

## The favourable effect of catechin in electrochemotherapy in human pancreatic cancer cells

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Until recently, green tea polyphenols were considered strong antioxidants. However, the latest reports have revealed that bioflavonoids can play a multiple role in anticancer therapy, including the inhibition of cell proliferation and generation of the oxidative stress in a dose-dependent manner. The presented research was designed to examine the potential of the green tea ( $\pm$ )-catechin as a reinforcement of the electrochemotherapy (ECT) with cisplatin in pancreatic cancer *in vitro*. The study was performed on two cell lines of the pancreatic ductal adenocarcinoma (PDA) – parental EPP85-181P and multidrug-resistant EPP85-181RNOV. Prior to the ECT protocol the cells were preincubated with high or low concentration of catechin for 2 or 24 hours, respectively. We assessed the influence of preincubation on the cisplatin toxicity with and without electroporation (EP), the electrosensitivity of PDA cell lines and the uptake of the daunorubicin and propidium iodide. Additionally, we evaluated the antioxidative properties of catechin by the measurement of the ROS-related fluorescence and the immunoreactivity of the oxidative stress-related enzymes superoxide dismutase (SOD2) and glutathione S-transferase (GST). We found that co-treatment with catechin can firmly enhance the efficacy of electroporation with cisplatin *in vitro*. More favorable effect was obtained for 2-hour incubation, which indicates the involvement of the transcriptional-independent mechanisms of catechin action. The effect may be partially explained by the increased oxidative stress level, which was higher in multidrug-resistant cells. However, further studies on cisplatin-catechin interplay and the thorough examination of the catechin-cell membrane interaction need to be performed.

**Key words:** electroporation, cisplatin, catechin, pancreatic cancer, *in vitro*

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**Abbreviations:** ECT, electrochemotherapy; EP, electroporation; GST, glutathione S-transferase; PDA, pancreatic ductal adenocarcinoma; SOD2, oxidative stress-related enzymes superoxide dismutase

### INTRODUCTION

#### Epidemiology of pancreatic cancer

Pancreatic cancer is the seventh most common cause of death due to cancer and one of the most lethal ma-

lignant neoplasms across the world. The disease has a wide range of genetic alterations, including germ line and somatic mutations (Zhang *et al.*, 2016). According to the GLOBOCAN 2012 estimates, pancreatic cancer causes more than 331 000 deaths per year (Ferlay *et al.*, 2015). The rates of incidence and mortality from pancreatic cancer were slightly higher in men than in women (Zhang *et al.*, 2016). The main risk factors for pancreatic cancer are smoking, obesity, positive family history and genetics, diabetes mellitus, dietary factors, alcohol use and lack of physical activity (Ilic & Ilic, 2016). The average survival time in patients with advanced pancreatic cancer is about 6 months (Long *et al.*, 2014). At the time of diagnosis as many as 80–85% of patients are not amenable to surgery due to local vascular invasion and/or distant metastases (Kommalapati *et al.*, 2018). Systemic palliative treatment slightly extends the survival time and does not increase the percentage of long-term experiences. Radical surgical treatment is the only method to obtain recovery due to the high resistance of pancreatic cancer to treatment with chemotherapeutics (Wong *et al.*, 2017). Severe complications and lack of effects after the use of cytostatics put modern medicine in front of the search for more effective and less severe side effects-abundant therapies. One approach is to include the substances of natural origin, such as green tea polyphenols, into the pancreatic cancer therapy.

#### Cellular effect of catechin

The green tea catechins constitute almost a three fourths of extracts derived from green tea leaves (Sang *et al.*, 2011). Each catechin consist of polyphenolic backbone with the dihydroxyl or trihydroxyl substitutions on the B ring and the m-5,7-dihydroxyl substitutions on the A ring (Balentine *et al.*, 1997a). The catechin backbone may be enlarged by the trihydroxyl structure in the D ring (gallate) forming (–)-epigallocatechin-3-gallate (EGCG) and (–)-epicatechin-3-gallate (ECG). Tea extracts have been shown to react with reactive oxygen species (ROS), such as superoxide radical, singlet oxygen, hydroxyl radical, peroxy radical, nitric oxide, nitrogen dioxide, and peroxynitrite (Hussain *et al.*, 2005; Tao *et al.*, 2015; Chen *et al.*, 2016). It is known that EGCG and other catechins can directly scavenge ROS and chelate free transition metals, thereby reducing oxidative stress (Forester & Lambert, 2011). However, recent studies have demonstrated that the tea polyphenols could also generate ROS that stimulate upregulation of the endogenous antioxidant systems through activation of the Nrf2 signaling pathway (Shen *et al.*, 2005). High concentrations

of generated ROS could in turn lead to toxicity. Some of the related studies will be discussed subsequently.

Tea polyphenols have significant potential as cancer chemopreventive agents against chemical carcinogen- or UV radiation-induced carcinogenesis in internal body organs, such as stomach, lung, liver, pancreas, esophagus, duodenum, breast, and colon, when assessed in specific tumor bioassay models. Several theories have been put forward, including inhibition of UV- and tumor promoter-induced ornithine decarboxylase, cyclo-oxygenase, and lipoxygenase activities, antioxidant and free radical scavenging activity; enhancement of antioxidant (glutathione peroxidase, catalase, and quinone reductase) and phase II (glutathione-S-transferase) enzyme activities; inhibition of lipid peroxidation, and anti-inflammatory activity (Schuijjer & Berns, 2003; Zhang *et al.*, 2009; Hagen *et al.*, 2013; Hosseinimehr *et al.*, 2013; Song *et al.*, 2014; Chen *et al.*, 2016). These properties of tea polyphenols make them effective chemopreventive agents against the initiation, promotion, and progression stages of multistage carcinogenesis (Katiyar & Mukhtar, 1997). Polyphenols from green tea extract can exert a growth-suppressive effect on human pancreatic cancer cells *in vitro*. Qanungo *et al.* (2005) observed that the antiproliferative action of epigallocatechin gallate on pancreatic carcinoma is mediated through programmed cell death or apoptosis as evident from nuclear condensation, caspase-3 activation and poly-ADP ribose polymerase (PARP) cleavage (Qanungo *et al.*, 2005). EGCG induced apoptosis of pancreatic cancer cells is accompanied by growth arrest at an earlier phase of the cell cycle (Basu & Haldar, 2009). Catechins also invoke Bax oligomerization and depolarization of mitochondrial membranes to facilitate cytochrome *c* release into cytosol (Roy *et al.*, 2010). EGCG-induced down-regulation of IAP family member X chromosome linked inhibitor of apoptosis protein (XIAP) might be helpful to facilitate cytochrome *c* mediated downstream caspase activation (Qanungo *et al.*, 2005). Furthermore, green tea polyphenols elicited the production of intracellular reactive oxygen species (ROS), as well as the c-Jun N-terminal kinase (JNK) activation in pancreatic carcinoma cells. Interestingly, the inhibitor of JNK signaling pathway as well as the antioxidant N-acetyl-L-cysteine (NAC) blocked polyphenols-induced apoptosis (Qanungo *et al.*, 2005). Appari and coworkers (2014) examined the efficacy of various catechins and the combination of these bioactive agents in the primary PDA cell line and non-malignant pancreatic ductal cells. Whereas each agent strongly inhibited colony formation, epicatechin gallate and catechin gallate were more effective than EGCG. A mixture of green tea catechins significantly induced apoptosis and inhibited viability, migration, expression of MMP-2 and -9, ALDH1 activity, colony and spheroid formation (Appari *et al.*, 2014).

