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RESEARCH

Therapeutic efficacy of candidate antischistosomal drugs in a murine model of schistosomiasis *mansoni*

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Abstract

Schistosomiasis is a neglected tropical disease associated with considerable morbidity. Praziquantel (PZQ) is effective against adult schistosomes, yet, it has little effect on juvenile stages, and PZQ resistance is emerging. Adopting the drug repurposing strategy as well as assuming enhancing the efficacy and lessening the doses and side effects, the present study aimed to investigate the in vivo therapeutic efficacy of the widely used antiarrhythmic, amiodarone, and diuretic, spironolactone, and combinations of them compared to PZQ. Mice were infected by *Schistosoma mansoni* "*S. mansoni*" cercariae (Egyptian strain), then they were divided into two major groups: Early- [3 weeks post-infection (wpi)] and late- [6 wpi] treated. Each group was subdivided into seven subgroups: positive control, PZQ, amiodarone, spironolactone, PZQ combined with amiodarone, PZQ combined with spironolactone, and amiodarone combined with spironolactone-treated groups. Among the early-treated groups, spironolactone had the best therapeutic impact indicated by a 69.4% reduction of total worm burden (TWB), 38.6% and 48.4% reduction of liver and intestine egg load, and a significant reduction of liver granuloma number by 49%. Whereas, among the late-treated groups, amiodarone combined with PZQ was superior to PZQ alone evidenced by 96.1% reduction of TWB with the total disappearance of female and copula in the liver and intestine, 53.1% and 84.9% reduction of liver and intestine egg load, and a significant reduction of liver granuloma number by 67.6%. Comparatively, spironolactone was superior to PZQ and amiodarone in the early treatment phase targeting immature stages, while amiodarone had a more potent effect when combined with PZQ in the late treatment phase targeting mature schistosomes.

Keywords *Schistosoma mansoni* · Amiodarone · Spironolactone · Praziquantel · Drug repurposing

Introduction

Schistosomiasis is a parasitic infection caused by *Schistosoma* spp. (McManus et al. 2018). According to recent estimates, 440 million people suffer from chronic schistosomiasis, about one-third of whom suffer from either current or complications of past *S. mansoni* infection (Tamarozzi et al. 2021). As a result, WHO classifies schistosomiasis as a target tropical disease second to malaria (Mawa et al. 2021). Egg deposition by female *S. mansoni* causes inflammation, granuloma, fibrosis, and progressive damage to organs (Mduluza and Mutapi 2017). In 2021, more than 75.3 million persons were treated out of an estimated minimum of 251.4 million people who needed preventive treatment. The World Health Assembly's new neglected tropical diseases roadmap 2021–2030 settled the eradication of schistosomiasis as a public health matter in all endemic countries and the

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stoppage of its transmission in certain countries as global objectives (WHO 2023).

Praziquantel (PZQ) has been the drug of choice for the treatment and control of schistosomiasis as it is given as a single oral dose with no severe side effects and at an affordable cost. However, the PZQ regime has several drawbacks, the most important being effective only against adult worms with low efficacy against the immature stages. For this reason, the juvenile schistosomula can continue the cycle causing damage to the host and resulting in lower cure rates in highly endemic areas (Fakahany et al. 2014). Moreover, PZQ shows poor bioavailability and insufficient concentration in the systemic circulation following oral administration (EL-Feky et al. 2015). Due to its low water solubility, higher oral doses are required, which increases the risk of side effects and the patient's non-compliance and encourages the emergence of resistant parasite strains (Borrego-Sánchez et al. 2020). In addition, the large size and bitter taste of PZQ tablets contribute to the patient's non-compliance and inconvenience for children (Olliaro et al. 2014). Furthermore, employing PZQ in mass drug administration campaigns for decades and its widespread use in humans and domestic animals contributed to the development of parasitic resistance (Pinto-Almeida et al. 2016).

The process of creating new medications is time-consuming, expensive, and requires several clinical trials. Subsequently, drug repurposing/repositioning is gaining increasing interest because employing drugs that are already on the market will cut the time, cost, and risk of traditional drug development (Abd El Hady et al. 2023).

Amiodarone is an antiarrhythmic medication used to treat and prevent cardiac dysrhythmias by blocking the voltage-gated potassium and calcium channels (Porto et al. 2021). It was proven that amiodarone has antiparasitic effects in addition to its cardiac activity (Dziduch et al. 2022). Amiodarone completely inhibited cercarial and schistosomular motility in vitro by depleting intracellular ATP levels and significantly damaging the worm's tegument with the disintegration of tubercles as visualized by SEM (Talaam et al. 2021).

Diuretics are the most frequently prescribed class of medications for reducing fluid congestion, particularly for patients with heart failure, renal failure, or liver cirrhosis. These compounds were investigated for their anti-schistosomal properties against *S. mansoni* due to their reasonably safety oral administration, tolerability, and affordability (Abd El Hady et al. 2023). Spironolactone is a potassium-sparing diuretic, and it is used as an antihypertensive drug. It showed 100% mortality of schistosomes in vitro (Guerra et al. 2019). As well, it caused the disintegration and sloughing of adult male tegumental tubercles, increased motor contractions, and reduction of body length, together with increased mortality in a concentration-dependent way (Aminou and Abdel Rahman 2020).

Considering the aforementioned, the present study aimed to investigate the therapeutic efficacy of amiodarone, spironolactone, and combinations of drugs in *S. mansoni*–infected mice compared to PZQ through parasitological and histopathological parameters.

Material and methods

Study type—experimental in vivo study.

Study setting: The study was conducted during the period from September 2022 to April 2023 at the Medical Parasitology Department, Faculty of Medicine, Ain Shams University, and Theodor Bilharz Research Institute (TBRI), Egypt.

Schistosoma mansoni strain

S. mansoni cercariae (Egyptian strain) were obtained from infected *Biomphalaria alexandrina* snails maintained in the Schistosome Biological Supply Center (SBSC) of TBRI.

Preparation of the study drugs

Each drug was prepared by grinding the tablet into a powder and then gradually suspending it in 2% cremophor-El (Sigma Chemical Co., St. Louis, MO, USA) until it was completely dissolved. The suspensions were freshly prepared before administration.

Each drug was orally given to each mouse in the corresponding group using oral gavage feeding needles in five divided doses for 5 consecutive days.

PZQ (Distocide®, Egyptian International Pharmaceutical Industries Company, EIPICO, Egypt) was prepared as a full dose of 1000 mg/kg body weight (200 mg/kg/D) according to El-Feky et al. (2015).

Spironolactone (Spectone®, Kahira Pharmaceutical & Chemical Industries Company, Cairo, Egypt) was prepared as a full dose of 500 mg/kg body weight (100 mg/kg/D) according to Guerra et al. (2019).

Amiodarone (Cordarone®, Global Napi Pharmaceuticals, GNP, Egypt under license of Sanofi Aventis, France) was prepared as a full dose of 500 mg/kg body weight (100 mg/kg/D) according to Porto et al. (2021).

Combined PZQ–Spironolactone: Half the dose of PZQ (100 mg/kg/D) was added to half the dose of spironolactone (50 mg/kg/D).

Combined PZQ–Amiodarone: Half the dose of PZQ (100 mg/kg/D) was added to half the dose of amiodarone (50 mg/kg/D).

Combined Spironolactone–Amiodarone: Half the dose (50 mg/kg/D) of each was combined.

Animal grouping

Seventy (70) male Swiss albino mice aged 6–8 weeks old and weighing 20–25 g were housed in clean polypropylene cages in a well-ventilated room (25 ± 2 °C) and maintained on a 12:12 h light/dark cycle at the animal care facility of SBSC of TBRI. Animals were fed on standard pellet food and water under strict hygienic conditions, and the bedding was changed every day. Animals were grouped as presented in Table 1.

Experimental infection

Mice were infected by 70 ± 10 Egyptian strain of *S. mansoni* cercariae by body immersion technique. They were separately divided (one in each jar) with a small amount of distilled water consisting of 70 ± 10 cercariae. They were left for 2 h and then transferred to cages (Abdel Menaem et al. 2022).

Experimental schedule

- Early-treated groups received treatment at 3 wpi and were sacrificed at 8 wpi.
- Late-treated groups received treatment at 6 wpi and were sacrificed at 8 wpi.
- The positive control group was sacrificed 8 wpi.

