Biosensor-controlled perfusion culture to estimate the viability of cells

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Abstract—*A perfusion cell culture is characterised by the continuous addition of fresh nutrient medium and the withdrawal of an equal volume of used medium, allowing the realisation of cell cultivation conditions that are approximated as closely as possible to the* in vivo *situation. The combination of a perfusion cell culture with an enzyme glucose biosensor allows the glucose consumption of the cell culture to be monitored continuously. The resulting biosensor-controlled perfusion cell culture is a complex biomonitoring system that is useful for checking the metabolic state of a perfusion cell culture continuously and non-invasively over several days. With this experimental setup, it has been possible to test detrimental external effects on living systems at early stages, in vitro, but under in vivo-like conditions.*

Keywords—*Biosensor, Copper, Cytotoxicity, Glucose consumption, Hydrogen peroxide, Perfusion cell culture*

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1 Introduction

IN CONTRAST to a stationary monolayer cell culture, fresh nutrient medium is added continuously to the cells in the case of an open perfusion cell culture. Because of the withdrawal of an equal volume of used medium, products of cell metabolism are removed continuously, too. In this way, stable cell cultivation conditions can be realised because of a stable nutrient supply, as well as prevention of an accumulation of metabolic products. With this *in vitro* cultivation technique, *in vivo* conditions can be approximated as closely as possible (MINUTH *et al.*, 1992; 1996; 2000).

Carbohydrates are the major energy source for cultivated cells. The most frequently used sugar in nutrient media is glucose (CARTWRIGHT and SHAH, 1994). Consequently, continuous monitoring of the glucose concentration in the perfusion medium after the passage of the cell culture should make it possible to draw conclusions concerning the metabolic state of the cultivated cells and, in this way, to control the cell viability in an effective and non-invasive manner.

Biosensors are devices that allow the detection of chemical species within a non-homogeneous matrix specifically without, or only with low-scale, sample processing. The analyte is the subject of an initial biological reaction, a product of which is continuously transduced into a physical signal (FISCHER *et al.*, 1994).

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This study was based at first on the idea to combine an open perfusion cell culture system with a glucose biosensor to examine whether continuous measurement of the glucose concentration in the nutrient medium would open up the possibility of monitoring the metabolic state of the cell culture. Furthermore, potential fields of application of such a biosensor-controlled perfusion cell culture should be tested.

Cultivated cells are the smallest self-regulating living systems. Cells are open systems characterised by a permanent substance and energy exchange with their environment. The special organotypic cultivation state that is reached with the perfusion culture can be used as an *in vitro* model to realise *in vivo*-like conditions to test the influence of external agents on living systems. In conventional cell-based toxicity test systems, stationary cell cultures are used. The cells are incubated with the agent, which has to be tested, and after the end of the exposition the number of cells is quantified compared with an untreated control. Established cell number determination methods are based on the visual counting of cells or nuclei under the microscope, densitometric analyses of fixed and stained cultures, determinations of the DNA content, colorimetric assays based on the cellular content of a specific enzyme or substrate, or the uptake of a dye (MCATEER and DAVIES, 1994). All these methods are time consuming and/or invasive, i.e. the integrity of the cell culture will be disturbed by the determination technique. Moreover, continuous monitoring is not possible. Even if such test systems are useful to estimate growth-inhibitory influences on the cells, it must be assumed that long before such lethal effects occur, the viability of the cells will be influenced, indicated by changes in the metabolic reactions of the cell. Such effects cannot be estimated by conventional cytotoxicity assays. Therefore our intention was to investigate

whether a change in the glucose consumption of a perfusion cell culture, which can be monitored by the biosensor, could be a measure for subtoxic or sublethal toxic reactions.

A first test substance was copper. The biological effects of copper are of particular interest because this substance is a vital component for living cells and tissues in several enzyme systems on the one hand, but has a substantial toxicity at higher concentrations on the other (GEORGOPOULOS *et al.*, 2001). In some cases, it is found among other heavy metals as an environmental pollutant in ground and surface water. According to current legal regulations, its concentration in drinking water has to be controlled.

In a second application, the influence of hydrogen peroxide (H_2O_2) on perfusion cell cultures was tested. In living organisms, H_2O_2 plays an important role as part of immunological defence processes, on the one hand. On the other hand, it has a high antimicrobial efficacy but is also very cytotoxic. However, because of its physiological occurrence, intact tissues possess effective protection mechanisms against the cytotoxic activity of H_2O_2, so that its tissue toxicity is very low. Therefore there should be investigation into whether it is possible to differentiate between cytotoxicity and tissue toxicity of H_2O_2 using the biosensor-controlled perfusion cell culture.

2 Materials and methods

2.1 *Perfusion cell culture*

Human amniotic epithelial cells (FL cells) were trypsinated. 100 µL each of a cell suspension (3×10^6 cells ml^{-1}) in nutrient medium (Dulbecco MEM with 10% neonatal calf serum, 0.1% sodium hydrogen carbonate and 1% penicillin/streptomycin)[1] were given on supports (13 mm round)[2] using Minusheet cell holder systems[3]. After a 24 h pre-cultivation time at 37°C and 5% carbon dioxide atmosphere, six supports grown over with cells were transferred into a perfusion chamber[3]. The cell-containing chamber was perfused continuously by nutrient medium (Dulbecco MEM with 1% Hepes and 0.1% sodium hydrogen carbonate and 1% N-acetyl-L-alanyl-L-glutamine)[1], with a perfusion rate of 1 ml h^{-1} at 37°C (MINUTH *et al.*, 1992). The basal glucose concentration of the nutrient medium was 5.55 mM (1000 mg l^{-1}), corresponding to the normoglycaemic blood glucose concentration.