### Electroporation in pancreatic cancer

Over the past 20 years, survival of patients grappling with pancreatic cancer has increased only by 3% (from 5 to 8%), therefore greatest hopes are connected to experimental regimens (Goess & Friess, 2018). Electroporation (EP) is a phenomenon occurring in cells after subjection to the action of external electric field. Currently, irreversible electroporation (IRE) is gaining importance in the treatment of the locally advanced pancreatic cancer either as a margin accentuation or as primary tumor treatment (Ruarus *et al.*, 2018). IRE is directed to a destruction of cellular membranes, whilst preserving the extracellular matrix, blood vessels and bile ducts (Vroomen

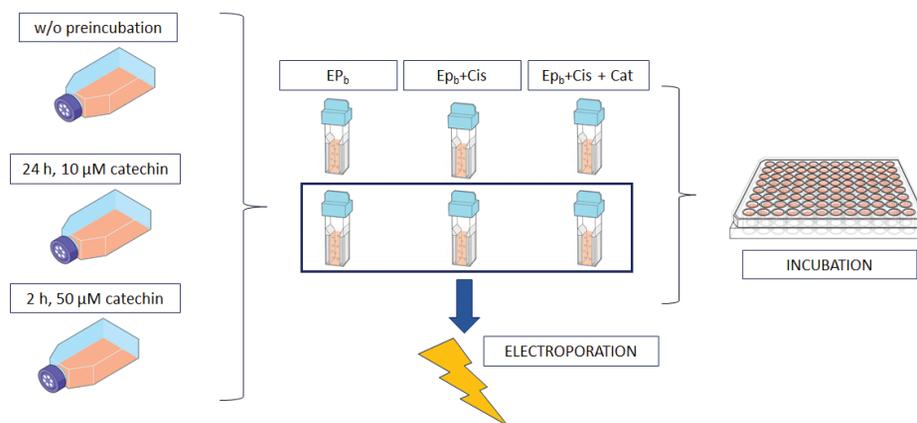
*et al.*, 2017). This was reflected by increased overall survival (20 *vs* 13 months) in comparison to standard treatment (Martin *et al.*, 2013). However, several confounders connected to IRE remain, such as the inconsistency of protocols or relatively high complication rate (Ruarus *et al.*, 2018). Promising approach in the PDA treatment is the electrochemotherapy (ECT) in which electric field is applied in order to temporarily increase the permeabilization (hence, it is often called “electropermeabilization”), allowing the membrane penetration by the hydrophilic therapeutic agents. Although the data on ECT application in pancreatic cancer are limited, preliminary results indicate the feasibility and safety of this method for the locally advanced disease (Granata *et al.*, 2015). According to recent reports, following ECT application in 19 patients with locally advanced pancreatic cancer, depending on the criteria, 60 to 100% patients showed a partial response to treatment (Granata *et al.*, 2017). However, the mechanisms of ECT application in pancreatic cancer remain poorly understood. Here, we propose a method for the enhancement of EP efficacy by means of catechin natural compound found in green tea. Our study aims to demonstrate the effect of catechin on the efficacy of electroporation in pancreatic cancer cells with different levels of drug resistance.

### MATERIAL AND METHODS

**Cell cultures.** The study was carried out on two cell lines of pancreatic ductal adenocarcinoma: the parental human cell line, sensitive to daunorubicin (EPP85-181P) and the MDR1/P-gp-independent atypical MDR cell line, selected against mitoxantrone (EPP85-181RNOV). The cultures were maintained in 37°C under high humidity in the Steri-Cult R Automated CO<sub>2</sub> Incubator (Thermo Fisher Scientific). As a culture medium we used modified Leibovitz (L-15) medium (Gibco, Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum, 1% antibiotics solution (10000 units penicillin and 10 mg streptomycin/mL, Sigma, Saint Louis, MO), 1.5% sodium bicarbonate (7.5%, Gibco), 1% MEM vitamin solution (Sigma), 0.5% ultraglutamine (Lonza, Basel, Switzerland), 0.1% glucose (45%, Sigma), 1% insulin (Sigma) and 0.7% aprotinin (5 TIU in 1mL PBS buffer; BioShop, Canada).

**Drugs.** Both drugs were freshly prepared each time prior experiments. (±)-Catechin hydrate (Sigma, cat no. C1788) was firstly dissolved in 99% ethanol to the 10 mM concentration and subsequently diluted to 5–100 μM concentration with culture medium for cytotoxicity tests or preincubation. The final concentrations of ethanol in samples was up to ~1%, which was proven to be non-toxic for cells prior to the study. For EP experiments catechin was diluted to 10 μM with EP buffer of a composition described previously (Kulbacka *et al.*, 2014). Cis-Diamineplatinum(II) dichloride (Sigma, cat. no. 479306) was dissolved in the PBS buffer to the concentration of 5 mM and then diluted with culture medium (5–100 μM) for cytotoxicity test or with EP buffer (5 and 10 μM) for the electroporation protocol.

**Electroporation protocol.** Prior to the electroporation (preceded or not by the catechin preincubation) cells were collected with trypsin and placed in dedicated cuvettes containing EP buffer of low conductivity (Kulbacka *et al.*, 2014). Then, for some cells drugs (cisplatin or mix of catechin/cisplatin) were added and part of the samples were electroporated. The parameters for the EP were as follows: 8 pulses of 100 μs duration with



**Figure 1.** The procedure of the electroporation preceded by catechin preincubation versus the procedure of electroporation not preceded by catechin preincubation (EP<sub>b</sub> – electroporation buffer, Cis – 10 μM cisplatin, Cat – 10 μM catechin). Electroporation was performed with the electric field intensity of 1000 V/cm.

0–1600 V/cm range of electric field intensity and 5 kHz frequency with the BTX 830 square wave electroporator (BTX Harvard Apparatus, Syngen Biotech, Wrocław, Poland). After electroporation, the cells were incubated for 10 minutes in 37°C, centrifuged (5 min, 1400 × *g*) and then seeded on multiwell plates. The scheme of the applied electroporation protocol is shown above (Fig. 1).

**MTT assay.** Following the experiments of chemosensitivity testing and electrochemotherapy *in vitro*, we determined the cells' viability *via* MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma) as a function of redox potential. After 24, 48- or 72-hour incubation, the medium was removed from the wells and replaced with MTT solution (0.5 mg/ml PBS buffer), then incubated in 37°C for 2 hours. The resulting formazan crystals were dissolved with the mixture of isopropanol (Archem, Poland) and 0.4% of hydrochloric acid (HCl, Archem, Poland). The absorbance was measured with a multiwell scanning spectrophotometer at 570 nm (EnSpire Multimode Plate Reader, Perkin Elmer, Waltham, Massachusetts/USA). The viability was expressed as a percentage of treated cells compared to the non-treated control.

