New Trends

Artificial tissues in perfusion culture

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ABSTRACT: In the stagnant environment of traditional culture dishes it is difficult to generate long term experiments or artificial tissues from human cells. For this reason a perfusion culture system with a stable supply of nutrients was developed. Human chondrocytes were seeded three-dimensionally in resorbable polymer fleeces. The cell-polymer tissues were then mounted in newly developed containers (W.W. Minuth et al, Biotechniques, 1996) and continuously perfused by fresh medium for 40 days. Samples from the effluate were analyzed daily, and the pH of the medium and glucose concentration remained stable during this period. The lactid acid concentration increased from 0,17 mg/ml to 0,35 mg/ml, which was influenced by the degradation of the resorbable polymer fibers used as three dimensional support material for the cells. This perfusion system proved to be reliable especially in long term cultures. Any components in the culture medium of the cells could be monitored without disturbances as caused by manual medium replacement. These results suggest the described perfusion culture system to be a valuable and convenient tool for many applications in tissue engineering, especially in the generation of artificial connective tissue. (Int J Artif Organs 1997; 20: 57-62)

KEY WORDS: Tissue engineering, Perfusion culture, Extracellular matrix, Chondrocytes, Metabolite

INTRODUCTION

Cell biology and biomedicine without cultured cells is unthinkable. In the last decade, isolated human cells cultured in flasks or petri dishes have been most important tools. Complex organs or tissues are often too difficult to investigate, but single human cells provide a good method for learning about vital and functional features. Thus, human cells *in vitro* have allowed countless experiments to investigate the pathogenesis of diseases including approaches for their treatment.

Although our knowledge of cell biology has increased enormously in recent years, the methods of handling human cells have hardly changed. As shown in recent experiments, conventional monolayer cultures have their limitations in generating highly differentiated structures (1). The cells lack the typical extracellular matrix, thus they are cultured on an unappropriate support (2).

The metabolic conditions within the culture medium are unstable (3), and finally high density and long term cultures are at risk of bacterial or fungal contamination during frequent manual medium replacement procedures. Recently, a novel perfusion culture system was developed and successfully applied to the typic tissue culture of renal duct cells (1-3) and also to artificial three-dimensional chondrocyte tissues (4). Differentiation and phenotypic development of three dimensional chondrocyte-polymer tissues have already been presented by our group (4). The present study describes culture conditions during the *in vitro* generation of artificial cartilage tissue using resorbable polymers and perfusion culture. We monitored the metabolic parameters of glucose and lactate during perfusion culture.

MATERIALS AND METHODS

Cell preparation

Hyaline cartilage was obtained from macroscopically normal femoral heads of humans between 30 and 65 years of age within 12h of death or from patients undergoing reconstructive surgery. Chondrocytes were isolated using an enzymatic solution as previously described (5). Briefly, the extracellular matrix of the cartilage slices were digested for 12-18 hours at 37°C in the presence of 2 mg/ml of type II collagenase (Seromed, Berlin, Germany),

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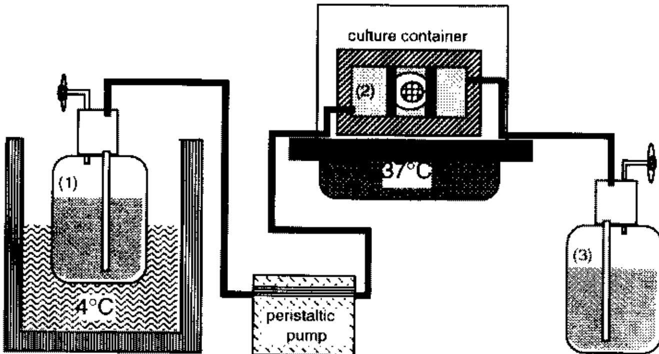

Fig. 1a - *Perfusion culture: Culture medium (1) is transported by a peristaltic pump into the culture container (2). The effluate is collected in a second bottle (3). A constant flow of fresh culture medium provides stabilized culture conditions and reduces risks of contamination in long term culture experiments. The system runs outside an incubator on a laboratory table.*