Evaluation parameters of the therapeutic potential of the study drugs

Estimation of TWB and calculation of its reduction percent

Recovery of worms was performed by perfusion of hepatic and porto-mesenteric vessels according to Duvall and DsWitt (1967); Ruppel et al. (1990); Smithers and Terry (1965). The percentage of reduction of TWB in all infected

Table 1 Animal grouping and drug doses

Animal groups (5 mice/each)		Treatment	
		Drug	Dose
1. Early-treated group (infected and treated 3 wpi)	**1.a.** Infected and treated with PZQ (PZQ control group)	**PZQ**	200 mg/kg/day for 5 days
	1.b. Infected and treated with amiodarone	**Amiodarone**	100 mg/kg/day for 5 days
	1.c. Infected and treated with spironolactone	**Spironolactone**	100 mg/kg/day for 5 days
	1.d. Infected and treated with combined PZQ/amiodarone	**PZQ and amiodarone**	100 mg/kg/day for 5 days for pzq 50 mg/kg/day for 5 days for amiodarone
	1.e. Infected and treated with combined PZQ/spironolactone	**PZQ and spironolactone**	100 mg/kg/day for 5 days for pzq 50 mg/kg/day for 5 days for spironolactone
	1.f. Infected and treated with combined amiodarone/spironolactone	**Amiodarone** and **spironolactone**	50 mg/kg/day of both for 5 days
2. Late-treated group (infected and treated 6 wpi)	**2.a.** Infected and treated with PZQ	**PZQ**	200 mg/kg/day for 5 days
	2.b. Infected and treated with amiodarone	**Amiodarone**	100 mg/kg/day for 5 days
	2.c. Infected and treated with spironolactone	**Spironolactone**	100 mg/kg/day for 5 days
	2.d. Infected and treated with combined PZQ/amiodarone	**PZQ and amiodarone**	100 mg/kg/day for 5 days for pzq 50 mg/kg/day for 5 days for amiodarone
	2.e. Infected and treated with combined PZQ/spironolactone	**PZQ and spironolactone**	100 mg/kg/day for 5 days for pzq 50 mg/kg/day for 5 days for spironolactone
	2.f. Infected and treated with combined amiodarone/spironolactone	**Amiodarone** and **spironolactone**	50 mg/kg/day of both for 5 days
3. Infected control group (positive control)	**3.a.** Infected untreated for the early-treated group		
	3.b. Infected untreated for the late-treated group		

groups was calculated according to the following equation (Abdel-Ghaffar et al. 2017).

TWB reduction %:

$$\frac{\text{mean of worms from control group} - \text{mean of worms from treated group}}{\text{mean of worms from control group}} \times 100$$

Tissue egg load in the liver and intestine

Pieces of intestine and liver were taken for the estimation of tissue egg loads, then the number of ova/g tissue was calculated (Moloney et al. 1982). The percentage of ova count reduction in the intestine and liver in all infected groups was calculated according to the following equation (El-Ansary et al. 2007).

Ova count reduction:

$$\frac{\text{mean of ova count from control group} - \text{mean of ova count from treated group}}{\text{mean of ova count from control group}} \times 100$$

Table 2 The count of adult worms, reduction % of TWB compared to the control group, and multiple comparisons between different groups regarding total adult count in the early-treated group (3 wpi)

Groups	Male	Female	Copula	Total adults	Reduction % of total adults	Multiple comparisons by Tukey's post-hoc test	
Control (infected untreated)	1.60 ± 1.52	1.20 ± 0.30	9.40 ± 1.67	21.60 ± 2.07		PZQ	0.231
						Amiodarone	< 0.001**
						Spironolactone	< 0.001**
						PZQ + amiodarone	< 0.001**
						PZQ + spironolactone	< 0.001**
						Amiordarone + spironolactone	< 0.001**
PZQ	4.20 ± 1.64	2.40 ± 0.55	6.40 ± 1.14	19.40 ± 3.85	10.2%	Amiodarone	0.007*
						Spironolactone	< 0.001**
						PZQ + amiodarone	0.003*
						PZQ + spironolactone	< 0.001**
						Amiordarone + spironolactone	< 0.001**
Amiodarone	2.60 ± 1.52	0.40 ± 0.29	5.60 ± 1.52	14.20 ± 3.70	34.3%	Spironolactone	< 0.001**
						PZQ + amiodarone	0.741
						PZQ + spironolactone	< 0.001**
						Amiordarone + spironolactone	0.106
Spironolactone	2.00 ± 1.00	0.60 ± 0.34	2.00 ± 1.00	6.60 ± 2.30	69.4%	PZQ + amiodarone	< 0.001**
						PZQ + spironolactone	0.912
						Amiordarone + spironolactone	0.016*
PZQ + amiodarone	1.40 ± 1.14	0.20 ± 0.15	6.00 ± 1.22	13.60 ± 2.51	37.0%	PZQ + spironolactone	< 0.001**
						Amiordarone + spironolactone	0.192
PZQ + spironolactone	2.20 ± 1.64	1.40 ± 0.55	1.40 ± 0.89	6.40 ± 3.13	70.4%	Amiordarone + spironolactone	0.012*
Amiodarone + spironolactone	5.20 ± 1.30	1.60 ± 0.55	2.20 ± 0.45	11.20 ± 1.48	48.1%		
F-value	5.057	3.877	30.586	21.090			
p-value	< 0.001**	0.006*	< 0.001**	< 0.001**			

Data are expressed as mean ± SD using *F*-one-way analysis of variance

p-value > 0.05 is insignificant; **p*-value < 0.05 is significant; ***p*-value < 0.001 is highly significant

Oogram pattern

The percentage of eggs at various developmental stages (mature, immature, and dead) was calculated, and the mean number of eggs at each stage/animal was determined (Pellegrino et al. 1962; Pellegrino and Faria 1965).

Histopathological examination of liver tissue

Liver sections from each group were examined for histopathological changes as distortion of hepatic architecture and presence of egg or worm granulomas as well as any associated inflammatory and fibrotic changes. The number, size, and type (cellular, fibrocellular, fibrous) of granulomas were calculated (Jacobs et al. 1997).

Statistical analysis of data

Recorded data were analyzed using the statistical package for social sciences, version 23.0 (SPSS Inc., Chicago, IL, USA). The quantitative data were presented as mean ± standard deviation (SD). Qualitative variables were presented as numbers and percentages. The following tests were done:

- A one-way analysis of variance (ANOVA) when comparing more than two means
- Post-hoc test: Tukey's test was used for multiple comparisons between different variables.
- Probability (p-value) ≤ 0.05 (significant), ≤ 0.001 (highly significant), and > 0.05 (insignificant)

Table 3 The count of ova/g in the liver, reduction % compared to control group, and multiple comparisons between different groups in the early-treated group (3 wpi)

Groups	Ova counts in the liver		Multiple comparisons by Tukey's post-hoc test	
	Mean ± SD	Reduction %		
Control (infected untreated)	15,136.21 ± 3629.74		PZQ	0.076
			Amiodarone	0.136
			Spironolactone	< 0.001**
			PZQ + amiodarone	0.004*
			PZQ + spironolactone	< 0.001**
			Amiordarone + spironolactone	< 0.001**
PZQ	12,469.40 ± 3187.05	17.6%	Amiodarone	0.395
			Spironolactone	0.003*
			PZQ + amiodarone	0.025*
			PZQ + spironolactone	0.007*
			Amiordarone + spironolactone	0.009*
Amiodarone	12,919.40 ± 1072.89	14.6%	Spironolactone	0.027*
			PZQ + amiodarone	0.144
			PZQ + spironolactone	0.051
			Amiordarone + spironolactone	0.063
Spironolactone	9288.80 ± 899.36	38.6%	PZQ + amiodarone	0.412
			PZQ + spironolactone	0.770
			Amiordarone + spironolactone	0.692
PZQ + amiodarone	10,583.00 ± 2006.51	30.1%	PZQ + spironolactone	0.596
			Amiordarone + spironolactone	0.669
PZQ + spironolactone	9748.20 ± 1990.82	35.6%	Amiordarone + spironolactone	0.917
Amiodarone + spironolactone	9911.60 ± 1803.98	34.5%		
F-value	3.660			
p-value	0.008*			

Expressed using F-one-way analysis of variance

p-value > 0.05 is insignificant; *p-value < 0.05 is significant; **p-value < 0.001 is highly significant

Table 4 The count of ova/g intestine, reduction % compared to control group, and multiple comparisons between different groups in the early-treated group (3 wpi)

Groups	Ova counts in intestine		Multiple comparisons by Tukey's post-hoc test	
	Mean ± SD	Reduction %		
Control (infected untreated)	20,194.20 ± 5132.60		PZQ	0.077
			Amiodarone	0.060
			Spironolactone	< 0.001**
			PZQ + amiodarone	0.002*
			PZQ + spironolactone	< 0.001**
			Amiordarone + spironolactone	< 0.001**
PZQ	16,833.80 ± 2664.49	16.6%	Amiodarone	0.522
			Spironolactone	< 0.001**
			PZQ + amiodarone	0.007*
			PZQ + spironolactone	< 0.001**
			Amiordarone + spironolactone	< 0.001**
Amiodarone	16,611.20 ± 2354.54	17.7%	Spironolactone	< 0.001**
			PZQ + amiodarone	0.031*
			PZQ + spironolactone	< 0.001**
			Amiordarone + spironolactone	0.002*
Spironolactone	10,415.60 ± 1711.54	48.4%	PZQ + amiodarone	0.078
			PZQ + spironolactone	0.809
			Amiordarone + spironolactone	0.467
PZQ + amiodarone	13,767.20 ± 2102.08	31.8%	PZQ + spironolactone	0.124
			Amiordarone + spironolactone	0.285
PZQ + spironolactone	10,862.40 ± 1723.50	46.2%	Amiordarone + spironolactone	0.625
Amiodarone + spironolactone	11,768.00 ± 3055.92	41.7%		
F-value	10.187			
p-value	< 0.001**			

Expressed using *F*-one-way analysis of variance

p-value > 0.05 is insignificant; **p*-value < 0.05 is significant; ***p*-value < 0.001 is highly significant

Table 5 Oogram pattern of the early-treated group (3 wpi)

