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The cytoprotective role of whey protein hydrolysates against $CdCl_2$-induced toxicity in fibroblasts by the Electric Cell-substrate Impedance Sensing (ECIS) method

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Abstract

Introduction and Objective. Cadmium (Cd) is a toxic mineral element with a long half-life that causes excessive accumulation in tissues, especially the kidney. It influences cell development, proliferation, and death by activating intracellular signalling pathways. Currently, treatment options include chelation agents and antioxidant therapy. Researchers are investigating natural compounds that might protect against Cd-induced toxicity while providing less negative effects. The aim of the study is to look into the protective properties of bovine milk whey protein hydrolysates against Cd-induced toxicity in *in vitro* studies.

Materials and Methods. The study was performed by testing different concentrations of $CdCl_2$ on fibroblast cell culture after the addition of different concentrations of whey peptides using the Electric Cell-substrate Impedance Sensing (ECIS) assay, in order to study intermediate-term exposure (20–25 hrs).

Results. By using the ECIS test, it was found that the higher the $CdCl_2$ concentration, the lower the impedance value, which indicates a decrease in cell viability. At the same time, it was found that the use of whey proteins in concentrations of 0.5%, 0.8% and 1.2% had a protective effect on cells treated with toxic cadmium. It could be stated that protein peptides present in the medium may be responsible for binding cadmium, preventing its interaction with the fibroblast cell membrane and cell organelles.

Conclusions. The study provided evidence that bovine whey proteins have a protective effect against $CdCl_2$ toxicity. This could be due to its enhanced antioxidant defence and metal chelating properties; therefore, whey proteins could be a useful nutritional supplement to reduce Cd toxicity.

Key words

toxicity, whey protein, ECIS, Cadmium, anti-cytotoxic effect

INTRODUCTION

Cadmium (Cd) is a mineral element that cannot be broken down into less toxic substances with long biologic half-life time (more than 30 years) and has no biological functions. Thus, it prolongs low-level exposure and leads to excessive accumulation in certain human and animal tissues, especially in the kidney. Thus, it is highly toxic even at low concentrations [1]. In the priority list of hazardous substances of the Agency for Toxic Substances and Disease Registry, Cd is ranked at No. 7 [2]. It occurs in the earth's crust, along with the other heavy metals lead, zinc, and copper. When certain heavy metals are released into water and soil, due to their high solubility, they become exceedingly poisonous and hazardous contaminants. When these metals are discharged into the environment, they might be absorbed into subsurface waters and deposited in aquifers, or they can flow-off into surface waters and soils, causing pollution. As a result, heavy metals constitute a potential environmental pollutant capable of facilitating trophic transmission in food chains [3].

Cadmium affects cell differentiation, proliferation, and induction of apoptosis. These activities influence on DNA repair mechanism and the formation of reactive oxygen species (ROS). At low concentrations, this chemical element can bind to the mitochondria and inhibit cellular respiration [4, 5]. Research shows that at low concentrations cadmium can cause apoptosis while an increase in concentration can lead to necrosis in a cell culture system [3, 5]. Furthermore, Cd in micromolar (mM) concentrations activates various intracellular signalling pathways (particularly NF-κB and AP-1) in immune cells, resulting in the up-regulation of inflammatory markers and mediators [6].

The treatment strategies for Cd toxicity include chelating agents and antioxidant therapies [4]. Until now, in treating intoxication with this metal, chelating compounds have been

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used which, however, are burdened with numerous undesirable symptoms. For this reason, many researchers worldwide are attempting to find natural compounds that help in protection against cadmium-induced toxicity, with fewer or no-side effects [7]. Among the milk protein products, whey proteins are now produced in large quantities as protein ingredients for the food industry, which provide specific health benefits beyond its basic nutritional value. Whey proteins appear promising as a functional food due to its anti-inflammatory, antioxidant, blood pressure-lowering, anti-obesity and appetite-suppressing properties [8]. The curative properties of whey have been known for centuries. Hippocrates (466–377 BC), the father of medicine, recommended whey to his patients [9]. The bioactive peptides released from whey proteins possess very important biological functionalities, including antioxidative, anticytotoxic [10] and improvement in the function of the intestinal barrier [11]. Thus, one may suppose that the peptides resulting from whey proteins during the digestion process in the gastrointestinal tract could alleviate Cd toxicity in chronic exposure in humans and animals. Bioactive peptides have a potential use in the production of therapeutic and prophylactic functional products [12].

There is an essential need for the determination of the cytotoxic potential of chemicals in toxicological research. Therefore, one of the established areas of *in vitro* cytotoxicity assays performed on cells in tissue culture is Electric Cell-substrate Impedance Sensing (ECIS) assay. This method involves measurements of the ability of cytotoxic compounds to inflict damage to cells, and can provide quantitative information concerning cell morphological changes, cell movements, and alteration in cellular function under various drug, chemical, and biochemical treatments. Thus, the development of ECIS opens wide prospects as a tool for *in vitro* toxicity testing [13]. ECIS is a method based on real-time impedance measurement, is non-invasive, and is a precise test of cell activity in tissue culture. The recording of electrical parameters allows for the assessment of morphological changes, cell migration and several other behaviours. This impedance-based cell monitoring technology was invented by Drs. Ivar Giaever and Charles R. Keese, the founders of Applied BioPhysics, Inc., where the assessment of cytotoxicity is a key element of the project. Thanks to ECIS technology, the toxicology and viability of cells can be quantified in real time, continuously and without labels, all under incubation conditions. ECIS technology has long been used to detect cell proliferation and viability by measuring changes in electrical impedance as cells grow over gold electrodes at the bottom of culture dishes. With ECIS software, as cells attach and spread, the impedance level will rise and plateau, representing confluence. After the introduction of a potentially cytotoxic substance, the impedance level will drop as cells die and detach from the gold electrode substrate [14].