**Immunocytochemical staining ABC.** For the examination of the immunoreactivity of superoxide dismutase (SOD2) and glutathione S-transferase (GST) cells were seeded on 12-well diagnostic slides (Thermo Fisher Scientific). After 24 hours in CO<sub>2</sub> incubator, the medium above cells was replaced with a culture medium containing catechin. The cells were subsequently incubated in 37°C for 24 hours with 10 μM catechin or for 2 hours with 50 μM catechin. After this time, the cells were fixed in 4% paraformaldehyde (Roth, Germany) and stained with the EXPOSE Mouse and Rabbit Specific HRP/DAB Detection IHC kit (Abcam, United States; cat. no. ab80a36). For the overnight incubation we used following antibodies: mouse monoclonal anti-SOD2 (Thermo Fisher Scientific; cat. no. LF-MA0030) and rabbit polyclonal anti-GST (Z-5) (Santa Cruz, cat. no. sc-459), both diluted 1:200 with the PBS buffer. After the two-day procedure samples were analyzed in a double-blinded manner with the upright microscope (Olympus BX51, Japan). The immunoreactivity was determined semiquantitatively as a percentage of positive cells stained (from a total of 150 cells per sample). The intensity of immunocytochemical staining was evaluated from five different fields of view per sample and marked as: (–) negative, (+) weak, (++) moderate and (+++) strong, according to scale developed in the laboratory.

**DCF assay.** The level of the reactive oxygen species (ROS) in cells was determined with the DCF (2,7-dichlorofluorescein) assay (Life Technologies, Poland). The DCF assay is conducted with 6-carboxy-2,7-dichlorodihydrofluorescein diacetate 2,7-dichlorofluorescein (carboxy-H<sub>2</sub>DCFDA) which is a chemically reduced form of fluorescein used as an indicator for the quantitative measurement of ROS in cells. After cleavage of the acetate groups by intracellular esterase and oxidation, non-fluorescent H<sub>2</sub>DCFDA transforms into highly fluorescent 2',7'-dichlorofluorescein (DCF). The intensity of DCF fluorescence in cells determines the level of the intracellular ROS synthesis, proportionally to the intensity of DCF fluorescence in cells. The cells were trypsinized and seeded on black 96-well plates (Perkin Elmer) for 24 hours. Then, the medium was replaced with the medium-catechin solutions and cells were preincubated for 24 h with 10 μM catechin or 2 h with 50 μM catechin. After that time medium was removed afresh and the cells were incubated for 4 h with 5 μM cisplatin, 10 μM cisplatin, or mixture of 10 μM cisplatin with 10 μM catechin. Then, the DCF assay was performed. For experiments, the stock solution of carboxy-H<sub>2</sub>DCFDA (50 μg/mL in sterile DMSO; Sigma, Poland) was established at the RT in the dark and then diluted in a cell culture medium without FBS. After washing out with PBS the reagent was added to cell culture to a final concentration 1 μM and was incubated at 37°C in darkness for 30 min. After the incubation, the fluorescence of DCF in wells (five replicated per sample) was detected every 15 minutes for 90 minutes total (with excitation wavelength of 495 nm and the emission wavelength of 530 nm).

**FACS analysis.** Flow cytometry analysis was performed for the assessment of the cell ability to internalize propidium iodide (PI) and daunorubicin (DB) into the pancreatic cancer cells. The cells were collected with Trypsin-EDTA and subjected to EP protocol described in the previous subsection. Immediately before EP, propidium iodide (Sigma; cat. no. P4170) or daunorubicin at a 10 μM concentration was applied to the cell suspension. After electroporation and 10-minute incubation in 37°C in CO<sub>2</sub> incubator, the cells were washed in PBS and resuspended in 0.5 ml of PBS. Flow cytometric analysis was performed on a FACS Calibur flow cytometer (Becton Dickinson). The fluorescence of PI was measured with FL-3 detector with 670LP emission filter. At least 10000 viable cells were measured from each sample at a rate of up to 1000 cells/s.

**Statistical analysis.** Each experiment was performed three times, in triplicate for each parameter (for a total of 9 replicates per parameter), unless pointed differently. The data was presented as the mean  $\pm$  S.D. The experiment on catechin cytotoxicity was analyzed *via* Student's *t*-test where following samples were compared to non-treated control. For the experiments with catechin preincubation multiple comparisons were performed where each parameter was compared to the same parameter of non-treated control. Statistically significant differences between treated samples and non-treated controls were placed on graphs with *P*-value of  $p \leq 0.05$  considered significant. All statistical calculations were performed with Microsoft Excel, GraphPad Prism 7 and Statistica 13.1 software.

## RESULTS

### The study on the catechin influence on PDA cell lines

The cytotoxicity of catechin towards sensitive and resistant cell lines

The parental cell line sensitive to daunorubicin EPP85-181P and the multidrug-resistant cell line EPP85-181RNOV showed similar response to the low concentrations of catechin (Fig. 2). The viability of sensitive cells was slightly more disturbed, in particular after 72 hours of incubation, when the mitochondrial activity was decreased by approximately 37% in comparison to the non-treated control. Two concentrations were selected for the subsequent experiments: 10  $\mu$ M for the electroporation protocol and 24-hour preincubation and 50  $\mu$ M for 2-hour preincubation.

The influence of catechin preincubation on the cytotoxicity of cisplatin.

The effect of preincubation on the cisplatin cytotoxicity was unequal for the tested cell lines (Fig. 3). Whilst for the parental cell line EPP85-181P the long-term incubation had no impact on the viability and the short-term incubation reduced the toxicity of cisplatin, for the drug-resistant cell line EPP85-181RNOV the cisplatin's concentrations at a range of 15 to 50 were more toxic when the cells were preincubated prior to the cytotoxicity assay. However, for the highest cisplatin concentration the protective role of 10  $\mu$ M catechin preincubation was evident in both cell lines.

### The involvement of the catechin preincubation in the oxidation and detoxication

The production of the reactive oxygen species (ROS)

To examine the effect of catechin preincubation on the cells' ability to neutralize ROS we performed the DCF assay. For both cell lines of pancreatic cancer preincubation with 50  $\mu$ M catechin was supporting the process more efficiently, which was especially visible in cells not subjected to the subsequent incubation with cisplatin (Fig. 4, cis 0  $\mu$ M). However, when cisplatin was combined with catechin (cis 10  $\mu$ M + cat 10  $\mu$ M), the level of ROS increased, which indicates an additive effect of catechin and cisplatin resulting in the oxidative stress occurrence (Fig. 4B, D).

The immunoreactivity of the mitochondrial superoxide dismutase (SOD2) and glutathione S-transferase (GST)

The immunoreactivity of the superoxide dismutase increased in daunorubicin-sensitive cell line EPP85-

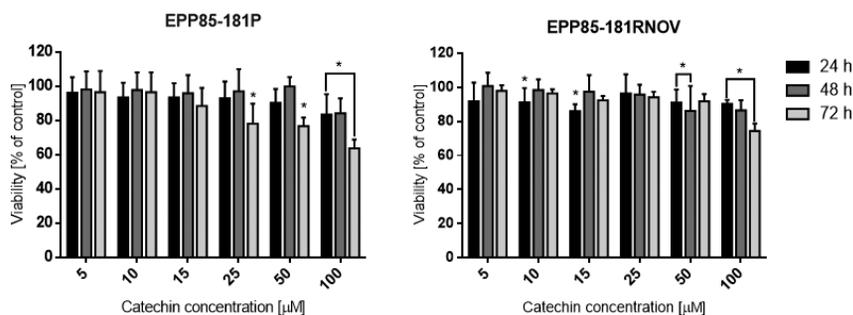


Figure 2. The viability of PDA cell lines EPP85-181P (parental) and EPP85-181RNOV (multidrug-resistant) measured with MTT assay following the 24, 48 and 72-hour incubation with different concentrations of catechin.