2.2 *Amperometric enzyme glucose biosensors*

Pencil-shaped Clark-type amperometric electrodes[4] consisting of a central platinum (Pt) anode and a surrounding silver/silver chloride (Ag/AgCl) cathode as a reference were layered by glucose oxidase (GOD)[5] immobilised covalently onto sepharose microspheres[6] using an established procedure (WEETALL, 1970; FLEMMING *et al.*, 1974). This enzyme layer was covered by a plane membrane made from regenerated cellulose[7]. The overall enzyme glucose sensor reaction involves the oxidation of glucose by oxygen as mediated by GOD, with H_2O_2 arising as a reaction product. The principle of the amperometric sensor comprises the subsequent electrochemical oxidation of H_2O_2 at a polarisation voltage of +700 mV, generating a current that, because of the stoichiometric interrelation, is a measure of the glucose concentration at the sensor (ABEL *et al.*, 1984; 1993; 1999)

$$\beta\text{-D-glucose} + O_2 \xrightarrow{GOD} \beta\text{-D-gluconic acid} + H_2O_2 \qquad (1a)$$

$$H_2O_2 \xrightarrow{700mV} O_2 + 2e^- + 2H^+ \qquad (1b)$$

With this laboratory-scale prepared glucose sensor, a linear glucose measurement was possible up to about 1.5 mM maximum glucose concentration. After covering the amperometric electrode with the enzyme membrane preparation, the sensor was connected to a constant polarisation voltage of +700 mV (Pt anode against Ag/AgCl reference) and stored at room temperature in glucose-free imidazole buffer pH 7.0[8]. After an electrochemical adaptation time of at least 24 h, the sensor was calibrated using solutions of glucose in imidazole buffer pH 7.0 at 37°C. To supply the constant polarisation voltage, for data registration and processing, a self-designed and self-constructed multi-channel amplifier[9] was connected with a data logger[10].

2.3 *Biosensor-controlled perfusion cell culture*

The glucose biosensor was arranged in the nutrient medium downstream from the cell culture (Fig. 1). Before measuring the glucose concentration, the nutrient medium was diluted at a ratio of 1:6 by imidazole buffer pH 7.0 using a bypass arrangement.

The glucose concentration was measured continuously in the used nutrient medium, i.e. after it had passed the cell culture. The moving average of the values measured during each hour was subtracted from the constant basal glucose concentration of 5.55 mM in the fresh nutrient medium. In this way, one value per hour of the glucose consumption of the cells was calculated and registered. However, it is possible to vary the profile of data processing and registration by reprogramming the data logger

To test external effects on the perfusion cell culture, test substances were added to the perfusion medium in the scheduled test concentration, 20–24 h after the perfusion culture had been started. To make possible a direct comparison of data from different experiments, the absolute glucose consumption was converted into a standardised glucose consumption (SGC) by setting the absolute glucose consumption, which was registered 20–24 h after the start of the perfusion, at 100%. Subsequently,

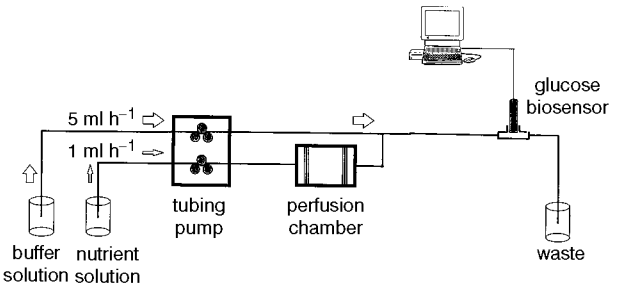

Fig. 1 *Schematic setup of biosensor-controlled perfusion cell culture system*

[1]Biochrom KG, Berlin, Germany

[2]Thermanox Nunc Inc., Naperville, IL, USA

[3]Minucells and Minutissue Vertriebs GmbH, Bad Abbach, Germany

[4]SME/S3, ELBAU Elektronische Bauelemente GmbH, Berlin, Germany

[5]EC 1.1.3.4., Arzneimittelwerk, Dresden, Germany

[6]Sepharose® CL-6B, Pharmacia Fine Chemicals, Uppsala, Sweden

[7]PT 150 Cuprophane®

[8]Imidazole puriss p.a., Fluka AG, Buchs, Switzerland

[9]Institute of Pathophysiology, Karlsburg, Germany

[10]HC-LOG, TIS Technische Informations-Systeme GmbH, Bocholt, Germany

all other data registered during the experiment were related to this value.

2.4 *Scanning electron microscopy*

Microscopic characterisations of the cells have been performed using scanning electron microscopy (SEM). For this purpose, after different perfusion periods, cell supports with adhering cells were removed from the perfusion chamber. The cells were prepared following an established procedure described elsewhere (JÜLICH *et al.*, 1998). To explore the cell morphology, a scanning electron microscope DSM 940[11] was used, with an acceleration voltage of 10 kV and a 1000 fold magnification. As a characteristic parameter of cell morphology, the relative density of microvilli on the cell surface was evaluated in a semi-quantitative manner by visual inspection of microscopic photographs by two independent examiners.

2.5 *Test substances*

Copper-containing test solutions were prepared, based on a copper sulphate solution with a copper content of 3900 $\mu g l^{-1}$, by dilution with distilled water.

H_2O_2 test solutions were prepared from a 30% stock solution (Ph.Eur., stabilsed)[12], by dilution with distilled water.

Aqueous solutions of two inclusion compounds of H_2O_2 and surfactants in urea[13] were tested in this study. The substance EO 210 contained 15% H_2O_2 and 5% lauryl sorbitan ester (Span 20); the substance EO 216 contained 16.5% H_2O_2 and 4% lauryl sorbitan ester (Span 20) (JÜLICH *et al.*, 1999).

