Flavonoid silymarin potentiates antihelmintic effect of praziquantel via down-regulation of oxidative stress and fibrogenesis in the liver.

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Abstract—Silymarin, a mixture of bioactive flavonolignans isolated from Silybum marianum, exhibits anti-inflammatory, cytoprotective and anticarcinogenic effects. In this study impact of silymarin on larvicidal effect of praziquantel and on fibrogenesis in the liver of mice infected with Mesocestoides vogae (Cestoda) tetrathyridia was investigated. Co-administration of bioactive flavonoid silymarin with antihelmintic drug praziquantel on the liver infection with Mesocestoides vogae larvae (Cestoda) was investigated in vitro experiments revealed no larvicidal effect of silymarin on M. vogae larvae and demonstrated its powerful ROS-scavenging activity on peritoneal macrophages. Co-administration of praziquantel and silymarin to infected mice significantly enhanced the efficacy of the treatment in comparison with administration of the drug alone. In the light of the in vitro findings we believe that silymarin potentiated larvicidal effect of PZQ in the liver indirectly via suppression of oxidative stress and normalisation of GSH redox balance. Elimination of reactive oxygen species in the course of combined therapy resulted also in the significant decrease of total collagen synthesis as well as in decrease expression of genes for collagen type I and III in the liver. Thus, potentiation of larvicidal activity of praziquantel with silymarin is mediated via its antioxidant effect, resulting in the down-regulation of fibrogenesis and consequently in higher availability of drug for parasite. Keywords—liver, silymarin, parasitic helminth, praziquantel.

INTRODUCTION

Fibrosis and cirrhosis represent the consequences of a sustained wound healing response to chronic liver injury from a variety of causes including drug-induced and metabolic diseases, viral or parasitic infections [1]. The composition of fibrotic scar tissue is similar regardless of etiology and consists of extracellular matrix components (EMCs), namely collagen types I and III, sulphated proteoglycans and glycoproteins [2]. Praziquantel (PZQ) is a broad-spectrum antihelmintic used in the control of larval stages of human trematode and cestode infections such as Schistosoma mansoni [3], Echinococcus multilocularis [4], Taenia solium [5], Mesocestoides corti [6], [7]. Whereas it is highly effective against adult stages, the higher concentrations and/or longer treatments are required for PZQ to be effective against larval stages of helminths mostly due to the encapsulation of parasites in the fibrous granulomas. The larval stage (tetrathyridium) of the cestode Mesocestoides vogae (syn. M. corti) multiplies asexually in the liver and peritoneal cavity of mice and was recommended as a suitable experimental model for slowly developing human metacestode infections in pharmacological and biological studies [8]. Migration and multiplication of larve cause severe damage to the liver parenchyma, which results in oxidative stress, hepatocyte dysfunctions, severe inflammation and progressing fibrogenesis. The key issue in the pathogenesis of liver fibrosis is activation of hepatic stellate cells (HSCs) from quiescent phenotype to proliferative fibrogenic and myofibroblast-like phenotype, which is responsible for synthesis of extracellular matrix components (EMCs) [9]. The activation is a complex process involving interactions among hepatocytes, endothelial cells, Kupffer cells and inflammatory cells [10] and is mediated by various cytokines and reactive oxygen species [11]. The general approach to the treatment of liver fibrosis involves protection of hepatocytes from injury, suppression of oxidative metabolism of Kupffer cells and reduction of HSCs activation [12]. Although it seems that, at least in metacestode infections, fibrogenesis is a host-protection process preventing parasite multiplication/migration, it markedly decreases bioavailability of drug for larvae enclosed in fibrous capsules [13], [14]. In pathological conditions, the high levels of ROS are produced by neutrophils, eosinophils and macrophages as a part of their nonspecific defence mechanism against pathogens [15] and ROS are released also from damaged liver parenchymal cells. The flavonoid silymarin, isolated from the seeds of milk thistle (Silybum marianum L. Gaertn.) has a high human acceptance, since it is used around the world for the treatment of liver diseases and its hepatoprotective properties...
have been reported by many authors, for example [16], [17]. It has been discovered that silymarin can act as (i) antioxidant, scavenger of free radicals in damaged tissue and regulator of intracellular content of GSH; (ii) cell membrane stabiliser and permeability regulator; (iii) promoter of ribosomal RNA synthesis and proteosynthesis [18]. It was shown that silymarin induced apoptosis in the selected types of cancer cells via Akt pathway, but did not affect normal cells in vitro [19]. In our previous studies on the experimental pathway, we have found that co-administration of PZQ and silymarin markedly decreased serum levels of hyaluronic acid, a marker of fibrogenesis and positively modulated serum activities of ALT and AST liver enzymes, which are considered to be the indicators of liver damage [20]. Recently we demonstrated that at the same experimental mouse model, co-administration of praziquantel with silymarin markedly increased concentration of tripeptide GSH, the major non-enzymatic antioxidant system in the cells and decreased lipid peroxidation [21]. Silymarin administration to the dogs with protozoan Giardia infection did not reduce parasitic load [22], but direct effect of silymarin on parasites in vitro was not examined. Therefore, in the present study we investigated the impact of silymarin co-administration on the anthelmintic efficacy of PZQ against liver tetrahyridia M. vogae in vitro and in vivo in relation to modulation of liver fibrogenesis and direct antioxidant capacity of silymarin.

