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Original paper

Antiprotozoal effect of steroid hormone 20-hydroxyecdysone in giardiosis patients

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ABSTRACT. The World Health Organization reports that approximately 200 million people are infected with *Giardia* (*G.*) *lamblia* worldwide. Taking into account the emergence of resistance and the high toxicity of conventional drugs, research into new strategies to fight against *G. lamblia* is increasing. The aim of the study was to assess the antiprotozoal activity of 20-hydroxyecdysone in water sports athletes with giardiosis. A randomized, double-blinded, placebo-controlled clinical study was conducted. Seventy-six athletes with *G. lamblia* infection participated in the study and were divided into 20-hydroxyecdysone, metronidazole and placebo groups. Clinical, parasitological, haematological and biochemical analyses were performed. Positive results for antiprotozoal therapy were revealed in the 20-hydroxyecdysone and metronidazole groups. After therapy, elimination of *G. lamblia* was observed in 100.0% of the athletes included in the 20-hydroxyecdysone group. However, *G. lamblia* was resistant to metronidazole in 4.0% of athletes included in the metronidazole group. A positive clinical response to the therapy occurred in the 20-hydroxyecdysone and metronidazole groups. Our study reveals high antiprotozoal activity of 20-hydroxyecdysone against *G. lamblia*. Further clinical studies are necessary to evaluate the antiprotozoal efficacy of 20-hydroxyecdysone.

Keywords: 20-hydroxyecdysone, ecdysteroids, antiprotozoal therapy, giardiosis

Introduction

20-hydroxyecdysone, an ecdysteroid hormone found in invertebrates and plants [1], exhibits a broad range of biological properties in various *in vitro* and *in vivo* models, including anabolic [2,3], antioxidant [4], anti-inflammatory [5], immunomodulatory, anti-obesity and antidiabetic activities [6], in addition to acting as a neuroprotective and hepatoprotective agent [7,8]. Ecdysteroids are widely used by athletes as dietary supplements to increase strength and muscle mass during resistance training, to reduce fatigue and to ease recovery. Mhashilkar et al. [9] reported the anti-parasitic activity of 20-hydroxyecdysone against human filarial parasites. According to recent searches in academic databases and medical search engines (Google Scholar, PubMed and MEDLINE), rare study has been conducted to assess the antiprotozoal activity of 20-hydroxyecdysone.

Water sport athletes are at risk of waterborne intestinal protozoa and helminths. Intestinal pathogenic protozoa play a key role in outbreaks, likely because of their small size, low infectious dose [10] and high tolerance to chlorine [11], the major disinfectant used in swimming pools. The average volume of water swallowed per minute by adult men and women during an average visit to a

swimming pool of 81 min, is 0.50 and 0.36 ml/min, respectively [12]. In one study, the risk of infection with *Cryptosporidium parvum* was approximately tenfold higher in a given swimming pool than that in recreational lakes, though the risk of infection with *Giardia* (*G.*) *lamblia* was alternately higher, lower or equal [13]. The World Health Organization reports that approximately 200 million people are infected with *G. lamblia* worldwide [14]. Moreover, *G. lamblia* has been associated with increasing outbreaks of swimming-associated gastrointestinal diseases [15,16]. Despite the increasing resistance of *G. lamblia* to anti-parasitic drugs, there are several classes of medications with good efficacy. Currently, metronidazole is the most commonly used drug to treat giardiosis globally, but with respect to efficacy or adverse effects, there is no ideal anti-parasitic drug. Taking into account the emergence of resistance and the high toxicity of conventional drugs, research into new strategies to fight against *G. lamblia* is increasing [17,18].

The aim of the study was to assess the antiprotozoal activity of 20-hydroxyecdysone in water sports athletes with giardiosis.

Materials and Methods

Study center

This randomized, double-blinded, placebo-controlled clinical study was conducted by the Uzbek State University of Physical Education and Sport, Institute of the Chemistry of Plant Substances named acad. S.Yu. Yunusov and Republican Specialized Research and Practical Medical Center of Epidemiology, Microbiology, Infectious and Parasitic Diseases, Tashkent, Uzbekistan, during the period from January 2019 until September 2021.

Participants

The study participants included 241 athletes engaged in water sports (all males) aged 19–24 years. To assess the antiprotozoal activity of 20-hydroxyecdysone, only athletes with confirmed giardiosis were enrolled. They were assigned to three groups: the 20-hydroxyecdysone group (n=27), metronidazole group (n=25), and placebo group (n=24). All study participants were residents of the Republic of Uzbekistan. The following were exclusion criteria: 1) use of any drugs; 2) having a history of infectious (except giardiosis) or non-infectious disease.