Groups	Stages of ova		
	Immature	Mature	Dead
	Mean ± SD	Mean ± SD	Mean ± SD
Control (infected untreated)	57.40 ± 8.71	36.20 ± 8.04	6.40 ± 1.34
PZQ	50.00 ± 0.71	41.20 ± 1.79	8.80 ± 1.30
Amiodarone	52.80 ± 9.18	39.20 ± 7.85	8.00 ± 2.12
Spironolactone	32.00 ± 7.58	30.00 ± 11.73	38.00 ± 17.89
PZQ + amiodarone	50.00 ± 12.25	38.40 ± 7.92	11.60 ± 4.77
PZQ + spironolactone	35.00 ± 12.25	48.00 ± 4.47	17.00 ± 9.75
Amiodarone + spironolactone	38.00 ± 4.47	40.00 ± 7.91	22.00 ± 11.51
F-value	6.317	2.491	7.549
p-value	< 0.001**	0.047*	< 0.001**

Expressed using *F*-one way analysis of variance

p-value > 0.05 is insignificant; **p*-value < 0.05 is significant; ***p*-value < 0.001 is highly significant

Table 6 The mean number, size, and type of liver granulomas, and the percent of reduction compared to control in the early-treated group (3 wpi)

Groups	Pathology of liver granuloma								
	Number			Size			Type		
	Mean ± SD	Reduction %	*p*-value	Mean ± SD	Reduction %	*p*-value	Cellular (%)	Fibrocellular (%)	Fibrous (%)
Control (infected untreated)	23.20 ± 2.59			359.00 ± 132.68			40	60	0
PZQ	19.40 ± 3.36	16.4%	0.020*	296.00 ± 73.82	17.5%	0.065	30	70	0
Amiodarone	15.00 ± 2.12	35.3%	< 0.001**	351.50 ± 68.80	2.1%	0.824	25	75	0
Spironolactone	11.80 ± 2.05	49.1%	< 0.001**	229.00 ± 54.25	36.2%	< 0.001**	20	70	10
PZQ + amiodarone	13.20 ± 1.92	43.1%	< 0.001**	353.33 ± 76.22	1.6%	0.854	20	75	5
PZQ + spironolactone	13.60 ± 2.88	41.4%	< 0.001**	298.50 ± 33.67	16.9%	0.076	35	65	0
Amiodarone + spironolactone	16.20 ± 1.64	30.2%	< 0.001**	354.55 ± 45.47	1.2%	0.892	20	80	0
F-value	13.478			4.362			x^2, 19.211	x^2, 201.72	x^2, 5.002
p-value	< 0.001**			< 0.001**			< 0.001**	< 0.001**	0.025*

Expressed using *F*-one-way analysis of variance

p-value > 0.05 is insignificant; **p*-value < 0.05 is significant; ***p*-value < 0.001 is highly significant

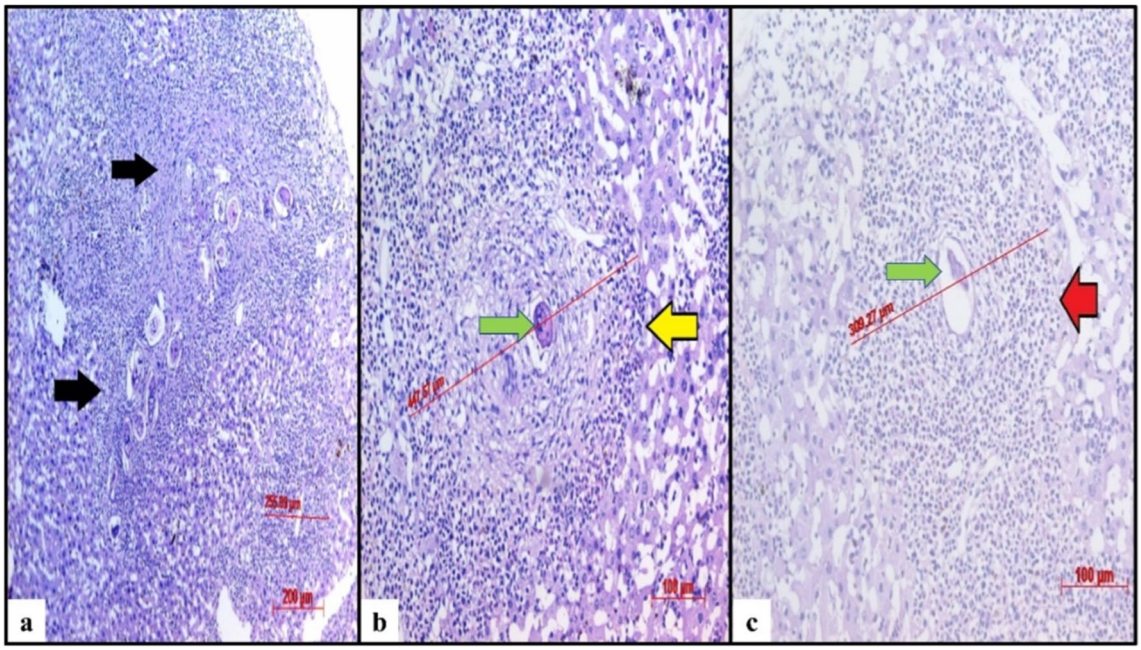

Fig. 1 Hepatic sections from the infected untreated control group (3 wpi). Black arrows indicate amalgamated granuloma. The yellow arrow indicates fibrocellular granuloma. The red arrow indicates a large cellular granuloma. Green arrows indicate central ova. (H&E stain **a** and **b** 100 × and **c** 200 ×; scale bar, 100 µm)

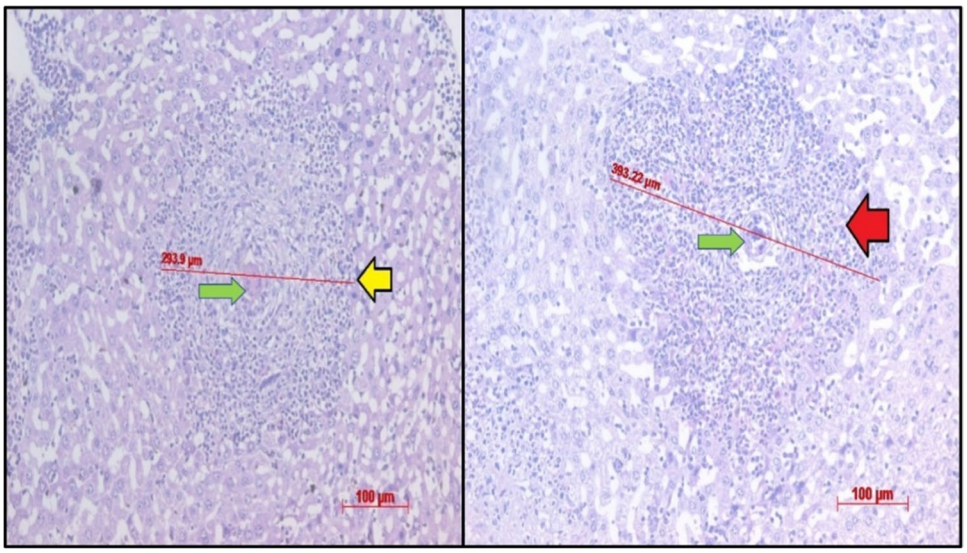

Fig. 2 Hepatic sections from PZQ early-treated group (3 wpi). The yellow arrow indicates fibrocellular granuloma. The red arrow indicates cellular granuloma. Green arrows indicate central ova. (H&E stain 200×; scale bar, 100 µm)

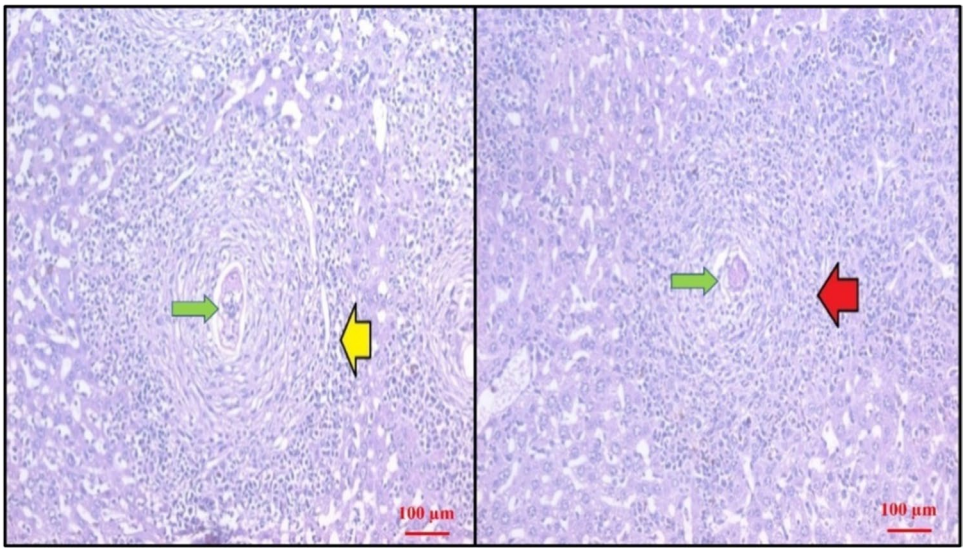

Fig. 3 Hepatic sections from the amiodarone early-treated group (3 wpi). The yellow arrow indicates fibrocellular granuloma. The red arrow indicates cellular granuloma. Green arrows indicate central ova. (H&E stain 200×; scale bar, 100 µm)