I. MATERIAL AND METHODS

A. Animals, drug formulations and experimental design

In the experiments outbred ICR male mice were used of which one group represented uninfected control and four groups were infected and treated either with PZQ alone, silymarin alone or with PZQ in combination with silymarin. Eleven infected individuals per each experimental day and group were used for either larval recovery (n = 6) or for other assays (n = 5). Experiments were carried out according to the guidelines for the care and use of experimental animals No. 289/2003 (Legislation of Slovak Republic) and were approved by the State Veterinary Administration of Slovak Republic. In the experiment, each mouse was infected orally with 55 – 60 tetrahyridia in warm (32°C) Hanks’ Balanced Salt Solution (HBSS) (Sigma Chemical Co., St. Louis, Missouri USA). Praziquantel (Sigma) was suspended in 1% cremophor oil (Sigma) in deionized water, giving rise to a suspension with drug concentration 7 mg/ml. Silymarin (Sigma) is a partially water-soluble natural product with limited bio-availability and that is why it was suspended in 1% cremophor oil with concentration 7 mg/ml. Administration of drug formulations started on day 15 post infection (p.i.) for ten consecutive days up to day 44 p.i. PZQ or silymarin was given orally (p.o.) at a dose 35 mg/kg body weight (b.w.) once a day and animals in group 3 received the same dosage of PZQ and ten doses of silymarin, group 4 served as the control. Treated animals were examined on the corresponding days post infection representing day 1, 4, 11 and 20 post therapy (p.t.), which are indicated as days 25/1, 28/4, 35/11 and 44/20 p.i/p.

B. Effect of PZQ and silymarin on larvae in vitro

In order to evaluate the drug interactions with tetrahyridia M. vogae in vitro, a drug exposure study was performed using either single drugs (PZQ, silymarin) or their combination. Larvae were aseptically isolated from the peritoneal cavity of infected mice, washed with several times with sterile 0.9 % saline solution and transferred to medium M199 (Sigma) supplemented with 10% Penstrep antibiotic solution (Sigma) and 5 % fetal bovine serum (FBS, Sigma). To test the effects of drugs in vitro, approximately 500 larvae were transferred per each of 50 ml cell culture flasks (Falcon) with 20 ml of M199 medium. Stock of PZQ and silymarin was prepared in DMSO in the concentration of 30 mg/ml and then the aliquots were added to the in vitro culture flasks so as to obtain the final concentrations of 3.0, 0.3 and 0.03 µg/ml medium.

C. Efficacy of treatment in vivo

The efficacy of the therapy on larval reduction in the livers of mice was examined on day 1, 4, 11 and 20 p.t. For this purpose, six mice from each group were killed by cervical dislocation and livers were excised. Liver samples were digested in 0.25 % trypsin solution (pH 7.8 - 8.0) at 37°C, larvae were collected and counted. Liver granulomas resistant to the trypsin digestion were further treated with solution of collagenase type I (0.5 mg/ml, Sigma) in Hank’s balanced salt solution for up to 4 h at 37°C. The final larval counts obtained after trypsin digestion (non-encapsulated larvae) and collagenase I digestion (encapsulated larvae) were calculated for the mean weight of the livers (3.2 g ± 0.5). The efficacy of treatment was calculated as described previously [24].

