Current situation and new possibilities in pharmacology of parasitic infections

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Abstract: - Parasitic worms are exceptionally successful infectious agents and as many as two billion individuals harbour these parasites, mostly in developing countries. Millions of humans are simultaneously infected with filarie, hookworms, whipworms, large round worms and/or schistosomes. The most dangerous parasitic infection of the Northern hemisphere is alveolar echinococcosis and a closely related cystic echinococcosis. Only a few classes of anthelmintic drugs were discovered by an empirical approach in the last century. Of these benzimidazole carbamates and praziquantel are still used to treat various human infections. However, their limited efficacy against the larval stages of helminths and increasing drug resistance indicate the need of an alternative treatment approaches and searching of the novel drugs among the natural-product extracts. In our study we focussed on medically important larval stages of several nematode and cestode species using laboratory model *Mesocestoides vogae*. Our findings demonstrated that the efficacy of benzimidazole carbamates and praziquantel can be markedly improved after their incorporation into the suitable drug carriers, which changed pharmacokinetic properties of drugs such as short plasma circulation time and the bioavailability. Application of the immunomodulatory substances such as polysaccharide glucan and flavonoid silymarin offered a very effective tool to activate the host immune defence, which is down-regulated by parasite-derived molecules. Finally, our approach employing a combined therapy of drugs with natural substances entrapped in the carriers proved to have multiple advantages over the classical therapy, regarding the drug efficacy and host pathophysiology.

Key-Words: - parasitic infections, anthelmintic drugs, cestodes, glucan, silymarin, liposomes, combined therapy

1 Introduction

Approximately one fourth of human population suffers from parasitic helminth infections with relatively high morbidity [1, 2]. Diseases caused by larval stages of cestodes from genus *Echinococcus* and in the lesser extension *Mesocestoides* are dangerous as the morbidity of untreated or mistreated humans ranges between 94-100% in the period of 10 – 15 years after diagnosis [3]. The asexually developing metacestode stage, tetrathyridium, of the cestode *Mesocestoides vogae* is recommended as a suitable model for the slower developing metacestode infections such as *E. multilocularis* in pharmacological and immunological experiments [4].

In the therapy of larval cestode infections anthelmintic drugs, such as benzimidazole carbamates and praziquantel exert parasitostatic effect only, and extended period of therapy with high doses of drugs can negatively influence immune system [5]. Developing of new antiparasitic drugs is very costly process and pharmaceutical research began to focus more on natural compounds from plants, yeasts and lichens. Moreover, suppression of host immune responses is a well-described phenomenon in parasitic infections and stimulation of the host immunity with immunomodulatory compounds has been proven to increase the therapeutic effects of drugs [6, 7]. Glucans are glucose polymers found in the cell walls of plants, fungi and bacteria and belong to a class of drugs known as biological response modifiers [8]. Flavonoids and flavonolignans form a large group of natural compounds with many biological activities, however their full therapeutic
potential and mechanisms of activities are little understood. Silymarin (SIL) is a mixture of flavonolignans isolated from the herb Silybum marianum and was known for its hepatoprotective effects. It has also antioxidant and antimutagenic properties and can induce selective apoptosis of leukemic and prostatic tumour cell lines [9]. Immunomodulatory effect of SIL was demonstrated but is specific regarding the type of disease.

Good solubility and extended presence of the active drug formulation in the plasma of patients is a prerequisite for the high efficacy of therapy, but anthelmintic drugs do not fulfill these criteria. Liposomal drug formulations allow solubility problems to be overcome and act as slow-releasing drug reservoirs of entrapped substances with the fast clearance. Liposomal carriers with drugs could serve as the secondary circulating depots for incorporated anthelmintics, releasing it slowly to the circulation [10]. In our experimental studies performed on model infectious disease caused by asexually dividing larvae of cestode Mesocestoides vogae, we showed that combined therapy with drugs and natural substances silymarin or glucan, free or entrapped in liposomes is significantly more effective than treatment with drug alone and improve host pathophysiology, therefore we propose it as promising alternative treatment option for chronic parasitic diseases.

2 Material and Methods

2.1 Animals and infection

In our experimental studies mice of ICR strain were infected with larval stage of laboratory cestode model Mesocestoides vogae and different treatment regimes using anthelmintic drug praziquantel (PZQ) free or incorporated into liposomes, glucan and silymarin were investigated. Mice were infected with 60 larvae orally and pharmacological experiments started from day 14 post infection (p.i.). Larvae were isolated from the peritoneal cavity of mouse with the chronic infection, maintained in the home animal unit.