This aim of the study is to determine, by using the ECIS method, whether the whey protein has a cytoprotective effect against $CdCl_2$-induced toxicity in the fibroblast cell line.

MATERIALS AND METHOD

Bovine whey proteins and cadmium. Whey Proteins Concentrate powder (WPC 80 INSTANT®) fresh whey protein was obtained from sweet cheese production (Spomlek, Poland). Its nutritional analysis physical chemical and microbiological characteristics were inspected and confirmed by J. S. Hamilton Quality Services Sp. z o.o. Poland (Tab. 1).

Table 1. Physico-chemical and microbiological characteristics of whey proteins

PHYSICAL-CHEMICAL CHARACTERISTICS			MICROBIOLOGICAL CHARACTERISTICS		
Item	Target	Acceptable	Item	Target	Acceptable
Moisture	5.0 %	Max 6.5 %	Total plate count	10,000 in cfu/g	Max 50,000 in cfu/g
Fat	6.0 %	Max 10.0 %	*Salmonella*	Absent in 25g n=5 c=0	Absent in 25g n=5 c=0
Protein in dry matter	81 %	Min 80 %	*L. monocytogenes*	Absent in 25g n=5 c=0	Absent in 25g n=5 c=0
Lactose	5 %	Max 8 %	*Staph. aureus* in cfu/g	< 10	100
PH	6.5	Min 6.0 max 7.0	*Enterobacteriaceae in* cfu/g	< 10	10
Solubility index	0.2 cm3	Max 1.0 cm3	Coliforms in cfu/g	< 10	10
Antibiotic or inhibitory substances	absent	absent	Yeast and mould in cfu/g	Max 50	Max 100

Preparation of whey proteins hydrolysates. Whey proteins hydrolysates with alkaline protease were prepared according to methodology described by Li et al. [15]; EC – 3. 4.21.63 (Amano Inc. Nishiki. Nagoya. Japan). Whey proteins (5.4 g) were dissolved in 100 mL of demineralized preheated water to obtain an original solution with protein mass per volume fraction of 5 % (w/v). The aqueous suspensions of whey proteins were incubated in a water bath at 45 °C for 10 min. The solution was adjusted to pH= 8.5 with 1 mol/L of NaOH. When the whey suspension reached 45 °C, the enzyme was added to the substrate in order to obtain the E/S ratio of 332 IU/g whey (1g of protease contains 204000 IU). The recommended weight of enzyme for the 5.4 g of whey proteins amounts to 8.08 mg of enzymes preparation. The hydrolysis process was carried out at 45 °C and pH= 8.5 and the pH of the reaction mixture was maintained at a constant through the continuous addition of 1 mol/L of NaOH monitored with a pH meter (pH/Conductivity Meter CPC-500 ELMETRON®). After 4h of the hydrolysis process, samples were withdrawn and centrifuged (10,000 g. 30 min). The supernatant was ultra-filtered with a 0.22 μm, fi 13 mm (STARGARD Chemland, Poland) to remove enzyme and non-hydrolyzed proteins from the reaction mixture. After the filtration was finished, whey proteins hydrolysates were heated in 80 °C water for 20 min to inactivate the proteases and stop the reaction. The hydrolysates were cooled at room temperature, frozen, lyophilized, and stored at –20 °C until used.

Preparation of whey protein peptides concentrations. The addition of whey peptides to the media containing cells were prepared by adding (different concentration (0.5, 0.8 and 1.2%) of protein from a sterile concentrated solution into the serum-free culture medium. Whey protein peptides (0.8%) were incubated with each of cadmium concentrations (2.2, 4.2 and 6.2 μM) and compared with the controls (cells with media and cells with whey peptides and media alone). Media

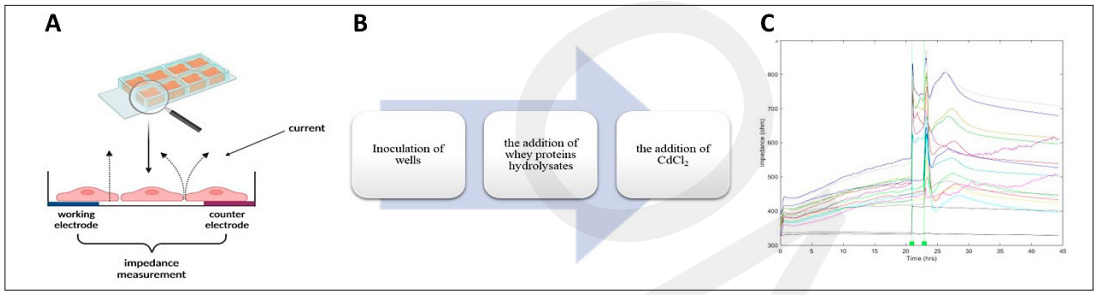

Figure 1. A – Scheme of ECIS measurements [15] B – Scheme of the experiment – inoculation of wells within 20 hours, followed by incubation with whey proteins and cadmium chloride within 24 hours. C – Record of impedance from the ECIS apparatus (own measurements)

supplemented with whey protein peptides were sterilized through a membrane filter (0.22 µm, fi 13mm) (STARGARD Chemland, Poland).

Preparation of cadmium concentrations. A stock solution of cadmium chloride (8.89 mM of cadmium) was sterilized through a membrane filter (0.22 µm, fi 13mm), (STARGARD Chemland, Poland) and kept at 4 °C for use in all the assays. To obtain the desired concentration of cadmium in each experiment, the appropriate amount of the stock solution was added to the culture medium. The culture medium was checked before addition of the metal – no detectable amount of cadmium was found. Cadmium chloride was dissolved in distilled water to a concentration of 10 mM and used as the stock solution.