3 Results

The monitoring of the glucose concentration in the nutrient medium after the passage of the perfusion chamber was started immediately after the transfer of the pre-cultivated cell supports into the perfusion chamber. Following this, a phase of increasing absolute glucose consumption was registered, i.e. the glucose concentration in the used medium behind the perfusion chamber decreased continuously in

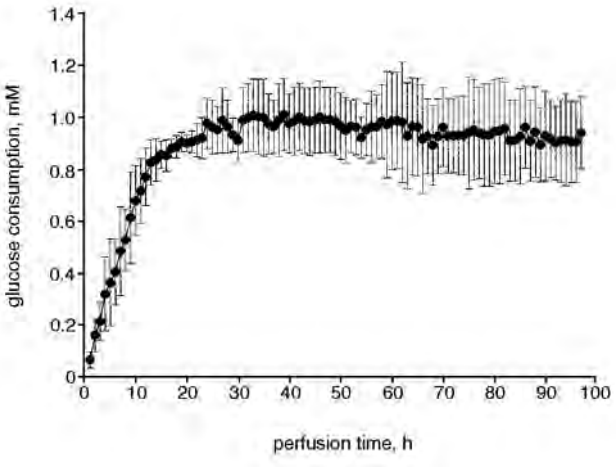

Fig. 2 *Absolute glucose consumption of perfused FL cell cultures (mean ± SD, n = 9)*

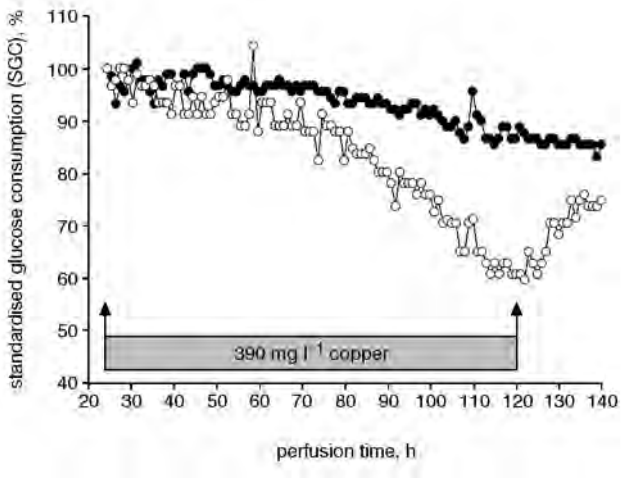

Fig. 3 ○*Standardised glucose consumption (SGC) of a perfused FL cell culture during 96 h exposure to 390 $\mu g l^{-1}$ copper, compared with (•) untreated control*

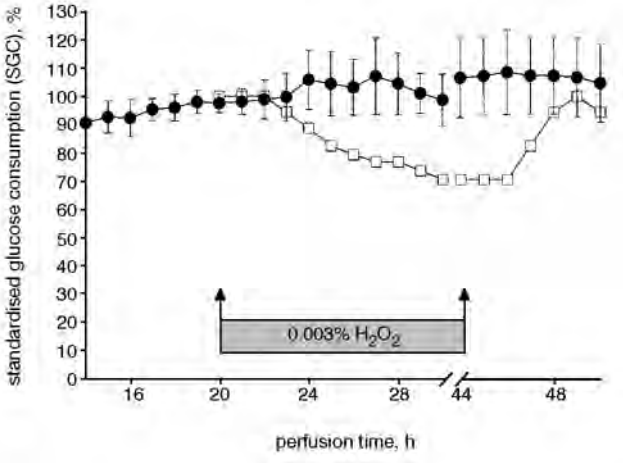

Fig. 4 □*Standardised glucose consumption (SGC) of a perfusion cell culture during 24 h exposure to 0.003% H_2O_2, compared with (•) untreated controls (mean ± SD, n = 9)*

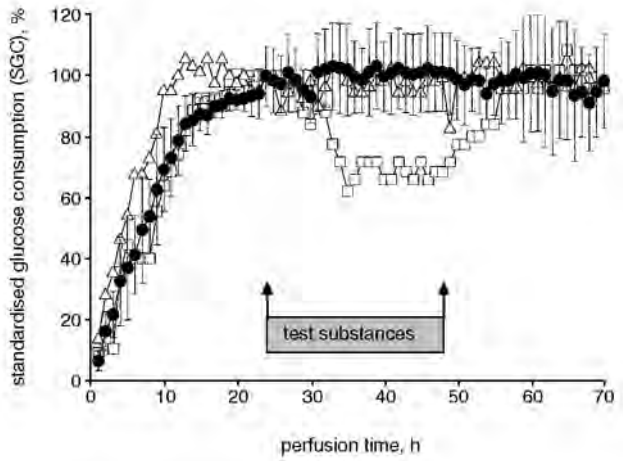

Fig. 5 *Standardised glucose consumption (SGC) of perfused FL cell cultures during 24 h exposure to (△) 0.005% EO 216 (corresponding to 0.0008% H_2O_2) as well as (□) 0.02% EO 210 (corresponding to 0.003% H_2O_2), compared with (•) untreated controls (mean ± SD, n = 9)*

[11]Zeiss, Oberkochem, Germany

[12]Roth GmbH, Karlsruhe, Germany

[13]Institute of Applied Chemistry, Adlershof, Berlin, Germany

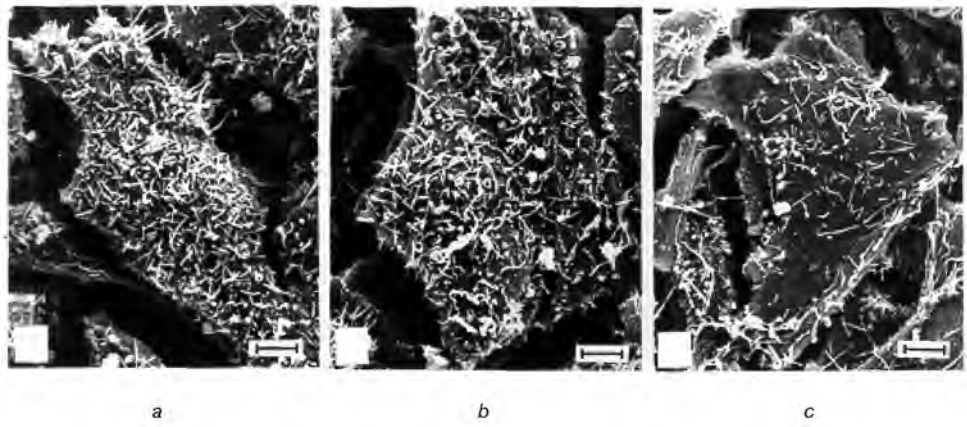

Fig. 6 *Scanning electron microscopic pictures of FL cells (DSM 940A, 10 kV; bar: 5μm): (a) high, (b) medium, and (c) low density of microvilli on cell surface*

