Abstract – Chondrocytes from 3-6 months aged pigs were seeded at high density (100x10^6/ml) into cylindrical culture chambers and were maintained between two membranes in culture for 6 days on ground and a subsequent 12 days in microgravity during the mission 7S to the ISS. The aim was to compare the results from the RPM with those gathered in an actual micro-gravity environment.

I. Introduction

Recent studies [2] have shown that cartilage cultivated on a Random Positioning Machine (RPM) exhibits a much more regular distribution of cells within the cartilage matrix, like in native cartilage, than the layered structures produced under normal conditions without scaffold. The aim of the present project was to compare the results of the artificial micro-gravity as simulated on the RPM, with results of tests in an actual micro-gravity environment. To this end we designed hardware suitable to be flown on the ISS (International Space Station) during a 10 day flight (see appendix D), which functioned as an in flight incubator. To the best of our knowledge this was the first time, that a biological space flight experiment was run in parallel to an RPM reference experiment, both from the same batch of cells.

II. Methods and Materials

Cell Culture Chambers (CC): The so called cell culture chamber used to cultivate the cartilage (see [1]), consisted of a 60ml polycarbonate cylinder with a neoprene o-ring (in direct contact with the nutrient solution) in which a Centerpulse “de novo” insert was fixed with two thin metal plate springs. (see Figure 6).

Experiment Protocol: Porcine Neo-cartilage was produced in three different environments:

1. International space station (ISS)
2. Random Positioning Machine (RPM),
3. Normal gravity 36°C.

Chondrocytes were extracted from slaughter-aged pigs (3-6 months) as described in the protocol found in the appendix B and seeded into the inserts. All three batches, each consisting of eight CCs, subsequently spent 36 hours on the RPM at 25°C. All batches were then removed from the RPM, one transferred to a transportation container (portable incubator) the other two were left in an incubator, all still at 25°C. To partially simulate transport disturbances the latter batches were turned every eight hours and lightly shaken in irregular intervals. The transport from Zurich to Baikonur lasted three days. Upon arrival in Baikonur nutrient solution was exchanged (see appendix C for formula). The solution in the reference batches was also changed at the same time in Zurich. Two out of the eight CCs were selected and integrated into the specially designed flight incubator that regulated the temperature at 36°C. Parallel to this, the two reference batches were also placed in an incubator at the same temperature. At L-18 (18 hours before the launch) the experiment was handed over to Russian technicians who installed it in the Soyuz Capsule. Then, at the time of Launch, one of the reference batches was mounted on the RPM, the other left in the incubator, both still at 36°C. After 48 hours in orbit, the Soyuz
spacecraft docked to the ISS, 3 hours later the experiment was transferred from the Soyuz to the ISS and the battery pack was replaced. After eight days in orbit on the ISS the experiment was disconnected from its power supply and returned to earth in the Soyuz capsule six hours later. Twelve hours after landing the neo-cartilage tissue was transferred to a transport vessel filled with PBS and Proteinase Inhibitor. At ambient temperature the samples were transported back to Zurich, where they arrived 24 hours later and were handed over to the Osteoarticular research Group, University of Bern together with the ground samples for further analysis.

**Evaluation of neo-cartilage:** The probes were processed by the Osteoarticular Research Group, University of Bern employing the following methods:

- **Histology:** Haemotoxylin Eosin (H&E: for an overview of structure and cell distributions), Alcian blue (collagens and glycosaminoglycans), Safranin O-Fast Green (sulfated glycosaminoglycans), Masson Trichrom (collagens).

- **Immunohistochemistry:** Collagens type I and type II. Collagen type II is the principle collagen subtype in the cartilage matrix and denotes the chondrocyte phenotype. Collagen type I is normally absent or expressed at very low levels in cartilage. This subtype is a classical marker of de-differentiated chondrocytes.

- **Real Time PCR (gene expression):** Collagen type I, collagen type II, and cartilage oligomeric matrix protein (COMP). COMP is an extracellular matrix protein whose expression is closely linked with chondrogenesis and collagen type II expression. Chondrogenic

---

**Figure 1:** Histology and IHC of neo-cartilage produced on the ISS, in the RPM and at 1g (reference sample).  
A-C: Alcian Blue  D-F: Safranin O.  G-H: Collagen type II IHC. (All images at 40 x)
phenotype is commonly established by denoting the expression of collagen type II in proportion to the expression of collagen type I. 18s-ribosomal RNA (18s-rRNA) was used as the reference gene to calculate the relative aforementioned gene expression levels.