D. Determination of hydroxyproline in the liver

Total liver collagen content was examined by means of biochemical determination of hydroxyproline (HP) concentration, which represents the most abundant amino acid forming collagens fibres. Livers were excised, homogenized on ice and tissue samples weighing approximately 100 mg each were hydrolyzed in 4 ml of 6 M HCl for 20 h at 110°C. For each experimental day and each group, livers from five mice were used of which two samples of homogenates were subjected to the analysis as was described previously [24].

E. Immunohistochemical localisation of collagen type I

Lever specimens were fixed in 4% paraformaldehyde in PBS for 24 h and samples were processed for embedding in paraffin. For immunohistochemical staining, endogenous
peroxidase activity was quenched by soaking the sections in methanol containing 0.8 % hydrogen peroxide (v/v) for 20 min at room temperature (RT). Some sections were also pre-treated with 0.1 % collagenase type I (Sigma, Saint Louis, USA) in PBS (w/v) for 10 min at 37°C to increase the availability of antigenic epitopes for antibody binding. This step was followed by intensive washing with PBS (pH 7.2). After blocking of non-specific binding sites with 3% BSA for 30 min at 37°C, sections were incubated with primary antibodies for 2 h at 37°C. The primary antibodies for collagen I were monoclonal anti-collagen type I clone col-1 (mouse ascites, 1:300) (Sigma, USA). Rinsed sections were then exposed to anti-mouse IgG secondary antibodies conjugated with Peroxidase (Px) for 1 h at 37°C. DAB solution (3',3' Diaminobenzidine) (0.05 %) in 0.1 M Tris-HCl, 0.15 M NaCl (pH 7.8) and 0.02% H2O2 (v/v) was used as a chromogenic substrate. Sections were counterstained with Gill’s haematoxylin for 5 min, washed for 5 min and mounted.

F. RNA isolation and reverse transcription

Quantitative transcription profiles of genes for collagen type I and type III and housekeeping gene β-actin in the livers were determined by real-time PCR. On each experimental day, RNA was isolated from a sample of homogenized liver lobes (large right) dissected from five mice. Total RNA was extracted using Trizol reagent (Invitrogen Corporation, USA) and 3 µg of total RNA I were reverse transcribed with RevertAid H Minus M-MuLV Reverse Transcriptase and oligo dT primers (both from Fermentas, St. Leon-Rot, Germany). Real-time quantitative analyses of the relative abundance of mRNA species was assessed on a BioRad iCycler (BioRad, Hercules, CA, USA) as was described previously [25]. The following primers, their optimal annealing temperatures and amplicons size were for β-actin 56 °C, 370 bp [26], collagen I were 56°C, 90 bp [27] and for collagen type III were: forward 5’-TGATCCTAACCAAGGGCTGCA-3’; reverse 5’-TGCTTACGTGGGACAGTCATG-3’ (54°C, 103 bp). Data from real-time assays were analysed using iSequencer BioRad Software (BioRad, Hercules, CA, USA) and the relative gene expression (RGE) of mRNAs was calculated using the comparative Ct (threshold cycle) method [28]. Data were normalised with β-actin and results are expressed as fold amplification of target gene compared with its expression in uninfecte mice. The concentration of prepared cDNAs in the final volume of 30 µl was measured spectrophotometrically at 260 nm, so that the same concentration of cDNAs was used in semi-quantitative PCR.

G. Antioxidant capacity of silymarin
Peritoneal exudate cells (PEC) from uninfecte mice (n=5) were harvested aseptically into RPMI-1640 medium (Sigma), centrifuged at 170 g for 5 min and cell pellets were washed with RPMI medium. The cell suspensions were further diluted with RPMI medium (pH 7.2) supplemented with heat-inactivated bovine foetal serum (Sigma) 10% (v/v) and antibiotics so as to obtain a final concentration of 1 x 10^6 cells.