relation to the fresh medium (Fig. 2). This increase continued until a nearly stable mean value around 1 mM was reached 20–24 h after the start of the cell perfusion, indicating that the glucose consumption of the cell culture became more or less stabilised. This equilibrium state of glucose consumption could be maintained stable over several days, apart from a slight decrease in glucose consumption in the course of the cultivation time.

In the following experiments, test substances were not added to the perfusion medium until this metabolic steady state was reached.

During the first 24 h following the addition of 390 $\mu g\,l^{-1}$ copper, the glucose consumption of the perfusion cell culture remained nearly unchanged compared with an untreated control tested simultaneously. However, in the further course of the experiment, an increasing reduction in the glucose consumption was found. However, this change in the glucose consumption of the cells was reversible if copper was removed from the perfusion medium after 96 h (Fig. 3).

Within about 10 h after the addition of 0.003% H_2O_2 to the perfusion medium, the glucose consumption of the cell culture decreased to reach a new steady state at a lower level. The mean values of standardised glucose consumption of nine control experiments served as references for this test. After H_2O_2 was removed from the perfusion medium, the glucose consumption of the perfusion cell culture increased, again reaching the level of untreated controls (Fig.4).

The same phenomenon was registered if the H_2O_2 inclusion compound EO 210 was added to the perfusion medium at a final concentration of 0.02%, corresponding to an effective H_2O_2 concentration of 0.003%. A lower, effective H_2O_2 concentration did not influence the glucose consumption of the perfusion cell culture, which was demonstrated with 0.005% of the H_2O_2 inclusion compound EO 216 corresponding to 0.0008% effective H_2O_2 concentration. In this test series, the mean values of standardised glucose consumption estimated during nine control experiments served as references again (Fig.5).

SEM investigations of the cell culture found a complete and non-disturbed cell monolayer during the whole period of nearly steady-state glucose consumption. Altogether, 2780 cells in the control experiment, 2644 cells in the course of incubation with 0.003% H_2O_2, 1272 cells in the course of incubation with 0.02% EO 210 and 1419 cells in the course of incubation with 0.005% EO 216, were evaluated using SEM pictures. Generally, the cells were classified into three categories: with high, with medium and with low density of microvilli on the cell surface (Fig. 6). At the end of the phase of steep increase in glucose consumption, i.e. 18 h after the start of the perfusion culture, 24–31% of the cells were found to have a high density of microvilli on their surface (Table 1). Without the addition of external substances, i.e. in the control experiment, the number of cells with a high density of microvilli was halved after another 12 h of cell perfusion. This value remained more or less stable up to the end of the period of investigation (Table 1). In parallel, the fraction of cells with a low density of microvilli increased in the same relationship. Only the relative portion of cells with a medium density of microvilli remained more or less stable (data not shown).

A corresponding reduction in cells with high density of microvilli on their surface has been found during a 24 h incubation with 0.003% H_2O_2. However, 24 h after the removal of the test substance, a further reduction in the microvilli-rich cell fraction was found (Table 1)

In contrast to that, incubation with H_2O_2 inclusion compounds did not result in a substantial reduction in the number of cells with a high density of microvilli on their surface. Only 24 h after the removal of EO 210, this cell fraction was reduced, whereas the number of cells with a high number of microvilli did not decrease permanently during the whole investigation period in the experiment with 0.005% EO 216 (Table 1).

Table 1 Percentage of cells with high number of microvilli on their surface (semi-quantitative classification based on SEM pictures)

Experimental conditions	Initial value before addition of test substance (18 h cell perfusion time)	Value 12 h after addition of test substance (30 h cell perfusion time)	Value 24 h after addition of test substance (42 h cell perfusion time)	Value 24 h after removal of test substance (66 h cell perfusion time)
Control (without test substance)	27.9%	12.7%	13.5%	13.2%
0.003% H_2O_2	30.9%	12.0%	15.2%	9.6%
0.02% EO 210	24.0%	25.8%	21.1%	16.3%
0.005% EO 216	24.1%	16.5%	31.8%	25.0%

4 Discussion

During the last decade, efforts have been made to develop alternatives for on-line and real-time monitoring of cultivated cells based on non-invasive methods, such as continuous pH detection or impedance measurement (McConnell *et al.*, 1992; Giaever and Keese, 1993; Keese and Giaever, 1994; Ehret *et al.*, 1997; Wolf *et al.*, 1998, Lehmann *et al.*, 2000; 2001). Based on such parameters, a continuous pattern of integrated cell characteristics can be registered. However, because of the well-known and defined composition of the nutrient media used for cell cultivation, it should be possible to detect at least partial aspects of the cell metabolism by control of the consumption of a specific nutritious substance, or the appearance of a specific metabolic product. This is considered to be valid for more specific control of the cell culture.

The instrumentation of an open perfusion cell culture by an enzyme glucose biosensor, as realised in this study, allowed the detection of the glucose concentration in the nutrient medium after the passage of the cell-containing perfusion chamber in relation to the constant glucose content in the fresh medium. The resulting glucose consumption is a useful parameter to reflect the metabolic state of the perfusion cell culture.

