

Original Article

Oxidative modulation of marcaine and lekoptin in H9C2 rat myoblasts

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Aim: The cytotoxicity of marcaine was estimated in combination with a calcium channel blocker. In addition, the influence of marcaine and marcaine plus lekoptin on a model system using the H9C2 cardiac cell line was investigated.

Methods: Cells were incubated for five hours with marcaine, lekoptin, or with both drugs simultaneously. Apoptotic cells were detected using the TUNEL assay and the alkaline comet assay. Mitochondrial cell function after drug uptake was examined using the MTT assay. The concentration of MDA (malondialdehyde) – the final product of fatty-acid peroxidation, was quantified spectrophotometrically. The expression of glutathione S-transferase π (GST- π) was detected by immunofluorescence (IF) and Western blotting (WB) and inducible nitric oxide synthase (iNOS) was assessed by immunocytochemical staining (ABC).

Results: Incubation with marcaine resulted in the highest number of apoptotic cells. After incubation with both marcaine and lekoptin, moderate damage to cells (54.2%±1.775% of DNA destruction) was observed. The highest levels of iNOS and GST- π expression were observed in cells treated with marcaine and marcaine plus lekoptin. The characteristic nuclear GST- π expression was observed in cells treated with both drugs.

Conclusion: Lekoptyn stimulated cells to proliferate. Marcaine caused membrane damage and ultimately cell death.

Keywords: heart myoblasts; marcaine; lekoptyn; oxidative stress; apoptosis; GST- π ; iNOS
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Introduction

Calcium acts as an intracellular messenger to modulate many aspects of cell physiology. Intracellular calcium signaling plays an essential role in cardiac physiology and modulates cardiac gene expression. However, the role of intracellular calcium signaling during cardiac development and the ability of calcium to modulate the differentiation of striated muscle cells are poorly understood^[1–4].

The primary target for marcaine's cardiotoxic action is generally considered to be voltage-gated sodium channels. Results from isolated heart studies as well as other studies suggest that the actions of bupivacaine (marcaine) on sodium channels probably contribute to the cardiotoxic

effects^[5–7]. Some authors have noted that nitric oxide (NO) acts in calcium signaling as an inter- and intracellular messenger in various types of cells. Pathogenic stimulation, such as inflammatory response, may induce the production of NO, which is mediated mainly by inducible nitric oxide synthase (iNOS). The role of NO generation by cardiomyocytes and the expression of NOS isoforms is still unclear. Cardiomyocytes have been reported to express two NOS isoforms, endothelial and inducible, in disease states^[8–10]. It was also reported that cardiac cells express neither iNOS nor eNOS immunoreactivity in both infarcted and normal myocardium^[11–13].

The crucial antioxidant enzymes that protect cells from oxidative stress are SOD, glutathione S-transferase, catalase, and glutathione peroxidase^[14]. Glutathione-S-transferase π (GST- π) is involved in a variety of cell detoxification processes. We investigated the level of expression of GST- π in cells treated with commonly used medicines. Increased

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expression of GST- π in different normal and tumor cells is associated with reduced sensitivity to cytostatics. Oxidative stress is an important component of cytostatic-induced apoptosis. GSTs are believed to detoxify endogenous substances that are formed as a consequence of oxidative stress, including lipid hydroperoxides and hydroxyalkenals. Therefore, increased GST- π expression in cells may protect them against drug-induced oxidative stress and, thus, apoptosis^[5]. Isomoto *et al* hypothesized that some antiarrhythmic drugs might cause cardiotoxicity through the induction of apoptosis^[15].