One ml of cell suspension was added into each well of 24-well plate (n=9 for each cell group) and incubated at 37°C in 5 % CO2 for 2 h to allow the adherence of cells. Non-adherent cells were removed by washing with Earl’s balanced salt solution (EBBS) (pH 7.2). Generation of extracellular superoxide anion (O2•-) from adherent cells was asayed as superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome C as was described previously [21]. Direct antioxidant activity of silymarin was examined after stimulation of the respiratory burst in the cells for 90 min with either PMA (phorbol myristyl acetate, Sigma; 160 nmol/well) or LPS (lipopolysacharide, Sigma; 100 ng/well). Then silymarin solution in DMSO (60 µg/ml and 120 µg/ml) was added to the cell monolayers in triplicates, incubated for further 30 min and concentrations of superoxide anions were calculated.

H. Statistical analysis

Results are expressed as mean values ± SD and numbers of replicates for each data set are indicated in the text. Multiple comparisons of groups were performed by non-parametric Kruskal-Wallis test. When significant differences were found, differences between each two groups (time-paired) were tested by post-hoc Tukey’s HSD (Honestly Significant Difference) test using Statistica 6.0 software (Stat Soft Inc., Tulsa, Oklahoma, USA). P level is indicated in the text.

II. RESULTS

A. In vitro effects of drugs on M. vogae larvae

Direct effects of praziquantel (PZQ) and silymarin (SIL) on the morphology, viability and motility of M. vogae larvae were investigated after 24 h exposure to three concentrations of both drugs (Fig. 1a-f). Larvae in the control culture were actively moving by typical rhythmic longitudinal contractions and scolecites were either invaginated or only slightly exposed. Exposure to PZQ concentrations of 3.0 and 0.3 caused rapid damage to the tegument and body vacuolization (b, c), neck extension, loss of motility and after 24 h all larvae were dead. Larvae survived at the concentration of 0.03 µg/ml PZQ (Fig. 1d) at which their morphology and motility were only slightly impaired. In contrast, incubation with the same three concentrations of SIL for 24 h had no deleterious effect on either morphology and tegument or larval motility (Fig. 1e, f) and larvae appeared as those in the control culture.

B. Efficacy of the treatment in vivo

The larvicial activity of PZQ administered alone and in combination with SIL in a total dose of 350 mg/kg b.w. for each of the drugs were examined in the livers of mice infected with Mesocestoides vogae larvae (Table 1). Although no direct larvicial effect of SIL was shown in our in vitro experiments, its co-administration with PZQ significantly increased efficacy (in %) of drug PZQ in the larval killing. In both treated groups, non-encapsulated larvae were more susceptible to the therapy, however co-administration of SIL significantly potentiated efficacy against encapsulated larvae (P < 0.05).
Table 1: Efficacy (%) of antihelmintic drug praziquantel (PZQ) administered alone or in combination with flavonoid silymarin (SIL) on the larvae, non-encapsulated or surrounded by the fibrous capsules (encapsulated) in the livers of mice.

<table>
<thead>
<tr>
<th>Days p.i./p.t.</th>
<th>PZQ-treatment</th>
<th>PZQ + SIL-treatment</th>
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<tbody>
<tr>
<td></td>
<td>non-encapsulated</td>
<td>encapsulated</td>
</tr>
<tr>
<td>25/1</td>
<td>34.4%</td>
<td>25.0%</td>
</tr>
<tr>
<td>28/4</td>
<td>23.1%</td>
<td>47.7%</td>
</tr>
<tr>
<td>35/11</td>
<td>10.9%</td>
<td>43.6%</td>
</tr>
<tr>
<td>44/20</td>
<td>0%</td>
<td>58.2%</td>
</tr>
</tbody>
</table>

* Significantly higher efficacy after PZQ+SIL versus PZQ treatment (P<0.05) for non-encapsulated larvae
♦ Significantly higher efficacy after PZQ+SIL versus PZQ treatment (P<0.05) for encapsulated larvae

D. Immunohistochemical localisation of collagen type I on liver sections

Distribution and localisation of main collagen type I was examined on paraffin sections from the livers of control and treated mice. In general, collagen staining was seen in the hepatic stellate cells (HSC) and myofibroblasts localised in the inflammatory lesions, borders of the larval penetration tracts and in the walls of capsules which surrounded larvae (Fig. 2a). In PZQ-treated group, a very intense collagen I-immunoreactivity was present in the capsules walls (Fig. 2b) and in the fibrous inflammatory lesions in the liver parenchyma forming the scar tissue on all examined days post therapy. In agreement with our data from hydroxyproline assay, collagen staining occupied lower areas in the livers of mice treated with PZQ and SIL when comparing with control and PZQ-treated mice. Very low immunoreactivity was associated with HSC in the inflammatory infiltrates and capsules on day 1 p.t. (Fig. 2c) indicating that majority of these collagen-producing cells are in quiescent stage. During the follow-up of the combined therapy, intensity of collagen staining increased having the same pattern of distribution as in other groups (Fig. 2d). In all groups distribution of collagen type III mirrored that of collagen type I, but the overall staining was much less intense (data not shown).