\*statistically significant differences between catechin-treated groups and the non-treated control ( $p \leq 0.05$ ).

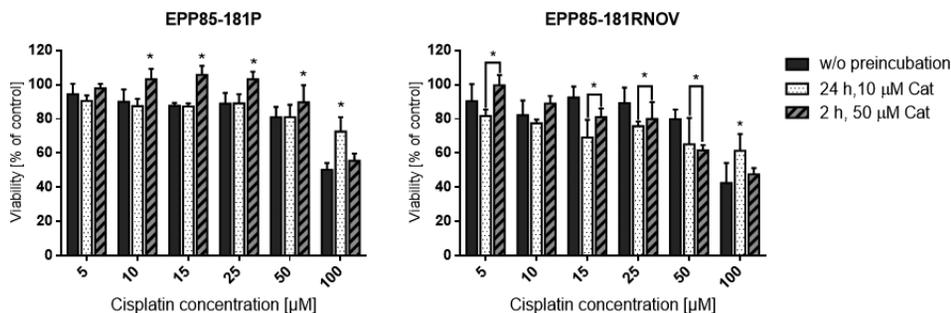


Figure 3. The viability of PDA cell lines EPP85-181P (sensitive to daunorubicin) and EPP85-181RNOV (multidrug-resistant) measured with MTT assay, following 48-hour incubation with cisplatin not preceded by catechin preincubation *versus* preceded by preincubation with 10  $\mu$ M catechin for 24 hours or with 50  $\mu$ M catechin for 2 hours.

\*statistically significant differences between groups treated with catechin and non-treated control ( $p \leq 0.05$ ).

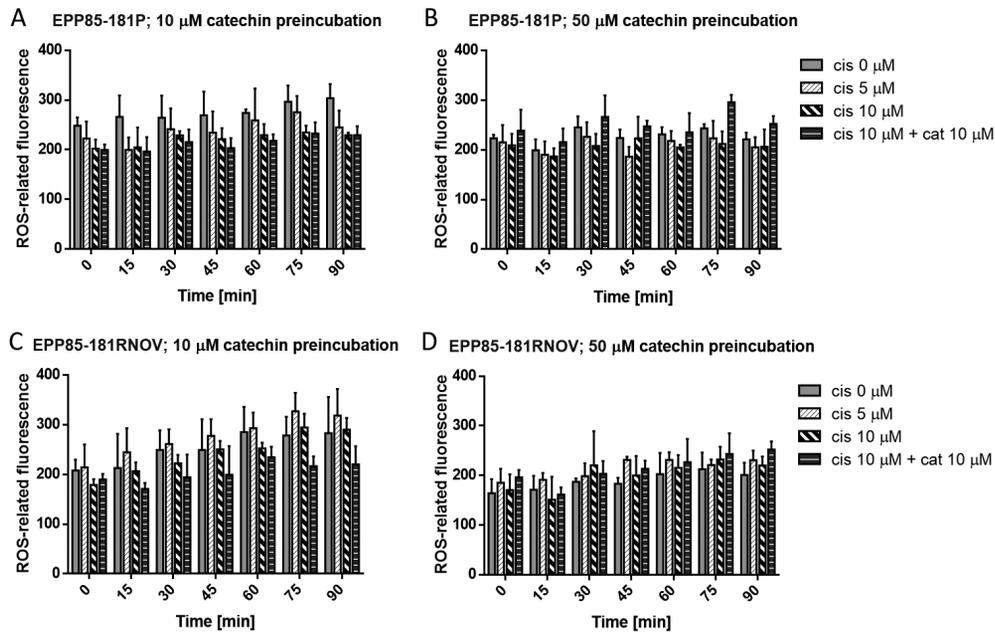


Figure 4. The results of DCF assay following the 4 h incubation with cisplatin preceded by catechin preincubation (10  $\mu$ M for 24 hours or 50  $\mu$ M for 2 hours) in PDA cell line EPP85-181P (sensitive to daunorubicin; A, B) and EPP85-181RNOV (multidrug-resistant; C, D) measured every 15 min for 90 minutes.

Table 1. The comparison of the immunoreactivity of the mitochondrial superoxide dismutase (SOD2) and glutathione S-transferase (GST) in PDA cell lines EPP85-181P (sensitive to daunorubicin) and EPP85-181RNOV (multidrug-resistant) not preceded by catechin preincubation versus preceded by incubation with 10  $\mu$ M catechin for 24 hours or with 50  $\mu$ M catechin for 2 hours.

	w/o incubation		24 h, 10 $\mu$ M Cat		2 h, 50 $\mu$ M Cat	
	% of stained cells	reaction strength	% of stained cells	reaction strength	% of stained cells	reaction strength
EPP85-181P						
SOD2	100	+	100	++	100	++/+++
GST	100	++	100	++	100	++
EPP85-181RNOV						
SOD2	85	+	97	+/++	100	++/+++
GST	40	+/-	100	++/+++	100	++/+++

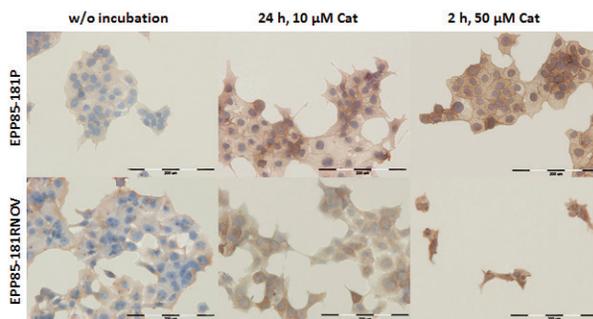


Figure 5. The representative photographs of the immunoreactivity of the mitochondrial superoxide dismutase (SOD2) in PDA cell lines EPP85-181P (sensitive to daunorubicin) and EPP85-181RNOV (multidrug-resistant) not preceded by catechin preincubation versus preceded by incubation with 10  $\mu$ M catechin for 24 hours or with 50  $\mu$ M catechin for 2 hours. The scale bars in the bottom right corner of each microphotography refer to 200  $\mu$ m.

