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ORIGINAL PAPER

Should human chondrocytes fly? The impact of electromagnetic irradiation on chondrocyte viability and implications for their use in tissue engineering

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Abstract A significant logistic factor as to the successful clinical application of the autologous tissue engineering concept is efficient transportation: the donor cells need to be delivered to tissue processing facilities which in most cases requires air transportation. This study was designed to evaluate how human chondrocytes react to X-ray exposure. Primary cell cultures were established, cultured, incubated and exposed to different doses and time periods of radiation. Subsequently, quantitative cell proliferation assays were done and qualitative evaluation of cellular protein production were performed. Our results show that after irradiation of chondrocytes with different doses, no significant differences in terms of cellular viability occurred compared with the control group. These results were obtained when chondrocytes were exposed to luggage transillumination doses as well as exposure to clinically used radiation doses. Any damage affecting cell growth or quality was not observed in our study. However, information about damage of cellular DNA remains incomplete.

Keywords Tissue engineering · Chondrocytes · X-ray · Cartilage · Cell culture

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List of abbreviations

DMEM	Dulbecco's modified essential medium
ECM	Extracellular matrix
FCS	Fetal calf serum
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H- tetrazolium bromide
PBS	Phosphate-buffered saline

Introduction

Today tissue engineering of human chondrocytes has become an emerging field in regeneration and reconstruction of cartilage defects. Since Cima et al. published the first report on tissue engineering of cartilage by cell transplantation in 1991 [1], much effort has been taken to understand the precise mechanisms of

engineering biocompatible chondrocytes with adequate morphology and function. The laboratory-scale establishment of chondrocyte cell cultures has been well documented by several in vitro studies [1–6]. Furthermore, in vivo experiments have been able to show potential clinical applications of engineered cell cultures and demonstrated the impact of biological environments and mechanical force (e.g. motion of human joints) on these cultures once they are implanted in the human body [7]. At present, ongoing research needs to be done requiring multi-international and interdisciplinary cooperation. This requires safe and rapid transportation between research centres and stability of autologous cell cultures during these journeys needs to be guaranteed. Air transportation is always associated with increased radiation. In an altitude of 10,000 m the radiation already amounts for about 5.7 mSv per year. Additionally, security standards today require hand baggage X-ray transillumination at airports.

Damage to cellular components like deoxyribonucleic acid (DNA) by cosmic and ionizing radiation is well known; therefore, the ''International Commission on Radiological Protection'' (ICRO) developed a consensus statement with determination of threshold values for parenchymal organs in 1980 [8]. However, our current knowledge base about the impact of radiation on cell cultures used in tissue engineering is limited. Considering the effective annual radiation dose in Switzerland of 4, 2.8 mSv is attributed to natural causes and 1.6 mSv to radon. Keeping these doses in mind, a negative influence on the viability of cell material seems obvious in Switzerland [9]. Moreover, cosmic radiation in general amounts for 0.3 mSv resulting in a total radiation (terrestical and cosmic) of 2.1 mSv per year at sea level in further dependency on latitude [10]. Interestingly, radiation doses of conventional thoracic X-rays are approximately 0.02 mSv per examination [10].

We hypothesized that primary chondrocyte cell cultures may be adversely influenced by these radiation influences. Therefore, in the present study human isolated chondrocytes were analysed by functional tests and statistically evaluated.

Materials and methods

All chemicals or solvents were purchased from Sigma-Aldrich Inc. (Buchs, Switzerland) unless stated otherwise. The components for MTT cell proliferation assay were obtained from Sigma- Aldrich Inc. (Buchs, Switzerland) and Fluka/ Riedel- de Häen Inc. (Buchs, Switzerland). Technical equipment came from NuAire (Corning/Costar USA, Distributor Vitaris AG, Baar, Switzerland) and medium, fetal calf serum and antibiotics by PAN Biotech (Germany, Distributor Teco Medical, Switzerland).

Cell culture

Chondrocytes were isolated from an amputate derived from a traumatic foot amputation of a 4-year-old child (male). Articular cartilage was harvested surgically under sterile conditions from the specimen and conserved in DMEM cell culture medium containing 10% fetal calf serum (FCS) and gentamicin solution. Subsequently, the cells were stored at 4°C for 24 h and then either snap-frozen for later applications or directly used for growing of a cell culture.