Table 2: Concentration of hydroxyproline (HP) in the livers of mice infected with Mesocestoides vogae larvae, untreated and treated with praziquantel (PZQ) alone and in combination with flavonoid silymarin (SIL).

<table>
<thead>
<tr>
<th>Days p.i./p.t.</th>
<th>Hydroxyproline concentration (µg/g) in the livers of mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control-untreated</td>
</tr>
<tr>
<td>25/1</td>
<td>279.6 ± 13.7</td>
</tr>
<tr>
<td>28/4</td>
<td>355.7 ± 25.7</td>
</tr>
<tr>
<td>35/11</td>
<td>417.5 ± 20.2</td>
</tr>
<tr>
<td>44/20</td>
<td>541.3 ± 28.8</td>
</tr>
</tbody>
</table>

Concentration of HP (µg/g) in the liver of uninfected mice was 76.4 ± 5.8, on day 7 p.i. = 110.4 ± 10.2, on day 14 p.i. = 160.1 ± 18.4, on day 21 p.i. = 242.5 ± 17.8. Significantly lower values (♦ P< 0.01) or higher values (♦♦ P< 0.05) to the control in the corresponding days p.t. HP was determined in the homogenates of the large liver lobes.
E. Gene expression of collagen type I and III in the livers
In order to investigate whether administration of PZQ alone or in combination with SIL can modulate collagen synthesis on the molecular level, the relative abundance of mRNA transcripts for collagen type I and III and housekeeping gene β-actin were determined by semi-quantitative real-time (RT)-PCR (Fig. 3A,B) and real-time PCR (Fig. 3a,b). Five RNA samples were analysed per each day/group. In comparison with control mice, gene expression activity of collagen type I was elevated following administration of PZQ on day 1 and 11 p.t. (A, a). Co-administration of SIL resulted in a temporary down-regulation of gene transcription, shown as the less intense 90 bp specific amplicon and RGE data (P < 0.05). Transcription of the collagen III encoding gene seen as 103 bp band in semi-quantitative PCR (Fig. 3B, b) was only slightly down-regulated following PZQ therapy within two weeks p.t. and significant decrease of gene expression was recorded after combined therapy with PZQ and SIL (P < 0.05) during two weeks of the follow-up therapy.

![Image of immunohistochemical localisation of collagen type I positive areas on liver sections from Mesocestoides vogae infected mice after therapy with PZQ alone or in combination with SIL. Collagen-IR was localised in the inflammatory lesions (black arrow), penetration holes (white arrow) and in the walls of capsules (arrowhead) which surrounded larva (a, control). In PZQ-treated group, a very intense collagen I staining was present in the capsules walls (b). After PZQ and SIL therapy collagen collagen I–IR was very scarce on day 1 p.t. (white arrow), (c) and was more intense around larvae (black arrow) and inflammatory lesions (white arrow) on day 20 p.t.](image)

Fig. 2 (a-d): Immunohistochemical (IHC) localisation of collagen type I positive areas on liver sections from Mesocestoides vogae infected mice after therapy with PZQ alone or in combination with SIL. Collagen-IR was localised in the inflammatory lesions (black arrow), penetration holes (white arrow) and in the walls of capsules (arrowhead) which surrounded larva (a, control). In PZQ-treated group, a very intense collagen I staining was present in the capsules walls (b). After PZQ and SIL therapy collagen collagen I–IR was very scarce on day 1 p.t. (white arrow), (c) and was more intense around larvae (black arrow) and inflammatory lesions (white arrow) on day 20 p.t.