Continuous measurement of glucose in a flow-through system using a biosensor is an old and well-established principle (Abel *et al.*, 1983). The glucose biosensor used in this study consisted of an electrochemical Clark-type electrode, a reaction layer containing immobilised glucose oxidase and an outer covering membrane made from regenerated cellulose. The function of this hydrophilic membrane was a protective one, to exclude substances with higher molecular weight than proteins and to fix the enzyme layer on the electrode surface.

By this simple biosensor construction, a measuring tool was realised with a short response as well as a high sensitivity to glucose concentration changes. Nevertheless, because of the simplicity of the membrane system, the upper range of linear glucose detection was limited to 1.5 mM. However, the basal glucose concentration in the fresh nutrient medium was as high as 5.55 mM ($1000\,mg\,l^{-1}$). An additional problem arose from the perfusion rate of the nutrient medium of only $1\,ml\,h^{-1}$. With this very slow perfusion rate, a stable flow-through measurement is difficult to realise, because of the possibility of local glucose concentration gradients and the risk of insufficient oxygen concentration at the sensor site. Both problems have been solved in a very simple way by diluting the nutrient medium before the measurement of glucose concentration. This dilution was realised by pumping imidazole buffer into the nutrient medium stream using a bypass arrangement with a five-fold higher flow rate. With the resulting dilution ratio of 1:6, the glucose detection range was extended without substantial loss of sensitivity on the one hand. On the other hand, with this bypass pump arrangement, an acceleration of the sample medium flow rate at the sensor site was reached, minimising the possibility of local disturbances of the glucose measurement. Additionally, a sufficient oxygen supply at the sensor site could be guaranteed by this bypass system.

In the cell perfusion experiments presented in this paper, human amniotic epithelial cells (FL cells) were used. Conventional stationary FL cell cultures have been proved and well established for several years as a useful model for cytotoxicity testing and are used now, as before, to test the biological effects of active substances *in vitro* (Kramer *et al.*, 1987; Kramer *et al.*, 1993; Jülich *et al.*, 1999; Awadh Ali *et al.*, 2001; von Woedtke *et al.*, 2002*a*; Fenske *et al.*, in press). Consequently, it was an obvious idea to use this cell line first of all to estimate possible influences of external noxious substances on the glucose consumption of cells in perfusion culture, with the main objective being to predict possible cytotoxic reactions at an early stage.

After the transfer of the pre-cultivated cells into the perfusion chamber, a phase of decreasing glucose concentration in the perfusion medium was monitored. Besides a technically caused initial decrease in the measured values immediately after the start of the perfusion, this was attributed mainly to an increase in the glucose consumption of the cells, because of a new phase of cell growth following the change in the cultivation conditions.

After about 18 h, the increase in glucose consumption slowed down to reach a plateau after 20–24 h, indicating a metabolic equilibrium state with a nearly stable consumption of about 1 mM of the glucose, which was supplied by the perfused nutrient solution. Despite a slight and time-dependent decrease in glucose consumption, this equilibrium state could be kept stable over several days, which is in accordance with recent experimental experience indicating stable perfusion cultures of other cell types over prolonged periods of time using the same perfusion technique (Sittinger *et al.*, 1997, Kreklau *et al.*, 1999, Kloth and Suter-Crazzolara, 2000; Temenoff and Mikos, 2000; Minuth *et al.*, 2000). Consequently, the instrumentation of such a perfusion system by a glucose biosensor will open up the possibility of monitoring the stability of cultivation conditions as well as the metabolic state of cells, continuously and non-invasively, over the whole time of cell cultivation on the one hand. On the other hand, it is possible that any changes in the glucose consumption could indicate low-scale detrimental effects of external noxious substances below the level of acute toxic reactions. To test this, different substances were added to the perfusion medium after the cell culture had reached the stable plateau phase of glucose consumption, i.e. 20–24 h after the perfusion was started.

As a first test substance, copper was chosen because of its ambivalent character for living organisms. Using the biosensor-controlled perfusion cell culture, a copper concentration of $390\,\mu g\,l^{-1}$ induced a significant but reversible decrease in glucose consumption within 96 h of incubation time. When stationary monolayer FL cell cultures were exposed to the same copper concentration over 24 h, the cell growth was not affected. Under these test conditions, an inhibitory concentration $IC_{50} = 980\,\mu g\,l^{-1}$ was found for copper (Fenske *et al.*, in press). Consequently, the continuously monitored glucose consumption was a sensitive means to demonstrate detrimental effects on the metabolism of perfused FL cells below the level of acute toxicity. Moreover, in the perfusion cell culture test, the beginning of the reduction in glucose consumption was detected up to 24 h after the heavy metal exposition was started, i.e. this effect could be registered only because of the longer test period realisable with this biomonitoring system. Obviously, it is a useful tool to register retarded as well as sublethal and reversible effects, especially.

The importance of such low-scale effects on the metabolism of living systems of concentrations of biologically active substances, which do not cause acute toxicity, is not completely clear yet. However, in our view, it should not be underestimated, especially in the case of a heavy metal such as copper, which occurs ubiquitously in drinking water. Threshold values for copper in drinking water are much higher than those of other heavy metals, e.g. $2\,mg\,l^{-1}$ is allowed according to the revised EU drinking water directive (98/83/EC). In other guidelines, it is in the same range (Fewtrell *et al.*, 2001). Against the background of the test results presented here, these values seem to be much too high. Consequently, an *in vitro* test system such as the biosensor-controlled perfusion cell culture could be helpful in future to estimate threshold values of hygienic or environmental relevance on a firm scientific basis.

