrogenic media

Name of components	**Specific components**
Amino acids	Glycine, L-Arginine hydrochloride, L-Cystine 2HCl, L-Glutamine, L-Histidine hydrochloride-H_2O, L-Isoleucine, L-Leucine, L-Lysine hydrochloride, L-Methionine, L-Phenylalanine, L-Serine, L-Threonine, L-Tryptophan, L-Tyrosine disodium salt dihydrate, L-Valine
Inorganic salts	Calcium Chloride ($CaCl_2$), Ferric Nitrate ($Fe(NO_3)_3 \cdot 9H_2O$), Magnesium Sulfate ($MgSO_4$), Potassium Chloride (KCl), Sodium Bicarbonate ($NaHCO_3$), Sodium Chloride (NaCl), Sodium Phosphate monobasic ($NaH_2PO_4 \cdot H_2O$)
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Table C.1 – continued from previous page

Name of Components	Specific Components
Vitamins	Choline Chloride (perform as Vitamin B4), D-Calcium pantothenate (Vitamin B5), Folic Acid (Vitamin B9), Niacinamide (Vitamin B3), Pyridoxine hydrochloride (Vitamin B6), Riboflavin (Vitamin B2), Thiamine hydrochloride (Vitamin B1), i-Inositol (perform as Vitamin B8), Ascorbic acid-2-phosphate (Vitamin C)

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