Cell Line Culture. Mouse fibroblast line L929 NCTC clone 929 [L cell. L-929. Derivative Strain L] (ATCC® CCL-1™) were obtained from the American Type Culture Collection (ATCC) and were cultured in Eagle's Minimum Essential Medium media, supplemented with 10% heat-inactivated foetal bovine serum (FBS, Gibco, Waltham, MA, USA), penicillin G (100 U/mL) (Sigma-Aldrich, St. Louis, MO, USA), and streptomycin (100 µg/mL) (Sigma-Aldrich, St. Louis, MO, USA). Cultures were kept in a Galaxy 170R incubator under a constant humidified atmosphere of 5% CO_2 and 95% air at 37 °C. When the culture reached at least 75% confluence, the next step was to administer whey proteins at the appropriate concentrations, and then treat the cells with different cadmium concentrations according to the scheme below.

Experiment schemes. The aim of the experiment (Fig. 1) was to cultivate L929 cells in an ECIS device under standard conditions. After 20 hours measurement of cells adhesion and stabilization of the system, whey proteins were added to the appropriate wells until the final concentrations of the hydrolysate preparation tested reached 0.5%, 0.8% and 1.2%. The next step was the administration of cadmium chloride at the following concentrations: 2.2 µM, 4.2 µM and 6.2 µM. Following cell manipulation, real-time measurements were initiated for 24 hours. The entire experiment was conducted in three replicates.

Electric Cell-Substrate Impedance Sensing (ECIS). The impedance measurements were made using ECIS system's Z theta instrument (Applied Biophysics Ltd., Troy, NJ, USA). The device contained two separate units: the station controller Zθ, located outside the incubator, and a docking station containing two 8-well plates, placed in the incubator space. In the standard 8-well ECIS disposable arrays gold electrodes delineated with an insulating film and mounted on a 20 mil optically-clear Lexan® polycarbonate substrate. Gold washers on the die edge ensure that the electrode is connected to the ECIS electronics by contacting the spring pins in the die station. The electrodes used were 8W10E (Applied Biophysics Ltd., Troy, NJ, USA), which comprised 8 wells and 10 active electrodes in each well. Before the inoculation, the arrays were incubated for 24–48 h with Eagle's Minimum Essential Medium in a Galaxy 170R incubator at 37 °C and 5% CO_2. After incubation, the culture medium was removed from the wells and replaced with fresh medium supplemented with 10% FBS to perform the stabilization process. Following stabilization, the arrays was removed from the array station and inoculated with cells. Suspension off cells was ~1.2 x 10^5 cells/mL in 600 microlitres per well. After cell manipulation, the arrays were placed in an incubator and real-time measurements were initiated. The maximum response for Z and C used were impedance (Z) 32,000 Hz and capacitance (C) 64,000 Hz. The changes in cellular response to the different combination of whey protein hydrolysate and $CdCl_2$ were recorded as impedance signals, and the data obtained were processed through ECIS software (Applied Biophysics Ltd., Troy, NJ, USA).

Statistical analysis. Data were analyzed using GraphPad Prism version 7.01 (GraphPad Software, San Diego (CA), USA), with at least three replications of each analysis. The results were presented as mean ± SD. Statistical significance was evaluated with the two-way ANOVA and Bonferroni post-test at *- $p < 0.05$; **-$p < 0.01$, ***-$p < 0.001$. Tables of exact statistic are available in supplementary data (S1 – for whey proteins, S2 – for cadmium with whey proteins).

RESULTS

Using the ECIS system, mouse fibroblast L929 cell line were monitored continuously for up to 45 h. Significant changes were noted in impedance and capacitance after the administration of whey protein hydrolysates and cadmium chloride in selected concentrations 0.5%, 0.8%, 1.2% of whey proteins and 2.2; 4.2; 6.2 µM. The obtained results allow the conclusion that both the addition of whey proteins and cadmium in various combinations affects the electrical parameters during cultivation. Each cell type has a different type of adhesion and growth curve. During the experiment, manipulation with stimuli, such as the chemical structure of the substance or its concentration in the medium, will be reflected in changing electrical parameters.

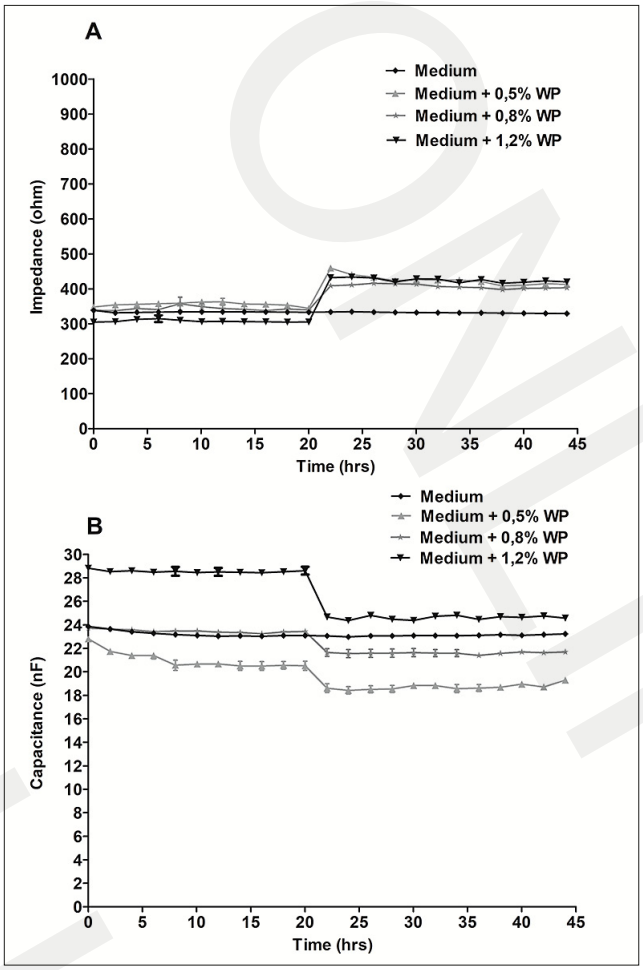

Figure 2. Effect of the addition of whey proteins (WP) to the culture medium on the electrical parameters of the experimental system. A – Changes in impedance. B – Changes in capacitance. Data presented as mean value ± SEM