As another physiologically important and biologically active agent, H_2O_2 was investigated in a second experimental series. Using stationary monolayer FL cell cultures, a substantial cytotoxicity of H_2O_2 was found. An H_2O_2 concentration of 0.0005% only caused a 50% growth inhibition, and, after incubation with 0.0015% H_2O_2, a mean cell growth of about 7% only, compared with untreated controls, was measured (ADRIAN *et al.*, 1998; JÜLICH *et al.*, 1999). However, in our experiments with an H_2O_2 concentration as high as 0.003%, a significant but reversible reduction in the glucose consumption of a perfused FL cell culture was induced. The concentrations tested here were in the same range as those occurring in the micro-environment of macrophages (KRAMER *et al.*, 1995).

Under physiological conditions, 0.003% H_2O_2 will cause an inhibition of the activity of natural killer cells without killing them (EL-HAG and CLARK, 1984). However, because of the physiological occurrence of H_2O_2 as part of the immune response, some protection mechanisms are active in living tissue to prevent healthy cells from toxic H_2O_2 effects. In our experiments, the same FL cells tolerated a much higher H_2O_2 concentration if they were cultivated in the perfusion system compared with the stationary cultivation. This is taken as another indicator that the state of perfused cells is approximated much more closely to the intact tissue situation, as can be realised by conventional stationary cultivation techniques. From this point of view, the lower H_2O_2 sensitivity of the perfusion cell culture can be explained. Obviously, protective functions against excessive H_2O_2 are much more intact in the perfusion culture than under stationary conditions. Consequently, *in vitro* investigations of biological effects of such low H_2O_2 concentrations can be realised only using a perfusion cell culture. Moreover, the biosensor-controlled perfusion cell culture could be a good model to estimate, not only the cytotoxicity of low substance concentrations in a sensitive manner, but also their compatibility with living tissue. For example, such differentiation is relevant, as antimicrobially active preparations containing H_2O_2 are scheduled for use as preservatives (JÜLICH *et al.*, 1999) and additives in cosmetics (HANSCHKE and JÜLICH, 2000), or as components of multi-step procedures to sterilise biologically active implants as biosensors (VON WOEDTKE *et al.*, 2002*b*).

Because of the direct contact of implants with living tissue following the sterilisation procedure, not only the microbiological activity but also the tissue compatibility must be regarded much more than the cytotoxicity. Based on the results presented here, it could be proved by a simple experimental model that antimicrobially active H_2O_2 concentrations can be used for the treatment of implant materials, because the risk of tissue toxic reactions of H_2O_2 residues is much lower than would be concluded based on conventional cytotoxicity tests.

In further tests, we investigated H_2O_2 inclusion compounds, which were found to be antimicrobially more effective but less cytotoxic in relation to pure H_2O_2, if the stationary test model was used (ADRIAN *et al.*, 1998, JÜLICH *et al.*, 1999). A concentration of 0.02% of the inclusion compound EO 210, which corresponded to an effective H_2O_2 concentration of 0.003%, induced nearly the same reversible disturbance of the glucose consumption of a perfusion cell culture as was found with 0.003% pure H_2O_2. 0.005% of the similar inclusion compound EO 216 had nearly the same antimicrobial efficacy as 0.02% EO 210 (JÜLICH *et al.*, 1999). However, because of the lower effective H_2O_2 concentration (0.0008%), no influence on the glucose consumption of the perfused FL cell culture was found in the presence of 0.005% EO 216. Consequently, besides a lower cytotoxicity, better tissue compatibility of these inclusion compounds could be concluded, based on these test results.

In addition to the detection of the glucose consumption, morphological investigations of the cells were performed. However, a disappearance of microvilli on the cell surface could be detected by scanning electron microscopy to be the only morphological change. Transmission electron microscopic investigations of the cell ultra structure, e.g. the size of mitochondria, did not point at other influences on cell morphology.

Generally, it has been found that the mean number of microvilli tended to decrease in the course of cell perfusion without any influence of external test substances. During the incubation with 0.003% H_2O_2, the fraction of cells with a high density of microvilli on the surface was reduced in the same scale as had been found within the control experiment. Consequently, besides a reversible change in glucose consumption, H_2O_2 had no additional effect on cell morphology.

These results are somewhat different to the estimations with H_2O_2 inclusion compounds. During the EO 210 incubation, no reduction in the number of microvilli on the cell surface was found. The disappearance of microvilli seemed not to start until the test substance was removed from the cell culture. Moreover, in the case of EO 216, the formation of a microvilli-rich fraction seemed to be promoted substantially. Even 24 h after removal of the test substance, a larger number of microvilli-rich cells could be detected.

Generally, it is assumed that microvilli serve to increase the cell surface available for substance exchange. Depending on cell activity, variations in the number as well as the length of microvilli are possible. The formation of microvilli is an active metabolic process that can be reduced under adverse environmental conditions as well as in the course of cell ageing. The density of microvilli on the cell surface was estimated in a semiquantitative manner, only. The high number of cells inspected has minimised the influence of artifacts possibly resulting from the cell preparation technique. Despite the preliminary character of these first data presented here, the disappearance of microvilli seemed to be only a result of cell ageing in the course of the perfusion cultivation. Short-term, reproducible influences on the cell metabolism indicated by changes in glucose consumption did not find any counterpart in changes in cell morphology.

On the other hand, the lower cytotoxicity of antimicrobially effective H_2O_2 inclusion compounds, compared with pure H_2O_2, has been accompanied not only by a lesser influence on the glucose consumption of perfused FL cells, but also by reduced disappearance of the microvilli.

To summarise, the metabolic state of a cell culture reflected by its glucose consumption can be taken as representative of its metabolic state and its viability. Moreover, through control of the glucose consumption, even slight changes in the cell metabolism can be detected. This aspect especially may open interesting fields of application for this biomonitoring system. If the stability of cell cultivation is monitored by a glucose biosensor, any changes in the glucose consumption could serve as a kind of alarm criterion on the one hand. On the other hand, biological effects can be monitored *in vitro* in a sensitive but non-expensive manner. Not only are concentration ranges of active agents of interest, which induce lethal effects with irreversible changes in cell morphology. The effects of such substance concentrations, especially, may have a predictive character for hygienic or ecotoxicological evaluations, which cause low-scale sublethal or subtoxic, as well as reversible, disturbances to the cell metabolism also over longer periods of time. Such effects will be detectable using this biomonitoring system.