There is little known about drug oxidation and toxicity in the heart. Cardiotoxicity occurs during therapy with several cytotoxic drugs and may be the dose-limiting factor in treatment and cell response^[16]. Lekoptin is often used for the treatment of ventricular arrhythmia and is a potent blocker of calcium channels. Walles *et al* showed that heart tissue successfully metabolized verapamil^[17]. Other authors have demonstrated an increase in cardiotoxicity when doxorubicin was given in combination with verapamil and several different mechanisms have been proposed for this effect. One mechanism is based on the capacity of verapamil to inhibit the function of P-glycoprotein and therefore increase intracellular cytotoxic drug concentrations. This effect may be useful for cancer chemotherapy, but it could also lead to toxic effects in normal cells, such as cardiac cells^[16]. However, some studies show increased doxorubicin accumulation in rat cardiomyocytes incubated in doxorubicin in combination with verapamil^[18, 19]. In another study, inhibition of L-type Ca²⁺ channels was implicated in the cardiac depression induced by bupivacaine (marcaine), which causes a stronger arrhythmogenic effect and is probably related to enantiomer-specific binding to Na⁺ or K⁺ channels^[20]. The authors found an increased cardiotoxic (arrhythmias) effect caused by R(+) bupivacaine, compared with S(-) bupivacaine^[20]. Marcaine is one of the most commonly used anesthetics in contemporary medicine, especially in obstetrics. It blocks sodium channels, with consequent retardation of stimulus production and conduction, and also causes oxidative stress, leading to cell death^[21, 22]. Cesselli *et al* concluded that this might constitute the most proximal event in heart failure^[23]. However, Lenfant *et al* indicated that bupivacaine was effective in protecting erythrocytes against oxidative stress^[24, 25].

We performed studies on H9C2 cells, which are a rat myoblast cell line that differentiates *in vitro*. We examined the influence of the L-type calcium channel blocker lekoptin (verapamil), the local anesthetic marcaine, and a combination of these two agents. Both drugs are used in common medical practice; thus, it is important to analyze their actions

in the human body. One of the first events that can be studied is oxidative stress, a disturbance of the pro- and antioxidative balance. The aim of this study included determination of the relative toxicities of marcaine and lekoptin, factors predisposing cells to toxicity, the mechanisms of these toxic effects, and the actions of these drugs.

Materials and methods

Cell culture

H9C2 rat heart myocardium cells (a gift from Prof Fluvio URSINI of University of Padova) were grown in DMEM medium (Sigma) with the addition of 10% fetal bovine serum (FBS), 2 mmol/L glutamine, 10⁴×diluted 10 000 U/mL penicillin, 10 mg/mL streptomycin (Sigma), and 0.05 mmol/L selenite (Sigma). For experiments, cells were removed by trypsinization and washed with PBS. The cells were maintained in a humidified atmosphere at 37 °C in 5% CO₂.

Culturing with marcaine and lekoptin

Marcaine and lekoptin (Polfa, Poland) were obtained from Dr Z SACZKO of the A. Falkiewicz Specialists' Hospital of Wroclaw, Poland. Cells were incubated for five hours in three combinations of clinically used concentrations: with marcaine (C_M=6 µg/mL), with lekoptin (verpamili hydrochloridum) (C_L=952 µg/mL), and both drugs simultaneously. The five hour incubation time was established from previous experiments. This time was optimal for observing the interaction between drugs and cells.

Lipid peroxidation

After *in vitro* treatment with the drugs, the cells were removed by trypsinization and resuspended in PBS. The final product of fatty-acid peroxidation, malondialdehyde (MDA), reacts with TBA to form a colored complex. The level of TBARS was measured by the absorbance at a wavelength of 535 nm. The concentration of MDA was quantified spectrophotometrically based on a set of MDA standards of known concentration.

Alkaline comet assay (ACA)

For the detection of DNA fragmentation associated with apoptosis, the alkaline comet assay method was used^[26]. Cells at a concentration of 1×10⁵/mL were mixed with low-melting-point agarose (Sigma) at a ratio of 1:10 (v/v) and spread on a slide. The slides were submerged in precooled lysis solution (2.5 mol/L NaCl, 100 mmol/L EDTA, pH 10, 10 mmol/L Tris base, and 1% Triton X-100) at 4 °C

for 60 min. After being lysed and rinsed, the slides were equilibrated in TBE solution (40 mmol/L Tris/boric acid, 2 mmol/L EDTA, pH 8.3), electrophoresed at 1.0 V/cm² for 20 min, and stained using the silver staining method^[27]. To score the comet pattern, 100–200 nuclei from each slide were counted by two independent investigators.

Apoptosis - TUNEL assay

DNA fragmentation was visualized using an ApopTag® (Qbiogene) kit. In the TUNEL assay the enzyme terminal deoxynucleotidyl transferase labels the 3'-OH ends of DNA generated during apoptosis with biotinylated nucleotides. These fragments are detected by immunoperoxidase staining. The apoptosis detection kit distinguishes apoptosis from necrosis by specifically detecting the DNA cleavage and chromatin condensation associated with apoptosis. Experiments were performed on 8-well slides. Cells with stained nuclei were determined by counting 100 cells in 3 randomly selected fields. The counting was performed by two independent investigators. Samples were examined with an upright microscope (Olympus BX51).