Effect of medium composition on impedance and capacitance. In the starting phase of experiment, it could be noted that administration of whey proteins to standard medium altered the electric parameters of the research system (Fig. 2). From the data obtained, the addition of whey proteins to the standard culture medium changed the impedance in the test system from about 330 ohms (impedance of Eagle's Minimum Essential Medium alone) to over 400 ohms (Fig. 2A) Moreover, a decrease in capacitance from 23 to 21 nF under the mentioned conditions was noted (Fig. 2B).

Effect of whey protein supplementation on fibroblast culture. Apart from the above mentioned changes in substrate impedance and capacitance, a significant effect of whey proteins on fibroblast cells was observed (Fig. 3). The highest values of impedance of about 700 ohms were recorded in the case of fibroblast cultures with the highest applied concentration of the supplement 1.2%. The lower concentrations (0.8% and 0.5%) had a correspondingly smaller effect on the cells.

Protective effect of whey peptides on cadmium-treated cell. The aim of this study was to demonstrate, using ECIS, the protective effect of whey proteins on normal L929 cells treated with the toxic element cadmium. As expected, the impedance increased when ECIS plates were inoculated with L929 cells. During the first 20 hours of the experiment, there was proliferation of cells and intensive migration. After stabilization was achieved, selected three concentrations of whey proteins were administered. As expected, the administration of whey proteins to fibroblast cultures resulted in an increase in the impedance value in all tested variants. The most significance change was recorded at 1.2% whey proteins, from about 500 ohms to over 800 ohms at 26 hours. The high impedance values were maintained until the end of the experiment. Correspondingly lower impedance values were recorded in cultures enriched with 0.5 and 0.8% whey proteins, about 600 and about 700 ohms, respectively. In the case of fibroblast cultures treated with cadmium chloride, it was found that the highest concentrations of cadmium (6.2 μM) limited the growth of fibroblasts the most, which is reflected in the lowest impedance in the culture system containing cells and cadmium (Fig. 4E, 5E, 6E). At the same time, a clear protective effect was observed of whey proteins in all the concentrations used. The impedance in the L929 cultures in variant cadmium plus whey proteins was higher than the variants with cadmium alone by about 300 ohms for cadmium concentrations of 4.2 and 2.2 μM and by 200 ohms for the highest concentration (Fig. 4AC, 5AC, 6AC).

In the case of the protective doses of the tested proteins, an effect related to the dose of the preparation was found. The weakest protective effect was exerted by the preparation at a concentration of 0.5%, which is in line with the initial expectations (Fig. 4). For all options, there was a decrease in capacitance in line with the corresponding increase in impedance. The decrease in capacity (Fig. 4, 5, 6 BDF) achieved during the initial 18 hours with increasing impedance, should be interpreted as cell proliferation, and in this respect both values complement each other and should be analyzed in parallel as a standard.

DISCUSSION

Depending on the sensitivity of a given cell line, the cytotoxic effect of cadmium may produce different results. Exposure to Cd may cause oxidative stress which may result in the appearance and increase in the concentration of reactive oxygen species and increase the number of free radicals [5,16]. This process can contribute to an increase in the level of lipid peroxidation, which can ultimately lead to the disruption of cell membranes, damage to DNA structure, and ultimately cell death by apoptosis or necrosis [17]. Its high concentration, e.g., 50 mM led to inducing cell death in almost 70% of monocytes and lymphocytes. On the other hand, after a lower Cd concentration, such as 5.0 mM exposure, LDH release and damage to cell membrane in renal tubular epithelial cells, were observed. At the same time, the IC_{50} dose of cadmium in lung epithelial treated cells was 45 mM [18].

Cow milk has many health-promoting and bioactive properties. One of the most interesting areas of milk action is the antioxidant activity of several peptides which can be linked to their radical scavenging, the preservation from lipid peroxidation or metal ion (such as cadmium) chelation properties of those peptides [10, 19]. Research confirms that whey includes proteins and peptides that allow the formation of bioactive ion-complexes that can be exploited in the pharmaceutical and food sectors [12].

The antioxidant activity of Fe(II), Ca(II), and Mg(II) complexed with whey peptides was revealed [20], angiotensin