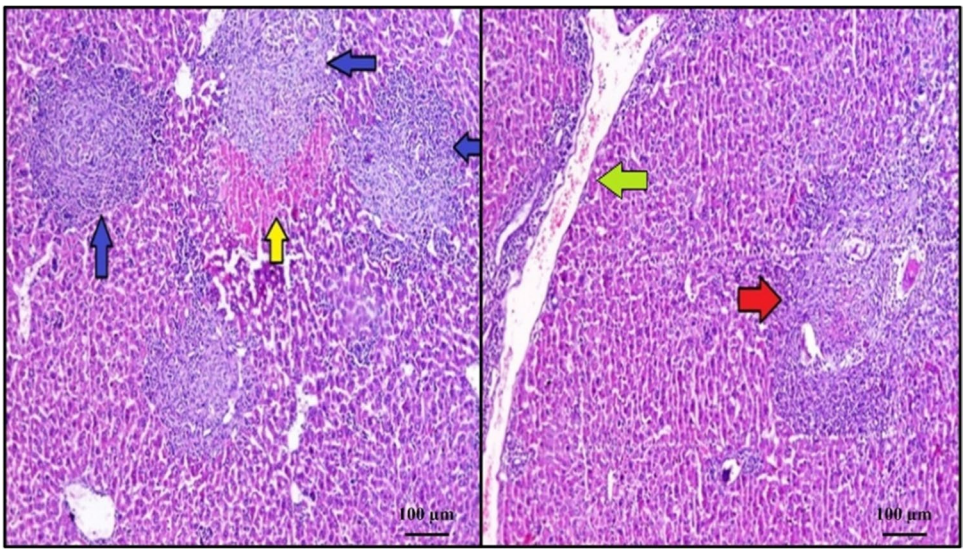

Fig. 4 Hepatic sections from the spironolactone early-treated group (3 wpi). Distorted hepatic architecture was revealed with many fibrocellular granulomas (blue arrows), focal hepatocellular necrosis (yellow arrow), portal egg granuloma (red arrow), and dilated congested blood sinusoids (green arrow). (H&E stain 100×; scale bar, 100 µm)

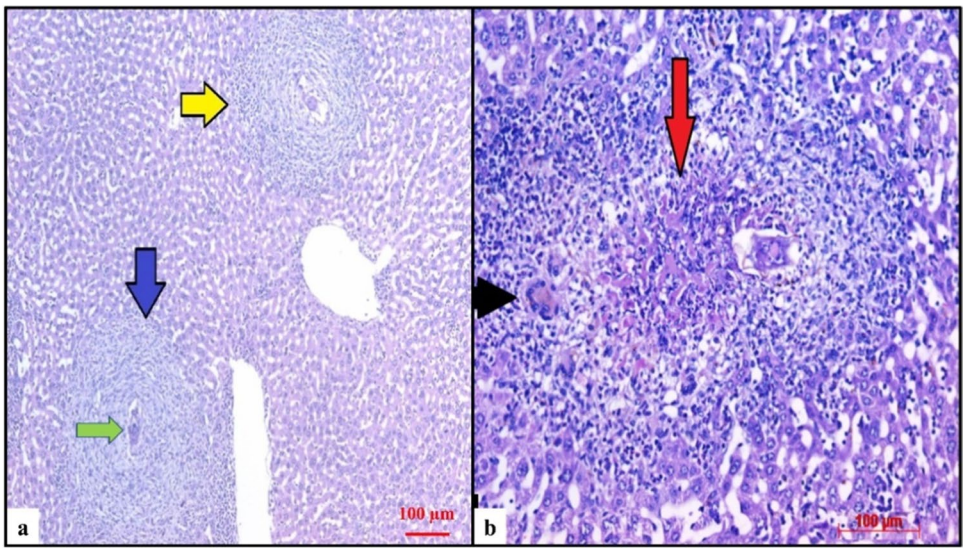

Fig. 5 Hepatic sections showing from PZQ and amiodarone early-treated group (3 wpi). Distorted hepatic architecture was revealed with many fibrocellular granulomas (yellow arrow), fibrous granulomas (blue arrow) with central ova (green arrow), and areas of central necrosis (red arrow) and giant cell reaction (black arrow). (H&E stain **a** 100× and **b** 200×; scale bar, 100 µm)

Results

The early-treated groups (3wpi)

TWB

The number of total adults recovered from the hepatic and porto-mesenteric vessel perfusion in all treated groups was reduced compared to the control group. Comparing TWB reduction percentages among the study groups revealed that spironolactone, singly and combined with PZQ, gave the highest percent of reduction (69.4% and 70.4%, respectively), while PZQ produced only 10.2% (Table 2).

Tissue egg load (Ova count/g in the liver and intestine)

Ova counts/g in the liver and intestinal tissue were demonstrated in Tables 3 and 4.

Oogram pattern

The mean count of different developmental stages of *S. mansoni* eggs is presented in Table 5. As shown in the table, spironolactone showed the best outcome with the highest number of dead ova and the lowest numbers of mature and immature ova.

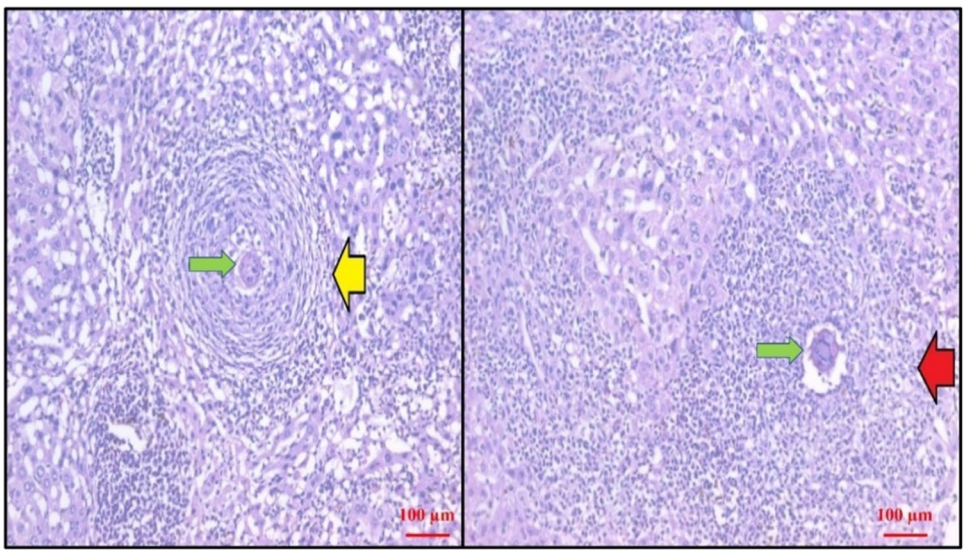

Fig. 6 Hepatic sections from PZQ and spironolactone early-treated groups (3 wpi). Distorted hepatic architecture was revealed with small fibrocellular granulomas (yellow arrow) and cellular granulomas (red arrow) with central ova (green arrows). (H&E stain 200×; scale bar, 100 µm)

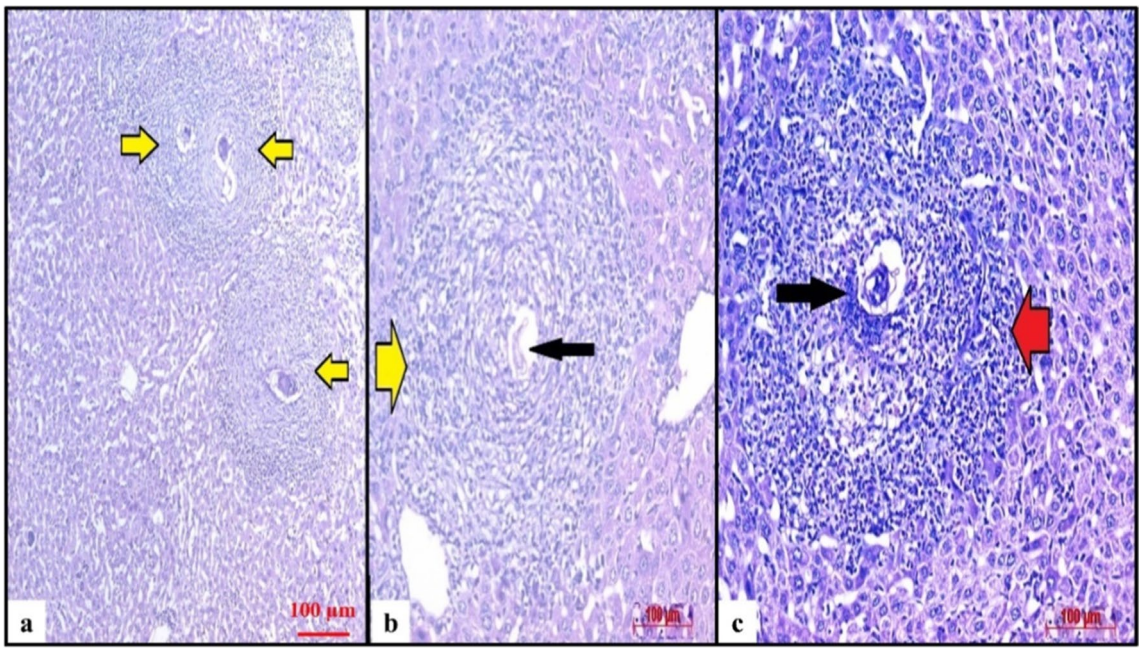

Fig. 7 Hepatic sections from amiodarone and spironolactone early-treated groups (3 wpi). Distorted hepatic architecture was revealed with multiple fibrocellular granulomas (yellow arrows) and cellular granulomas (red arrows) with central degenerated ova (black arrow). (H&E stain **a** 100× and **b** and **c** 200×; scale bar, 100 µm)

Histopathological examination of liver tissue

The pathological features of liver granulomas regarding the number, size, and type are summarized in Table 6. As shown in the table, spironolactone exhibited the highest percentage of reduction of granulomas' number and size (49.1% and 36.2%, respectively) with predominant fibrocellular type (70%).