Parasitological diagnosis

Stool samples were taken for parasitological examination from all the participants before antiprotozoal therapy and after 5, 10, 20 and 30 days of therapy completion to confirm parasite elimination. Turdiev's preservative (80 ml of 0.2% aqueous solution of sodium nitrite, 10 ml of formaldehyde, 2 ml of glycerine, 8 ml of Lugol's solution and 250 ml of distilled water) was used to conserve and stain protozoan cysts in stool samples for one year. The diagnosis of *G. lamblia* was performed by triple coproscopy using the formalin-ethyl acetate concentration technique and iodine stained smear.

Haematological and biochemical analyses

Five millilitres of peripheral venous blood was taken (after 8–12 hours of fasting) from all participants before therapy and immediately after therapy. Blood samples were collected into Human Tube Serum Gel-C/A containing a clot activator and serum gel separator for serological and biochemical analysis and Human Tube Serum-EDTA for haematological analysis (HUMAN Diagnostics, Germany). Biochemical and haematological analyses were performed at 2 hours after blood sample collection.

Serum samples were assayed for general biochemical markers (*i.e.*, glucose, total protein, blood urea nitrogen, creatinine, aspartate aminotransferase, alanine aminotransferase, albumin, total bilirubin and alkaline phosphatase). Whole blood samples were assayed for standard cell blood counts with percentage differentials (*i.e.*, haemoglobin, platelet, red blood cell counts, red blood cell distribution width, white blood cell counts (neutrophils, lymphocytes, monocytes, eosinophils, basophils).

Study protocol

The steroid hormone 20-hydroxyecdysone was isolated from *Rhaponticum carthamoides* and prepared at the Institute of the Chemistry of Plant Substances named acad. S.Yu. Yunusov (Tashkent, Uzbekistan). Each tablet contains 100 mg of 20-hydroxyecdysone and auxiliary substances (sucrose, starch and calcium stearate). The placebo tablet was identical to the 20-hydroxyecdysone and metronidazole tablets except that it did not contain an active ingredient.

After obtaining informed consent for therapy, water sports athletes with *Giardia* infection (n=76)

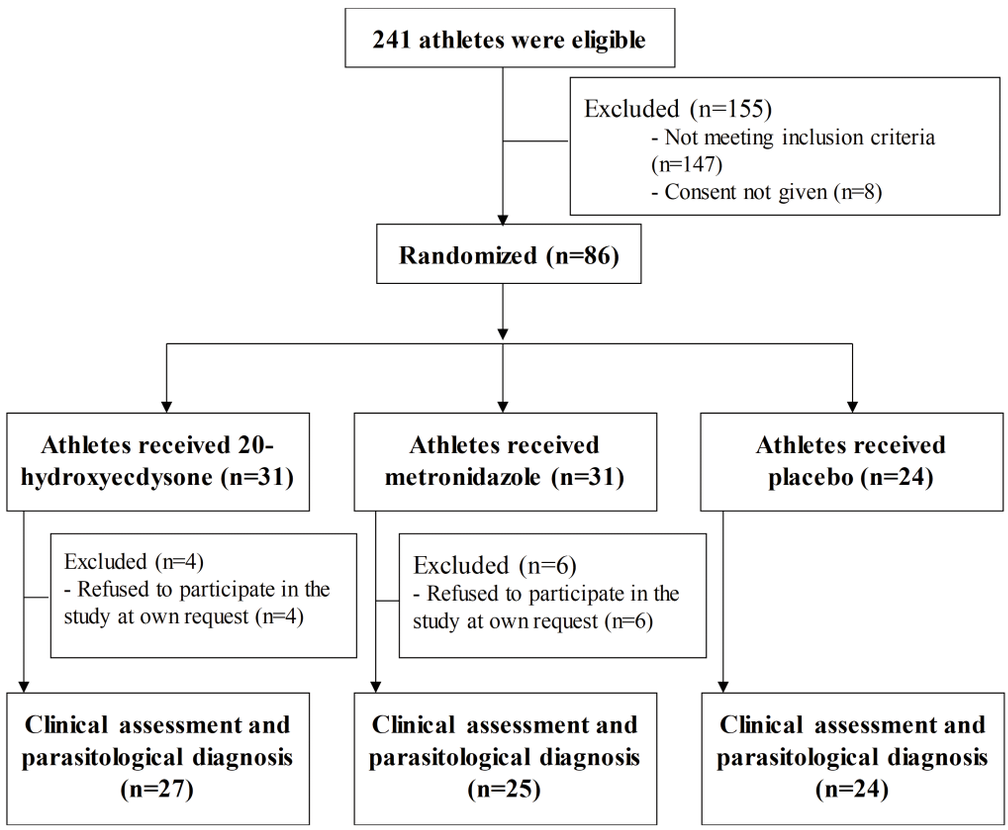


Figure 1. The selection process of the participants

received one of the following antiprotozoal therapies:

1. 20-hydroxyecdysone group: twenty-seven water sports athletes with giardiosis were treated with 20-hydroxyecdysone (100 mg twice a day orally for 10 days);

2. metronidazole group: twenty-five water sports athletes with giardiosis were treated with metronidazole (500 mg twice a day orally for 10 days);

3. placebo group: twenty-four water sports athletes with giardiosis were treated with placebo-sugar pills (100 mg twice a day orally for 10 days).