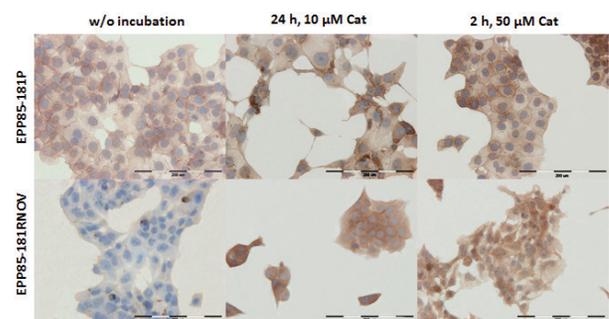
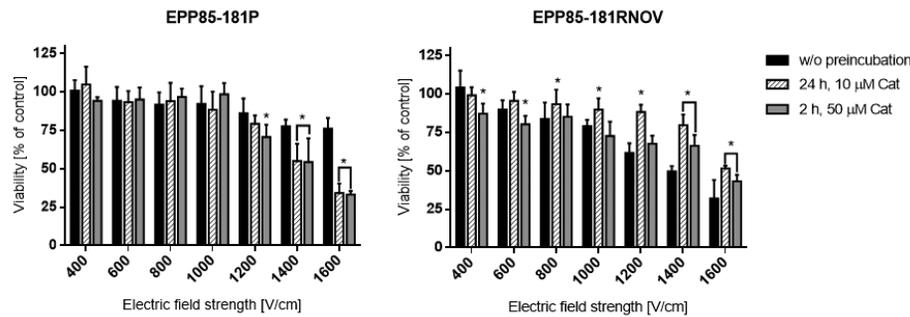


Figure 6. The representative photographs of the immunoreactivity of the glutathione S-transferase (GST) in PDA cell lines EPP85-181P (sensitive to daunorubicin) and EPP85-181RNOV (multidrug-resistant) not preceded by catechin preincubation versus preceded by incubation with 10  $\mu$ M catechin for 24 hours or with 50  $\mu$ M catechin for 2 hours. The scale bars in the bottom right corner of each microphotography refer to 200  $\mu$ m.



**Figure 7.** The viability of PDA cells EPP85-181P (sensitive to daunorubicin) and EPP85-181RNOV (multidrug-resistant) measured with MTT assay following exposition to the electric field strength at a range of 0–1600 V/cm after 48 hours of incubation when not preceded by catechin preincubation *versus* preceded by incubation with 10  $\mu\text{M}$  catechin for 24 hours or with 50  $\mu\text{M}$  catechin for 2 hours.

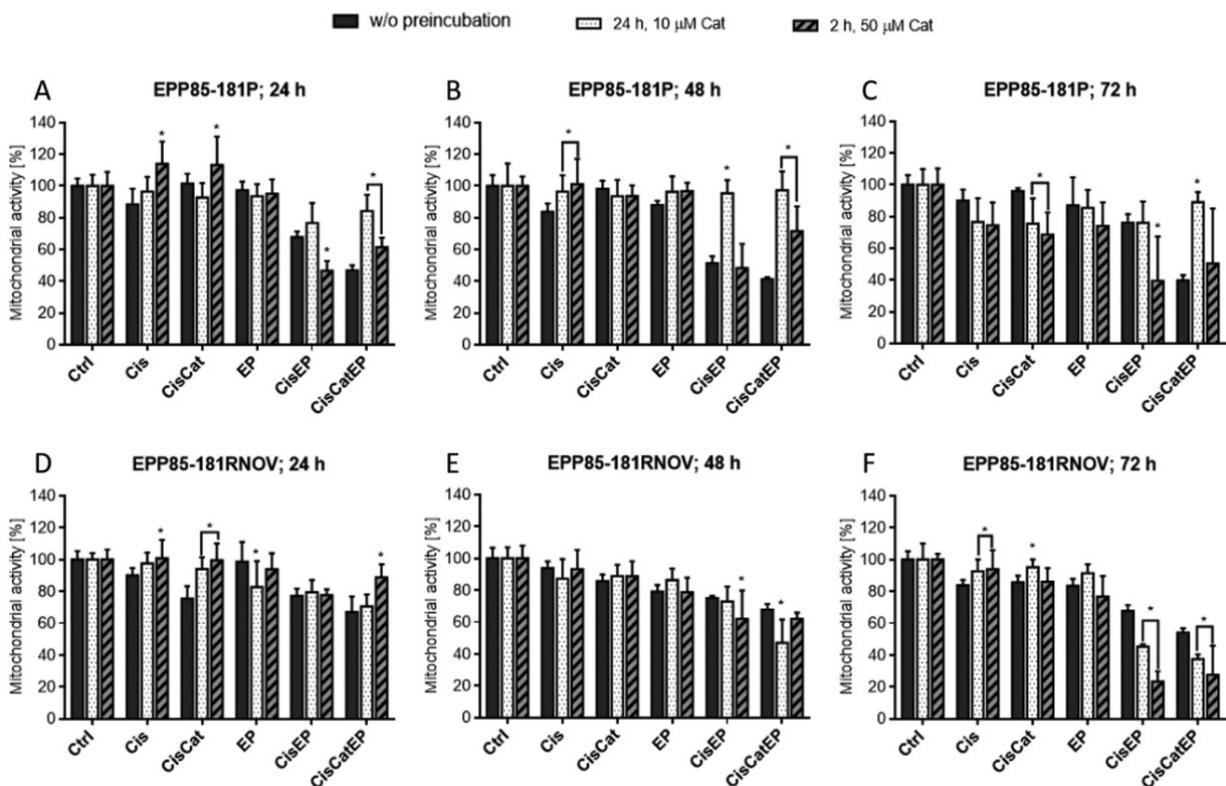
\*statistically significant differences between groups treated with catechin and non-treated control ( $p \leq 0.05$ ).

181P (Table 1) and the effect was stronger for the shorter incubation with 50  $\mu\text{M}$  catechin (Fig. 5). At the same time, the immunoreactivity of the glutathione S-transferase remained stable, regardless of the time of incubation and concentration of catechin (Table 1). More apparent changes occurred in the atypical drug-resistant cells EPP85-181RNOV, in which the increase of the GST exceeded the increase of the SOD2 immunoreactivity, especially after incubation with 50  $\mu\text{M}$  catechin, which was reflected by the percentage of stained cells and strength of the reaction (Table 1). Additionally, short incubation with the higher concentration of catechin resulted in the disturbed morphology of the multidrug-resistant cells,

which was manifested inter alia via shrinkage of the cytoplasm and the appearance of the atypical protrusive structures (Fig. 5 and 6).

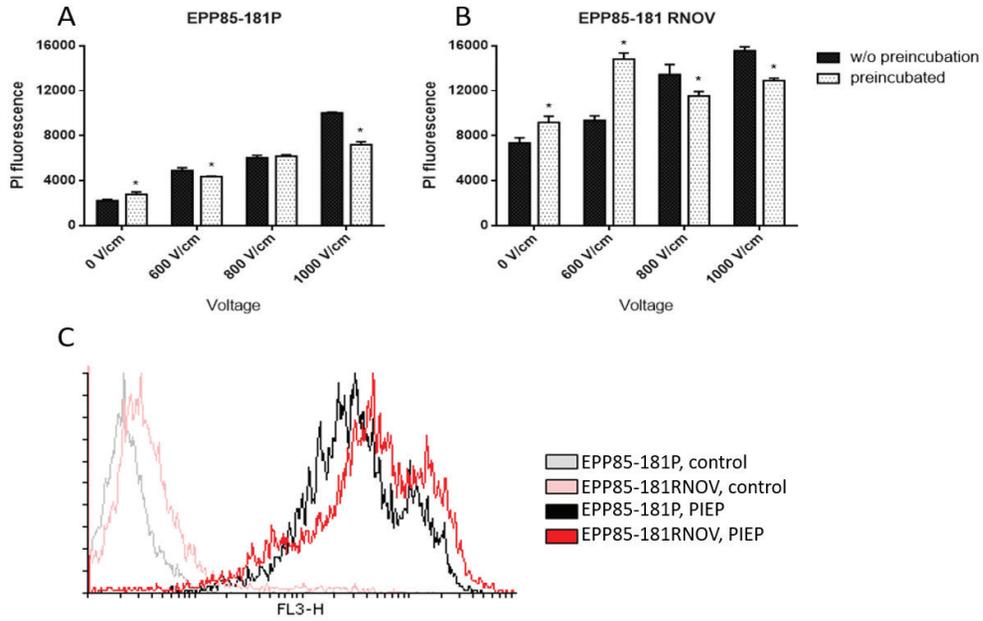
The electrosensitivity of PDA cells with and without catechin preincubation