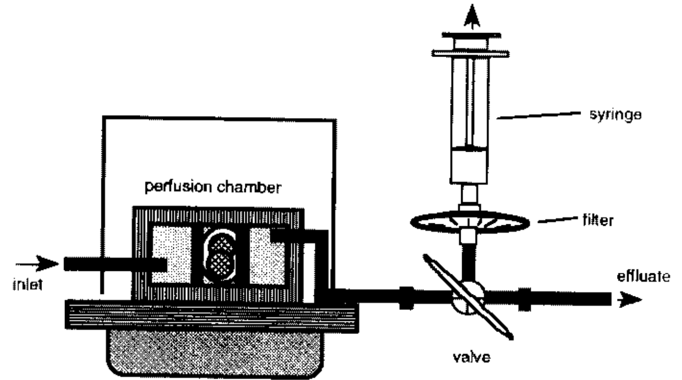

Fig. 1b - *To control the quality of the culture medium, samples of the perfusate were taken daily from the effluate using a syringe.*

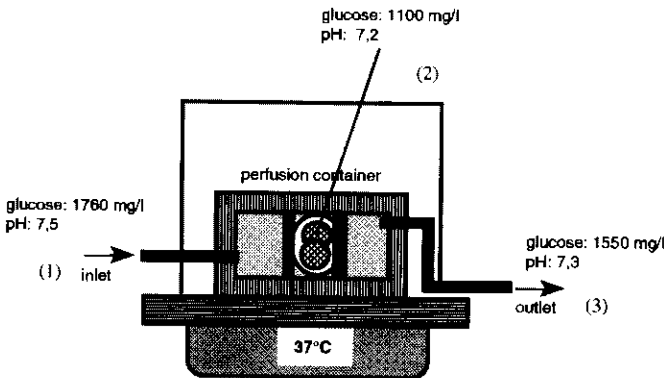

Fig. 2 - *Stabilized culture conditions: Typical measurements of glucose and pH in the perfusion container with generated bioengineered artificial cartilage tissue.*

0.1 mg/ml of hyaluronidase (Serva, Berlin, Germany) and 0.15 mg/ml of DNase II (Paesel, Frankfurt, Germany) in Ham's F12 medium. The resulting cell suspension was filtered through a nylon sieve with a pore size of 88 µm. Chondrocytes were suspended in Ham's F12 with 10% fetal calf serum (BRL Life Technologies, Eggenstein, Germany) in a spinner flask for 15 hours. The resulting cell suspension was centrifuged at 1500 rpm for 7 min and resuspended in Ham's F12 containing 20% fetal calf serum.

Tissue engineering and culture

Bioresorbable copolymer fleeces of vicryl and polydioxanon (Ethicon, Norderstedt, Germany) were soaked in poly-L-lysine or type II collagen and then lyophilized. The isolated chondrocytes ($10x10^6$) were loaded into the polymer fleeces (ca 2x10x30 mm) by overlaying the biomaterial with the chondrocyte suspension in a tube. The biomaterials containing the cells were encapsulated with 4% low-melting agarose solution (Sigma, Deisenhofen, Germany). The prechilled agarose solidified within a few minutes by cooling in cold water. The encapsulated cell-polymer tissues were placed in perfusion culture containers (Minucells and Minutissue, Bad Abbach, Germany) and hooked to a permanent flow of fresh medium (6). In general, the peristaltic pump IPC cartridges (Ismatec, Wertheim-Mondfeld, Germany) were adjusted to deliver 1 ml of Ham's F12 culture medium per hour (Seromed; Berlin; Germany) with 2% fetal calf serum, ascorbic acid (50 µg/ml) and 1760 mg/ml glucose, 0,63 mg/ml lactate). A termperature of 37°C was maintained on the heating table (Amersham, Braunschweig, Germany).

Monolayer cultures as controls were established in 75 cm^2 flasks.

Sampling procedure

About 500 µl medium were taken daily from the effluate under sterile conditions using a syringe. After 10 days of perfusion culture, additional samples were taken from the medium reservoir and from between the two tissues inside the chamber (2). At least three measurements were always performed per experimental sample presented. Figures 1a and 1b illustrate the means of measurement. Serum specimens and control solutions provided by the manufacturers were used daily to calibrate the Stat profile 9 plus analyzer (Nova Biomedical, Rödermark, Germany).

