Cell Cultures in Pharmacological and Toxicological Research

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Abstract

High-throughput cell based assays became essential and standard in pharmacological and toxicological research. In this paper we are introducing two ongoing projects based on cell cultures to illustrate the power and typical applications of these *in vitro* methods. Oxidative stress plays a crucial role in several diseases including ischemic heart disease. It was also indicated as a major toxicity mechanism of pyrethroid pesticides. We have developed an oxidative stress model based on hydrogen peroxide application to cultured C2C12 skeletal muscle cells or primary chicken cardiac myocytes. Quantification of cytotoxicity was performed by a high-throughput Alamar blue metabolic assay. The reference compound adenosine partially reversed the cytotoxic effect of hydrogen peroxide with a bell shaped concentration/effect curve. Cyfluthrin, a representative of pyrethroids, showed similar toxicity as hydrogen peroxide, except that adenosine rather augmented than reversed its toxic action. Ascorbic acid, an antioxidant, partially reversed the action of cyfluthrin. Joint application of sub-toxic concentrations of hydrogen peroxide and cyfluthrin showed an additive effect. Our oxidative stress model will be a useful tool to characterize drugs intended to reverse reperfusion injury following cardiac ischemia. The same cell culture system could be used for toxicological evaluation of pesticides.

Keywords: cell culture, C2C12, cardiac myocytes, pharmacology, toxicology, *in vitro*, cell based assays

Introduction

Alternative methods in biological research obtained importance in connection with the ethical problems associated with animal testing (*KANDÁROVÁ,* 2011). After a century of development, these practical applications are finding their place in basic research and in clinical therapies (*MERICKO* 2002). Utilization of these methods increased rapidly over the last 20 years. Nowadays, *in vitro* methods have a crucial role in drug development, hazard identification and toxicological testing. Several alternative methods have already been validated and can be used for the reduction or the replacement of living animals in toxicological experimentation (*in vitro*, *ex-vivo* or *in silico* systems). The accepted alternative methods are valuable and appreciated tools of modern toxicology (*KANDÁROVÁ*, 2011).

There was a paradigm change in pharmacological and toxicological research at the end of the 20th century (*AN 2010, BHATTACHARYA 2011*). Target oriented drug development became the standard which required the screening of thousands of compounds on one single target. *In vitro* test systems became essential in drug development and also in toxicology to reduce time and costs. Cell culture based assays have several benefits compared to biochemical tests (*NATARAJAN* 2006, 2011). They could measure functional effects such as activation of signal conduction pathways or complex behavior of cells. Several primary cultures (from different species and different tissues) and cell lines have already been developed for different purposes, for example for physiological or pharmacological studies or

as disease models. Special importance should be given to human stem cell based systems (*LUDWIG 2006, STANSLEY 2012, FRANK 1984*). For the high-throughput screens several methods have been adapted to detect physiological changes in the status of cells (receptor activation, cell death, metabolic activity changes, etc.) in a high-throughput (multi-well) format (*KEPP2011, RISS 2013*).

The ideal test for *in vitro* cytotoxicity is a simple, reliable, sensitive, safe and cost effective measurement of cell viability. Certainly, it is important that is should not interfere with the tested compound and physiological activity of the cells. Since 1993, a new dye – Alamar Blue - has been available that can fulfill these conditions. (*FIELDS 1993*) Alamar Blue dye has gained popularity in scientific experiments as a very simple, versatile, efficient and rapid way of measuring cytotoxicity (*O'BRIEN 2000*).

Ischemic heart disease is one of the leading causes of death in Europe. In the treatment of myocardial infarction it is a huge problem that after restoration of circulation, called reperfusion phase, a considerably amount of cardiac myocyte damage occurs, especially because of sudden high degree of oxidative stress *(IBANEZ 2011, SANADA 2011, MINAMINO 2012, FROEHLICH 2013, OERLEMANS 2013*). The pharmacological intervention is possible in the reperfusion phase therefore it is a great importance to examine compounds that protect against the harmful effects of oxidative stress. One of our research's aims was to examine cardioprotective effects of selected compounds on a high throughput functional *in vitro* oxidative stress model. As reference compound we used adenosine. The protective effect of adenosine is well-known in the case of a heart attack, but its effect is not fully understood in the reperfusion phase (*JACOBSON 2000, SAFRAN 2001, MCINTOSH 2012*).