Sections of liver tissue from early-treated (3 wpi) study groups are depicted in Figs. 1, 2, 3, 4, 5, 6 and 7. Liver sections from the infected untreated control group showed many amalgamated granulomas, large cellular and fibrocellular ones (Fig. 1). Liver tissue from PZQ-treated (Fig. 2) and amiodarone-treated (Fig. 3) groups exhibited cellular and fibrocellular granulomas with central ova. Liver sections from the spironolactone-treated group demonstrated distorted hepatic architecture with many fibrocellular granulomas, focal hepatocellular necrosis, portal egg granuloma, and dilated congested blood sinusoids (Fig. 4). On the other hand, sections from PZQ combined with the amiodarone-treated group showed many fibrous granulomas and fibrocellular ones with central ova as well area of central necrosis and giant cell reaction (Fig. 5). Sections from PZQ combined with the spironolactone-treated group showed smaller and fewer cellular and fibrocellular granulomas with central ova (Fig. 6). Sections from combined amiodarone and the spironolactone-treated group showed multiple cellular and fibrocellular granulomas with central degenerated ova (Fig. 7).

The late-treated groups (6 wpi)

TWB

The TWB in all treated groups was reduced compared to the control group. Comparing the TWB reduction percentages among the study groups revealed that PZQ combined with amiodarone and with spironolactone groups gave the highest percent of reduction (96.1% and 95.1%, respectively), followed by PZQ-treated group (94.1%). In addition, it was noted that combinations of PZQ with amiodarone and with spironolactone caused the total disappearance of female and copula in the liver and intestine, and PZQ alone caused the disappearance of copula in the liver and intestine (Table 7).

Tissue egg load (Ova count/g in the liver and intestine)

Ova counts/g in the liver and intestinal tissue are demonstrated in Tables 8 and 9.

Oogram pattern

The mean count of different developmental stages of *S. mansoni* eggs is presented in Table 10. As shown in the table, PZQ alone and combined with amiodarone had the

Table 7 The count of adult worms, reduction % of TWB compared to control group, and multiple comparisons between different groups regarding total adult count in the late-treated group (6 wpi)

Groups	Male	Female	Copula	Total adults	Reduction % of total adults	Multiple comparisons by Tukey's post-hoc test	
Control (infected untreated)	1.60 ± 0.44	0.80 ± 0.24	9.00 ± 1.00	20.40 ± 1.14		PZQ	< 0.001**
						Amiodarone	< 0.001**
						Spironolactone	< 0.001**
						PZQ + amiodarone	< 0.001**
						PZQ + spironolactone	< 0.001**
						Amiordarone + spironolactone	< 0.001**
PZQ	0.60 ± 0.89	0.80 ± 0.24	0.00 ± 0.00	1.20 ± 0.44	94.1%	Amiodarone	< 0.001**
						Spironolactone	< 0.001**
						PZQ + amiodarone	0.770
						PZQ + spironolactone	0.890
						Amiordarone + spironolactone	< 0.001**
Amiodarone	1.20 ± 0.44	0.20 ± 0.10	2.40 ± 1.67	6.20 ± 3.35	69.6%	Spironolactone	< 0.001**
						PZQ + amiodarone	< 0.001**
						PZQ + spironolactone	< 0.001**
						Amiordarone + spironolactone	0.890
Spironolactone	5.00 ± 1.58	0.60 ± 0.29	2.60 ± 1.52	10.80 ± 3.03	47.1%	PZQ + amiodarone	< 0.001**
						PZQ + spironolactone	< 0.001**
						Amiordarone + spironolactone	< 0.001**
PZQ + amiodarone	0.80 ± 0.24	0.00 ± 0.00	0.00 ± 0.00	0.80 ± 0.24	96.1%	PZQ + spironolactone	0.890
						Amiordarone + spironolactone	< 0.001**
PZQ + spironolactone	1.00 ± 0.41	0.00 ± 0.00	0.00 ± 0.00	1.00 ± 0.41	95.1%	Amiordarone + spironolactone	< 0.001**
Amiodarone + spironolactone	1.00 ± 0.00	0.60 ± 0.25	2.40 ± 1.52	6.40 ± 2.88	68.6%		
F-value	10.267	1.630	42.262	52.812			
p-value	< 0.001**	0.176	< 0.001**	< 0.001**			

Data are expressed as mean ± SD using *F*-one-way analysis of variance

p-value > 0.05 is insignificant; **p*-value < 0.05 is significant; ***p*-value < 0.001 is highly significant

best outcome with the highest number of dead ova and the lowest numbers of mature and immature ova.

Histopathological examination of liver tissue

The pathological features of liver granulomas regarding the number, size, and type are summarized in Table 11. As shown in the table, PZQ combined with amiodarone exhibited the highest percentage reduction of granulomas' number and size (67.6% and 40.6%, respectively) with predominant fibrocellular type (80%).

Sections of liver tissue from late-treated (6 wpi) study groups are depicted in Figs. 8, 9, 10, 11, 12, 13 and 14. Liver sections from the infected untreated control group showed many amalgamated large-sized egg granulomas mostly of the fibrocellular type with central intact ova and areas of liver cell necrosis (Fig. 8). Liver tissue from the PZQ-treated group showed mostly preserved hepatic architecture with remnants of fibrocellular reaction and focal intra-portal dead worm granuloma (Fig. 9). Sections from the amiodarone-treated group revealed many variable sized egg-granulomas mostly of the fibrocellular and cellular type with central intact ova (Fig. 10). Sections from the spironolactone-treated group demonstrated markedly distorted hepatic architecture with large number of fibrocellular granulomas (Fig. 11). On the other hand, sections from the combined PZQ and amiodarone-treated group showed fewer smaller-sized egg granulomas mostly of the fibrocellular type and few cellular ones with central mostly degenerated ova (Fig. 12). Sections from the combined PZQ and spironolactone-treated group showed mostly preserved hepatic architecture with fewer fibrocellular granulomas with central degenerated ova (Fig. 13). Sections from the combined amiodarone and spironolactone-treated group showed distorted hepatic architecture with many fibrocellular granulomas with central intact ova and degenerated ones (Fig. 14).

Table 8 The count of ova/g in the liver, reduction % compared to control group, and multiple comparisons between different groups in the late-treated group (6 wpi)

Groups	Ova counts in the liver		Multiple comparisons by Tukey's post-hoc test	
	Mean ± SD	Reduction %		
Control (infected untreated)	11,418.92 ± 1366.41		PZQ	< 0.001**
			Amiodarone	< 0.001**
			Spironolactone	< 0.001**
			PZQ + amiodarone	< 0.001**
			PZQ + spironolactone	< 0.001**
			Amiordarone + spironolactone	< 0.001**
PZQ	4230.40 ± 1424.05	63%	Amiodarone	< 0.001**
			Spironolactone	< 0.001**
			PZQ + amiodarone	0.160
			PZQ + spironolactone	0.040*
			Amiordarone + spironolactone	< 0.001**
Amiodarone	7082.00 ± 852.74	38%	Spironolactone	0.310
			PZQ + amiodarone	0.040*
			PZQ + spironolactone	0.140
			Amiordarone + spironolactone	0.760
Spironolactone	7896.80 ± 435.14	30.8%	PZQ + amiodarone	< 0.001**
			PZQ + spironolactone	0.020*
			Amiordarone + spironolactone	0.190
PZQ + amiodarone	5358.60 ± 1973.31	53.1%	PZQ + spironolactone	0.490
			Amiordarone + spironolactone	0.070
PZQ + spironolactone	5908.60 ± 810.04	48.3%	Amiordarone + spironolactone	0.240
Amiodarone + spironolactone	6839.80 ± 1152.31	40.1%		
F-value	17.413			
p-value	< 0.001**			

Expressed using *F*-one-way analysis of variance

p-value > 0.05 is insignificant; **p*-value < 0.05 is significant; ***p*-value < 0.001 is highly significant

Discussion

The WHO calls for the exploration of new drugs active against all stages of schistosomes alternative to PZQ to eliminate schistosomiasis (Basha and Mamo 2021). Drug repurposing has been a promising tactic as it saves time, cost, and risks compared to the de novo drug development process (Abd El Hady et al. 2023).