Before and during the follow-up period, all participants underwent a medical examination and completed a comprehensive health screening questionnaire. If necessary, participants were contacted by telephone. The presence of clinical signs of giardiosis and adverse effects of antiprotozoal drugs during the 30-day follow-up period was assessed. Adverse events were classified as mild, moderate, severe, or life-threatening.

Randomization

A simple randomization method was performed to assign the participants into three groups using computer-generated randomization schedules. The sequence was generated by an individual not directly involved in the study. The randomization list was sealed and maintained in the files of the institution's administrator until the study was completed. The study participants, investigators, laboratory staff, and observers were blinded to treatment assignment.

Follow-up and outcome measures

The purpose of follow-up was to monitor and evaluate the response to induct ion therapy, adverse events and recurrence of symptoms. Clinical examination and parasitological diagnosis were conducted before therapy, on the fifth day of therapy, immediately after the completion of the therapy, at the twentieth and thirtieth days of observation. Haematological and biochemical tests were conducted before and immediately after completion of the therapy. The participants were

Table 1. Baseline characteristics of the participants

Characteristics	20-hydroxyecdysone group (n=27)	Metronidazole group (n=25)	Placebo group (n=24)	F-value	*P*-value
Age, years	21.31±1.14	23.10±1.76	20.29±1.32	26.50	<0.001
Height, m	1.794±0.78	1.740±3.01	1.774±2.82	25.91	<0.001
Body mass, kg	74.16±6.32	71.34±5.17	67.93±9.76	6.02	0.003
BMI, kg/m^2	23.11±3.17	23.43±2.39	21.72±2.91	2.20	0.117
Years of swimming	6.17±1.08	5.87±1.90	6.43±1.75	1.48	0.233
Amount of swimming, h/week	17.03±0.32	17.07±0.95	17.04±0.41	0.003	0.967
Amount of gym training, h/week	6.02±1.22	6.09±0.84	6.06±0.66	0.01	0.981
Blood pressure, mmHg					
systolic	124.10±4.13	128.06±5.71	128.06±5.71	5.06	0.008
diastolic	78.05±2.45	81.04±3.97	72.09±4.63	11.21	0.001

Explanations: values are expressed as mean±standard deviation; one-way analysis of variance was used to establish the significance of differences between the groups; BMI: body mass index

followed-up for 30 days.

The primary outcomes of antiprotozoal therapy effectiveness were measured by the rate of *Giardia* infection radication during the observation period and the positive clinical response to therapy. The secondary outcome was tolerability of antiprotozoal therapy, assessed by the frequency and severity of adverse events, and by changes in haematological and biochemical parameters during the follow-up period.

Statistical analysis

Means and standard deviations (SD) for all variables were calculated. One-way analysis of variance was used to assess differences between the means of three independent groups. Paired t-tests measured changes within each group before and after therapy. Where appropriate, Pearson's chi-square test was used to establish the significance of differences between the groups. All statistical procedures were performed using Origin 8 software (OriginLab, Northampton, MA), and the level of statistical significance was set at $P<0.05$.

Ethics approval and informed consent

All participants provided written informed consent, and ethical approval was obtained from the Ministry of Health of the Republic of Uzbekistan in accordance with the Declaration of Helsinki. The trial was registered (#NCT04827537) at the U.S. National Institutes of Health (ClinicalTrials.gov).

Results

Two hundred forty-one athletes from the Uzbek State University of Physical Education and Sports were selected for parasitological diagnosis. However, 8 participants refused to participate and 147 athletes were excluded from the study due to the absence of *Giardia* infection. During the study, only 86 athletes were found to have *Giardia* infection, and 10 of them refused to participate in the study at their own request. Thus, seventy-six water sports athletes were enrolled and randomized into 20-hydroxyecdysone (n=27), metronidazole (n=25) and placebo (n=24) groups (Fig. 1). The baseline characteristics of the participants are presented in table 1.