Cell proliferation

Cytotoxicity was determined by the MTT assay (Sigma, *In Vitro* Toxicology Assay). Cells were seeded onto 96-well plates at a concentration of 5×10^3 cells/well. For the viability assay the cells were exposed to a combined-drug therapy. Following incubation for 5 and 24 h at 37 °C, the medium was removed and 100 µL 0.04 mol/L HCl in 2-propanol (Sigma) was added. The absorbance at 570 nm was measured spectrophotometrically (Multiscan MS microplate reader) and results were expressed as a percentage of the untreated control (% of control).

Immunocytochemistry

Immunocytochemistry was performed using the ABC method. Briefly, cultures were fixed and dehydrated using 4% paraformaldehyde and an ethanol gradient, respectively. The samples were then permeabilized and blocked by incubation with 0.1% Triton X-100 in PBS. Inducible nitric oxide synthase was visualized with mouse monoclonal antibody (1:100, NOS2 and Ki-67, Santa Cruz). For conventional bright-field microscopy (peroxidase-ABC labeling), the samples were incubated with a diaminobenzidine-H₂O₂ mixture to visualize the peroxidase label and counterstained with hematoxylin for 30 s. The samples were examined using an upright microscope (Olympus BX51). Stained cell numbers were determined by counting 100 cells in 3 randomly selected fields. The counting was performed by two inde-

pendent investigators. The result was judged to be positive if staining was observed in more than 5% of cells. The intensity of immunohistochemical staining was evaluated as (-) negative (no reaction), (+) weak, (++) moderate, and (+++) strong. All experiments were repeated three times.

Immunofluorescence

To visualize the expression of glutathione S-transferase π after marcaine and lekoptin treatment, H9C2 cells were plated in 600 µL of growth medium into an eight-chambered coverglass (Nunc) and grown for two days. The cells were then incubated for five hours with both drugs. After incubation, the cells were fixed with 99.9% methanol. The intracellular localization of GST- π (rabbit polyclonal antibody, 1:100, Novocastra) labeled with TRITC (tetramethylrhodamine isothiocyanate, excitation wavelength of 541 nm and emission wavelength of 572 nm) was monitored using a confocal laser scanning microscope (Olympus FluoView FV1000).

Subcellular fractionation

Cells were harvested in 700 µL of lysis buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, pH 8.0) containing a cocktail of protease inhibitors (Roche) and sonicated with a Vibra-Cell YC-130PB (Labo-Plus) at level 60 for 40 s. Cell lysates were centrifuged for 20 min at 600×g in a Sigma 3K18 centrifuge using the 12154-H rotor to pellet nuclei. The supernatant was further centrifuged at 100 000×g for 60 min in a UP-65 centrifuge. The final supernatant was designated the cytosolic fraction. The proteins contained in the supernatant and pellet were subjected to SDS-PAGE/Western blot.

SDS-PAGE and immunoblotting

Whole-cell lysates were characterized by SDS-PAGE, according to the methods of Laemmli, using 10% mini gels (Bio-Rad). A dual Color molecular weight marker (Bio-Rad) was used as a size standard. For immunoblotting, after SDS-PAGE, the gel was electrophoretically transferred to an Immobilon P (Millipore) membrane with transfer buffer (10 mmol/L Tris, 150 mmol/L glycine, 20% methanol) at 4 °C for 1.5 h and at 1.2 mA/1 cm² of membrane. The transfer and immunoblotting were performed according to the procedure described in the Millipore manual. Briefly, the membrane with transferred proteins was incubated at 37 °C for 1 h with a rabbit antibody against GST (Santa Cruz Biotechnology) diluted at 1:200 in 1% BSA in Tris-HCl buffer saline containing 0.05% Tween-20 (TBS-T, 20 mmol/L Tris-HCl, pH 7.0, 50 mmol/L NaCl, 0.05% Tween-20). The

membrane was then incubated at 37 °C for 1 h with goat anti-rabbit IgG conjugated with horseradish peroxidase (Sigma) diluted 1:1000 in TBS-T. The membrane was washed four times in TBS for 10–15 s after each incubation with antibodies. Immunoblots were developed with a 4-chloro-1- α -naphthol substrate solution in the presence of H₂O₂.