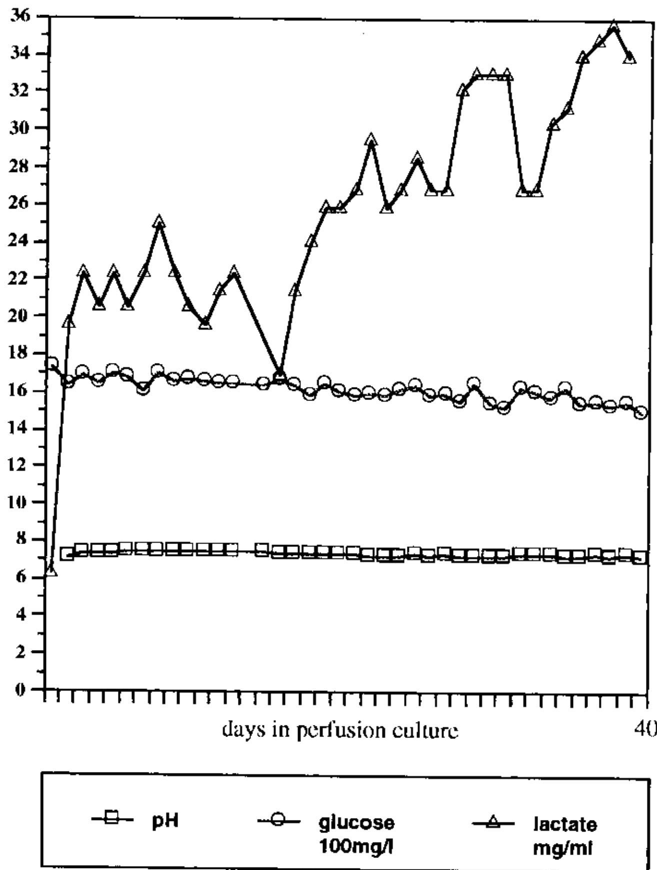

Fig. 3a - *Glucose, lactate and pH of a three-dimensional chondrocyte-fleece culture in perfusion. Values of 40 days (n=3, 10x10⁶ cells).*

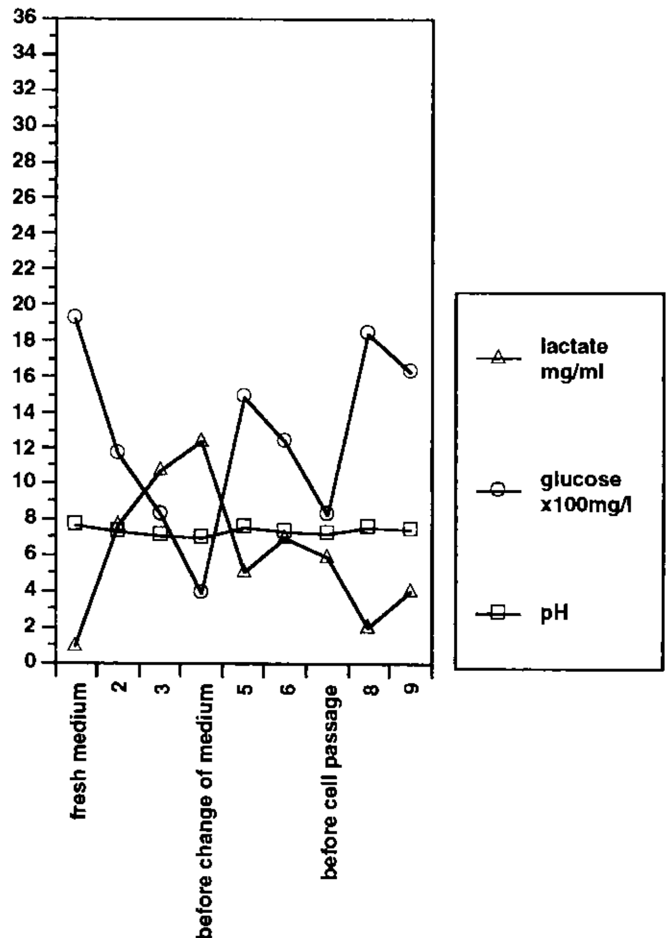

Fig. 3b - *Fluctuating conditions for chondrocytes cultured as monolayer in the stagnant medium environment of flasks. Glucose and lactate concentrations were measured in a culture of 5x10⁶ cells.*

Measurements

Undiluted medium samples of 200 µl were analyzed immediately after collection with a Stat Profile 9 Plus apparatus from Nova Biomedical GmbH, Rödermark, Germany. By this method, pH, pCO_2, pO_2, Na^+, K^+, Cl^-, Ca^{++}, lactate, glucose and osmolality can be determined in undiluted samples. Solutions with defined glucose and lactate concentrations served as controls.