There are an increasing number of substances that we meet day to day, such as pollutants and chemicals (in other words environmental stressors). They have detrimental effects on our health and contribute substantially to a lot of diseases (cancer, chronic lung disease, diabetes and neurodegeneration) that is a significant concern of public health (*KÜNZLI 2000, SMITH 2013*). Better understanding of how environmental factors influence the development of diseases could lead to corrections and improvements in public health. (Franco 2009).

There are several reports indicating that oxidative stress could be one of the factors behind the toxic actions of pyrethroids (*KALE 1999, GIRAY 2001*). Our second aim was in this study to examine the toxicity of cyfluthrin (the agent of Bulldock 25 pesticide) and the reversal effect of ascorbic acid (vitamin C, it is an antioxidant) on chicken cardiomyocytes and C2C12 skeletal muscle cells. The growing presence of environmental hazards (such as synthetic pesticides) in everyday life is a serious and actual problem that concerns everyone. Clarification of mechanism of possible toxic actions of pesticides is in public interest.

Methods

Cell culture

Cardiac myocytes

Fertilized chicken eggs were obtained from Bábolna-Tetra Kft, Uraiújfalu, Hungary. Eggs were incubated for 14 days at 38 ºC. Cardiomyocytes were obtained from the ventricular parts of the hearts, from 14 day old chicken embryos. The hearts were treated with Trypsin (Sigma 1x) for 16-18 hours at 4 ºC, in order to facilitate the enzymatic dissociation of cardiac tissues. Thereafter cardiomyocytes were centrifuged in culture media (DMEM plus 10% FBS, SIGMA) at 1000 rpm for 5 minutes. Cardiomyocytes were cultured on 24-well plates for 3 days (5 hearts per 24-well plate) in a 5 % CO_2 incubator at 37°C. 50 percent of the media was replaced with fresh media one day after plating and every 2 days after that.

C2C12 skeletal muscle cells

C2C12 mouse skeletal muscle cell line was obtained from ATCC. Stock was kept frozen in liquid nitrogen. C2C12 cells were cultured in 25 cm^2 flasks until confluency. 3 ml trypsin was added to the flasks, cells were centrifuged at 2000 rpm for 5 minutes in DMEM containing 10% FBS and were replated in 24-well plate in one flask to one plate ratio in DMEM + 10% FBS. Skeletal muscle cells were incubated at 37°C in 5 % CO_2 for 3 days, until confluency. Undifferentiated cells were used for the toxicological experiments at this stage or cultured further in serum-free DMEM for another 5 days until well differentiated myotubes formed.

Administration of drugs

We applied all of the drugs from a 100x pre-mixed stock solution directly into the cell culture media into the culture wells. We used at least 4 parallels in each experiment. Then we put back the plates into the CO_2 incubator for 24 hours. We applied H_2O_2 (Sigma) at concentrations of 1, 2, 4 and 8 mM; Cyfluthrin (from Bulldock 25, a 25 g/l solution) at 1, 3, 10, 30 and 100 µM; ascorbic acid (Sigma) 1 µg/ml and adenosine (Sigma) at 1, 3, 10, 30 and 100 µM.

Evaluation of cell survival

After the 24h incubation period we took phase contrast photographs of each well. Thereafter we added 0.01 mg/ml Alamar blue dye (Sigma) (from a 100x stock solution) to each well and measured the amount of produced fluorescent metabolite with a fluorescent plate reader (Cytofluor 4000, Molecular devices, excitation: 530 nM, emission: 580 nm) in every 5 minutes for 30 min. The slope of the fitted line was calculated and used as a relative measure of the number of live cells. Cell death was expressed as decrease in cell number compared to control in percentage of control. Data was given as mean±SEM. Microsoft Excel and Sigmaplot was used for data evaluation and illustrations. .

Results

Cell cultures

The C2C12 mouse skeletal muscle cell line is an "easy to culture" line. It does not require expensive medium additives; it is a fast growing line which can be used either in the undifferentiated or in the differentiated state (Figure 1).