Positive results of antiprotozoal therapy were observed in both the 20-hydroxyecdysone and metronidazole groups. On the 5th day of follow-up, elimination of *G. lamblia* was higher in the 20-hydroxyecdysone group (37.03±9.29%) than in the metronidazole group (16.00±7.33%) ($\chi^2(1)=2.92$, $P=0.08$). On the 10th and 20th days of follow-up, parasitological diagnosis in the 20-hydroxy-

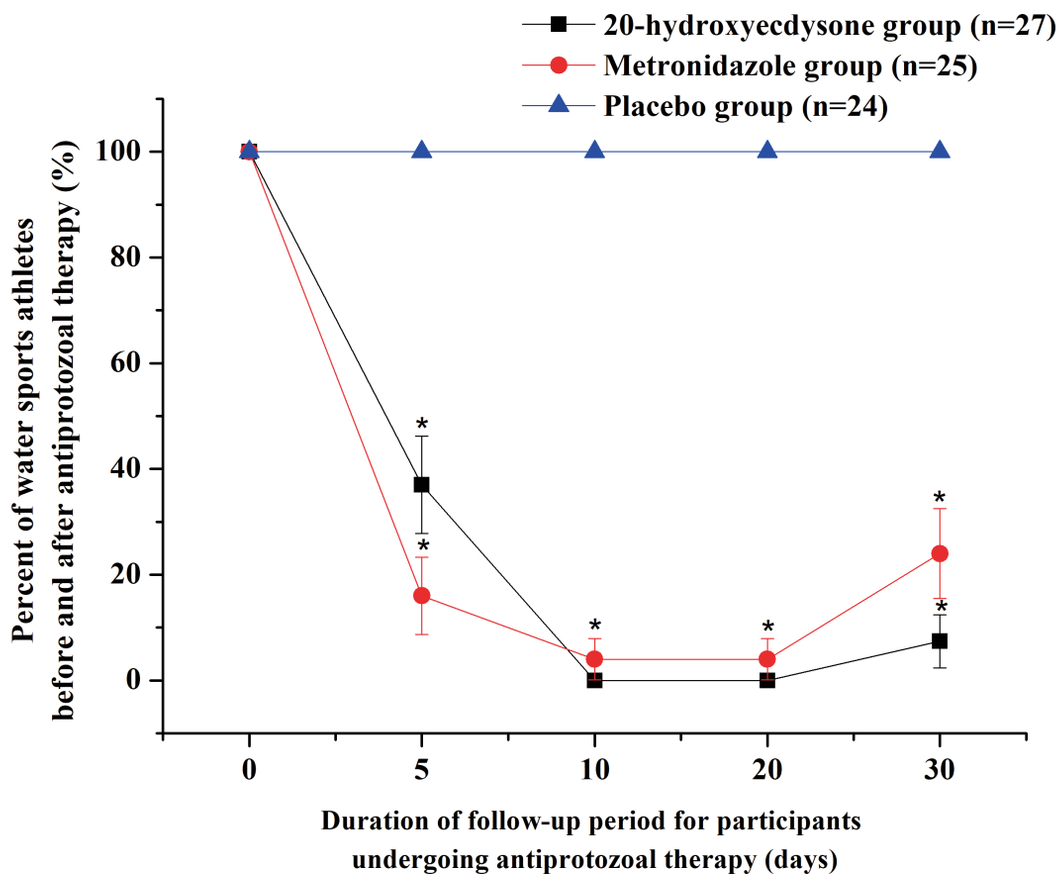


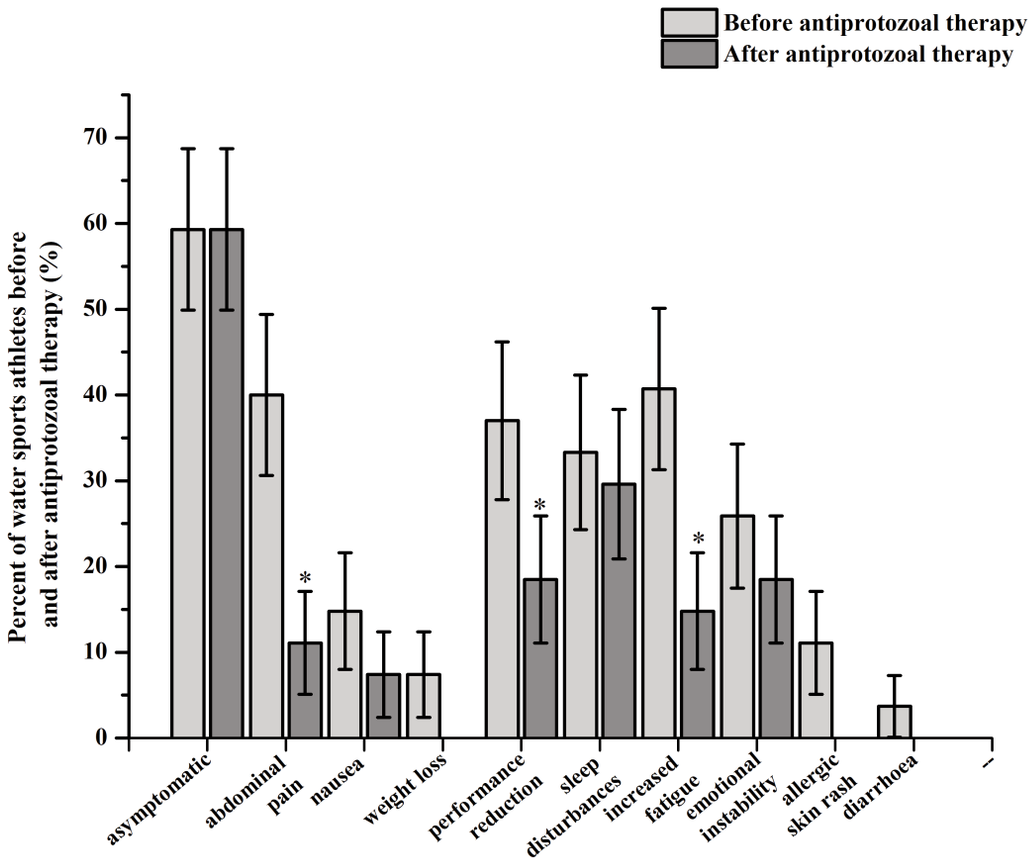
Figure 2. Antiprotozoal efficiency of therapy in water sports athletes infected with *Giardia lamblia.* Data on the assessment of the antiprotozoal efficacy of therapy during the follow-up period. Data was expressed as percentage (%) of athletes before and after antiprotozoal therapy. Bars are the standard deviations. Paired t-test was used to establish the significance of differences before and after therapy. * Significant difference with placebo group (P<0.0001)