Statistical analysis

The normality of the continuous variables was checked by the Saphiro-Wilk test. The significance of the difference between mean values of different groups of cells compared with the control group (untreated cells) was assessed by Student's *t*-test with values of $P \leq 0.05$ taken as statistically significant. Statistical analysis was performed using Statistica 6 software.

Results

Lipid peroxidation

Rat myoblasts were incubated for five hours with marcaine or lekoptin (as a calcium channel blocker) and simultaneously with both drugs. The high level of MDA (Figure 1) shows that marcaine increases MDA concentration in H9C2 cells to $0.726 \pm 0.45 \mu\text{mol/L}$. However, we also observed an increase in cells treated with lekoptin ($0.565 \pm 0.369 \mu\text{mol/L}$). Incubation with both drugs resulted in a lower level of MDA than in the control cells (Figure 1). No statistically significant differences were seen between the groups.

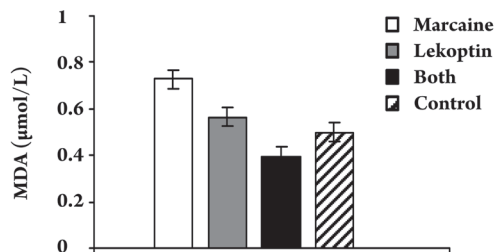


Figure 1. The levels of MDA in H9C2 cells treated with marcaine ($C_M=6 \mu\text{g/mL}$) and marcaine plus lekoptin ($C_M=6 \mu\text{g/mL}$ and $C_L=952 \mu\text{g/mL}$) compared with controls. Error bars indicate standard deviations.

Alkaline comet assay (ACA)

The results of ACA are shown in Figure 2. The highest number of apoptotic cells was seen in cells treated with marcaine only ($65\% \pm 5.7\%$ of cells). Results obtained for marcaine were statistically significant. Cells treated with lekoptin

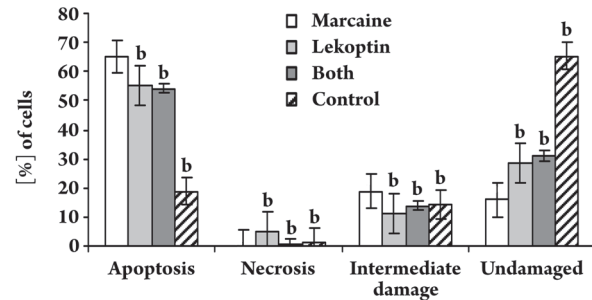


Figure 2. The percentage of nuclei with DNA damage in H9C2 cells treated with marcaine ($c_M=6 \mu\text{g/mL}$) and marcaine plus lekoptin ($c_M=6 \mu\text{g/mL}$ and $c_L=952 \mu\text{g/mL}$) compared with controls. Error bars indicate standard deviations. ^b $P < 0.05$ vs marcaine treated group.

and treated simultaneously with both drugs showed the same level of apoptosis ($54.2\% \pm 1.775\%$ of cells). Surprisingly, some necrotic cells ($5.25\% \pm 6.625\%$) were observed among the cells treated with lekoptin. The same level ($13.95\% \pm 1.6\%$ of cells) of intermediate damage was seen for the drug combination and control cells ($14.4\% \pm 4.825\%$).

MTT assay

The oxidoreductive mitochondrial function is shown in Figure 3. The 24-h incubation with marcaine resulted in the greatest damage to mitochondrial function ($16.29\% \pm 0.075\%$ compared with control cell level). The levels of viability after 5 and 24 h with lekoptin were comparable and exceeded 100% of the control cells. In the cells treated with both drugs, we observed a statistically significant decrease in viability after 24 h, to $17.57\% \pm 0.086\%$ of that of control cells. The 5-h incubation post therapy was the first stage in which changes in cells were observed. The 24 hour period after therapy was established for the cytotoxicity assay. After that period, no significant changes were detected.

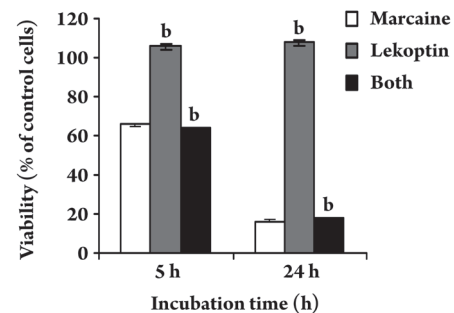


Figure 3. The effect of lekoptin and marcaine on the proliferation of H9C2 cells as evaluated by the MTT assay. Error bars indicate standard deviations (maximum $SD \pm 1.02$; minimum ± 0.15). ^b $P < 0.05$ vs marcaine treated group.