To the best of our knowledge, this is the first Egyptian study investigating the in vivo therapeutic efficacy of amiodarone and spironolactone, singly and combined, in *S. mansoni*–infected mice compared to PZQ in the early (3 wpi) and late (6 wpi) phases of infection. The currently investigated drugs were previously reported to have antiparasitic effects. Amiodarone was proven to be effective against *Trypanosoma cruzi* and *Leishmania mexicana* (Benaim et al. 2021; Dziduch et al. 2022; Pinto-Martinez et al. 2018), *Plasmodium falciparum* and *P. berghei* (Bobbala et al. 2010; Boulet et al. 2021), and *Acanthamoeba castellanii* (Baig 2020). Furthermore, amiodarone exhibited in vitro and in vivo antischistosomal activity. Amiodarone may exert its effect on *S. mansoni* by blocking potassium, sodium, and calcium ion channels of the schistosome's neuromuscular system (Porto et al. 2021). The drug was assumed to be *S. mansoni* respiratory chain inhibitor because it depleted intracellular ATP levels by acting on the mitochondrial membrane potential or generating ROS leading to a reduction in oxygen consumption rate (Talaam et al. 2021). Regarding spironolactone, it showed potency against *Leishmania amazonensis* and *L. infantum* (Andrade-Neto et al. 2021). Additionally, it had in vitro and in vivo antischistosomal activity. Spironolactone is a potassium-sparing diuretic, an action that may affect the neuromuscular system of adult *S. mansoni* leading to decreased motility, induced contractions, and tegumental sloughing (Abd El Hady et al. 2023; Aminou and Abdel Rahman 2020; Guerra et al. 2019). Given that adrenal hormones influence the survival and oviposition of schistosomes, the anti-schistosomal action of spironolactone could be linked to inhibition of aldosterone hormone production because it is a competitive aldosterone receptor antagonist (Abd El Hady et al. 2023).

Table 9 The count of ova/g intestine, reduction % compared to control group, and multiple comparisons between different groups in the late-treated group (6 wpi)

Groups	Ova counts in the intestine		Multiple comparisons by Tukey's post-hoc test	
	Mean ± SD	Reduction %		
Control (infected untreated)	17,853.06 ± 2579.29		PZQ	< 0.001**
			Amiodarone	< 0.001**
			Spironolactone	< 0.001**
			PZQ + amiodarone	< 0.001**
			PZQ + spironolactone	< 0.001**
			Amiordarone + spironolactone	< 0.001**
PZQ	3868.40 ± 1113.94	78.3%	Amiodarone	0.020*
			Spironolactone	< 0.001**
			PZQ + amiodarone	0.190
			PZQ + spironolactone	0.680
			Amiordarone + spironolactone	0.040*
Amiodarone	6034.00 ± 1000.65	66.2%	Spironolactone	0.340
			PZQ + amiodarone	< 0.001**
			PZQ + spironolactone	0.049*
			Amiordarone + spironolactone	0.810
Spironolactone	6893.00 ± 915.32	61.4%	PZQ + amiodarone	< 0.001**
			PZQ + spironolactone	0.010*
			Amiordarone + spironolactone	0.240
PZQ + amiodarone	2695.20 ± 993.42	84.9%	PZQ + spironolactone	0.090
			Amiordarone + spironolactone	< 0.001**
PZQ + spironolactone	4237.60 ± 939.40	76.3%	Amiordarone + spironolactone	0.080
Amiodarone + spironolactone	5824.00 ± 1434.23	67.4%		
F-value	66.504			
p-value	< 0.001**			

Expressed using *F*-one-way analysis of variance

p-value > 0.05 is insignificant; **p*-value < 0.05 is significant; ***p*-value < 0.001 is highly significant

Table 10 Oogram pattern of the late-treated groups (6 wpi)

Groups	Stages of ova		
	Immature	Mature	Dead
	Mean ± SD	Mean ± SD	Mean ± SD
Control (infected untreated)	59.40 ± 5.86	34.60 ± 5.55	6.00 ± 1.41
PZQ	0.00 ± 0.00	1.40 ± 2.19	98.60 ± 2.19
Amiodarone	26.00 ± 15.17	50.00 ± 10.00	24.00 ± 10.25
Spironolactone	24.00 ± 15.35	47.00 ± 23.35	29.00 ± 9.45
PZQ + amiodarone	0.00 ± 0.00	18.00 ± 10.90	82.00 ± 24.90
PZQ + spironolactone	8.00 ± 4.89	24.60 ± 4.56	67.40 ± 15.77
Amiodarone + spironolactone	31.00 ± 18.84	44.00 ± 11.40	25.00 ± 8.66
F-value	9.807	7.572	22.205
p-value	< 0.001**	< 0.001**	< 0.001**

Expressed using *F*-one-way analysis of variance

p-value > 0.05 is insignificant; **p*-value < 0.05 is significant; ***p*-value < 0.001 is highly significant

Remarkably, the findings of the present study revealed a more potent activity of spironolactone than PZQ against the juvenile stage of the parasite in the early phase of infection (3 wpi) where spironolactone significantly reduced TWB by 69.4%, while PZQ showed only 10% reduction. The TWB reduction percentage increased to 70.4% upon combining spironolactone with PZQ. In contrast, amiodarone gave only 34% and 37% reduction of TWB singly and combined with PZQ, respectively. Generally, all the tested drugs had a better overall impact on females than males and copula. Since females' eradication will stop oviposition, disease progression, and infection continuation, targeting an impact on females is a hope of the drug's potency. The contemporary results of TWB reduction were in accordance with Guerra et al. (2019) who reported a moderate reduction of TWB by 47.4% with a significant impact on female worms in the spironolactone-treated group, while PZQ produced only a 25–30% reduction. Likewise, Porto et al. (2021) illustrated the low insignificant reduction of TWB by 25% in the PZQ-treated group. Yet, the authors antagonized the herein study

Table 11 The mean number, size, and type of liver granulomas, and the percent of reduction compared to control in the late-treated group (6-wpi)

Groups	Pathology of liver granuloma								
	Number			Size			Type		
	Mean ± SD	Reduction %	p-value	Mean ± SD	Reduction %	p-value	Cellular (%)	Fibrocellular (%)	Fibrous (%)
Control (Infected-untreated)	29.60 ± 1.52	–		422.00 ± 60.17	–		40.00	60.00	0.00
PZQ	12.00 ± 2.65	59.5%	< 0.001**	285.00 ± 47.43	32.5%	0.020*	5.00	95.00	0.00
Amiodarone	23.20 ± 2.59	21.6%	< 0.001**	325.00 ± 153.26	23.0%	0.049*	30.00	70.00	0.00
Spironolactone	18.40 ± 2.70	37.8%	< 0.001**	314.55 ± 91.14	25.5%	0.030*	20.00	80.00	0.00
PZQ + Amiodarone	9.60 ± 1.14	67.6%	< 0.001**	250.67 ± 79.41	40.6%	< 0.001**	10.00	80.00	10.00
PZQ + Spironolactone	11.00 ± 2.24	62.8%	< 0.001**	269.33 ± 67.77	36.2%	< 0.001**	20.00	75.00	5.00
Amiodarone + Spironolactone	13.20 ± 2.86	55.4%	< 0.001**	308.67 ± 79.36	26.9%	0.020*	10.00	85.00	5.00
F-value	50.739			2.792			x^2: 32.593	x^2: 85.96	x^2: 4.982
p-value	< 0.001**			0.017*			< 0.001**	< 0.001**	0.037*

Using: F-One Way analysis of variance

*p-value > 0.05 is insignificant; *p-value < 0.05 is significant; **p-value < 0.001 is highly significant*

by claiming that amiodarone achieved a 52–60% reduction in TWB in the early-treated mice group. Still, we both agreed that amiodarone had a better effect than PZQ in the early phase of infection.

Upon assessing the ova count in the early-treated groups (3 wpi), spironolactone produced a statistically highly significant reduction of ova count/g intestinal tissue by 48.4%, 46.2%, and 41.7% (singly and combined with PZQ and with amiodarone, respectively). The oogram pattern revealed a substantial reduction in the mean number of immature and mature ova associated with an increased number of dead ova. That agreed with Guerra et al. (2019) who declared a highly significant reduction of intestinal ova count by 41.2%, particularly the immature ones, in spironolactone-treated mice. The reduction in ova count could be credited to the considerable reduction in TWB and/or inhibition of oviposition by adult females. The ova count reduction in the amiodarone-only-treated group was equivocal to that of the PZQ (17.7% versus 16.6%). From our point of view, this low activity might be referred to the ineffectiveness of amiodarone on the juvenile schistosomes in the early phase of infection like PZQ.