2.2 Drugs and treatment regimes

Anthelmintic drugs praziquantel and albendazole and flavonoid silymarin were purchased from Sigma Aldrich and suspended in 0.4% cremophor oil in distilled water. Glucan was dissolved in sterile PBS, all in desired concentration. Liposomes with incorporated PZQ, glucan or SIL were prepared from phosphatidylcholine, cholesterol and dicetyl-phosphate according to our modified method [11]. Liposomal formulations were administered to mice intraperitoneally and free compounds were given orally from day 14 p.i., once or twice a day for 3, 6 and 10 consecutive days.

2.3 Effect of treatments

Two separate experiments were performed, in each treatment started on day 14 p.i. In experiment A total dosage of drug was 210 mg.kg\(^{-1}\) given in 6 doses twice a day for 3 days. In experiment B total dose of 60 mg.kg\(^{-1}\) of body weight was given once a day for 6 days. After termination of therapy, mice were sacrificed and following biological material was collected: blood, larvae and inflammatory cells from peritoneal cavities, livers for histological analyses. Isolation of larvae from livers was following digestion of tissue with 0.25% trypsin and 1% collagenase A solutions as was described previously [12]. Due to continuous asexual division of larvae, efficacy of treatment was expressed as their reduction in comparison with untreated control group using the formula:

\[
\% \text{efficacy} = 100 \times \frac{N_{\text{control}} - N_{\text{treated}}}{N_{\text{control}}}
\]

N – numbers of larvae

2.4 Histological examinations

Samples of liver tissues from large lobe were either fixed in 4% paraformaldehyde in PBS or frozen after exposure to cryoprotection. Fixed tissue blocks were processed by the classical procedure: dehydrated and embedded in the wax and sections were cut and mounted on glass slides. Liver sections from livers of control mice and livers isolated from mice in pharmacological experiments were subjected to haematoxylin/eosin staining to assess overall liver pathology, picrosirius red/fast green for localization of extracellular matrix proteins, namely collagens, PAS staining for lamellar layers of Echinococcus multilocularis cysts from livers of Meriones unguiculatus. Connective tissue mast cells (CTMC) were visualized following Toluidine blue staining method as was described previously [12, 13].

2.5 Immunological assays

Peritoneal exudate cells comprising macrophages, eosinophils, neutrophils, lymphocytes and mast cells were collected from infected treated and untreated mice aseptically and examined for phagocytic capacity, ability to adhere and production of reactive oxygen species (ROS) \textit{in vitro}. Firstly, cells smears were prepared and stained with Giemsa staining.
Phagocytic activity of macrophages was evaluated after 2 h incubation with HEMA particles (/USOL, Praha, Czech Republic) and phagocytosed particles localized inside the cells were counted after Giemsa staining. Adherent capacity of cells was determined after 4 h of incubation in incubation trays on glass slides in RPMI medium. Nonadherent cells were collected, centrifuged at 1000g, placed on clean glass slides and analyzed under the microscope after Giemsa staining. Generation of ROS by adherent cells in vitro was measured by means of determination of superoxide anions (O$_2^-$) as superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome C with and without stimulation with PMA as was described in details previously [14].

3 Results

3.1 Histopathology of infected livers of mice

Asexually developing larval stages of medically important cestodes *Echinococcus multilocularis* and *Mesocestoides vogae* (syn. *M. corti*) persisted in the livers and peritoneal cavities of mice, where they caused severe pathological changes, similar in both control and treated livers. Migrating *M. vogae* larvae were surrounded with the massive inflammatory infiltrates (Fig.1A) which were gradually replaced with extracellular matrix proteins, mainly collagens (Fig.1C). Mechanical damage to the livers tissue was caused by migration (Mi) of larvae (Fig.1D) or growing cysts of *E. multilocularis*, filled with the antigenic liquid containing larvae called protoscoleces (Fig.1B).

Connective tissue mast cells (CTMC) are known to be involved in the progression of fibrosis. On paraffin sections from the livers of mice from our experiments, CTMC were localized in the chronic inflammatory lesions and fibrous capsules surrounding larvae (Fig.2A). Glucan administration resulted in the elevation of their numbers (Fig.3) and more intense fibrosis in comparison with control. Co-administration of glucan with PZQ lowered MC numbers and fibrosis to the level found in PZQ-treated group. In contrast, SIL co-administration did not stimulate directly immune cells in the liver but significantly decreased fibrosis and numbers of CTMC in infected liver was determined.

Administration of praziquantel (PZQ) alone or in combination with either glucan or silymarin significantly modified numbers and distributions of eosinophils, neutrophils and mast cells in the livers and peritoneal cavities. In the acute inflammatory lesions ROS producing cells were eosinophils, neutrophils and in the lesser extent macrophages (Fig.2B), which were localized on cryostat sections by means of peroxidase-sensitive staining (brown colour).