TUNEL assay

Figure 4 shows the H9C2 TUNEL assay. Cells incubated with marcaine and with both drugs showed the strongest TUNEL positivity (as assayed by stained cell nuclei) (Figure 4A, 4B). Single apoptotic cells were observed (not present) in the cells treated with lekoptin or in the control cells (Figure 4C, 4D, Table 1).

iNOS expression

The highest expression of inducible nitric oxide synthase was also observed for cells treated with marcaine and both marcaine and lekoptin (Figure 5A, 5B). In cells incubated only with lekoptin, no significant iNOS expression was observed (figures not included); the results were comparable to that of control cells (Table 2).

Table 1. Analysis of TUNEL reaction in H9C2 cells after applied drug therapy.

Cells	Intensity of ABC reaction*	[%] of stained cells**
Control	–	0
with marcaine	+++	95±1
with lekoptin	–/+	24±1
with marcaine+lekoptin	+++	80±2

* The evaluation of stained reaction: (–) negative, no reaction, (+) weak, (++) moderate and (+++) strong.

** Results presented as a mean of the cells number counted from 3 fields with ±SD.

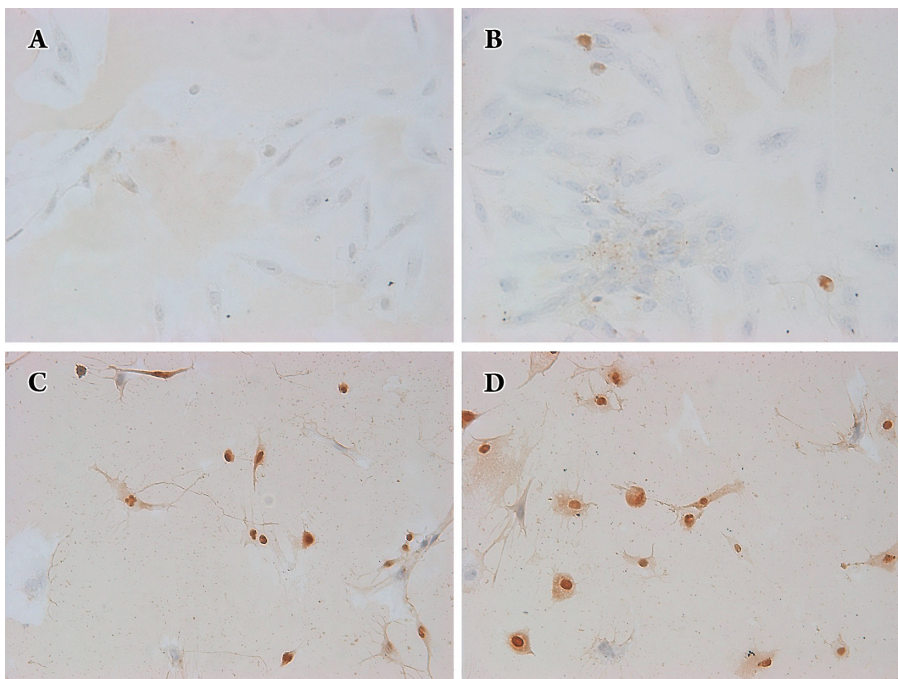


Figure 4. Apoptosis: TUNEL staining in H9C2 cells treated with marcaine (A), marcaine plus lekoptin (B), lekoptin (C), and control cells (D). (×400).