Cartilage samples were enzymatically digested with 0.3% collagenase II at 37°C for a maximum of 20 h with DMEM and antibiotics without FCS. The digested cartilage suspension was filtered using a sterile 250 nm nylon filter and centrifuged at 400*g* for 5 min. The cells were washed twice with DMEM medium containing 10% FCS. Cell number and viability were determined by cell counting using a hemocytometer and Trypane blue dye exclusion. Resulting cell pellets with viability percentage values of more than 85% were used for cell culture. The cells were resuspended in DMEM cell culture medium.

Chondrocytes were maintained using DMEM as a culture medium supplemented with 10% FCS, 50 μg/ml Gentamicin, 0.4 mM Prolin and 50 μg/ml ascorbic acid; morphologically, the cultures consisted of a two-dimensional chondrocyte monolayer in a 75 cm^2 cell flask (Corning/ Costar USA, Distributor Vitaris AG, Baar, Switzerland) which was seeded with about 50,000 cells [11]. The chondrocytes were expanded and incubated in monolayer culture about four passages (Nuaire IR Autoflow, 37°, 5% CO_2, 80% relative humidity).

After harvesting, isolation and conservation of chondrocytes the cell cultures were assigned to one of the following ten groups according to a randomization protocol. The *first three groups* of cell cultures were brought to the international airport Zurich (*airport group*) where they were passed through a baggage transillumination standard device produced by Smiths Heimann (Wiesbaden, Germany). [FRG designation: Smiths Heimann rear CAN 6040i, speed 0.2 m/sek, X-ray-dose:1.4 μSv (0.14 mrem), approx.. 0.26 μSv by inspection (controlled), X-ray power:140 kV/a; radiation arranged diagonally from down upwards.] All three groups were then irradiated using a fixed dose and X-ray power but different time intervals. Passing

through transillumination one time induces a radiation time of 1,500 ms. Group two and three were treated repeatedly in the same fashion (Table 1).

Another *six groups* were irradiated at the department for diagnostic radiology. We adopted a device by Siemens that is a modular remote and tableside-controlled overtable fluoroscopy system for universal applications (Siemens designation: Model Siregraph D). Three groups of the cultures were irradiated with three different doses over one time unit. The other groups were irradiated with a dose at three different time frames.

Additionally to the nine experimental groups we used one control group that was functionally tested without any prior radiological treatment. During irradiation the cell cultures of the experimental groups were always maintained at 4°C using ice cooling [12].

MTT assay

Cell cultures of each group were tested repeatedly within 10 days for metabolic activity. The MTT assay determines viable cell quantity and is based on the mitochondrial conversion of tetrazolium salt [3-(4,5-Dimethyl-2- thiazolyl)-2,5-diphenyl-2H- tetrazolium bromide (MTT)] [13]. In the present study we used a modified assay to quantitatively assess the viable chondrocytes as described by Zünd et al. [14]. 50,000 cells resuspended in 0.1 ml DMEM were placed in a 24-well culture plate. Subsequently, 250 µl DMEM and 20 µl MTT solution (5 mg/ ml MTT in PBS) were added to each well at eight different time points within the first 10 days and incubated at 37°C for 1 h. The supernatant was discarded and replaced by 400 µl isopropanol with 10% formic acid. Samples were again incubated at 37°C for additional 5 min and vortexed for another 10 min. Finally, the MTT absorbency values of the resulting solution were measured using an ELISA reader (Dynatech 5000, Dynatech, Billinghurst, UK) at a wavelength of 570 nm.

The samples were lyophilised and weighed. Subsequently, the protein digesting took place for the exposure of the cartilage with Proteinase K at 56°C overnight. Then, we initiated the colour reaction of the sulphated glycosaminoglycans (GAG) with 1,9 dimethyl-methylene blue chloride (Serva Electrophoresis Heidelberg, Germany). Quantification of protein production was obtained by comparison with a standard curve in accordance with a dilution series of chondroitin sulphate B from bovine cartilage (Sigma Aldrich Corporation, Switzerland).

Cell cultures were kept in 4.5% formaldehyde solution (pH 7) for further histological examination. They were stained with HE and cell viability was microscopically evaluated (dye exclusion technique and morphological parameters).