According to our first experimental results, this test system allows us to detect the effects of external noxious substances on cell cultures under conditions that approximate the *in vivo*

situation much more closely than conventional techniques, because the perfusion cell culture resembles organotypic conditions (MINUTH *et al.*, 1996; 2000). Therefore it may be possible to differentiate aspects of tissue toxicity or tissue compatibility from cytotoxicity. Beyond the data presented here, another application of the biosensor-controlled perfusion cell culture in the field of investigation of new drugs is described elsewhere (VON WOEDTKE *et al.*, 2002*a*).

Further developments of the biosensor-controlled perfusion cell culture will include the simultaneous monitoring of such additional parameters as pH, oxygen pressure and, above all, the concentration of lactate as a breakdown product of glucose metabolism under culture conditions. Moreover, further investigations will be carried out to prove whether other cell types can be controlled by this method, and to what extent, so that it can be used for special test purposes. Based on these first, encouraging results, we intend our future work to obtain a compact and flexible cell-based biomonitoring system for several fields of application.

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References

ABEL, P., WILKE, W., HÜBNER, G., and KRÜGER, I. (1983): 'Kontinuierliche Bestimmung der Blutglukose mittels Enzymelektrode', *Z. med. Labor-Diagn.*, **24**, pp. 264–270

ABEL, P., MÜLLER, A., and FISCHER, U. (1984): 'Experience with an implantable glucose sensor as a prerequisite of an artificial beta cell', *Biomed. Biochim. Acta*, **43**, pp. 577–584

ABEL, P., VON WOEDTKE, TH., REBRIN, K., SCHLOSSER, M., and FISCHER, U. (1993): 'Glucose biosensor for *in vivo* application' in ALCOCK, S. J., and TURNER, A. P. F. (Eds): '*In vivo* chemical sensors. Recent development' (Cranfield, 1993), pp. 16– 26

ABEL, P. U., VON WOEDTKE, TH., SCHULZ, B., BERGANN, TH., and SCHWOCK, A. (1999): 'Stability of immobilized enzymes as biosensors for continuous application *in vitro* and *in vivo*', *J. Mol. Cat. B: Enzymatic*, **7**, pp. 93–100

ADRIAN, V., JÜLICH, W.-D., ALHITARI, N., VON WOEDTKE, TH., and ABEL, P. (1998): 'Einfluß von antimikrobiell wirksamen Einschlussverbindungen des Wasserstoffperoxids auf humane Amnionepithelzellen', *Hyg. Med.*, **23**, p. 94

AWADH ALI, N. A., JÜLICH, W.-D., KUSNICK, C., and LINDEQUIST, U. (2001): 'Screening of Yemeni medicinal plants for antibacterial cytotoxic activities', *J. Ethnopharmacol.*, **74**, pp. 173–179

CARTWRIGHT, T., and SHAH, G. P. (1994): 'Culture media' in DAVIS, J. M. (Ed.): 'Basic cell culture. A practical approach' (IRL Press at Oxford University Press, Oxford, New York, Tokyo, 1994), pp. 57–91

EHRET, R., BAUMANN, W., BRISCHWEIN, M., SCHWINDE, A., STEGBAUER, K., and WOLF, B. (1997): 'Monitoring of cellular behaviour by impedance measurements on interdigitated electrode structures', *Biosens. Bioelectron.*, **12**, pp. 29–41

EL-HAG, A., and CLARK, R. A. (1984): 'Down regulation of human natural killer activity against tumors by the neutrophil myeloperoxidase system and hydrogen peroxide', *J. Immunol.*, **133**, pp. 391–397

FENSKE, CH., GÜNTHER, B., KNAUER, A., RUDOLPH, P., ADRIAN, V., VON WOEDTKE, TH., ROSSBERG, H., KRAMER, A., and JÜLICH, W.-D. 'Comparison of different biological methods for the assessment of toxicological risks', *Acta hydrobiol. hydrochim.* (in press)

FEWTRELL, L., KAY, D., and MACGILL, S. (2001): 'A review of the science behind drinking water standards for copper', *Int. J. Environ. Health Res.*, **11**, pp. 161–167

FISCHER, U., REBRIN, K., VON WOEDTKE, TH., and ABEL, P. (1994): 'Clinical usefulness of the glucose concentration in the subcutaneous tissue–properties and pitfalls of electrochemical biosensors', *Horm. Metab. Res.*, **26**, pp. 515–522

FLEMMING, CH., GABERT, A., and ROTH, P. (1974): 'Synthese und Eigenschaften trägerfixierter Enzyme. VI. Kovalente Bindung von Glucoseoxidase an Dialdehydepidex und Dialdehydzellulose', *Acta Biol. Med. Germ.*, **33**, pp. 15–20

GEORGOPOULOS, P. G., ROY, A., YONONE-LIOY, M. J., OPIEKUN, R. E., and LIOY, P. J. (2001): 'Environmental copper: its dynamics and human exposure issues', *J. Toxicol. Environ. Health B Crit. Rev.*, **4**, pp. 341–394

GIAEVER, I., and KEESE, CH. R. (1993): 'A morphological biosensor for mammalian cells', *Nature*, **366**, pp. 591–592

HANSCHKE, R., and JÜLICH, W.-D. (2000): Patent application DE 19851776 A 1, 11.05.2000

JÜLICH, W.-D., HANSCHKE,R., and ALHITARI, N. (1998): 'Gentle sterilisation of biologically active implants. Part IV: Comparative electron microscope observations on the influence of hydrogen peroxide and hydrogen peroxide/surfactant urea inclusion compounds on human amnion epithelial cells in a perfusion cell culture', *Hyg. Med.*, **23**, pp. 465–472