F. Direct scavenging capacity of silymarin for superoxide anions
To examine whether silymarin can exert direct antioxidant and free-radical scavenging capacity, peritoneal exudate cells were obtained from healthy (uninfected) mice and adherent cells comprising majority of macrophages were used. Respiratory burst in the cells was stimulated by PMA or LPS and elimination of O$_2^{-}$ following exposure to two different silymarin concentrations in vitro was measured (Fig. 4). Extracellular release of O$_2^{-}$ (nmol/mg of cell proteins) from macrophages was stimulated after PMA (75.0 ± 11.3) and LPS (87.2 ± 9.4) incubation for 90 min. SIL in concentration of 60 μg/well reduced concentration of O$_2^{-}$ to 7.5 ± 3.8 and 16.1 ± 4.7 nmol/mg proteins, respectively. Surprisingly, 30 min incubation with the twice as higher concentration (120 μg/well) completely modified chemistry of the reaction (reduction of ferricytochrome C to ferrocytochrome C due to presence of donor electrons from superoxide anions) recorded as the colour change. Thus calculations of superoxide concentrations produced negative data, indicating the very high potency of silymarin molecules to scavenge reactive oxygen species.

![Image of gene expression profiles measured by real-time PCR (a,b) and pictures from semi-quantitative PCR (A,B) of transcribed m-RNAs after the indicated number of cycles for collagen I (A, a, 30 cycles) and collagen III (B, b, 30 cycles). Results are expressed as the relative gene expression (RGE) value (mean ± S.D., n = 5) after comparison with uninfected liver following normalization with β-actin.](image)
III. DISCUSSION

Larval stage (tetrahyridium) of cestode *Mesocestoides vogae* proliferate rapidly in the liver and peritoneal cavity of vertebrate hosts (including humans) and provide good experimental model for immunological and pharmacological studies. Infection triggers intense inflammatory responses, which are manifested as hypergammaglobulinemia, shift to Th2 cytokine production and white blood cells infiltrates in infected tissues. In the liver parasitization of *M. vogae* larvae results in the damage of hepatic parenchyma, followed by hepatocyte dysfunction, granulomatous infiltrations and extensive fibrosis. Fibrosis and formation of collagenous capsules around parasites reduces availability of drugs for parasites [13].

In the present study we have shown that co-administration of PZQ and SIL can significantly enhance efficacy of the treatment in comparison with administration of PZQ alone. Development of new efficient drugs for the treatment of human and animal infections caused by the larval stages of cestodes is an urgent issue for pharmacologists. However until a new potent substance is discovered, a combined therapy may represent a promising approach. A several immunomodulatory compounds were tested in combination with anthelmintics in cestode models for example polysaccharide glucans isolated from cells walls of fungus [21], [24], [29], muramyldipeptid (MDP) [30] or Transfer factor [31]. Natural compounds belong to the class of drugs known as biological response modifiers [32] and can stimulate functional activity of native and adaptive arm of the hosts immunity. We have demonstrated on the same experimental model that combined therapy with PZQ and glucan was significantly more effective against liver and peritoneal infection than were the single compounds. Glucan contributed to the increased larvicidal activity directly as result of modulation of innate host defences through interactions with specific receptors on macrophages, neutrophils and NK cells [33-34] and modulation of IgG and IgM responses to the newly exposed larval tegumental antigens [35]. Although application of glucan was able to activate immune system of the mice being suppressed by the parasite-derived molecules [36-38], liver fibrosis was either no affected or slightly increased. Fibrosis represents the most severe pathological consequence of larval cestode infections in the liver [39,40], therefore we aimed to investigate the different approach to the treatment using combined therapy with silymarin. In the livers of mice progression of *M. vogae* infection correlated with the gradual accumulation of main collagens type I and III, areas of inflammatory lesions and expression of genes encoding main Th2 type cytokines [25]. We also demonstrated profound activation of HSC, probably by neighbouring cells, mainly Kupffer cells, eosinophils and neutrophils which were the dominant cells during the initiation of fibrogenesis (personal observations). In the present study amount of collagens determined by means of hydroxyproline concentrationwas increased moderately after PZQ administration. Elevated synthesis of collagen type I was confirmed on the level of gene transcription and also on the liver sections from PZQ-treated mice by immunohistochemical staining. As it was mentioned, activation of HSC and transformation to myofibroblasts is a very complex process, in which ROS and selected soluble mediators released from immune cells take part. Here we have shown that PZQ is a very potent drug in the larval killing *in vitro* and the primary site of anthelmintic action of PZQ is the larval tegument [41], [42]. However *in vivo*, much higher doses are required to achieve therapeutical efficacy, which is immune-dependent process mediated by the hidden surface antigens revealed after PZQ exposure [43]. In other parasitic model on *Opisthorchis viverrini* during the short-term PZQ treatment drug-induced dispersion of parasitic antigens produced a recruitment of inflammatory cells, transcription factor NF-kappa beta and iNOS mRNA expression [44]. Therefore we suppose that stimulation of collagen type I synthesis in the liver following PZQ-therapy may be attributed to the activation of effector functions of the immune cells in the liver, including ROS overproduction, mainly in the vicinity of larvae (unpublished data). This is in agreement with the higher number of encapsulated larvae and resulting lower efficacy in comparison with the effect of combined therapy with SIL.