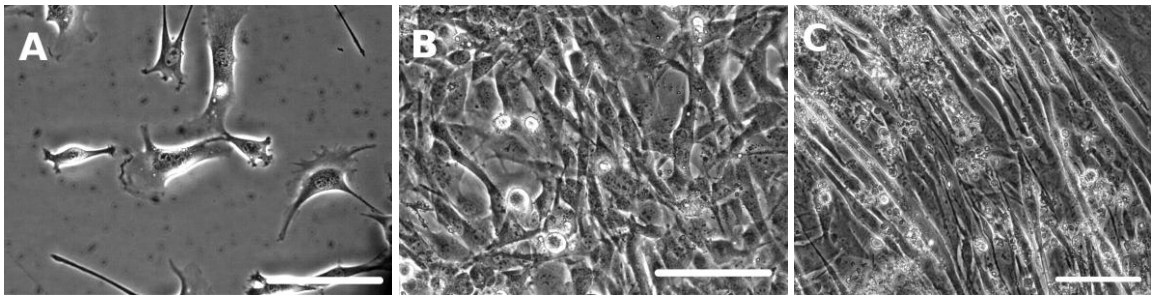

Figure 1. Development of C2C12 cells in culture. A: proliferating cells in DMEM+10% serum. B: confluent culture 3 days after plating. C: Differentiated myotubes 5 days after removing the serum from the medium in a confluent culture (scale bar: 100 µm, phase contrast).

The C2C12 line is a reliable source of a relatively homogenous cell population. We did find small differences in the effectiveness of some drugs on C2C12 cells and primary chicken cardiac myocytes, which is subject of further investigations, but the C2C12 line is a good choice for non-specific toxicity studies.

The benefit of primary cells is that they are not transformed, thus they are keeping most of the properties of the original tissues. We have chosen chicken cardiac cells for our tests because there are less ethical issues associated with this cell types and because of the

lower cost. There are clear interspecies differences between chicken and human cells, but in connection with cell death they might be less significant.

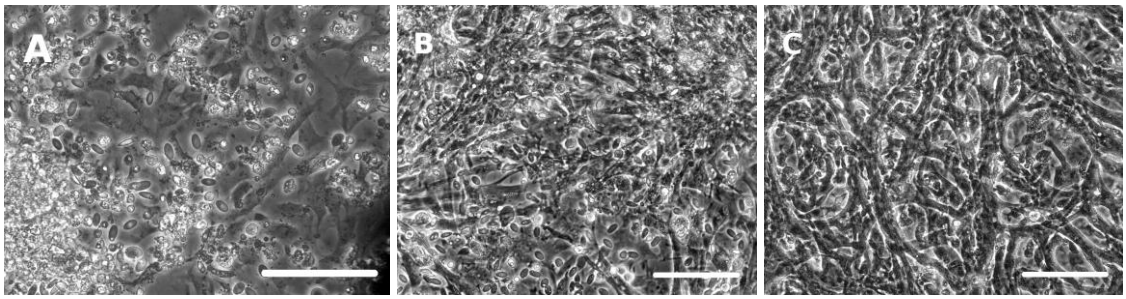

Figure 2. Primary chicken cardiac cells in culture. A: One day after plating. B: One week after plating. C: One month after plating (scale bar: 100 µm, phase contrast).

Primary chicken cardiac cells were physiologically active one day after plating. It took about one week for the culture to stabilize, although spontaneous activity decreased (possible dedifferentiation). The cultures survived more than a month (Figure 2).

Oxidative stress – H_2O_2 toxicity

Hydrogen peroxide treatment caused significant cell death in both primary chicken cardiac cultures and in undifferentiated and differentiated C2C12 cells (Figure 3).