ecdysone group revealed elimination of the parasite in 100.0% of cases; however, on the 30th day of follow-up, *G. lamblia* was re-identified in 7.40±5.03% of the athletes. In 4.00±3.91% of athletes in the metronidazole group, *G. lamblia* was stable on the 10th and 20th days of observation; the percentage of *G. lamblia* increased to 24.00±8.54% on the 30th day of follow-up. During all follow-up periods, no changes were found in the athletes in the placebo group (Fig. 2).

Clinical assessment showed an asymptomatic course of giardiosis in 59.26±9.45%, 52.00±9.99% and 54.16±10.17% of the athletes in the 20-hydroxyecdysone, metronidazole and placebo groups, respectively. Abdominal pain in the 20-hydroxyecdysone, metronidazole and placebo groups was detected in 40.74±9.45%, 32.00±9.32%, 45.83±10.17% and 11.11± 6.04%, 16.00±7.33%, 54.16±10.17% of cases before and after therapy, respectively ($\chi^2(1)=6.04$, $P<0.05$; $\chi^2(1)=1.71$, $P>0.05$; $\chi^2(1)=0.33$, $P>0.05$). Symptoms such as nausea, weight loss, diarrhea and allergic skin rush were found in a small number of athletes, with no significant changes before and after therapy in any group. Decreases in performance before and after therapy was attenuated in the 20-hydroxyecdysone (37.03±9.29% and 18.51±7.47%; $\chi^2(1)=2.31$, $P=0.12$) and metronidazole (32.00±9.32% and 12.00±6.49%; $\chi^2(1)=2.8$, $P=0.09$) groups. The opposite was found in the placebo group: the percentage of athletes with performance reduction increased after therapy (33.33±9.62%) than before therapy (20.83±8.28%) ($\chi^2(1)=0.93$, $P=0.33$). The same tendency was observed for emotional instability, but the changes were insignificant.

Most of the athletes complained of sleep disturbance. After therapy, the number of athletes with sleep disturbance decreased in all three groups, though the result was insignificant ($P>0.05$). Before and after therapy, emotional instability was stable in