The multidrug-resistant cell line EPP85-181RNOV is characterized by a stronger sensitivity to the higher pulsed electric field than the parental cell line EPP85-181P (Fig. 7). In case of the application of 1600 V/cm, the viability measured by the MTT assay in sensitive cells decreased by approximately 34%. The same EP parameter in the multidrug-resistant cells resulted



**Figure 8.** The viability of EPP85-181P and EPP85-181RNOV cells, measured with MTT assay, following the electrochemotherapy in vitro with cisplatin not supported *versus* supported by the catechin preincubation.

Ctrl – non-treated control, Cis – 10  $\mu\text{M}$  cisplatin, CisCat – mix of the 10  $\mu\text{M}$  cisplatin and 10  $\mu\text{M}$  catechin, EP – electroporation 1000 V/cm, CisEP – Cis supported by EP, CisCatEP – CisCat supported by EP. \*statistically significant differences between groups treated with catechin and non-treated control ( $p \leq 0.05$ ).



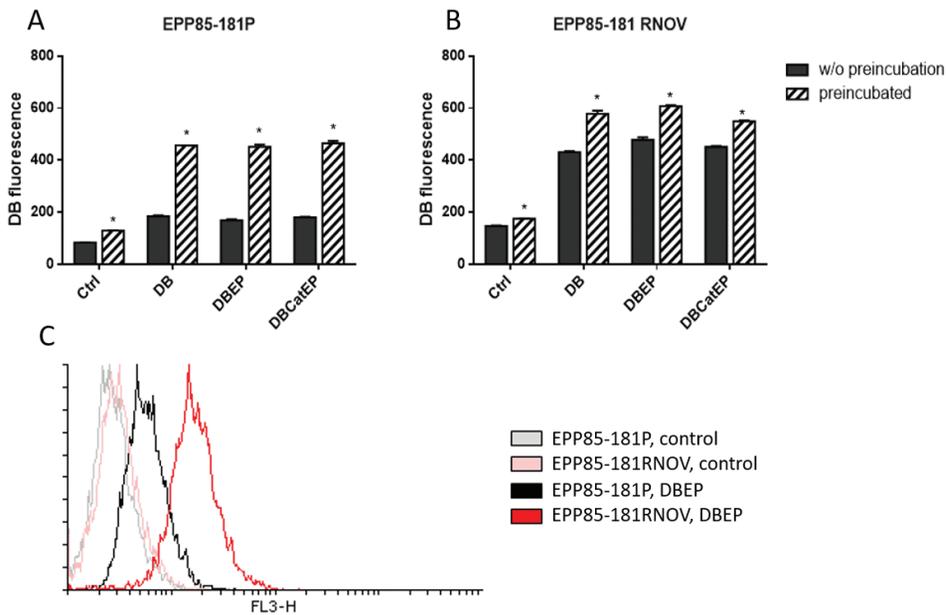
**Figure 9.** The fluorescence of the propidium iodide (PI) after EP with 10 μM PI concentration in PDA cell lines EPP85-181P (sensitive to daunorubicin) and EPP85-181RNOV (multidrug-resistant) not preceded by catechin preincubation *versus* preceded by incubation with 50 μM catechin for 2 hours. PIEP – electroporation (1000 V/cm) with 10 μM propidium iodide. \*statistically significant differences between groups treated with catechin and non-treated control ( $p \leq 0.05$ ).

in a significant drop by nearly 68% after 48-hour incubation ( $p \leq 0.05$ ). Interestingly, the preincubation with catechin influenced the electrosensitivity of PDA cells in a manner opposite to the one obtained in a cisplatin chemosensitivity testing – while preincubation seemed to protect resistant cells against negative effects of higher voltages, the same incubation time and concentrations of catechin evoked the decreased viability of the parental cells EPP85-181P.

**Electrochemotherapy *in vitro***

Electroporation with cisplatin preceded by catechin preincubation

The results of the electroporation with cisplatin clearly show that the influence of catechin concentration and time of incubation are not unequivocal for the multidrug-resistant and the drug-sensitive cell line. In the parental cells EPP85-181P the viability after CisEP meas-



**Figure 10.** The fluorescence of the daunorubicin in PDA cell lines EPP85-181P (sensitive to daunorubicin) and EPP85-181RNOV (multidrug-resistant) after electroporation (1000 V/cm) with daunorubicin (1 μM) not preceded by catechin preincubation *versus* preceded by preincubation with 50 μM catechin for 2 hours. Ctrl – non-treated control, DB – incubation with daunorubicin, DBEP – electroporation with daunorubicin, DBCatEP – electroporation with daunorubicin and 10 μM catechin. \*statistically significant differences between groups treated with catechin and non-treated control ( $p \leq 0.05$ ).

ured via MTT assay was significantly higher when the cells were subjected to 24-hour catechin preincubation (Fig. 8A, B). However, this effect was less detectable after 72 hours of incubation (Fig. 8C). The same parameters significantly reduced the viability of the multidrug-resistant cells after 72 hours of incubation (Fig. 8F). The effect of shorter incubation with 50  $\mu\text{M}$  of cisplatin is more comparable between cell lines after 72-hour incubation. In both cell lines cisplatin combined with EP induced higher cytotoxicity when cells were subjected to the preincubation with catechin (Fig. 8C, F). However, after 24 hours this effect was visible only in case of the parental cells and not for the EPP85-181RNOV cell line (Fig. 8A, D). Interesting results were obtained when 10  $\mu\text{M}$  catechin had been added to the cuvette with cisplatin just before the electroporation. Our results showed that the short exposition to the low concentration of catechin activated the protective mechanisms in PDA cells not subjected to the electroporation. However, when cisplatin and catechin (CisCatEP) were simultaneously delivered by electroporation to the cells not subjected to preincubation, there was revealed stronger anticancer effect in the sensitive cells ( $p \leq 0.05$ ). On the other hand, the differences between CisEP and CisCatEP in cells subjected to preincubation were minor. When cells were preincubated, the additional dose of catechin resulted in either slight protection in sensitive cells after the short preincubation with catechin (Fig. 8A, B) or by the slight enhancement in the cisplatin cytotoxicity in the resistant cells subjected to 24-hour preincubation (Fig. 8D, E, F).