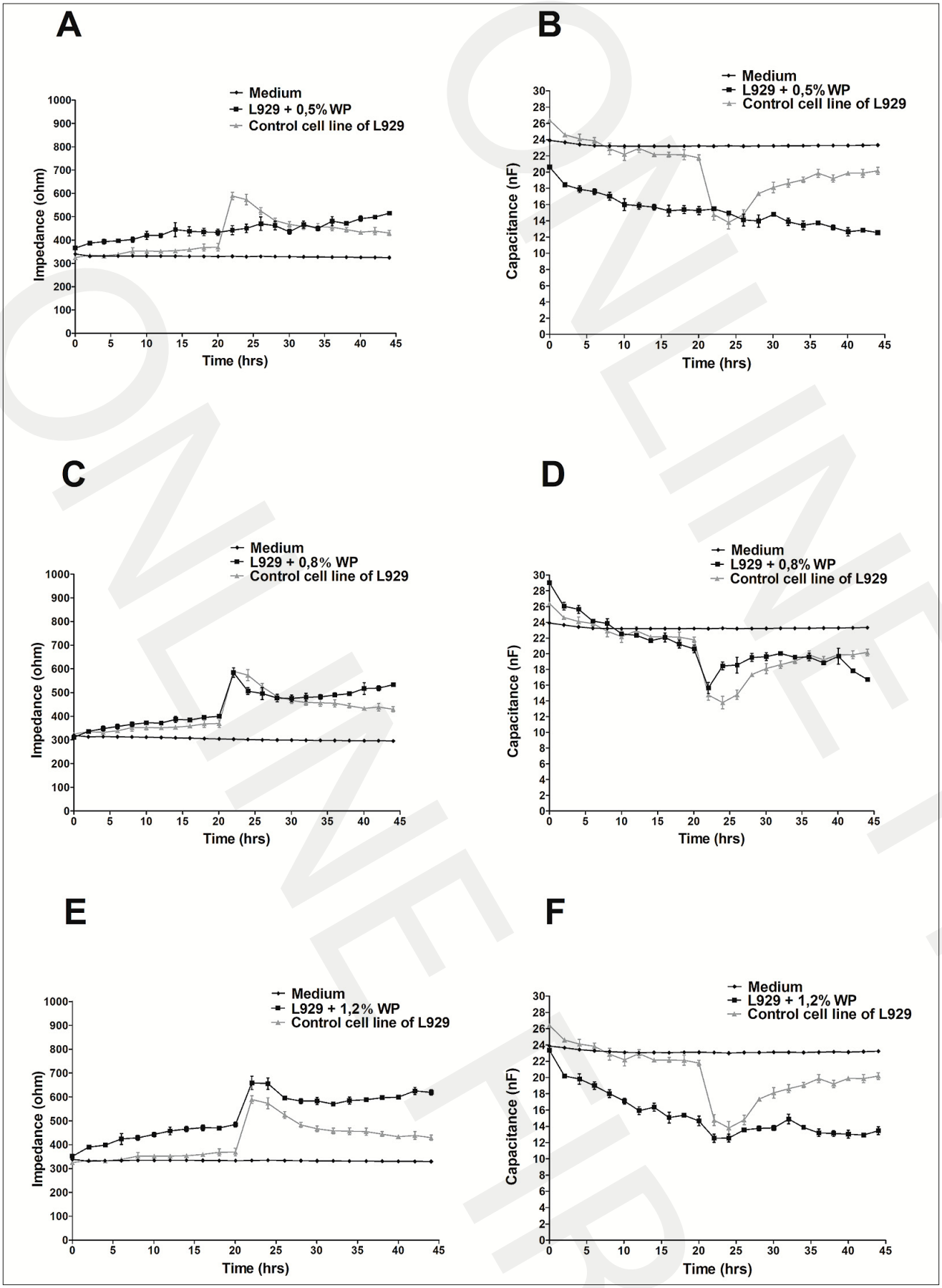

Figure 3. Impedance (A,C,E) and capacitance (B,D,F) changes monitoring of the cell line L929 during 44 h treatment with whey proteins (WP) in concentration 0.5% (A,B) 0.8% (C,D) and 1.2% (E,F). Data presented as mean value ± SEM