A


Before antiprotozoal therapy
After antiprotozoal therapy
Percent of water sports athletes before and after antiprotozoal therapy (%)
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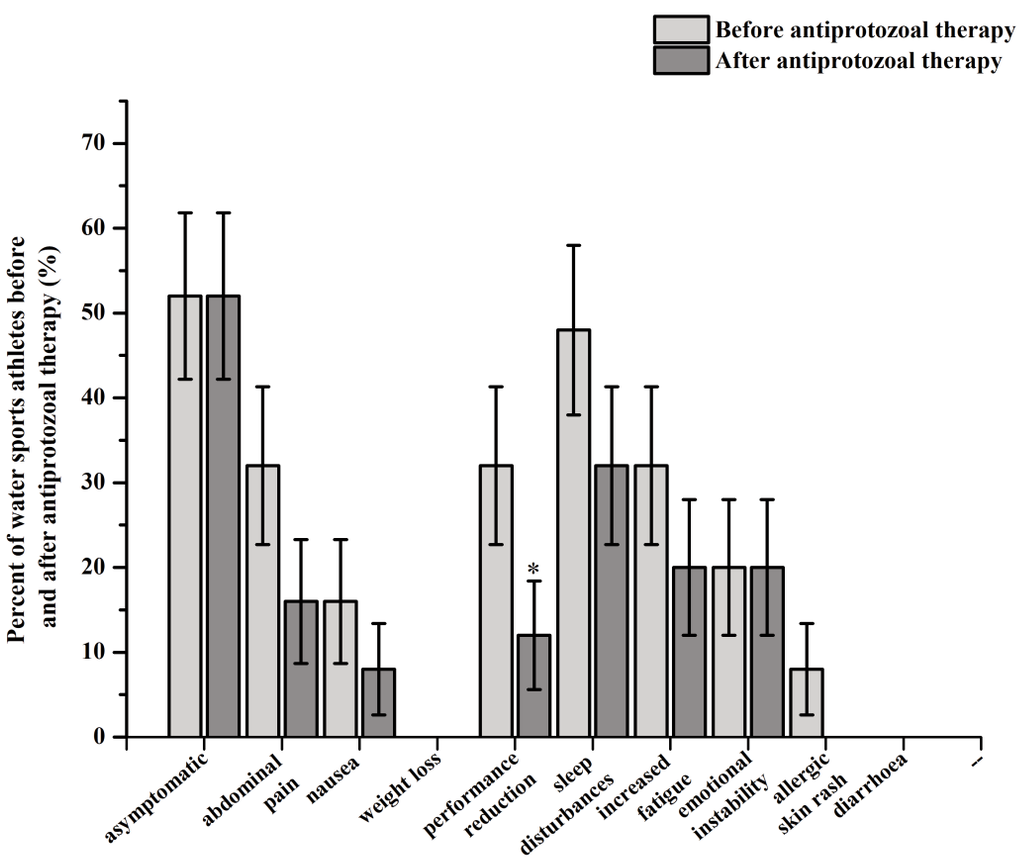
performance reduction
sleep disturbances
increased fatigue
emotional instability
allergic skin rash
diarrhoea

B


Before antiprotozoal therapy
After antiprotozoal therapy
Percent of water sports athletes before and after antiprotozoal therapy (%)
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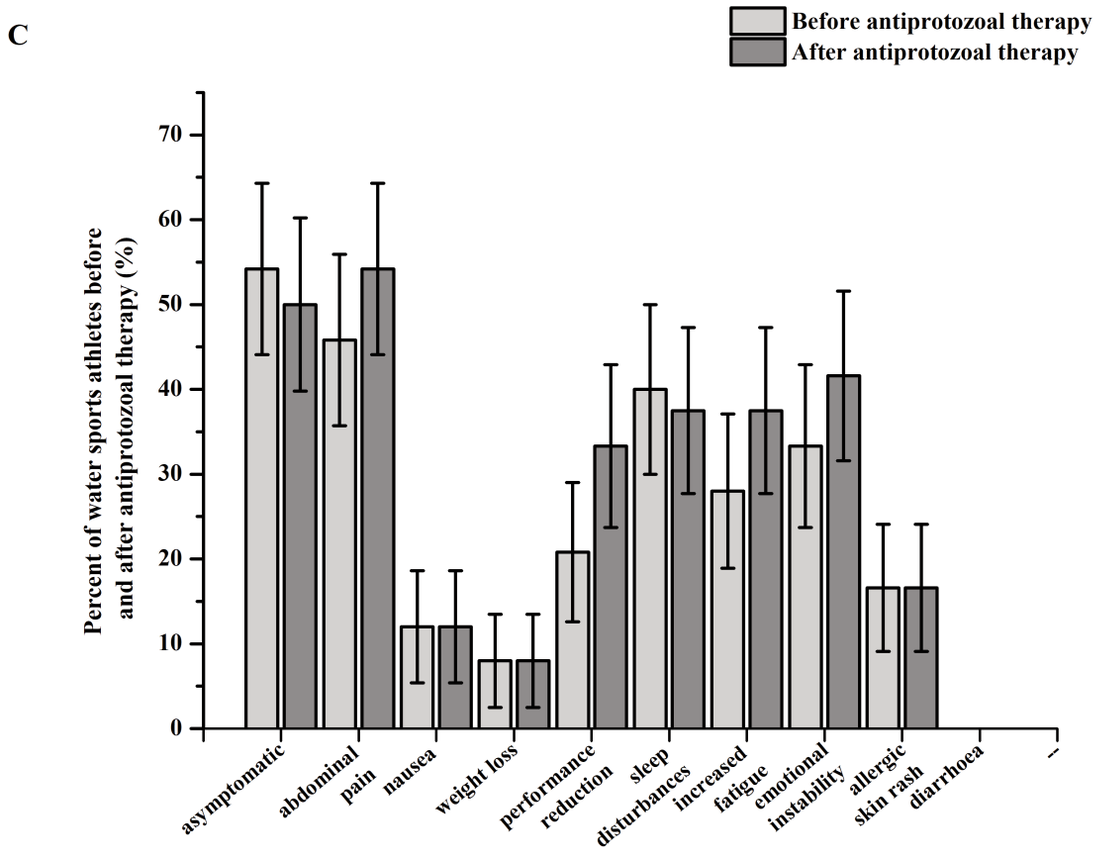