![Graph](https://via.placeholder.com/150)

**Fig. 4:** *In vitro* antioxidant effect of silymarin for superoxide anions (O$_2^-$) produced by peritoneal macrophages from uninfected mice. Cell monolayers were incubated with PMA (160 nmol/well) or LPS (100 ng/well) for 90 min, then SIL (60 µg/well or 120 µg/well) was added to the wells with adherent cells and reaction was carried out for further 30 min. (*, significantly lower values comparing to values without SIL treatment, P < 0.01).

ROS such as superoxide anions (O$_2^-$) and others, which are liberated by phagocytes recruited to sites of inflammation, are proposed to be a major cause of the cells and tissue damage associated with many chronic inflammatory diseases, including parasitic [45]. There are several enzymatic and non-enzymatic systems responsible for maintaining physiological levels of ROS, among which glutathione (GSH) is most important non-
enzymatic antioxidant and redox regulator [46], [47]. Decreased GSH levels were observed in many human fibrotic diseases. Ref. [48] demonstrated that in fibrotic livers activation of main pro-fibrotic mediator transforming growth factor β (TGF-β) increases ROS production in non-phagocytic cells and decreases the concentration of GSH, suggesting the crucial role of oxidative stress in fibrogenesis. In agreement with this, we have shown recently that GSH levels in the livers of PZQ-treated mice were markedly depleted following following end of therapy and co-administration of SIL was able to restore GSH levels to the physiological level [21]. Silymarin and silibinin exert antioxidant activity and both have been demonstrated to affect redox status, lipid peroxidation, and inflammatory cytokine release, see review [49].

The GSH redox status is critical for various biological events that include transcriptional activation of specific genes mediated by NF-kappa beta, regulation of cell proliferation, apoptosis and inflammation [45]. In our study we have demonstrated powerful antioxidant and ROS-scavenging capacity of silymarin in vitro similarly to [50]. However, no cytotoxic and larvicidal effect of silymarin was seen in our in vitro cultures of larvae indicating that significantly higher therapeutical effect of combined therapy with PZQ was not due to larvicidal effect of SIL. Studies on various animal models showed that silymarin is non-toxic and largely free of adverse side effects in subchronic and chronic tests even at large doses and there is not known LD_{50} in animal studies [51], [52]. In the light of present data we believe that SIL potentiated PZQ drug effect indirectly via suppression of oxidative stress and normalisation of GSH redox balance in the liver. Elimination of ROS and ROS-induced activation of HSC is down-regulated resulting in the decreased collagen synthesis and larval encapsulation. Our in vitro study showed that the free larvae are very susceptible to the drug action. Indeed, lower numbers of both non-encapsulated and encapsulated larvae found after combined therapy, expressed as the higher efficacy, in contrast with PZQ-therapy supports our hypothesis. In line with this is significantly decreased expression of genes for collagen type I and III in the liver during the follow-up the combined therapy.

We conclude that in the chronic liver diseases caused by helminth infections, combined therapy with antihelminitic drug PZQ and flavonoid antioxidant silymarin, is the perspective way of alternative treatment approach. The main beneficial effect of silymarin-co-administration is the potentiation of the drug larvicidal action, which is mediated indirectly via its antioxidant capacity, decreasing chronic inflammatory reactions what results in the down-regulation of fibrogenesis and thus higher availability of antihelminitic drug for parasites.

IV. REFERENCES