III. Results

It was not possible to handle tissue from the ISS with forceps. Although the structural integrity of the normal gravity cultured neo-cartilage was not notably stable, it was more so compared to both reduced gravity produced tissue. Neo-cartilage formed in normal gravity was regular in shape and form. ISS and RPM tissues consisted of aggregates of cells that were not continuous in structure, especially less so in the ISS cultures. No striking visual difference in cell distribution was immediately apparent, although on closer inspection, there may be greater distance between cells created in the RPM (This needs further examination see Figure 2.D-F). Extracellular matrix staining, particularly illustrated by Safranin. O-Fast Green and Alcian blue stains, of neo-cartilage cultivated on the ISS was weaker compared to both Earth cultivated tissues (Figure 1). Collagen type II immuno stains reveal substantial collagen formation in all conditions, albeit visually weaker in ISS cultivated tissue and confirms the other histological observations (Figure 1). Immuno stains for collagen type I was extremely faint to negative for all constructs examined (Figure 2) and reflected at the gene expression level.

**Figure 2:** Histology of neo-cartilage produced on the ISS, in the RPM and at 1g (reference sample). A-C: H&E stain (40x) D-F: H&E stain (80x) showing a lower density of cells in the RPM produced tissue G-H: Masson Trichrom (40x).
The collagen profile most probably reflects the undifferentiated state of the cells since they were not expanded in culture. Low staining with masson trichrom indicates immature collagen formation that is expected in such immature cartilaginous tissue (Figure 2, G-I). Real-time PCR (Figure 3) revealed that ISS cultivated neo-tissue had substantially higher gene expression levels of collagen type II and COMP compared to the other treatments and to normal cartilage. Although it not clear why this would be so, it may be either a response of the ISS cultivated tissue’s return to 1g conditions. On the other hand, microgravity may induce a greater expression at the RNA level if gravitational dependent pathways are critical to translate this into protein.

**IV. Discussion**

This study demonstrates that microgravity clearly impacts development of cartilaginous extracellular matrix products. This is consistent with other similar studies (Freed et al. 1997 and others) although the tissues formed here were only 16 days old compared to 3 to 7 months as reported by Freed et al. (1997). The reference results produced on the RPM and in the 1g environments correspond well with the previous study, Conza et al. (2001).

RPM cultivated chondrocytes created tissue with matrix composition characteristics in between the reference and ISS produced neo-cartilages, but more strikingly produced neo-cartilage with more evenly dispersed and further spaced apart cells. This characteristic may be a useful means of spacing chondrocytes closer to native tissue without the requirement of a scaffold and moreover, reduce the need for a large number of cells. The restriction of the number of cells obtained from a clinically relevant sized biopsy is not adequate for cell based therapies like ACI or to produce a functional implant. Thus the classic expansion phase is needed to increase cell numbers and adds to the probability of poor chondrogenic capacities. This is especially the case in scaffold free methods that rely on the volume of cells to act as their own scaffold support and tissue volume. The RPM therefore offers the opportunity to have the best of both

**Figure 3:** Real time PCR of collagen type II and COMP in relation to collagen type I in native porcine cartilage and neo-cartilage cultivated on the ISS, in the RPM and at 1g (Reference sample). Both RPM and the reference sample have normal gene expression profiles compared to normal cartilage. ISS cultivated neo-cartilage higher collagen type II (2-fold) and COMP (6-fold) gene expression level compared to normal cartilage.
worlds: reduction in the amount of cell expansion needed and the prefabrication of implants without the complications associated with scaffolds. The effect of gravity on the muscular-skeletal system at the gene expression level has been demonstrated by Taylor et al. [4] using microarrays on rats either subjected to normal or microgravity gravity. Interestingly, they found an increased expression of 12 genes in the reduced gravity animals compared to the 1g controls (and 38 decreased). This may be a similar circumstance in the present findings in neo-cartilage tissue produced on the ISS. Moreover, the theory that the change in gene expression profiles may be due to the “re-adaptation” to the normal gravity environment may be echoed in the results by Taylor and others since the rats exposed to microgravity were processed 24 hours after returning to earth. Understanding such re-adaptation to normal earth gravity or indeed other gravities at the gene and protein level may be critical in maintaining the health of humans in such environments.