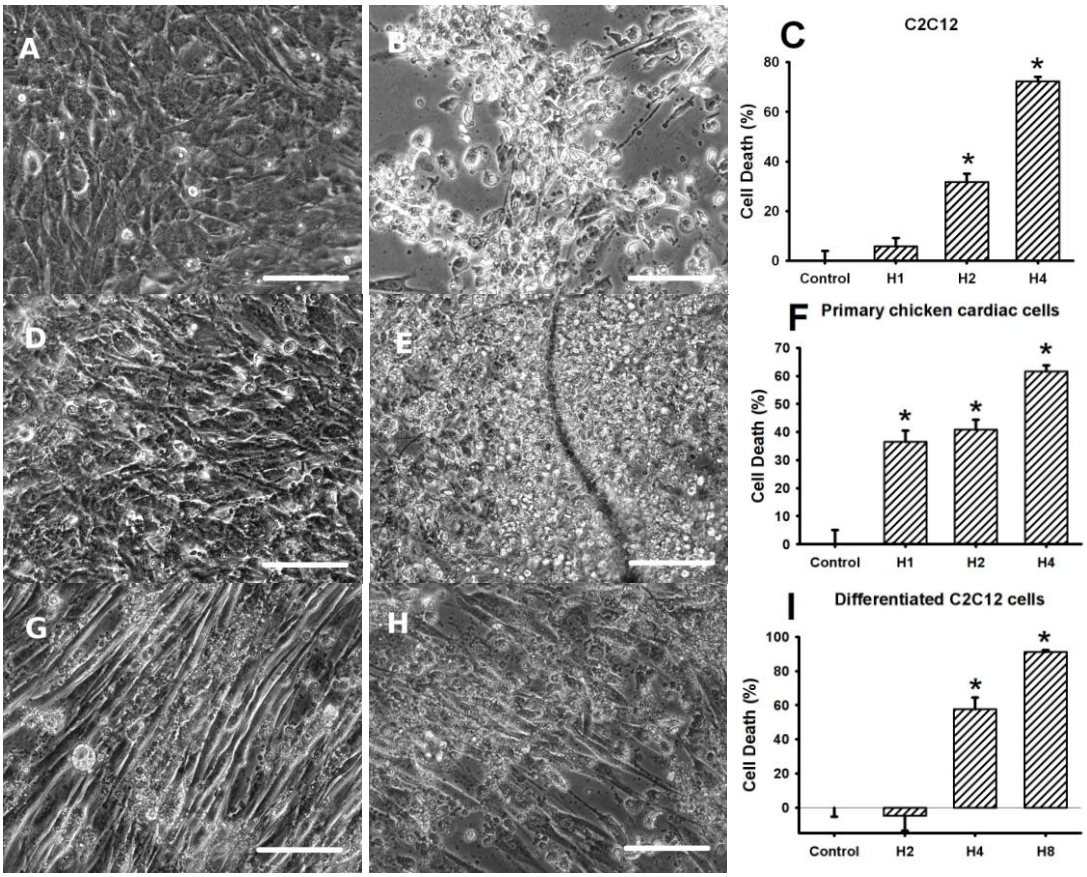

Figure 3. Effect of oxidative stress (H2O2 treatment) on the survival of cultured cells. A, B, C: C2C12 cells were treated with 4 mM H_2O_2 for 24h after reaching confluence. A: control, B: treated, C: evaluated using Alamar Blue metabolic assay. H1: 1 mM H_2O_2, H2: 2 mM, H4: 4 mM. D, E, F: similarly treated primary chicken myocytes. G, H, I: C2C12 cells were differentiated for 5 days in serum free medium and were treated with H_2O_2. H8: 8 mM. Hydrogen peroxide seemed to have weaker effect on differentiated than on non-differentiated C2C12 cells. Data was given as mean±SEM. n≥4.*: significantly differ from control (Two-sample Students t-test; scale bar: 100 µm, phase contrast)

Interestingly, the effectiveness of H_2O_2 in cardiac and undifferentiated C2C12 cells seemed to be the same, whereas it was slightly lower in differentiated C2C12 cells. The slope of the concentration/effect curve was very steep, 1 mM H_2O_2 did not cause observable effects, whereas 4 mM caused almost 100% cell death. The Alamar Blue assay gave reliable, quantitative measure for cell death, which was qualitatively confirmed by the photographs.

Effect of adenosine

Adenosine was tested only on C2C12 cells. At higher concentrations (10-100 µM) adenosine alone had some concentration dependent toxicity (Figure 4). Adenosine at concentrations of 3 and 10 µM partially, but significantly reversed the toxic effect of 4 mM hydrogen peroxide. At lower and at higher concentrations there was no significant effect.