![Fig.2A,B: Localisation of CTMC (A) and peroxidase-sensitive cells in the inflammatory lesions (B) and fibrous capsules around larvae.](image)

![Fig.3: Numbers of CTMC in large liver lobes obtained from control and mice treated with PZQ alone, PZQ in combination with silymarin or glucan, and glucan alone.](image)

Legend: Mean numbers of CTMC were calculated for 1mm$^2$ area. Total drugs dosages in experiment C were: PZQ, 350 mg/kg$^{-1}$ of body weight, 300mg SIL and 30mg of glucan.
3.2 Effect of liposomal drug formulations
In our experiments on mice infected with \textit{M. vogae} larvae praziquantel was incorporated into negatively-charged liposomal carriers in order to extend its circulation time and bioavailability for larvae. Based on the modified pharmacokinetic behavior of PZQ, a higher efficacy of the therapy upon various treatment schedules was achieved against larvae localized in the livers and also in peritoneal cavities, free from fibrosis. In the liver efficacy (%) of free PZQ and the same dose of drug given in liposomes in experiment A and B are shown in Fig.4. We found that after administration of free PZQ (in both experiments), the highest efficacy was seen on day 1 p.t. (empty arrow), whereas administration of PZQ in liposomes resulted in a significantly higher therapeutic effects which appeared later - on day 7 p.t. (black arrow). The explanation for this was found in our other studies where we showed that PZQ contributed to the acceleration of fibrogenesis and larval encapsulation in fibroblasts via decrease of glutation redox-balance \cite{14, 15} and antibody-dependent larvicidal effects \cite{16}. Similarly, in the peritoneal cavities, efficacy of liposomized PZQ was higher (black arrows) than the effect of free drug in both experiments, indicating extended concentration of drug in the tissues. There was no direct correlation between total dosage 60 mg.kg$^{-1}$ versus 210 mg.kg$^{-1}$ and the increase of efficacy.

3.3 Natural compounds in therapy
During tissue-dwelling helminth infections in humans and animals, the gradual down-regulation of effector functions of immune cells is triggered by parasites, aimed to protect them from the cytotoxic activity of immune system and in the same time to shift the pathophysiology to a chronic type of disease. Having this in mind in our studies we examined the immunostimulatory and antioxidant effects of two different natural compounds: glucan and silymarin, which were administered to mice free or entrapped in lipid carriers. We confirmed strong pro-inflammatory activity of glucan and activation effector functions of immune cells, resulting in the significantly higher therapeutical efficacy after its co-administration with PZQ in comparison with efficacy of free PZQ (Fig.6) in the livers and peritoneal cavities of treated mice in experiment A.

Flavonoid silymarin (SIL) has well-described antioxidant effects. In our study direct scavenging of ROS produced from activated granulocytes in the liver of infected mice resulted in down-regulation of collagen-producing hepatic stellate cells and fibroblasts and decrease of fibrosis. Larval encapsulation in fibrous tissue is host-protecting response to infection but it markedly reduced availability of drugs for larvae. Due to the reduced amount of collagen in livers of mice treated with PZQ in combination with SIL, significantly higher efficacy was achieved in the liver \cite{15}. There is no fibrogenesis in infected peritoneal cavities of mice, where larvae continue asexual proliferation what is associated with the massive accumulation of inflammatory cells, mainly macrophages and eosinophils. However they functions seem to be directly suppressed by the larvae so as to permit parasite survival \cite{17, 18}. In experiment C, where
total 10 doses of PZQ (one dose contained 35 mg.kg\(^{-1}\) of drug) was given once a day from day 15 up to day 24 p.i., co-administration of SIL (total dose of 300 mg.kg\(^{-1}\)) contributed to markedly elevated efficacy of treatment (Fig. 7).

![Fig 7: Efficacy of total dose of 350mg.kg\(^{-1}\) PZQ given alone and in combination with SIL in total dose of 300mg.kg\(^{-1}\) against larvae in the peritoneal cavities of infected and treated mice.](image)

**3.4 Immunomodulatory activities of glucan and silymarin in vivo and in vitro**

Polysaccharide glucan is a potent non specific activator of effector functions of mononuclear phagocytic cells, natural killer cells [19] and modulated proliferative activity and cytokines production of lymphocytes during *E. multilocularis* infection in mice [7] after co-administration with albendazole. Stimulation of T and B lymphocytes after therapy with glucan and anthelmintic drug was observed also in experimental larval toxocariosis [20]. Glucan has a weak antioxidant activity but markedly stimulated respiratory burst in macrophages. Phagocytic ability (PA) of macrophages is their important effector function involved in removing of dead tissue parasites and bacteria. Immunomodulatory activity of SIL was demonstrated in non-parasitic diseases in several studies and our data about modulation of immune cells PA, summarized in Table 1, are the first obtained in experimental helminth infection. In our experiments we showed that SIL modulated phagocytic activity towards normalization of peritoneal macrophages, stimulated proliferation of Th1 type of T helper lymphocytes and production of cytokines involved in killing of larvae (not shown).