Figure 3. Clinical efficiency of antiprotozoal therapy. Data comparing the clinical efficiency of antiprotozoal therapy between the 20-hydroxyecdysone, metronidazole, and placebo groups before and after therapy. A**.** The clinical efficacy of 20-hydroxyecdysone before and after therapy; B. The clinical efficacy of metronidazole before and after therapy; C. The clinical efficacy of placebo before and after therapy. Data was expressed as percentage (%) of athletes before and after antiprotozoal therapy. Bars are the standard deviations. Paired t-test was used to establish the significance of differences before and after therapy. * Significant difference before and after therapy (P<0.05)

the metronidazole group (20.00±8.00% and 20.00±8.00%; P=1.00) but increased in the placebo group (33.33±9.62% and 41.67±10.06%; $\chi^2(1)$=0.34, P=0.55). The percentage of athletes with increased fatigue decreased significantly after therapy only in the 20-hydroxyecdysone group (40.74±9.45% and 14.81±6.83%; $\chi^2(1)$=4.43, P=0.03). Overall, a positive clinical response to the therapy was found in the 20-hydroxyecdysone and metronidazole groups. Comparison of clinical efficacy after therapy between the 20-hydroxyecdysone and placebo groups showed decreased percentages of athletes with abdominal pain (11.11±6.04% and 54.16±10.17%, $\chi^2(1)$=10.74, P=0.001), fatigue (14.81±6.83% and 37.5±9.8%; $\chi^2(1)$=3.38, P=0.06) and emotional instability (18.51±7.47% and 41.67±10.06%; $\chi^2(1)$=3.20, P=0.06) in the first group (Figs 3A, 3B and 3C).

Biochemical and haematological markers before and after therapy were presented in table 2. Despite significant changes in some markers after therapy, all values remained within normal clinical ranges.

Both 20-hydroxyecdysone and metronidazole were well tolerated and did not cause serious adverse events; furthermore, no athletes needed to stop medication because of potentially drug-related adverse events. Vomiting (16.00%), bitter taste (8.00%), headache (4.00%) and dizziness (4.00%) were noted in the metronidazole group. No adverse events were reported by the participants taking 20-hydroxyecdysone. All adverse reactions were graded as mild or transient and did not require treatment for relief or hospitalization.

Table 2. Changes in blood biochemical and haematological markers before and after therapy

Variables	20-hydroxyecdysone group (n=27)		Metronidazole group (n=25)		Placebo group (n=24)	
	Baseline	After therapy	Baseline	After therapy	Baseline	After therapy
Glucose mg/dl	92.00±2.12	93.08±3.28	101.05±3.61	90.03±5.19*	94.78±1.90	93.17±2.34
Total protein g/dl	7.31±0.15	7.28±0.14	7.00±0.12	7.45±0.11	7.32±0.15	7.35±0.12
Urea nitrogen mg/dl	16.97±0.51	16.14±0.60	17.01±0.44	15.28±0.45*	16.24±0.77	16.16±0.56
Creatinine mg/dl	1.10±0.03	1.01±0.02	1.04±0.04	1.13±0.02	1.00±0.03	1.07±0.02
Total bilirubin mg/dl	1.00±0.24	0.62±0.11	0.62±0.08	0.71±0.17	1.06±0.13	0.72±0.07
Aspartate aminotransferase IU/l	26.00±2.68	23.03±2.19	23.12±4.91	22.97±3.29	28.38±5.15	20.14±1.41*
Alanine aminotransferase IU/l	29.13±5.20	28.36±3.65	30.96±2.15	26.72±3.25	28.85±4.80	33.06±2.44
Albumin g/dl	3.94±1.00	5.27±0.87	4.17±1.24	4.54±0.57	4.07±1.58	4.37±0.90
Alkaline phosphatase IU/l	72.00±7.41	70.09±5.63	74.50±9.73	74.51±6.16	71.05±5.96	71.54±4.35
White blood cells $\times 10^3/\mu l$	5.71±0.36	5.04±0.37	5.32±0.48	4.17±0.21*	4.76±0.32	5.15±0.41
Red blood cells $\times 10^6/\mu l$	5.68±0.08	5.31±0.05	4.77±0.07	5.29±0.06*	4.58±0.05	4.82±0.07
Haemoglobin g/dl	15.16±0.17	15.05±0.24	14.64±0.43	15.00±0.27	15.15±0.14	14.88±0.37
Platelet $\times 10^3/\mu l$	321.00±9.51	378.06±11.42*	409.00±10.18	389.06±7.15*	345.09±11.70	356.04±8.24
Red cell distribution width	13.50±0.30	13.25±0.12	13.17±0.14	13.20±0.27	13.35±0.23	13.07±0.13
Absolute neutrophils μl^{-1}	3242.00±311.42	2981.07±288.66	3035.02±325.80	3124.05±278.90	3560.04±309.70	3241.03±175.00
Absolute lymphocytes μl^{-1}	2056.00±112.11	1876.11±95.28	1902.76±84.16	1978.36±90.00	1821.61±126.41	1757.54±129.28
Absolute monocytes μl^{-1}	454.62±27.12	367.38±16.78*	409.14±22.32	370.37±34.65	412.16±29.84	424.56±33.51
Absolute eosinophils μl^{-1}	207.91±34.13	154.51±27.10	314.67±57.29	294.80±43.21	160.47±17.51	278.48±39.16*
Absolute basophils μ^{-1}	24.80±3.39	22.66±1.73	31.18±2.85	28.11±3.20	26.46±2.17 22.	17±1.62*