RESULTS

Compared to experiments in petri dishes, the applied perfusion culture system provided major advantages in handling three-dimensional connective tissue cultures. In previous experiments it has been shown that the high cell densities in cartilage engineering requires a permanent renewing of culture medium. As shown in three experiments, the slowly maturing cultures could be left untouched for several weeks in the described perfusion system. No contamination was observed during this time in culture.

The content of glucose, lactate and the pH of the perfused culture medium from a chondrocyte-fleece culture effluate was analyzed daily. Figure 2, shows the sample taken at the inlet (1) within the tissue container (2) and at the perfusion outlet (3). This procedure allowed the measurement of the fresh medium, the medium in the tissue environment and the effluate. Over a 40 day culture period, the pH and the glucose concentration remained stable (Fig. 3). When $10x10^6$ chondrocytes in a ca 2x10x30 mm polymer fiber fleece were placed in a chamber with a perfusion rate of 1 ml/hour with culture medium, glucose was found to be 1760 mg/L at the inlet, decreasing to 1100 mg/L in the tissue environment and 1550 mg/L in the outlet. An average glucose take up of

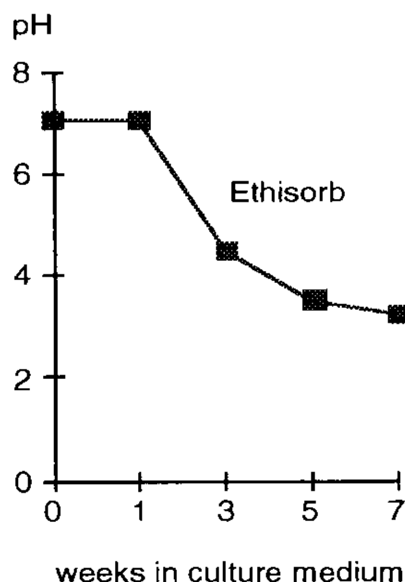

Fig. 4 - *Decrease of the pH due to the degradation of a resorbable fleece carrier Ethisorb 210 during incubation without cells with culture medium at 37°C in a petri dish (10 mg/ml) (n=1).*

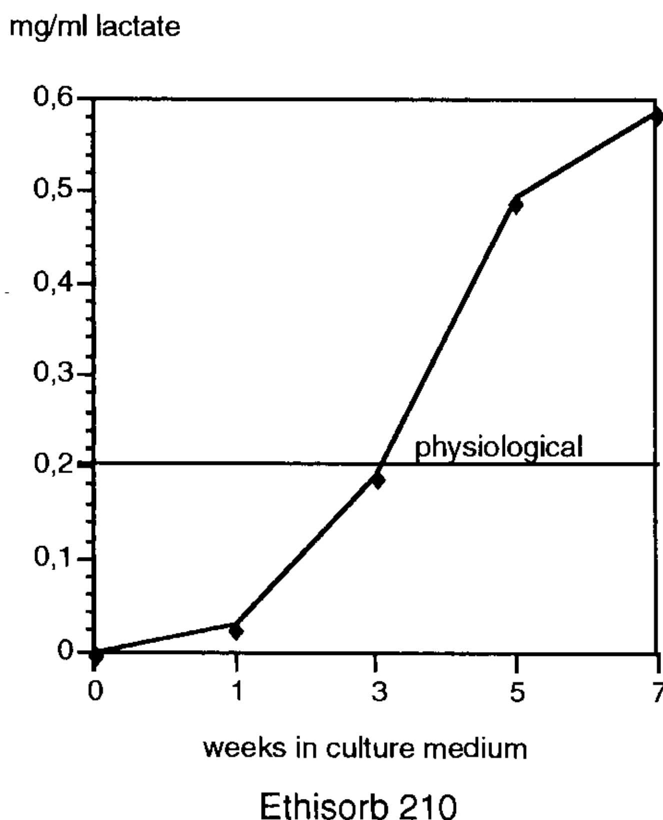

Fig. 5 - *Lactic acid release of a resorbable fleece carrier Ethisorb 210 during incubation without cells with culture medium at 37°C in a petri dish (10 mg/ml) (n=1).*

21ng per cell and hour can therefore be calculated from these results. However, the glucose concentration actually measured between the two tissue constructs in the chamber revealed a lower value of 1100 mg/L. A slightly lower pH of 7.2 was also found as compared to samples taken from the outlet.