Statistics

Groups were statistically analysed using the Student's *t* test for paired samples (with Bonferroni correction). Differences between groups was tested by repeated ANOVA analysis. Significance threshold was set at a *p* value of equal or less than 0.001.

Results

Results of MTT analysis showed a clearly positive continuous cell growth in all groups. The steepest gradient of increase in cell growth can be found between 120 and 192 h after X-ray. The maximum mitochondrial activity of chondrocytes was found at day 10 (216 h culture time). After that 216 h all groups showed a constant decline of cell growth. During the first 3 measurements (0–72 h) a comparable growth is recognizable. There are two slight irregularities along the growth curve. One experimental group showed a temporarily decline in growth at day 5 after 120 h. Two further experimental groups point their maximum of cell number at the 6th measurement (192 h) with subsequent decreasing results. In order to maintain a comparison of the individual test groups, the respective MTT values were analysed for the corresponding

Table 1 Effective dose during irradiation of the chondrocytes with specification of irradiation times, each group divided into four subgroups

Group	Airport			Radiology					
	1	2	3	4	5	6	7	8	9
kV	140			141	141	141	40	81	141
mAs				2.8	5.6	11.0	2.8	2.8	2.8
ms	1,500	3,000	4,500	280	12.2	24	4.28	3.55	6.15
1	µG			256	460	914	8.52	62.9	213
2	µG			257	464	950	11.8	77.1	229

measurement using a growth curve (Fig. 1). No statistical difference for $p < 0.001$ of cell density can be found in all groups at any time.

In the groups of the cells irradiated at the airport, a low growth deficit could be documented in contrast to the groups treated at the Department of Diagnostic Radiology. Again no statistical significance ($p < 0.001$) for all groups in the comparison to the control-group and between all experimental groups (airport and department for radiology) was reached.

The measured glycosaminoglycane concentration was 120.50 µg/mg (95.13%) in the airport group and 120.00 µg/mg (94.73%) in the radiology group. In relation to the lyophilised sample with a weight of 0.0048 mg, a GAG value of 126.67 µg GAG per µg sample (100%) can be calculated. Hence, a quantitative evidence of matrix production indicates the presence of viable chondrocytes.

Evaluation of light microscopy showed similar morphology of the cells between all groups. There were sheets of fibroblast-like spindle cells with a moderately wide eosinophilic cytoplasm and oval-shaped nuclei with small nucleoli. Occasional mitotic figures could be observed in our samples. Figure 2 shows a representive picture of chondrocytes after irradiation at day 10 (Fig. 2).

Discussion

Cartilage tissue engineering requires adequate expansion of isolated donor chondrocytes, because a stable and functioning engineered construct within a physiological environment will always depend on sufficient cell quality and growth capacity [15, 16]. Today, there is an increasing knowledge regarding repair mechanisms of these isolated and cultured chondrocytes, however any damage to these cells needs to be avoided during cell processing [4, 16, 17].

In this study we were able to show that the growth of chondrocyte cultures is not significantly impaired by radiation with different and repeated X-ray doses. All groups demonstrated an increase in cell numbers within the first 10 days after irradiation.

One experimental group showed a minimal decline of MTT count at the 4th measurement after 120 h. The exact cause of these findings remains unclear. The following measurements in this group revealed again a steep gradient in growth adapting to the growth pattern of the other groups. This excludes the hypothesis that the culture medium was insufficient or wasted. The highest cell numbers were noticed at 216 h after X-ray except two experimental groups which already had their maximum at the 6th measurement (192 h). We hypothesize that the waste of MTT counts at day 8 and 9 appears to be caused by the lack of available culture medium as this is only sufficient for a certain cell number within the defined drawing spaces in the 24-well plates.