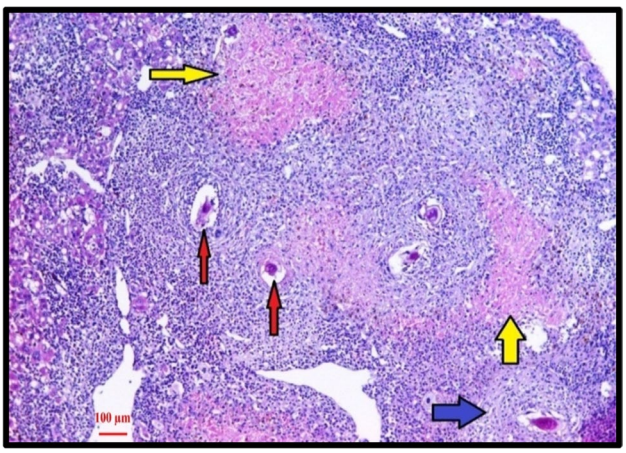

Fig. 8 Hepatic section from the infected untreated control group (6 wpi). Amalgamated large-sized egg granulomas mostly of the fibrocellular type (blue arrow) with central intact ova (red arrows) and areas of liver cell necrosis (yellow arrows). (H&E stain 100×; scale bar, 100 µm)

The present histopathological findings demonstrated that spironolactone substantially alleviated liver granulomatous lesions with a predominance of fibrocellular type (70%). These findings aligned with Guerra et al. (2019) who examined the weights of spleen and liver as an indication of the histopathological changes revealing that spironolactone had statistically significant reductions in weights of spleen and liver compared to infected untreated mice. The authors stated that the pharmacological properties of spironolactone as a diuretic used to treat edematous conditions added to the advantageous histopathological impact. Also, Abd El Hady et al. (2023) attributed the improvement in granulomatous lesions to the reduction of TWB and consequently

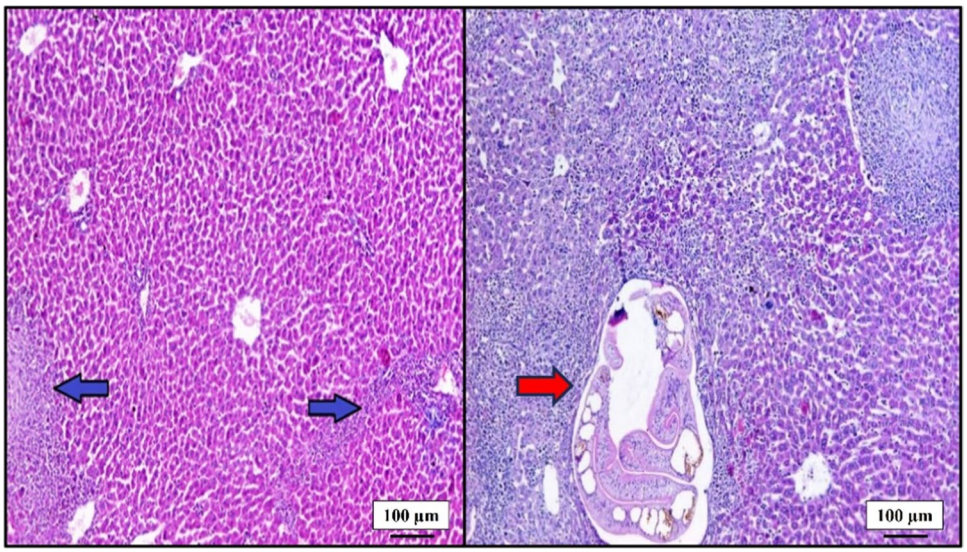

Fig. 9 Hepatic sections from the PZQ late-treated group (6 wpi). Mostly preserved hepatic architecture with remnants of fibrocellular granuloma (blue arrows) and intra-portal dead worm (red arrow). (H&E stain 100×, scale bar: 100 µm)

diminished oviposition and ova count that trigger granuloma formation.

Among the late-treated groups (6 wpi), PZQ combined with amiodarone and spironolactone significantly reduced the TWB by 96.1% and 95.1%, respectively, associated with the total disappearance of females and copula. PZQ alone showed a statistically significant reduction of TWB by 94.1% with the disappearance of the copula. Emphasizing the impact of the drug combination, these findings went along with Hegazy et al. (2018) who declared that PZQ combined with artesunate gave the highest percentage of reduction of TWB by 95.4% compared to control and the drugs monotherapy at 6 weeks post-treatment. Amiodarone alone caused a statistically significant moderate reduction of TWB by 69.6%, disagreeing with Porto et al. (2021) who claimed that amiodarone had an insignificant low TWB reduction by 18–23% in the late phase of infection. However, we both agreed that PZQ achieved highly significant TWB reductions exceeding amiodarone-alone treated groups.

Upon assessing the ova count/g intestinal tissue, PZQ combined with amiodarone produced the highest percentage of ova reduction (84.9%). Amiodarone alone caused a statistically significant moderate reduction of ova count by 66.2% consequent to TWB reduction, disagreeing with Porto et al. (2021) who claimed that amiodarone had insignificant low intestinal ova count of 16.81% in the late phase of infection. This slight

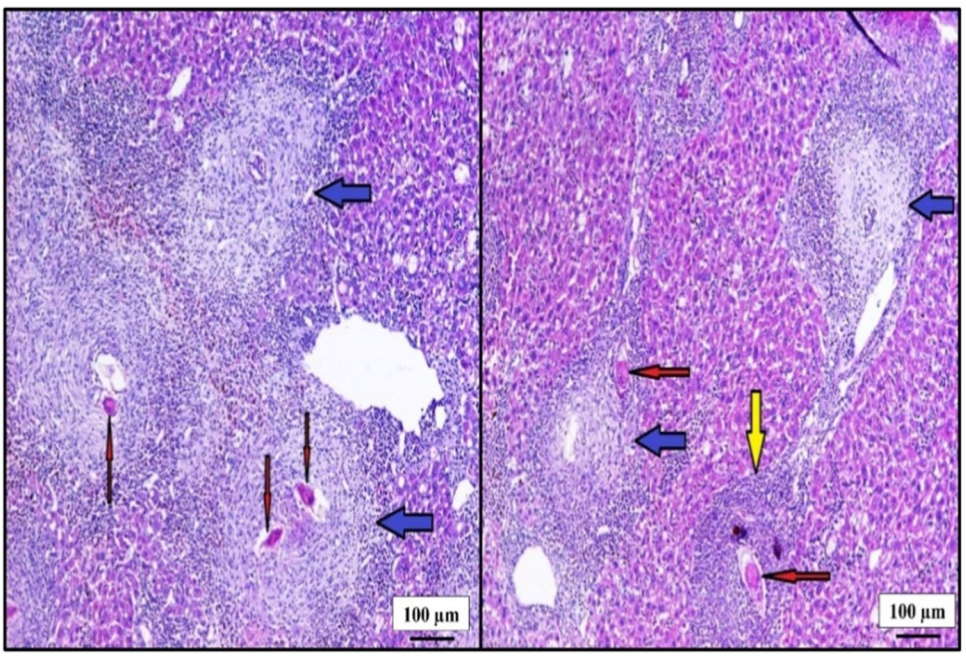

Fig. 10 Hepatic sections from the amiodarone late-treated group (6 wpi). Many variable-sized egg granulomas mostly of the fibrocellular (blue arrows) and cellular types (yellow arrow) with central intact ova (red arrows). (H&E stain 100×; scale bar, 100 µm)

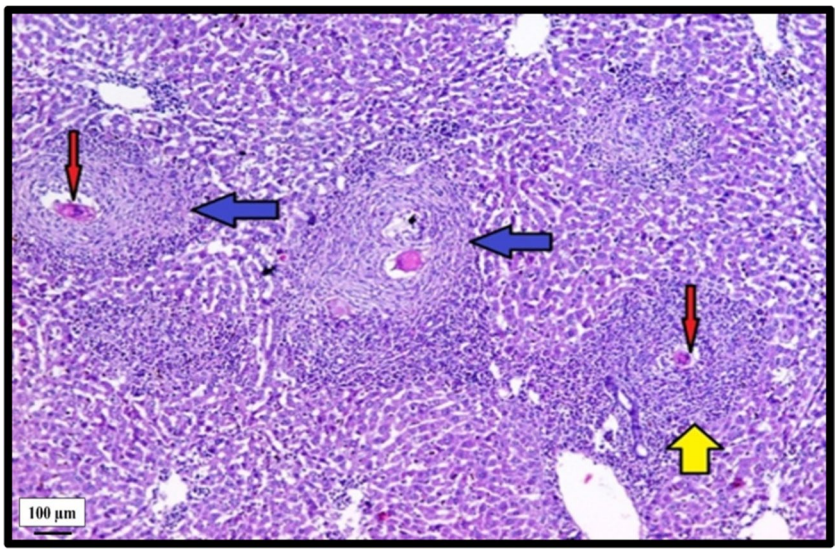

Fig. 11 Hepatic sections from the spironolactone late-treated group (6 wpi). Markedly distorted hepatic architecture with a large number of fibrocellular (blue arrows) and cellular granulomas (yellow arrow) with central intact ova (red arrows). (H&E stain 100×; scale bar, 100 µm)