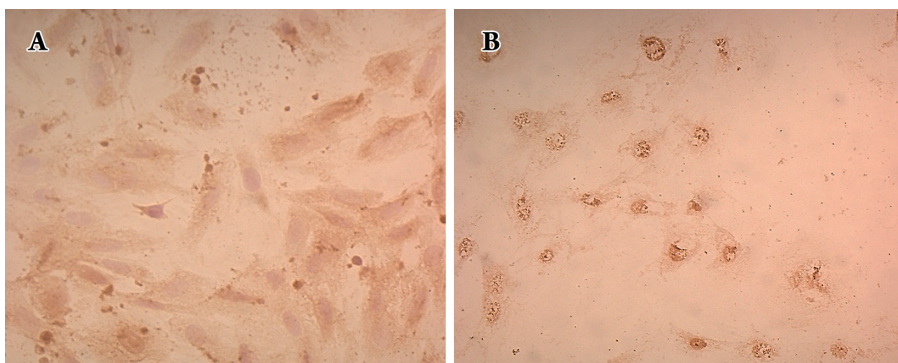


Figure 5. iNOS expression in H9C2 cells treated with marcaine (A) and marcaine plus lekoptin (B). (×400).

Table 2. Analysis of iNOS expression in H9C2 cells after applied drug therapy.

Cells	Intensity of ABC reaction*	[%] of stained cells**
Control	-	0
with marcaine	++	95±2
with lekoptin	+	12±3
with marcaine+lekoptin	++	98±1.5

* The evaluation of stained reaction: (-) negative, no reaction, (+) weak, (++) moderate and (+++) strong.

** Results presented as a mean of the cells number counted from 3 fields with ±SD.

Ki67 expression

The expression of a marker for cell proliferation (Ki-67) is presented in Figure 6A–6D). In the cells treated with marcaine the reaction was about 5%, comparable with that of control cells (Figures 6A, 6D). The strongest nuclear staining was seen in cells treated with lekoptin (Figure 6C) and both drugs (Figure 6B, Table 3).

GST- π expression and immunoblotting

The expression of S-glutathione transferase π demonstrated a specific nuclear localization in cells treated with both drugs (Figure 7B). In the cells treated with only marcaine, GST- π expression was observed in the cytoplasm and

Table 3. Analysis of Ki67 expression in H9C2 cells after applied drug therapy.

Cells	Intensity of ABC reaction*	[%] of stained cells**
Control	-/+	5±1
with marcaine	+	6±2
with lekoptin	++/++	78±1.5
with marcaine+lekoptin	++	54±2

* The evaluation of stained reaction: (-) negative, no reaction, (+) weak, (++) moderate and (+++) strong.

** Results presented as a mean of the cells number counted from 3 fields with ±SD.

the nuclear envelope (Figure 7A). The control cells and cells treated with lekoptin demonstrated very weak enzyme expression (data not shown). To complement the immunofluorescence results, immunoblotting of cytosolic and nuclear fractions was performed and presented in Figure 8. This permitted us to demonstrate a characteristic immunoreactive protein band at 26 and 50 kDa, particularly in the nuclear fraction of H9C2. In the cytosolic fraction we observed split bands of NF κ B p50-GST protein (50 kDa) for samples treated with marcaine and for control cells. The same probes also indicated no significant expression of GST fusion proteins (26 kDa). The staining was detectable in the nuclear fraction of all samples (it was the weakest band in the