Explanations: values are expressed as mean±standard deviation; paired t-test was used to establish the significance of differences before and after therapy; *$P<0.05$

Discussion

Several studies have reported a wide range of pharmacological effects of ecdysteroids in mammals, most of which are beneficial. Extensive investigations on the possible growth-promoting effects and stimulation of protein synthesis of ecdysterone in various animal species and a few in humans have been reported [19–22]. The purpose of this study was to examine the antiprotozoal effect of the steroid hormone 20-hydroxyecdysone.

Giardiosis is characterized by a high incidence rate in tropical countries and may also be an epidemic problem in developed countries [23]. In endemic regions with high levels of environmental pollution, the prevalence of *G. lamblia* ranges from 2.00% to 30.00%. In developed countries, its frequency is in the range of 2.0–7.0% [24–27]. Our results revealed a high prevalence of *G. lamblia* in water sports athletes (35.6%), more than two times higher than that in the general population (16.0%) of Uzbekistan [28]. The main reasons for the high prevalence of parasitic infection among water sports athletes are likely training in a swimming pool (17 hours/week) with contaminated *G. lamblia* cysts and regular swallowing of this water [29,30].

There is a lack of approved vaccines, and drugs are the main treatment for giardiosis. However, failures in treatment has been observed with all standard antiprotozoal drugs, including metronidazole, quinacrine, furazolidone, and albendazole. Ineffectiveness of therapy may result from an inadequate drug dose, resistance of *G. lamblia* to the drug, or, rarely, from *Giardia* sequestration in the gallbladder or pancreatic duct [31]. Resistance of *G. lamblia* and other pathogenic protozoa to the main antiprotozoal drugs is a global problem of growing concern. The results of parasitological diagnosis showed elimination of *G. lamblia* in 100.0% of cases in the 20-hydroxyecdysone group; however, resistance of *G. lamblia* was detected in 4.0% of cases in the metronidazole group. Nevertheless, no adverse events were observed among the athletes taking 20-hydroxyecdysone. Ogawa et al. [32] determined an LD_{50s} for ingested 20-hydroxyecdysone in mice of >9 g/kg and an LD_{50s} of 6.4 g/kg for intraperitoneally-injected 20-hydroxyecdysone. Thus, ecdysteroids are regarded as nontoxic to mammals.

Chronic giardiosis is usually associated with diarrhea and intestinal malabsorption, resulting in steatorrhea, lactase deficiency, and deficiency of vitamin A, vitamin B12 and folate [33]. Giardiosis can be asymptomatic or responsible for a broad clinical spectrum, with symptoms ranging from acute to chronic. In our study, most of the athletes were asymptomatic. The main symptoms were abdominal pain, performance decrement, sleep disturbances and increased fatigue. A positive clinical response was found after therapy in the 20-hydroxyecdysone group, especially abdominal pain, increased fatigue, performance reduction, and emotional instability decreased after therapy in comparison with the placebo group. Most of the symptoms of overtraining syndrome and giardiosis are similar. Kerimov et al. [34] showed that intestinal parasites imitate overtraining syndrome in elite athletes, and cytokine, hypothalamic, glycogen, and branched-chain amino acid hypotheses have been suggested to explain the aetiopathogenesis of overtraining syndrome [35]. Regardless, none of the presented hypotheses fully explains the pathophysiology of overtraining syndrome in athletes, and there is almost no study indicating the influence of intestinal helminths and pathogenic protozoa on the development or imitation of overtraining syndrome. Our results highlight the necessity of parasitological diagnosis of all athletes in the case of an assumption of overtraining syndrome.

In conclusion, the results of the study showed high antiprotozoal activity of 20-hydroxyecdysone against *G. lamblia*. No adverse events during the follow-up period were observed among water sport athletes receiving 20-hydroxyecdysone. Further clinical studies are necessary to assess the antiprotozoal efficiency of 20-hydroxyecdysone.

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