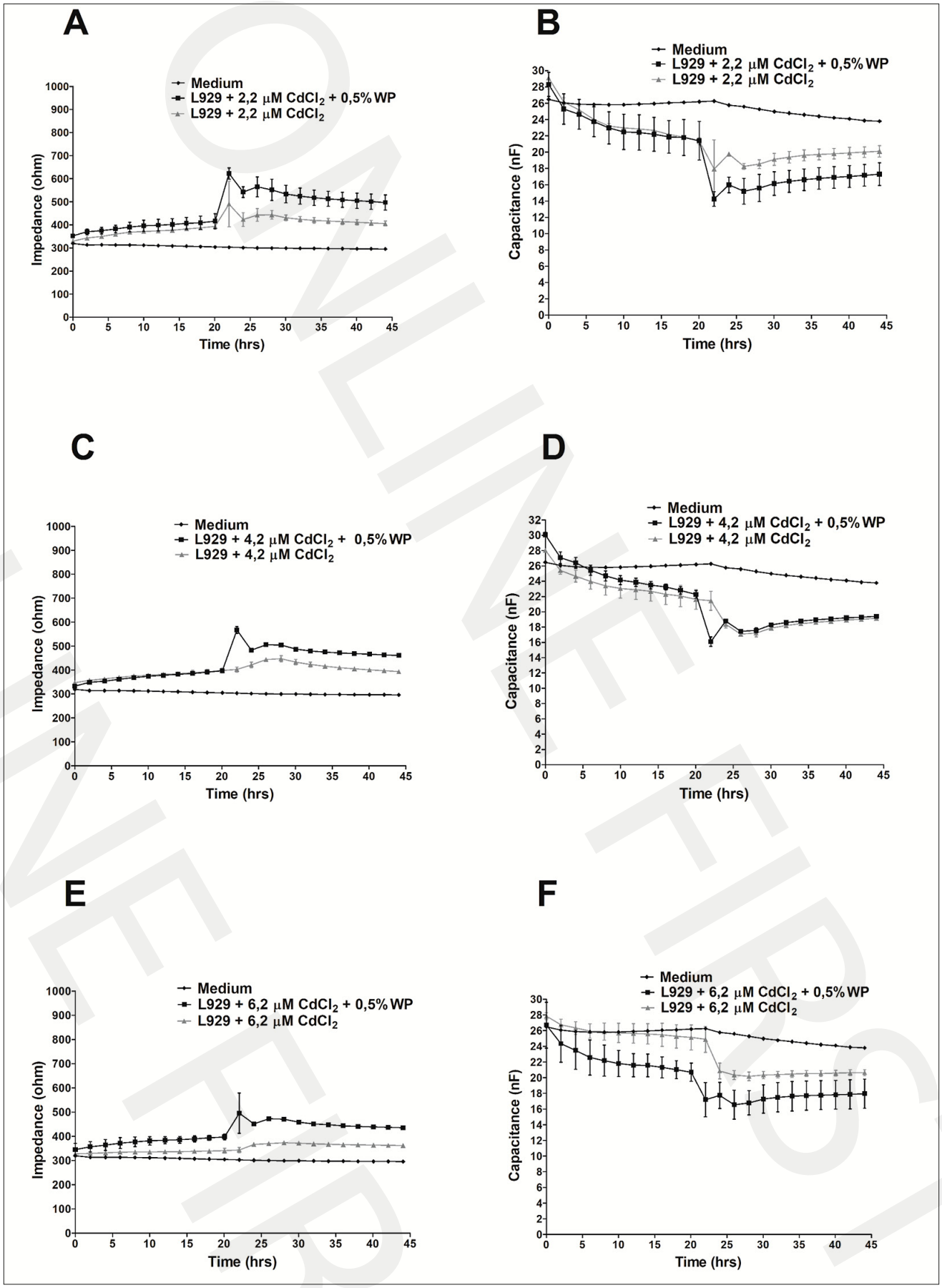

Figure 4. Impedance (A,C,E) and capacitance (B,D,F) changes monitoring of the cell line L929 during 44 h treatment with CdCl2 in concentrations 2.2 μM (A,B), 4.2 μM (C,D), 6.2 μM (E,F) and whey proteins (WP) in concentration 0.5%. Data presented as mean value ± SEM

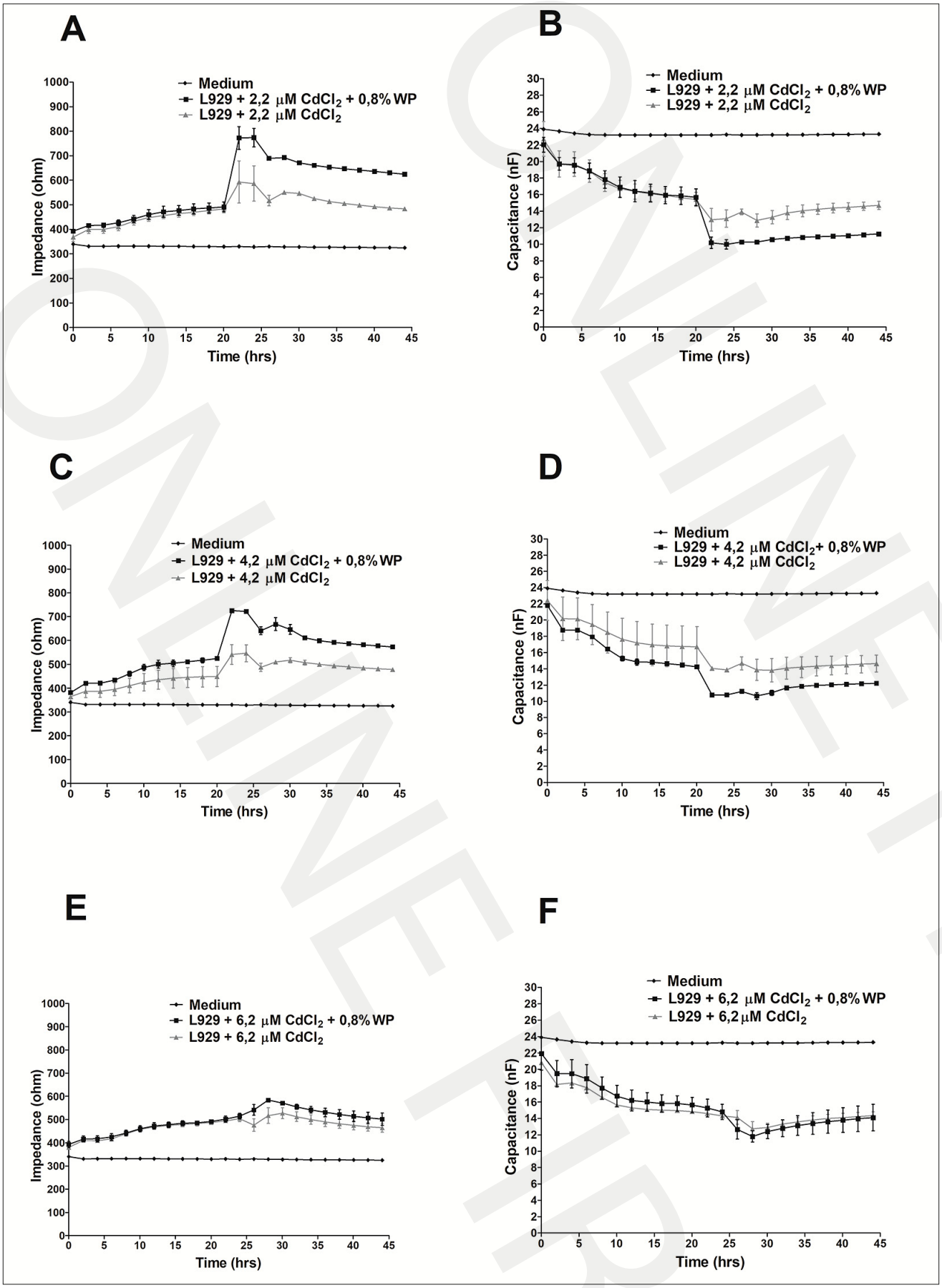

Figure 5. Impedance (A,C,E) and capacitance (B,D,F) changes monitoring of the cell line L929 during 44 h treatment with CdCl2 in concentrations 2.2 μM (A,B), 4.2 μM (C,D), 6.2 μM (E,F), and whey proteins (WP) in concentration 0.8%. Data presented as mean value ± SEM