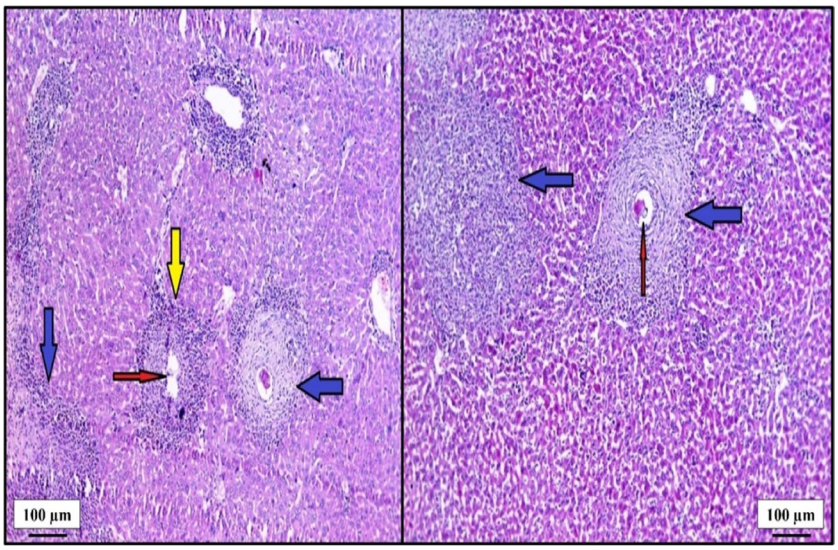

Fig. 12 Hepatic sections from the PZQ with amiodarone late-treated group (6 wpi). Fewer smaller-sized egg granulomas mostly of the fibrocellular (blue arrows) and few cellular granulomas (yellow arrow) with central mostly degenerated ova (red arrow). (H&E stain 100×; scale bar, 100 µm)

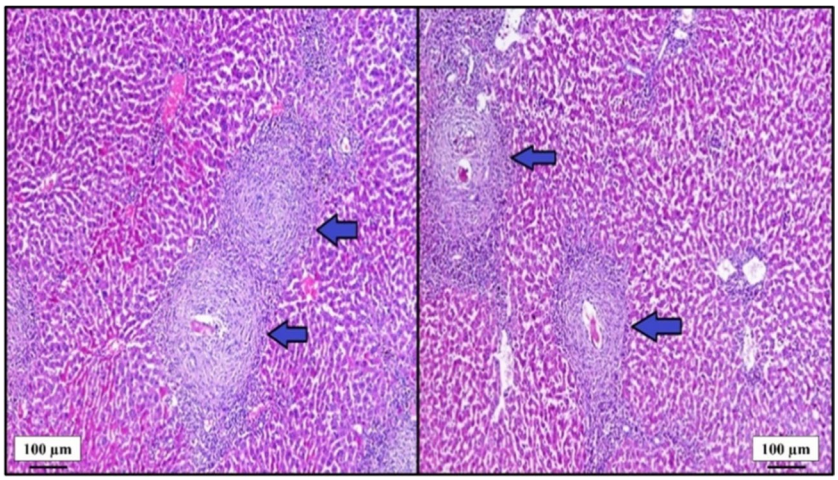

Fig. 13 Hepatic sections from the PZQ with spironolactone late-treated group (6 wpi). Mostly preserved hepatic architecture with fewer fibrocellular granulomas (blue arrows) with central degenerated ova. (H&E stain 100×; scale bar, 100 µm)

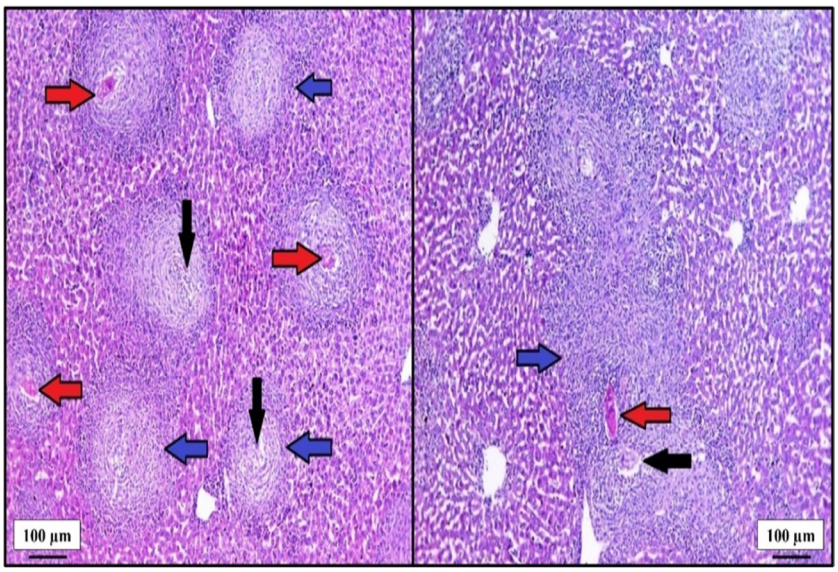

Fig. 14 Hepatic sections from the amiodarone with spironolactone late-treated group (6 wpi). Distorted hepatic architecture with many fibrocellular granulomas (blue arrows) with central intact ova (red arrows) and degenerated ones (black arrows). (H&E stain 100×; scale bar, 100 µm)

disagreement might be explained by using different strains of the parasite and laboratory mice. However, we both agreed that PZQ achieved significant ova count reductions exceeding amiodarone-alone treated groups. The lowest intestinal ova count reduction was obtained with spironolactone (61.4%) which was in accordance with Abd El Hady et al. (2023) as spironolactone caused a 48.81% reduction of intestinal ova count. Yet, the present findings disagreed with Guerra et al. (2019) who claimed that spironolactone had a more potent effect on late-treated groups than early-treated ones as it caused a 75.6% reduction of immature intestinal ova. This discrepancy might be justified by using different strains of the parasite and laboratory mice, or the different infecting dose.

The existing oogram pattern findings demonstrated that PZQ alone and combined with amiodarone caused the complete disappearance of immature ova, the lowest number of mature ova, and the highest number of dead ova. These results were concomitant with previous reports showing a complete absence of immature stages, decreased matures, and an increased number of dead ones upon treatment with PZQ combined with mefloquine (El-Lakkany et al. 2011), pentoxifylline (Ibrahim et al. 2019), and artesunate (Hegazy et al. 2018). Those data supported the assertion adopted by Pellegrino et al. (1962) that anti-schistosomal treatment was considered effective when the oogram pattern showed the disappearance of ≥ 50% of mature ova, or if there was a complete absence of immature stages and an increased number of dead ones.

The histopathological findings demonstrated that PZQ combined with amiodarone noticeably amended hepatic granulomas with a predominance of fibrocellular type (80%). Mahmoud et al. (2017) attributed the amelioration of the pathological lesions to progressive shrinkage of hepatic granulomas after using curable drugs due to the elimination of the adult worms and disappearance of females leading to diminished egg deposition and production of poorly developed ova unable to induce granuloma formation. In the same context, Yang et al. (2021) declared that the combination therapy not only attenuated the egg burden but also inhibited the formation of egg-induced granulomas.

For decades, PZQ has been the optimal drug for *Schistosoma* spp. even with a non-fully understood mechanism of action (Thomas and Timson 2020). Being effective, cheap, and easily administered as a single oral dose, PZQ is a cornerstone in the WHO roadmap to eliminate schistosomiasis as a public health problem by 2030 (Park et al. 2021). Nonetheless, its low efficacy against juvenile flukes, the inability to reverse tissue damage, and the possible existence of resistant strains are still questioned (Nogueira et al. 2022). The main event in the antischistosomal effect of PZQ is the dysregulation of calcium homeostasis as it antagonizes and disrupts voltage-gated calcium channels in adult schistosomes resulting in uncontrolled calcium ion influx followed by muscle contraction and spastic paralysis (Thomas and Timson 2020). Subsequently, the worms are shifted to the liver where they are finally destroyed by phagocytic cells (Abou-El-Naga 2020). In addition, PZQ causes tegumental damage that exposes the parasite surface antigens allowing the host immune system to recognize and eliminate the parasite, an action which explains the difference in drug sensitivity between juvenile and adult stages (Cupit and Cunningham 2015). Further, other than voltage-gated calcium channels, PZQ was shown to interact with other *Schistosoma* macromolecules, e.g., myosin regulatory light chains and transient receptor potential (TRP) channels (Bais and Greenberg 2018). The drug engages in a binding pocket within the TRP melastatin ion channel causing calcium entry and worm paralysis (Park et al. 2021). PZQ affects oviposition in adult worms (Nogueira et al. 2022). This justifies the maximum sensitivity of schistosomes to PZQ 6 weeks post-infection at the period of oviposition (Hegazy et al. 2018).

Conclusion

In this experimental paradigm, spironolactone exerted a significant anti-schistosomal effect on the immature stages of *S. mansoni* in the early infection phase of murine schistosomiasis. Meanwhile, a combination of amiodarone with PZQ surpassed the anti-schistosomal effect of PZQ alone against the adult stage of *S. mansoni* in the late infection phase of murine schistosomiasis. Both spironolactone and amiodarone showed better anti-schistosomal effects on immature stages of *S. mansoni* in the early phase of infection compared to PZQ making them potential candidates for drug repurposing in early diagnosed patients. Additionally, combined amiodarone and spironolactone with PZQ were more advantageous than PZQ alone in adult stages in the late phase of infection.

Author contributions Hussein H.M.: Conceptualization, supervision. Abdel-Sayed S.W. and Mohamed G.A.: Methodology, investigations, resources, formal analysis. Mohammad O.S. and Shehata M.A.: Writing-original draft, Writing-review &; editing.

Data availability No datasets were generated or analysed during the current study.

Declarations

Ethical consideration The study was approved by the Ethical Committee of Scientific Research, Faculty of Medicine, Ain Shams University under authorization number (FMASU MS 543/2022) with the regulations of the Egyptian Ministry of Higher Education and Helsinki declaration, 1964. The animal experiments were carried out according to the ILARC (Institute of Laboratory Animal Resources Commission) guidelines and principles for use of laboratory animals.

Consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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