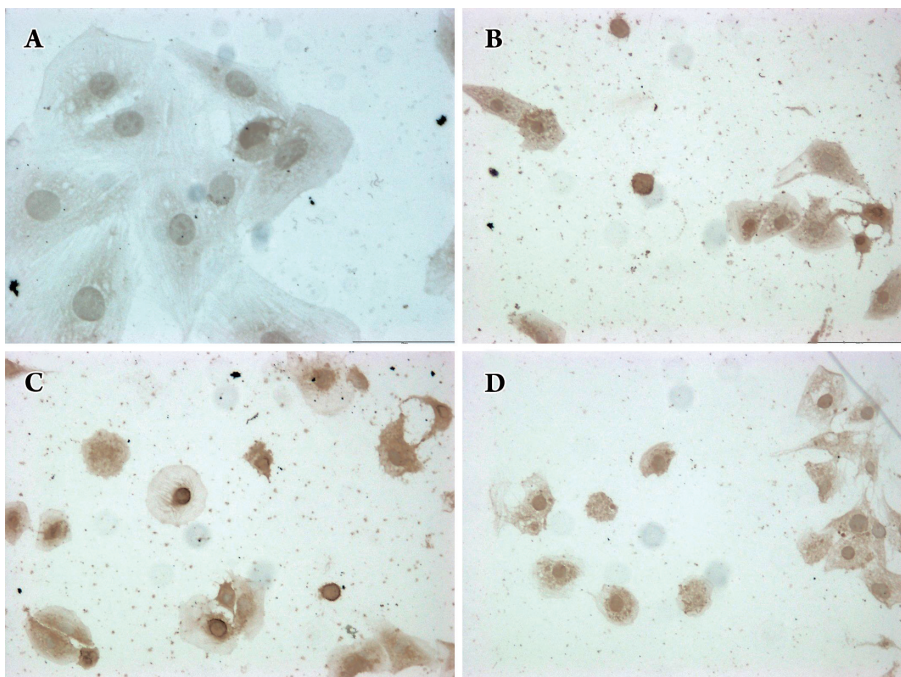


Figure 6. Ki-67 immunocytochemistry in H9C2 cells. (A) control cells, (B) marcaine plus lekoptin, (C) lekoptin and (D) marcaine.

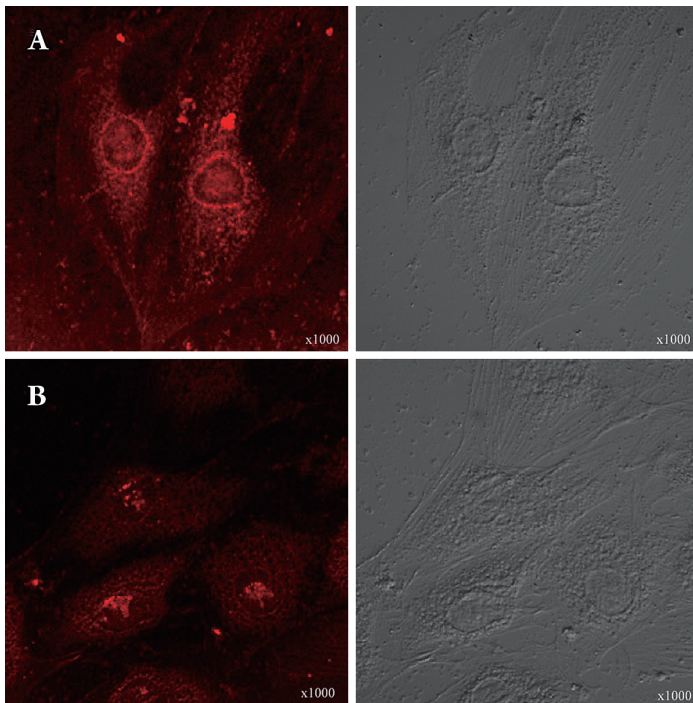


Figure 7. Fluorescent immunolabeling of GST- π in H9C2 cells with marcaïne (A) and marcaïne plus lekoptin (B). ($\times 1000$).

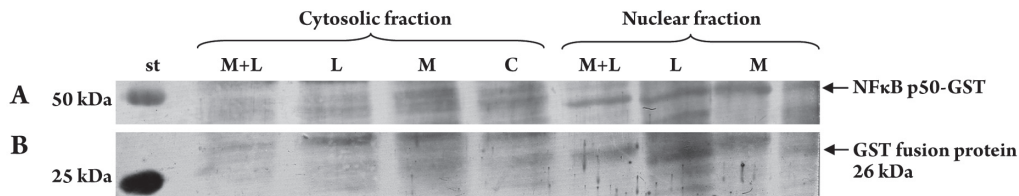


Figure 8. Immunoblotting (A–B) of 60 μ g of proteins. Immunoblotting was performed with rabbit antibody raised against a sequence of GST of *Schistosoma japonicum* origin. Cells were treated as indicated: M – marcaïne, L – lekoptin, M+L – marcaïne and lekoptin, C – untreated control cells; st – standard.

control probe). The strongest band appeared for cells treated with lekoptin, followed by marcaïne and both drugs. This is partially a confirmation of fluorescent immunolabeling in confocal microscopy, but this strong immunoblotting result is unexpected for lekoptin treatment.