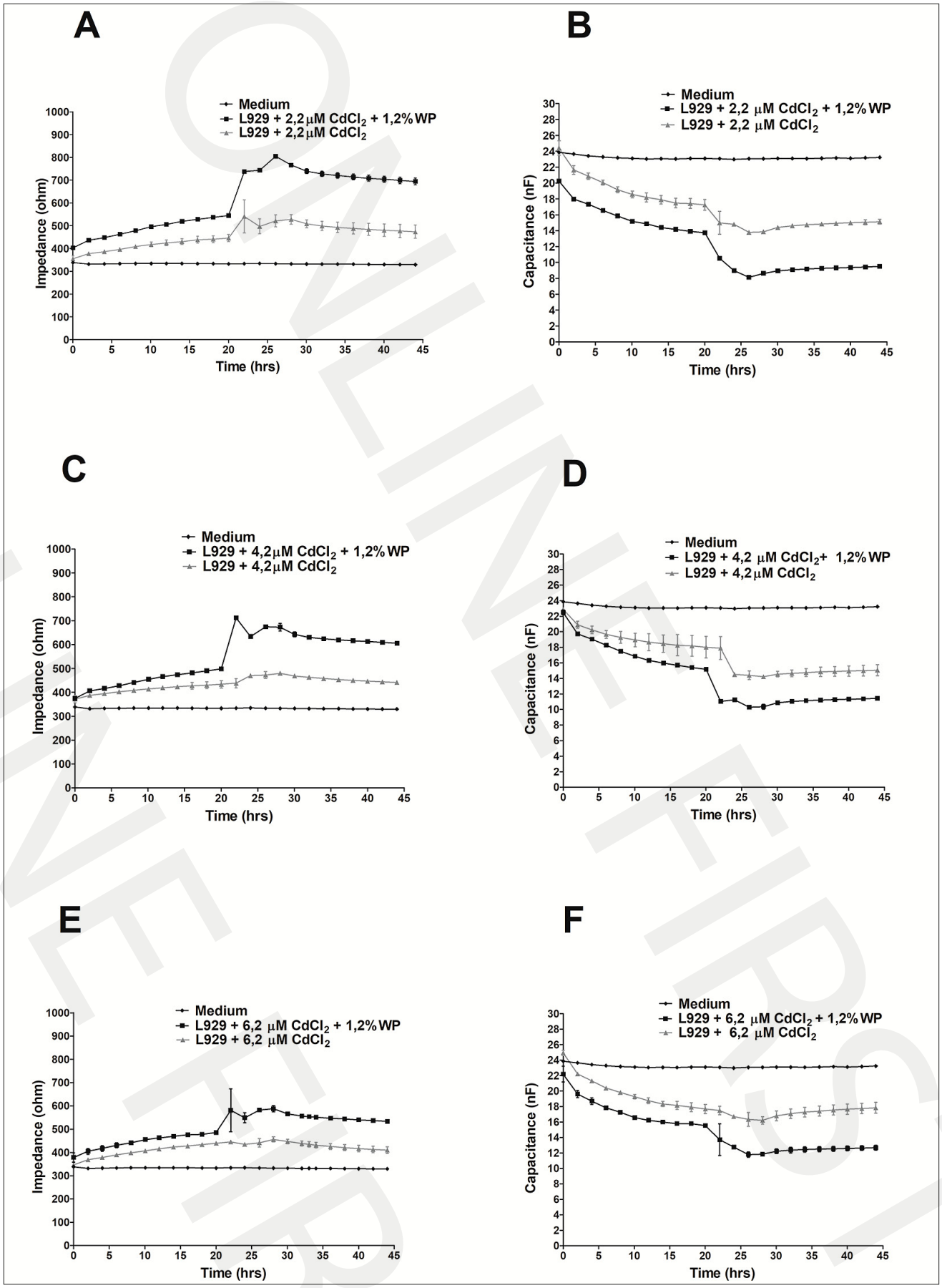

Figure 6. Impedance (A,C,E) and capacitance (B,D,F) changes monitoring of the cell line L929 during 44 h treatment with CdCl2 in concentrations 2.2 μM (A,B), 4.2 μM (C,D), 6.2 μM (E,F) and whey proteins (WP) in concentration 1.2%. Data presented as mean value ± SEM

I-converting enzyme inhibitory properties [21] and the protective effect on intestinal barrier [11]. On the other hand, studies performed on rats intoxicated with cadmium chloride in drinking water and fed a diet containing whey protein WP 80, showed a lower cadmium accumulation in liver, kidneys, and fur [22]. Whether chelation of cadmium by the resulting peptides from protein digestion in the intestinal lumen is the mode of action, remains to be clarified in future molecular studies Although, in the current study it was not possible to perform analysis of the resulting peptides in the whey protein hydrolysis study, information about the nature and kind of peptides are available in the literature.

Chelsey and Baraem [23], regarding a proteomics approach to characterizing limited hydrolysis of whey protein concentrate (WP80), using the same type of Amano enzymes and with the aid of SEHPL, MALDI-TOF MS and LC-MS/MS techniques, demonstrated that the resulting peptides were within the range 350–4,500 Da. In the literature, there are only a few research findings conducted on cell cultures; however, the results obtained in the current study are consistent with those by Mata et al. who found that the cadmium uptake by Caco-2 cells decreased by 25% after supplementing the medium with 10% cow's milk [24, 25]. This finding may be explained by the distribution of cadmium among the components of bovine milk. Mata et al. found that cadmium in human milk is mainly associated with substances with a molecular weight lower than 10,000 Da which are easily absorbed, while in bovine milk, most cadmium is associated with a compound with a molecular weight of more than 70,000 Da that cannot be internalized by the cells [26]. Another interpretation is that lactoferrin is an iron-binding protein which is considered one of the main components of whey proteins, and it is possible that lactoferrin could bind the cadmium present in the medium, avoiding its interaction with the cell membrane [24, 25]. He et al. evaluated the other beneficial effects of lactoferrin and milk on mice with spermatogenetic dysfunction induced by cadmium and bisphenol A (BPA).Cd (1.6 mg/kg) and BPA (50 mg/kg), both of which produce substantial testicular damage, including reduced germ cell numbers, low sperm quality, disorganize apoptosis, oxidative stress, and autophagy. Milk and lactoferrin supplements protected mice against spermatogenetic dysfunction caused by BPA and cadmium exposure. They protected the testis from contaminant-induced aberrant apoptosis, oxidation resistance, and autophagy via sustaining ERK1/2-mediated ubiquitin-dependent protein degradation of P62 [27].