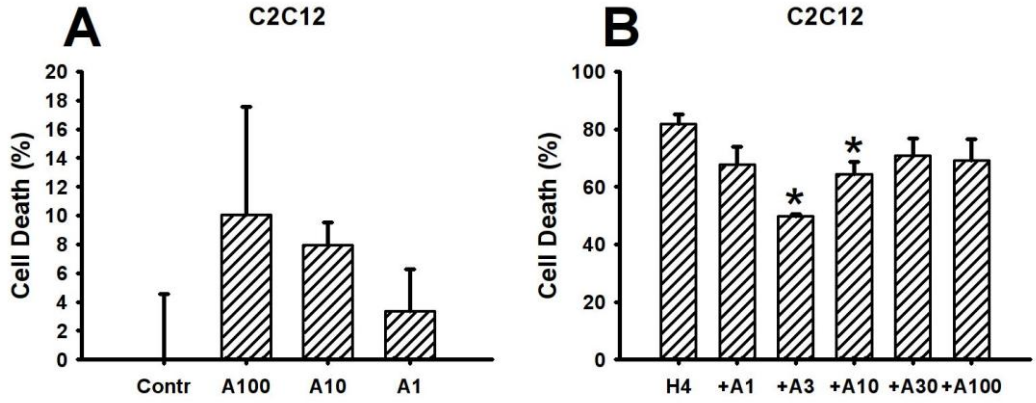

Figure 4. Reversal of H_2O_2 toxicity by adenosine in undifferentiated C2C12 cells. A: At higher concentrations adenosine (applied at 1, 10 and 100 µM concentrations) showed some toxic effects. B: 3 and 10 µM adenosine significantly reduced the toxic effect of 4 mM H_2O_2. Higher (30 and 100 µM) or lower (1 µM) concentrations were less effective.

Effect of cyfluthrin

Cyfluthrin was toxic to both cell types above 10 µM concentrations (Figure 5). 1 µg/ml ascorbic acid reversed the toxic effect of 10 µM cyfluthrin in C2C12 cells. In contrast, 3 µM adenosine, which was protective against hydrogen peroxide induced cell death, potentiated the cytotoxic effect of 3 µM cyfluthrin. Another interesting observation that 1 and 3 µM cyfluthrin apparently increased cell number in C2C12 cells compared to control. This was not the case for chicken cardiac myocytes.

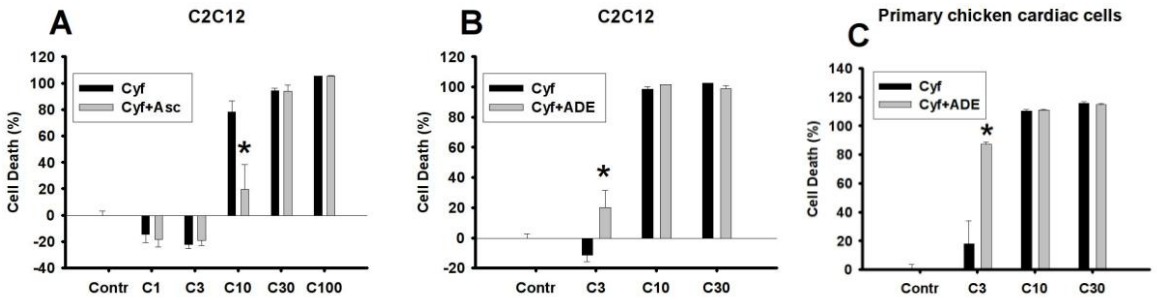

Figure 5. Effect of cyfluthrin on cultured cells. A, B: undifferentiated C2C12 cells. Different concentrations of cyfluthrin (from 1 to 100 µM) was given in the presence and in the absence of 1 µg/ml ascorbic acid or 3 µM adenosine. Cell death evoked by 10 µM cyfluthrin was significantly reduced by ascorbic acid. Interestingly, lower concentrations of cyfluthrin seemed to increase cell number (or activity of mitochondria). Adenosine significantly increased cyfluthrin toxicity. C: cardiac myocytes. Cyfluthrin was given in the presence and in the absence of 3 µM adenosine. Adenosine significantly increased cyfluthrin toxicity.