Generally, cell growth is strongly associated with various different and very complex molecular processes that can duplicate cell DNA. It is well known that some components of DNA are most sensitive to radiation and that repair mechanisms of the cell are often ineffective due to these irreversible damages [10, 12]. Löbrich et al. described radiation-induced damage to DNA and that aggregation of base damage can lead to cell death, mutations and even generation of cancer [10, 12]. Whether the temporary decline in cell growth at day 5 (120 h) in our study was due to the influences

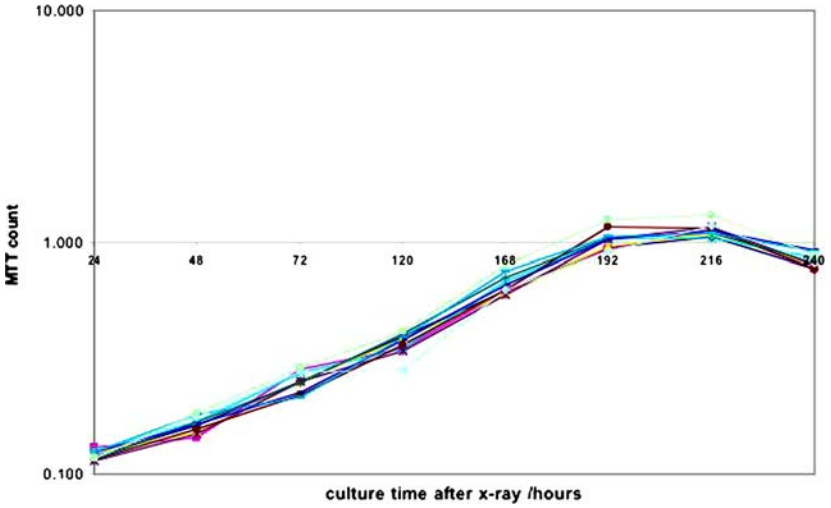

Fig. 1 MTT count of experimental groups and control group at eight different time points within the first 10 days after treatment

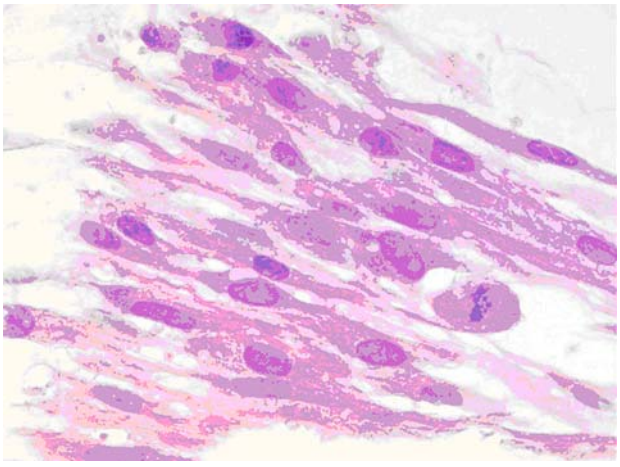

Fig. 2 Histologically the morphology of the cells was the same in all examined specimens: sheets of plump spindle cells with occasional mitotic figure (original magnification × 400; hematoxilin and eosin staining)

of radiation and subsequent repair mechanisms of the cell could not be determined. The lower cell losses in the group of the cells irradiated in the radiology with a higher dose corresponded also with the results of Ogawa, who determined an irradiation tolerance of 20 Gy when comparing lymphocytes and chondrocytes and then classified them by means of the reactive oxygen species (ROS) [18]. Furthermore, it has to be noted that the relative biological effectiveness (RBE) increases with decreasing X-ray energy. This increase of RBE induces a greater occurrence of double-strand breaks. If and to what extent these molecular pathomechanisms contribute to the insignificant differences in our growth curve remains unclear.

In addition to that, clinical X-rays and transillumination checks at airports only use indirect radiation. The impact of direct radiation, however, on isolated donor chondrocytes has not been evaluated in our study.

Some studies were able to show that an effective dose of 10 Gy has a significant effect on the formation of p38-MAPK and the metabolism of proteoglycane with different effects on the cell differentiation and growth. The formation of prostaglandin E2 could not be influenced within these experimental settings [19–21]. In another study by Ryan et al. the authors observed that the viability and the metabolism of chondrocytes were impaired by radiofrequency energy [22]. Damron et al. demonstrated a qualitative and quantitative effect of irradiation (17.5 Gy dose) on the growth plate of the tibia and femur which caused a decreased growth rate during the first week after irradiation. However, after 2 weeks growth rate had returned to baseline values and was constantly associated to proliferation of chondrocytes [17].

In conclusion, we believe that cell cultures of isolated donor chondrocytes can be exposed to lower doses of radiation as used for general X-rays today. Furthermore, transportation of these chondrocyte cultures with special regard to transillumination checks at airports seems reasonable as cell numbers and growth rates are unaffected by transillumination.

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