Another study demonstrates that whey protein concentrate is a possible functional nutritional dietary supplement that slows the advancement of age-related oxidative damage in Wistar rats [28]. Other rodent model studies suggest that whey protein supplementation reduces oxidative stress, protects central nervous system neurons, and improves the neurobehaviour of diabetic mice [29]. On the other hand, studies of Al-Malki et al. confirmed that whey protein combined with *Brassicaceae* extract, inhibited the development of neurodegeneration in mice and increased antioxidant activity after $CdCl_2$ treatment [30]. Further research has confirmed the idea of the protective qualities of whey proteins against cadmium toxicity. Whey proteins in combination with endogenous antioxidant molecules significantly improved the overall antioxidant status in cadmium-exposed male Wistar rats. This was demonstrated by increased the Total Antioxidant Capacity (TAC) which reduces oxidative stress and cell damage. It indicates that whey proteins show an antioxidant effect against cadmium toxicity [31].

Analysis of the characteristics and physiological functions of cells is an effective way to understand many biological and biomedical problems. Routine analysis conducted on cell lines allow, among other effects, for the evaluation of the count, morphology and phenotype of cells, their viability (proliferation, apoptosis), metabolic activity, synthesis of intracellular transcription factors, and the release of several substances (cytokines, chemokines). They allow for determination of the cellular response to specific culture conditions (drugs, toxins, extracts). Traditionally, the study of cell culture uses microscopy (inverted, confocal, fluorescence), spectrophotometry, colourimetry, ELISA, and flow cytometry. These methods are now standard in studies on cell cultures. They provide information on the mechanisms of alterations, but the inevitable 'sacrifice' of the examined cells prevents observation of the dynamics of these changes. The measurement of selected electrical parameters, i.e., impedance, resistance, and capacitance of the cell membrane in real time, may be an important complement to results obtained by traditional methods of analyzing cell cultures. If electric changes precede biochemical changes, the electric system of monitoring the above-mentioned parameters is a method allowing for analysis of cellular activity during grow thin cell culture.

The impedance method seems to be a promising, non-invasive technique for the detection of the whole cellular behaviour and morphology changes. Giaever and Keese were the pioneers of this technique, who developed ECIS as a continuous monitoring system for studying cell behaviour using a real-time, and label-free method [7]. Changes in electrical parameters that were the effect of cadmium concentrations and whey proteins peptides on fibroblasts monolayer, was monitored continuously for 45h by using the ECIS system for the first time in this research model.

Using the ECIS system in the current study, important changes were recorded in impedance and capacitance in L929 cells after administration of various doses of whey proteins, as well as the combination of the preparation with cadmium in different doses. From the obtained results, it seems obvious that different doses of protein preparation affect electrical parameters in different modes (Fig. 2–6). Every cell type has its characteristic adhesion and growth curve that can be manipulated by, e.g., varying seeding densities or other stimuli.

One of the major difficulties in studying these processes is differentiation between adhesion, spreading and proliferation. Wegener et al. [32] described in detail the use of a combination of resistance and capacitance to distinguish between those parameters. In the current study, the combination of impedance and capacitance was used, based on the dependence that cell function modulates cell morphology. ECIS is capable of detecting and quantifying morphology changes in the sub-nanometer to micrometer range. In ECIS a small alternating current (I) is applied across the electrode pattern at the bottom of the ECIS arrays (direct current DC cannot be used). This results in a potential (V) across the electrodes which is measured by the ECIS instrument. As cells grow and cover the electrodes, the current is impeded in a manner related to the number of cells covering the electrode, the morphology of the cells and the nature of the cell attachment. When cells are stimulated to change their function, the accompanying

changes in cell morphology alter the impedance. The data generated is impedance versus time [33]. The results obtained in the current study coincide with those by Wegener on resistance and capacitance, and complement each other (Fig. 4–6). According to the data obtained in the presented study, cell proliferation was suppressed by $CdCl_2$ in a dose dependent manner. Decrease in capacitance (Fig. 4–6 B,D,F) indicates cell proliferation while increase in the impedance corresponds to cell proliferation, hence the two values complement each other. Variation in cell behaviour after cell attachment, spreading, proliferation, micro-motion, demise, and/or responses to external substances, result in changes in impedance [32]. Electrical impedance is defined as the opposition to an electrical current within a circuit. In systems utilizing direct current, the impedance is simply the resistance, but in systems utilizing alternating currents, the changing electric and magnetic fields create additional and varying opposition to the applied current [34]. ECIS is a system using alternating current. In the current study, it was found that when L929 cells were treated with a combination of cadmium in different concentration with whey proteins, it changed their function, and the accompanying changes in cell morphology altered the impedance.

CONCLUSIONS

Whey peptides have a protective effect on fibroblast cells exposed to cadmium at various concentrations. It can be hypothesized that cadmium is bound by peptides of bovine milk whey proteins present in the medium. This might be due to its elevated antioxidant defence and metal chelating activities; therefore, whey proteins could be a valuable nutritional supplement to prevent Cd toxicity. However, in the future, more extended molecular analyses should be performed to determine the precise mechanism of action of whey proteins.

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