#### The uptake of the propidium iodide and daunorubicin

The experiment with the propidium iodide showed that in both cell lines not exposed to electroporation the uptake of the PI was bigger in cells preincubated with catechin prior to the procedure (Fig. 9). However, when the electroporation was applied, this effect occurred only in multidrug-resistant cells for the low voltage (600 V/cm), whereas for the higher parameters the outcome was the opposite in both – the drug-sensitive and the drug-resistant cell line.

The uptake of the daunorubicin was significantly increased via catechin preincubation (Fig. 10). On the other hand, when catechin was applied shortly during electroporation process – the daunorubicin transportation was not improved (Fig. 10A, B – DBCatEP).

It is noteworthy, that the multidrug-resistant cells “absorbed” more propidium iodide and daunorubicin than the cells from the parental cell line EPP85-181P (Fig. 9C; Fig. 10C).

## DISCUSSION

Numerous studies have demonstrated the possible role of green tea bioflavonoids in the anticancer therapy. The best studied catechin is epigallocatechin gallate (EGCG) in which green tea is the richest. The vast majority of studies is focused on the antioxidative activity of green tea polyphenols, which can include direct mechanisms, such as scavenging of reactive oxygen species (ROS) or indirect action, like activation of antioxidant enzymes, inhibition of oxidases, mitigation of oxidative stress caused by nitric oxide or the increase in the antioxidant properties of low molecular antioxidants (Procházková *et al.*, 2011). The activity of the flavonoid is determined by the configuration and the total number of hydroxyl groups (Heim *et al.*, 2002). The difference between catechin and EGCG is the lack of the trihydroxyl structure in the D

ring (gallate). The B ring hydroxyl configuration is the most significant determinant of ROS scavenging (Burda & Oleszek, 2001), whereas the substitution of rings A and C has little impact on superoxide anion radical scavenging rate constants (Taubert *et al.*, 2003; Amić *et al.*, 2007; Procházková *et al.*, 2011). Hence, we believe that antioxidant properties of catechin may be similar to EGCG.

Surprisingly, despite the huge popularity of green tea, data on the bioavailability of individual green tea products are still limited. Imai and coworkers revealed that a cup of green tea provides 80–106 mg of polyphenols (Imai *et al.*, 1997). Most of these polyphenols are methylated, glucuronidated and sulfated metabolites in blood plasma and urine. It has been found, that several presystemic processes contribute to the low oral bioavailability of green tea catechins (Lee *et al.*, 2002; Wu *et al.*, 2012). These include chemical degradation, intestinal metabolism, microbial metabolism, hepatic metabolism, poor membrane permeability and efflux transporter-mediated intestinal excretion (Sarma *et al.*, 2008). Almost all the orally-administered green tea catechins are retained in the stomach until 60 min, but a portion is absorbed into the bloodstream from the intestines, temporally accumulated in the liver, and then returned through the bile to the colon (Shimizu *et al.*, 2014). This indicates high role of the first-pass effect in catechin bioavailability (Yong Feng, 2006). Hence, catechin should be administered parenterally and that explains higher plasma concentration of green tea compounds when drug was administered intravenously (Lee *et al.*, 2002; Yong Feng, 2006; Janle *et al.*, 2008). For catechin hydrate, the maximum reported plasma concentration was 12.17  $\mu\text{g}/\text{mL}$  at an intravenous dose of 20 mg/kg in rats (Wu *et al.*, 2012). In our study, we used catechin concentrations ranging from 1 to 29  $\mu\text{g}/\text{mL}$ . Concentrations used for preincubations were 2.9  $\mu\text{g}/\text{mL}$  and 14.5  $\mu\text{g}/\text{mL}$  (for 24- and 2-hour, respectively), which are physiologically achievable with intravenous or intratumoral injection. However, our study did not include the examination of intracellular catechin concentration. Furthermore, the direct interaction between catechin molecules and cellular membrane should be examined in subsequent studies to fully understand the role of catechin on the electroporation process.

In our investigation, we examined the influence of catechin preincubation on the neutralization of the reactive oxygen species *via* DCF assay. In general, the cells subjected to the short preincubation with 50  $\mu\text{M}$  were characterized by a lower level of ROS than those incubated for 24 hours with 10  $\mu\text{M}$  catechin and the differences were more prominent for the multidrug-resistant cell line. In the study by Jun-ichi Ueda and coworkers (1996) catechol compounds including catechin greatly suppressed the generation of  $\bullet\text{OH}$  from the reaction of  $\text{Cu}(\text{en})_2$  with  $\text{H}_2\text{O}_2$  by forming ternary  $\text{Cu}(\text{en})(\text{L})$  complexes (Ueda *et al.*, 1996). However, EGCG can also autoxidize and produce hydrogen peroxide in culture media with and without cells (Kim *et al.*, 2014). The autoxidative action of the catechin *via* the production of the hydrogen peroxide may be limited because of the lack of the pyrogallol moiety; however, this is not the only mechanism leading to the ROS generation. EGCG was also found to trigger the Fenton reaction *via* the reduction of  $\text{Fe}(\text{III})$  to  $\text{Fe}(\text{II})$  and hereby creating more potent reactive oxygen species (ROS) such as hydroxyl radicals in the human T lymphocyte Jurkat cells, in which 50  $\mu\text{M}$  EGCG generated about 1  $\mu\text{M}$  of hydrogen peroxide (Nakagawa *et al.*, 2004). The study conducted on the non-small-cell lung

cancer-derived cell line H1299 has shown that EGCG led to the ROS formation in a dose-dependent manner, and the effect was attenuated by the addition of SOD/catalase (Li *et al.*, 2010). In the study 25 and 50  $\mu\text{M}$  concentrations of EGCG were applied for 12 and 24 hours. In our research, cells were incubated with 50  $\mu\text{M}$  catechin only for 2 hours, which could explain the differences in the ROS-scavenging activity. Importantly, we have found that catechin may also have an indirect impact on cells. In our study, the immunoreactivity of the superoxide dismutase increased in both – the parental and the multidrug-resistant cell line. The intensity of the immunocytochemical reaction of the glutathione S-transferase changed only in the EPP85-181RNOV cells. The most visible changes occurred in cells preincubated with 50  $\mu\text{M}$  catechin. This, in turn, may suggest that the catechin may be also involved in the oxidative stress generation and have a toxic effect on the pancreatic cancer cells. In the study conducted by Yamamoto and coworkers (2004) antioxidant N-acetyl-L-cysteine rescued the tumor cells from  $\text{H}_2\text{O}_2$ -induced damage only, but not from EGCG-induced mitochondrial damage, which suggests the existence of mechanisms for the mitochondrial damage not related to hydrogen peroxide (Yamamoto *et al.*, 2004). On the other hand, in the *in vivo* study, the EGCG prevented the free fatty acids-induced peripheral insulin resistance through its antioxidative action leading to the decreased plasma markers of oxidative stress: malondialdehyde (MDA) and 8-isoprostaglandin, and the increased antioxidant enzymes: superoxide dismutase and glutathione peroxidase (Li *et al.*, 2011). The discrepancies between EGCG activity in different *in vivo* and *in vitro* studies may be explained, among others, by the different amount of antioxidants, including glutathione, thioredoxin, catalase and SOD in various tissues and serum components (Kim *et al.*, 2014).