Cyfluthrin and oxidative stress

In order to decide whether hydrogen peroxide and cyfluthrin share a common cytotoxic mechanism we applied both compounds together in sub-lethal concentrations (2 mM and 3 μM, respectively). The compounds together caused significant cell death (Figure 6), a more than additive effect. H_2O_2 plus cyfluthrin amplified each other's effects on both chicken cardiac myocytes and C2C12 skeletal muscle cells.

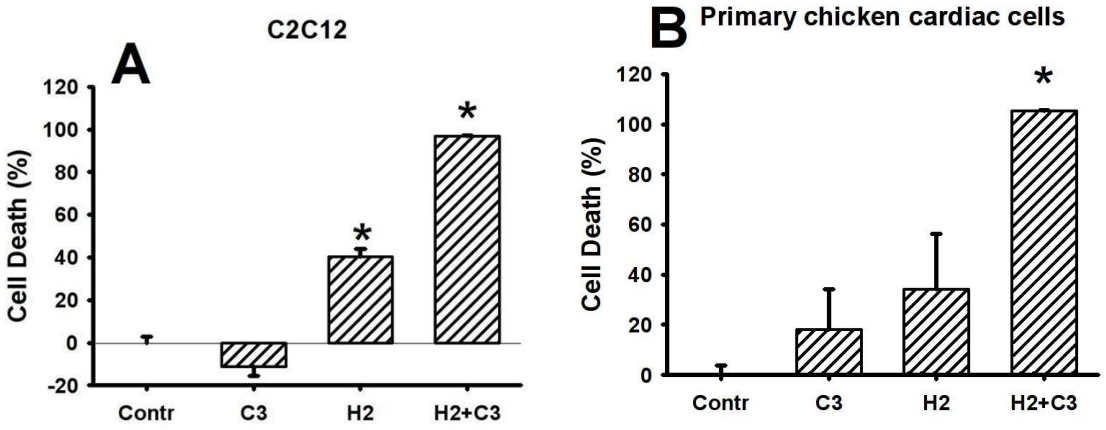

Figure 6. Joint effect of hydrogen peroxide and cyfluthrin. A: C2C12 cells. B: primary chicken cardiac myocytes. In both cases the effect of sub-toxic cyfluthrin and H_2O_2 was superadditive.

Discussion

In this series of experiments we showed that simple, low cost cell culture based systems could be used for potentially high-throughput pharmacological or toxicological studies. The C2C12 cell line offered the benefits of an almost inexhaustible, low cost, homogenous source of cells and the potential to study effects on muscle differentiation, whereas primary chicken cardiac myocytes were physiologically active terminally differentiated, functional cardiac cells. The Alamar blue assay enabled the fast and simple quantification of cytotoxicity with the possibility of easy scale up.

Oxidative stress plays a crucial role in several diseases such as neurodegeneration, hypoxia, ischemia, etc. We have adapted a simple oxidative stress model (hydrogen peroxide treatment) in our system. Both cell types showed reliable responses to H_2O_2 treatment, although the slope of the concentration/cell death curve was very steep. Differentiated C2C12 cells showed a slightly decreased sensitivity to hydrogen peroxide. Adenosine, as expected based on the literature, partially reversed the toxic effect of H_2O_2. Interestingly, higher concentrations of adenosine showed some signs of toxicity when administered alone. There are evidences in the literature for toxic effects of high concentrations of adenosine. Adenosine concentration/effect curve on hydrogen peroxide cytotoxicity was bell shaped with a maximum at 3 μM.

Oxidative stress was also suggested to be one of the mechanisms of pyrethroid toxicity. Cyfluthrin showed similar toxic effects than hydrogen peroxide. Cyfluthrin effect was partially reversed by ascorbic acid, an antioxidant. Surprisingly, low concentrations of cyfluthrin seemed to increase cell number compared to control. Whether proliferation of cells was really increased or only metabolic activity was augmented should be verified using different methods. Another surprise was that adenosine augmented and not reversed cyfluthrin toxicity. The effect of cyfluthrin and hydrogen peroxide, administered together in sub-toxic concentrations, was additive, the compounds seemed to even potentiated each other effects. With these experiments we were not able to prove that oxidative stress is the main mechanism

behind cyfluthrin toxicity, but the additive effect of hydrogen peroxide and cyfluthrin could indicate this possibility. Adenosine effect on cyfluthrin toxicity requires further clarifications.

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