Stabilization of medium conditions

When the glucose concentration in the effluate was monitored for five weeks, the outlet values slightly decreased in a linear fashion from about 1650 mg/L to 1550 mg/L (Fig. 3a) over the 40 day culture. These results suggest that the glucose consumption must have slightly increased during this culture time. However, compared to the variations of glucose levels found in the stagnant medium environment in culture flasks (Fig. 4), the glucose concentration in the applied perfusion conditions can be considered stable (Fig. 3a). This observation is also true for the pH of the medium which showed no change during the 40 days culture period. On the contrary, the lactate concentration did not reach a steady state, but increased continuously during the five weeks of culture (Fig. 3a). This increasing release of lactate was not due to the metabolic activity of the chondrocytes, but was most likely a result of the degrading resorbable polymer which contains lactic acid as one of the monomers. The degradation behavior of the resorbable fleece Ethisorb is shown in Figures 5 and 6. Fluctuations in lactate concentrations in the days to follow were much more prominent than with glucose.

The changes in pH, glucose and lactate of a typical culture medium from chondrocytes kept in monolayer flasks are shown in Figure 3b. An oscillating, not linear concentration of lactate and glucose was found.

For the bioengineering of cartilage tissue fiber structures are needed for the temporary mechanical replacement of the ECM. Ethisorb is a resorbable copolymer of lactic and glycolic acid. Therefore, the fleeces were placed in a culture dish without cells and the liberation of degradation products as a function of pH shift was registered (Fig. 4). It was observed that during a 20 day incubation the pH changed from 7 to 2.5. At the same time an increase in lactate from 0 to 0,18 mg/ml was seen. Thus, the pH decrease is caused by the release of degradation products.

Fig. 6 - *Sample of the cell-polymer construct forming artificial tissues. Chondrocytes were seeded three-dimensionally in a polymer fiber structure to allow the synthesis of a new extracellular matrix.*

DISCUSSION

Compared to conventional monolayer cultures, three-dimensional cultures in tissue engineering lead to rather high cell densities similar to the native tissue. Human articular cartilage normally contains about $15x10^6$ cell/ml (7) which is similar to the density chosen in the experiments. As shown by recent experiments, such cell densities require very frequent medium changes to maintain stable nutrient conditions. Using perfusion culture containers as a convenient method, a stable pH and constant concentrations of nutrients like glucose were provided (Fig. 2). In our experience with long term cultures, perfusion cultures prove easier to handle with a lower risk of contamination. A further advantage is that the culture containers are completely closed and a CO_2 incubation is not needed as long as the medium is buffered with HEPES (25 mM) or an other CO_2 independent media. During five weeks perfusion culture, the glucose concentration only slightly decreased from 1650 mg/ml to 1550 mg/ml (Fig. 2). This indicated that the glucose consumption of the culture increased by 6.25% during that time. The measured concentrations of lactate were unstable and significantly increased during the perfusion culture of cell-polymer tissues due to the hydrolytic degradation of the poly-α-hydroxy acids.

Analysis of glucose concentrations in typical monolayer culture conditions clearly demonstrated the enormous nutrient fluctuations affecting the cells even in much lower cell densities compared to tissue-like cultures (Fig. 3). Stable concentrations might be even more important for growth factors in the serum or when additionally supplemented. For example, the effects of bone morphogenic proteins (BMP) which play a key role in bone and cartilage development and have very different effects depending on their concentrations (8, 9). Perfusion not only stabilizes the components provided by the culture medium, it also stabilizes secreted autocrine factors at a certain level and does not allow an overshoot of synthesized paracrine factors similar to the situation *in vivo*. In addition, important advantages could be provided by novel gradient chambers to establish a concentration gradient of differentiating morphogenic factors across the artificial tissues. The experimental set up presented provided 1 ml of fresh medium per hour to 10 million cells. Further studies are needed to show whether this ratio can be optimized. The local concentration of factors produced by the cells certainly depend greatly on the flow of medium. In the above cell polymer tissues, a faster flow of medium would probably result in a slower increase in the lactate concentration. However, this would also lead to an elevated glucose level and most likely the autocrine factors would be shifted to lower steady state levels. The oxygenation of the tissue has not been studied here, however our tissues were only 2 mm thick and at least for cartilage tissue a slightly anaerobic metabolism was found *in vivo* because of a lack of vascularization.

In summary, for tissue engineering involving long term cultures with high cell densities and use of tissue inducing protein factors, modern perfusion culture systems allow a typical tissue simulation of the environment which results in a high degree of cell differentiation.

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