The study conducted on lung tumor xenograft mice model showed that oral administration of EGCG is associated with the induction of oxidative stress and DNA damage in tumor cells. Most importantly, this effect applied only to cancer cells and was not observed in normal cells (Li *et al.*, 2010). This difference was subsequently assigned to sirtuin 3 (SIRT3), an important mitochondrial redox modulator (Tao *et al.*, 2015). In our investigation, the most apparent differences after catechin incubation were obtained in a multidrug-resistant cell line EPP85-181RNOV. There are several mechanisms which can explain the increased effects of catechins on the particularly resistant cells. The first one is the impact of the green tea flavonoids on P-gp expression. The investigation carried out on the P-gp overexpressing cellosaurus cell line showed that catechins can decrease the accumulation of P-gp substrates (rhodamine-123 and daunorubicin) and this inhibitory effect was not correlated with their total hydrophobicity, but depended significantly on their chemical structure (Kitagawa *et al.*, 2004). Another study showed that the green tea polyphenols can inhibit the photolabeling of P-gp by 75% and increase the accumulation of rhodamine-123 3-fold in the multidrug-resistant cellosaurus and epithelial colorectal adenocarcinoma cells, indicating the interaction with P-gp (Jodoin *et al.*, 2002). Interestingly, this modulation was reversible. Tang and coworkers found that EGCG suppressed the MDR-1 expression in resistant cells (Tang *et al.*, 2017). The study by Zhu and coworkers revealed that tea polyphenol could improve the uptake of  $^{99\text{m}}\text{Tc}$ -tetrofosmin (a substrate for P-gp) in MCF-7/Adr cells (Zhu *et al.*, 2001). On the contrary, a different study on MDCKII cells transfected with hPgp, hMRP1, and hMRP2 showed

that EGCG and its methyl metabolites are substrates for MRP1 and MRP2, but not for P-gp (Hong *et al.*, 2003). In our examination, we used parental cell line of pancreatic cancer and atypical derivative with MDR1/P-gp-independent multidrug-resistance. Therefore, P-gp mitigation is clearly not the reason of the increased potency of catechin in resistant cells, and there have to be different mechanisms involved. Indeed, EGCG has been shown to inhibit the cell proliferation and induce apoptosis in various cancer cells *inter alia* by blocking the activation of the epidermal growth factor receptor (EGFR) family of RTKs (Shimizu *et al.*, 2011). Furthermore, EGCG inhibited fatty acid synthase in intact cells and selectively induced apoptosis in prostate cancer cells, but the epicatechin which does not inhibit the fatty acid synthase had no effect on cells' growth and viability (Brusselmans *et al.*, 2003). EGCG has been demonstrated to inhibit telomerase, topoisomerase II and DNA methyltransferase 1, thus ultimately affecting chromatin maintenance and remodeling (Patra *et al.*, 2008). Overexpression of the topoisomerase II is one of the leading mechanism of EPP85-181RNOV drug-resistance (Lage & Dietel, 2002), so this might explain the stronger response of the cell line to the catechin incubation. However, this issue should be examined in a separate investigation.

In our investigation, there was a synergistic effect of the low-dosage cisplatin application with and without electroporation, but the effect was visible only in the multidrug-resistant cells and not in the parental ones. The synergistic anti-cancer effect of green tea catechins in combination with other anti-tumor medicines such as cisplatin, docetaxel, 5-fluorouracil and paclitaxel on various tumors has been revealed in many reports (Ramadass *et al.*, 2015; Zhang *et al.*, 2015; Meng *et al.*, 2017; Yuan *et al.*, 2017). The combination of the cisplatin and the green tea polyphenols has been studied *in vitro* and *in vivo* (Chan *et al.*, 2006; Mazumder *et al.*, 2012). It has been shown, that polyphenols and cisplatin can synergistically inhibit the growth of various cancer cells, such as MCF-7 breast cancer cells and non-small cell lung cancer A549 cells (Cao *et al.*, 2016). The co-treatment of cisplatin and EGCG induced apoptosis of the resistant ovary and the lung cancer cells by targeting expression of the CTR1 (Wang *et al.*, 2015; Jiang *et al.*, 2016). Co-treatment of tea polyphenols and cisplatin can also decrease proliferation and induce apoptosis in breast cancer cells (Chen & Zhen, 2013). These reports suggest that catechins could be useful, multifunctional drugs in the treatment of chemo-resistant cancer cells. Our study revealed that catechin preincubation have increased the intercellular accumulation of doxorubicin. Unfortunately, the results from FACS analysis cannot be directly related to viability assay results because of different chemotherapeutics (doxorubicin and cisplatin). Interestingly, in both experiments Cat augmented drug uptake or effect, in particular in combination with electroporation. The main mechanism of doxorubicin resistance is connected to P-gp overexpression, which is clearly not the aim in our research. Hence, the obtained results suggest that Cat can sensitize cell membrane in different manners. Once by the modulation of drug resistance proteins (involving the P-gp expression and other MDR proteins) and second by sensitizing cell membrane, enhancing the intracellular drug accumulation.

Catechin preincubation exhibited a protective role towards EPP85-181P cells and enhanced drug toxicity in multidrug-resistant cells, which has been shown in chemosensitivity tests (Fig. 2). On the other hand, when cells were subjected to the action of pulsed electric field,

the effects were exactly opposite. Remarkably, multidrug-resistant cell lines appear to be more sensitive to higher voltage than sensitive cancer cells and normal cell lines which has been observed also during electrochemotherapy (Gehl, 2005; Kulbacka *et al.*, 2014), irreversible electroporation (Neal *et al.*, 2011) and on 3D culture models (Frandsen *et al.*, 2015). Frandsen *et al.* (2016) explains that phenomenon by the more effective membrane repair in normal, primary cells in comparison to the resistant cancer cells (Frandsen *et al.*, 2016). In case of our study, the protective effect apparent in multidrug-resistant cells especially for the low concentration of catechin does not have to be unfavorable. The ultimate aim is to increase the effectiveness of the electrochemotherapy, and not irreversible electroporation. The mitigation of the electrosensitivity by natural compounds enables the application of higher voltage while still promoting electroporation and not necrosis within the cell population. It is worth noticing, that in case of the internal cancers, such as pancreatic adenocarcinoma, the favored type of cell death is apoptosis.

In conclusion, our research revealed for the first time, that the pre-treatment with catechin increased the efficacy of cisplatin with electroporation which was reflected by the decreased viability of the human pancreatic cancer cells. Although transcriptional regulation is one of the explanations of the green tea polyphenol's effectiveness, that may not be the leading mechanisms responsible, as the best results were obtained only 2 hours after treatment. Short incubation with a higher concentration of catechin in general was more effective in direct scavenging the ROS but was simultaneously involved in the oxidative stress generation which was also found in different studies. Hence, it seems that the strict determination catechins as antioxidants loses relevance, especially in the face of its growing applicability in the anticancer studies. Our research demonstrates that co-treatment of standard cytostatic and natural compounds such as catechin from green tea can significantly improve the effectiveness of electrochemotherapy. We believe that catechin effects on cellular response and membrane permeabilization are worth a further examination.

### Conflicts of Interest

The authors declare that there are no conflicts of interest.

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