V. Appendix

A Hardware: The experiment hardware was designed to match the specifications and safety standards of both ISS and Soyuz manned missions. In addition to this the following physical specifications had to be fulfilled:

- Maximum size: 125mm x 110mm x 110 mm
- Maximum weight: 600g

This led to the Cell Cultivation System (CCS) as shown in Figure 4.

It consisted of three layers of containment, the two CC’s, containment 2 (C2), and containment 3 (C3) the outer aluminium cylinder, and a temperature regulation system (TRS).

![Figure 4: CCS fully assembled](image)

The TRS was made up of a heating foil surrounding the CC, a thermal controller and a thermostat as a safety measure to cut the power as soon as the surface temperature would reach 40°C. As additional safety measure a heat fuse set to 70°C was attached to the heat foil. The disassembled hardware is shown in Figure 5. The CC, C2 and C3 were tested to withstand inside overpressure of 1.1bar, 1.65bar and 3bar respectively.

B Isolation of chondrocytes: The protocol used to isolate the chondrocytes was the following:

- Cut open the hip joint and cut pieces of cartilage from the joint ball.
- Add pronase solution (pronase: Roche Cat. No. 1 459 643, solution: 1mg/ml, 10 ml per g cartilage) and transfer to incubator at 37°C 5% CO₂ for 2 hours on shaker.
- Wash with PBS and add collagenase P solution (ColP: Roche Cat. No. 1 231 865, solution: 0.25mg/ml, 10ml per g cartilage) and transfer to incubator at 37°C 5% CO₂ for 15-18 hours on shaker.
- Filtrate cell suspension (filter: 70µm) and wash with PBS
- Count and adjust concentration to 100 million cells per ml.

![Figure 5: Disassembled CCS](image)
C Nutrient Solution. The nutrient solution used is made on the basis of the commercially available solution DMEM / Nutrient Mix F12 (Life Technologies, Cat. 42400), with the additives: 1.125g/l NaHCO₃, 10% FCS, 1µg/ml Insulin, 50µg/ml Vitamin C and 100µg/ml Pen/Strep.

D Flight 7S. The Soyuz TMA-3 spacecraft, carrying a crew of three blasted off from Baikonur Cosmodrome’s Site 1, at 9:38:03 Moscow Summer Time on October 18, 2003, heading to the ISS. Onboard were the Russian commander Alexander Kaleri, NASA Science Officer Michael Foale, comprising the eighth long-term crew of the station, and European astronaut Pedro Duque, a visiting crew member carrying our experiment as well as performing the battery exchange, which was the only in flight procedure, later on. Duque returned to Earth after eight days onboard the outpost, in the company of the seventh long-term crew, which stayed in orbit since April 2003. The Soyuz TMA-3 successfully docked to the Pirs module of the ISS on October 20 at 11:15:58 Moscow Time (0715 GMT). (Scheduled time: 0716 GMT). After a week-long hand-over activities, Expedition 7 and Duque returned to Earth again carrying our experiment onboard Soyuz TMA-2 on Oct. 28, 2003.

VI. Acknowledgements

We are very grateful for the support and help of the whole team of the Space Biology Institute of the ETH Zurich, we also would like to thank the whole ESA Outreach Office for providing this unique flight opportunity, the High Energy Physics Institute of the ETH Zurich for building and designing most of the hardware. Our special thank goes out to Rogier Schonenborg who assisted greatly with the technical development of the hardware and Carmen Adusumalli who was responsible for making all this possible. We are also grateful for Ms Verena Winkelmann and Dr. Dobrila Nesic of the Osteoarticular Research Group (Institute of Pathology, University of Bern), for processing the histology and for performing the RT-PCR, respectively.

This research was funded by: ESA, ETH Zurich and Centerpulse.

VII. References