Discussion

The present study showed the cytotoxic effect of marcaïne on rat myoblasts *in vitro*. Five hours of incubation with marcaïne led to higher levels of lipid peroxidation, higher expression levels of iNOS and GST- π , and damaging action on mitochondrial function. In addition, marcaïne caused membrane damage and, finally, cell death. Mitochondria have recently been shown to be involved in apoptosis and have been implicated in the generation of reactive oxygen species

(ROS). Singh *et al* suggest that mitochondrial DNA determines the cellular response to cancer therapeutics and some drugs can induce mutations in the mitochondrial genome^[28]. There is a question of whether similar mutations could occur in normal cardiac cells. Our results show that marcaïne mainly induces the late phase of apoptosis (comet assay) and causes irreparable changes in cells. Contrary to the therapeutic effect of marcaïne, lekoptin application indicated a protective role for the H9C2 cell line and stimulation of further cell proliferation. These results were confirmed by the MTT assay and Ki67 labeling. In cells incubated with only lekoptin we observed single apoptotic nuclei (TUNEL assay). Thus, we conclude that this mechanism may account for the protective effect of lekoptin against oxidative stress in this model. The action of lekoptin has membrane stabilizing properties that may induce changes in membrane polarization. It blocks cal-

cium channels, which should cause a more intensive action of marcaine. Isomoto *et al* showed that selective blockers of Na⁺, K⁺, and Ca²⁺ channels or β -adrenergic receptor did not induce apoptosis in H9C2 cells, but the authors indicated that some antiarrhythmic drugs (eg, amiodarone) had a proapoptotic effect on cardiac cells^[15].

Similar to our study, others have shown that expression of this synthase was markedly increased in cardiomyocytes and endothelial cells in the hearts of spontaneously hypertensive rats, which indicates a pathophysiological significance of altered iNOS expression^[12].

Some studies have shown that bupivacaine induces apoptosis in the RT4-D6P2T cell line, which may be associated with ROS production^[29,30]. The ROS level was elevated in Schwann cells during the early period of bupivacaine-induced apoptosis. In contrast to our study, those authors did not observe a significant increase in NO level^[31]. Feinstein *et al* showed that bupivacaine causes cell death in cardiac myocytes and dibucaine induces apoptosis in HL-60 cells^[32]. In another study, verapamil and nimodipine, drugs that reduce cardiac automatism, were effective in decreasing cardiac toxicity induced by racemic bupivacaine^[24].

Glutathione S-transferase (GST) isoenzymes are important in the detoxification of lipid peroxidation products; they bind glutathione (GSH) to a variety of electrophiles, including carcinogens and cytotoxic drugs^[14]. The isoenzymes of class pi are highly sensitive to ROS. In the present study we demonstrated the increased expression of the detoxification enzyme GST π , after treatment with marcaine. Vander Heide *et al* have shown that regulated increased expression of GST in cardiac myoblasts protected against anthracycline-induced cell death^[33]. Our data indicate a correlation between oxidative stress and cell death after marcaine treatment, which was reduced by GST- π overexpression. This suggests that oxidative stress is important in both apoptotic and necrotic cell death. L'Ecuyer *et al* indicated that GST overexpression eliminated the oxidative stress generated by doxorubicin in the H9C2 cell line. The authors suggested that other factors in addition to oxidative stress played a role in DOX-induced cell death^[14]. In contrast to our study, some authors indicate only cytoplasmic expression of GST- π in normal cells^[34]. Our results showed an unexpected nuclear expression of this enzyme in cells incubated with marcaine and lekoptin. However, other authors have demonstrated that nuclear expression of this enzyme is characteristic of neoplastic cells^[35], which suggests that there are neoplastic changes in cells induced by applied drugs. However, some authors suggest that GST overexpression may be an important part of a protective strategy against drug-induced cell death^[14].

In conclusion, our study confirms evidence for an interaction between these two drugs, which could be of importance in clinical practice. The present *in vitro* study demonstrated an interaction between marcaine and lekoptin affecting the response of rat myoblasts to oxidative stress. We have shown that marcaine can induce apoptosis in cardiomyocytes, which can be mediated by the increase of intracellular levels of oxidative stress that involve the activation of inducible nitric oxide synthase. The protective action of lekoptin against marcaine and oxidative stress and the contribution of GST detoxification mechanism were verified. There is a need for further studies to explain the mechanism of this interaction.

Author contribution

Julita KULBACKA and Jolanta SACZKO designed research; Agnieszka CHWILKOWSKA, Malgorzata DUMANSKA, Malgorzata DRAG-ZALESINSKA, Teresa WYSOCKA, Kamilla STACH, Iwona BEDNARZ and Mateusz LUGOWSKI performed research; Julia BAR and Anna MARCINKOWSKA contributed new reagents or analytic tools; Julita KULBACKA, Jolanta SACZKO and Julia BAR analyzed data; Julita KULBACKA and Andrzej GAMIAN wrote the paper.

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