JÜLICH, W.-D., OHME, R., ALHITARI, N., ADRIAN, V., VON WOEDTKE, TH., and KRAMER, A. (1999): 'Synthesis, antimicrobial efficacy, and tolerability of new structures of urea, hydrogen peroxide and tensides' *Pharmazie*, **54**, pp. 171–178

KEESE, CH. R., and GIAEVER, I. (1994): 'A biosensor that monitors cell morphology with electrical fields', *IEEE Eng. Med. Biol.*, **June/July**, pp. 401–408

KLOTH, S., and SUTER-CRAZZOLARAR, C. (2000): 'Modulation of rebal blood vessel formation by glial cell line-derived neurotropic factor', *Microvascular Research*, **59**, pp. 190–194

KRAMER, A., HALLE, W., WEUFFEN, W., ADRIAN, V., HERRMANN, M., BREMER, J., FLECK, H., and STEIGER, E. (1987): 'Microbiostatic effectivity, cytotoxicity and phytotoxicity as a basic information for the compatibility of antimicrobial agents', *Z. gesamte Hyg.*, **33**, pp. 610–615

KRAMER, A., ADRIAN, V., and ADAM, CH. (1993): 'Comparison of the toxicity of Lavasept® and selected antiseptic agents', *Hyg. Med.*, **18**, pp. 9–16

KRAMER, A., JÜLICH, W.-D., and OHME, R. (1995): 'New functional supramolecular structures of urea, hydrogen peroxide and tensides suitable for use as preservatives. First communication: Working hypothesis, bacteriostatic efficacy and skin tolerability', *Hyg. Med.*, **20**, pp. 330–343

KREKLAU, B., SITTINGER, M., MENSING, M. B., VOIGT, C., BERGER, G., BURMESTER, G. R., RAMANZADEH, R., and GROSS, U. (1999): 'Tissue engineering of biphasic joint cartilage transplants', *Biomaterials*, **20**, pp. 1743–1749

LEHMANN, M., BAUMANN, W., BRISCHWEIN, M., EHRET, R., KRAUS, M., SCHWINDE, A., BITZENHOFER, M., FREUND, I., and WOLF, B. (2000): 'Non-invasive measurement of cell membrane associated proton gradients by ion-sensitive field effect transistor arrays for microphysiological and bioelectronical applications', *Biosens. Bioelectron.*, **15**, pp. 117–124

LEHMANN, M., BAUMANN, W., BRISCHWEIN, M., GAHLE, H.-J., FREUND, I., EHRET, R., DRECHSLER, S., PALZER, H., KLEINTGES, M., SIEBEN, U., and WOLF, B. (2001): 'Simultaneous measurement of cellular respiration and acidification with a single CMOS ISFET', *Biosens. Bioelectron.*, **16**, pp. 195–203

MCATEER, J. A., and DAVIES, J. (1994): 'Basic cell culture technique and the maintenance of cell lines' in DAVIS, J. M. (Ed.): 'Basic cell culture. A practical approach' (IRL Press at Oxford University Press, Oxford, New York, Tokyo), pp. 93–148

MCCONNELL, H. M., OWICKI, J. C., PARCE, J. W., MILLER, D. L., BAXTER, G. T., WADA, H. G., and PITCHFORD, S. (1992): 'The Cytosensor Microphysiometer: Biological applications of silicon technology', *Science*, **257**, pp. 1906–1912

MINUTH, W. W., STÖCKL, G., KLOTH, S., and DERMIETZEL, R. (1992): 'Construction of an apparatus for perfusion cell cultures which enables *in vitro* experiments under organotypic conditions', *Eur. J. Cell Biol.*, **57**, pp. 132–137

MINUTH, W. W., KLOTH, S., AIGNER, J., SITTINGER, M., and RÖCKL, W. (1996): 'Approach to an organo-typical environment for cultured cells and tissues', *BioTechniques*, **20**, pp. 498–501

MINUTH, W. W., SCHUMACHER, K., STREHL, R., and KLOTH, S. (2000): 'Physiological and cell biological aspects of perfusion culture technique employed to generate differentiated tissues for long term biomaterial testing and tissue engineering', *J. Biomater. Sci. Polymer Edn.*, **5**, pp. 495–522

SITTINGER, M., SCHULTZ, O., KEYSZER, G., MINUTH, W. W., and BURMESTER, G. R. (1997): 'Artificial tissues in perfusion culture', *Int. J. Artif. Organs*, **20**, pp. 57–62

TEMENOFF, J. S., and MIKOS, A. G. (2000): 'Review: tissue engineering for regeneration of articular cartilage', *Biomaterials*, **21**, pp. 431–440

VON WOEDTKE, TH., LINDEQUIST, U., ALHITARI, N., KUSNICK, C., JÜLICH, W.-D., and ABEL, P. U. (2002*a*): 'The usefulness of a biosensor controlled perfusion cell culture for the investigation of new drugs demonstrated with the marine fungus Kirschsteiniothelia maritima', *Pharmazie*, **57**, pp. 270–274

VON WOEDTKE, TH., JÜLICH, W.-D., HARTMANN, V., STIEBER, M., and ABEL, P. U. (2002*b*): 'Sterilization of enzyme glucose sensors: problems and concepts', *Biosens. Bioelectron.*, **17**, pp. 373–382

WEETALL, H. (1970): 'Storage stability of water insoluble enzymes covalently coupled to organic and anorganic carriers', *Biochem. Biophys. Acta*, **212**, pp. 1–7

WOLF, B., BRISCHWEIN, M., BAUMANN, W., EHRET, R., and KRAUS, M. (1998): 'Monitoring of cellular signalling and metabolism with modular sensor-technique: The PhysioControl-Microsystem (PCM®)', *Biosens. Bioelectron.*, **13**, pp. 501–509

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