<table>
<thead>
<tr>
<th>Days p.i/ p.t</th>
<th>Groups of treated mice</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
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<tr>
<td>0</td>
<td>51,8 ± 4,2</td>
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<tr>
<td>7/-</td>
<td>79,1 ± 2,5</td>
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<tr>
<td>14/-</td>
<td>76,6 ± 2,9</td>
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<tr>
<td>21/-</td>
<td>65,3 ± 1,8</td>
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<tr>
<td>25/ 1</td>
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<tr>
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<td>59,3 ± 2,8</td>
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<tr>
<td>44/ 20</td>
<td>55,4 ± 2,4</td>
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Table 1: Phagocytic activity of peritoneal macrophages from mice (experiment C) infected with *M. vogae* larvae (control) and from groups of mice treated between days 15 – 24 p.i. with PZQ alone, in combination with SIL or glucan.

Revealed that the treatment can stimulate hepatic fibrogenesis. Apart from significantly elevated mastocytosis in the infected livers, only moderate effects on the effector functions of cells of innate immunity was seen following PZQ treatment. Moreover, we revealed that PZQ administration caused short-term abolishment of redox-balance in the liver.

For the first time, effects of liposomized formulations of PZQ were investigated in a larval cestode infection caused by *M. voage*. Increased efficacy was found after lip.PZQ administration in the sub-chronic phase of infection with 5-7 days delay, in comparison with free drug. This corresponded to the time of lip. PZQ degradation in macrophages after their phagocytosis, which served as the second circulating drug depots. However, inhibition of larval multiplication was only temporal and was increased in mice treated with empty liposomes. Higher efficacy was achieved in case of liposomized formulations probably due to maintaining drug therapeutical levels in the

**4 Conclusions**

Using the experimental cestode model *Mesocestoides vogae* we revealed that free PZQ administered in a vehicle (oil in water) is larvicidal *in vivo* even in sub-curative doses, however it was...
tissues for a prolonged period of time. Subcutaneous administration of glucan in liposomes had a stronger immunostimulatory effect than had free glucan resulting in reduction of the larval numbers, but it partially enhanced fibrosis. Taking into account above findings we propose that the suitable therapy of larval cestode infections is combination of free praziquantel (or possibly other anthelmintics) and liposomized glucan given in well-tailored doses. In contrast to the effects of both compounds given alone, combined therapy reduced pathophysiological processes such as hepatocyte damage, liver fibrosis, immunosuppression and ascites in the peritoneal cavity. Fibrogenesis was initiated due to liver damage following penetration of larvae, which led to the gene activation of profibrotic genes for collagen I, III and α-SMA as well as genes expression of Th2 type cytokines IL-10, IL-5, IL-4 and IL-13 in T lymphocytes elevated. Eosinophils and neutrophils predominated in the acute inflammatory lesions, where contributed to the activation of hepatic stellate cells by means of high amount of released reactive oxygen species (ROS), and seem to participate in the initiation of fibrogenesis during the liver inflammatory diseases. In contrast, connective tissue mast cells appeared lately in the fibrous lesions and capsules indicating on their role in perpetuation of myofibroblasts surviving and collagen synthesis. Our findings led us to design a combined therapy using strong natural antioxidant natural substance, the flavonoid silymarin, which was used for the first time in the treatment of diseases caused by helminth parasites. Silymarin is a lipid soluble natural product available on the market and is well absorbed after oral administration with food. Neither cytotoxic effects towards primary cells, nor larvicidal effects were seen, however combined therapy with PZQ and silymarin was shown to be an excellent treatment approach, at least against experimental infection with M. vogae larvae. Suppression of fibrogenesis after combined therapy was our important observation demonstrated by decreased collagen content and suppressed gene expression of TGF-β1. Data indicated that antifibrotic effect of combined therapy was mediated by antioxidant activity of silymarin, resulting in the increased bioavailability of praziquantel and consequently, in elevated efficacy against larvae. However, administration of single silymarin is not a suitable approach, as reduction of fibrosis during the follow-up correlated with higher parasite migration and growth. We also proved the immunomodulatory effect of silymarin, directed to the restoration of immunological balance. During the follow-up of the combined therapy, mastocytosis and eosinophilia declined in the liver supporting their regulatory role in fibrogenesis.

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References:


