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Enhanced Role of Grapefruit Juice on the Anti-schistosomal Activity of Artemether on the Liver of *Schistosoma haematobium* Infected Hamsters

Madiha Mahmoud [1], Fatma Ebeid [1], Mona Nosseir [2]*

[1]Department of Pharmacology, Theodor Bilharz Research Institute, Imbaba, 12411, Guiza, Egypt

[2] Department of Pathology, Theodor Bilharz Research Institute, Imbaba, 12411, Guiza, Egypt

Abstract

Artemether (ART) is an efficacious anti-malarial drug that also displays antischistosomal properties. Laboratory studies have found that ART curtails the development of schistosoma worms and thus prevents morbidity. Grapefruit juice was found to interact with various drugs that have been metabolized by a form of Cytochrome P450, CYP3A4, thus increasing the plasma drug concentration. This work aimed to study the effect of grapefruit juice when administered before infection with *Schistosoma haematobium* and/or treatment with ART on its anti-schistosomal activity.

Golden hamsters were infected each with 300 *S. haematobium* cercariae and divided into 6 groups (A – F), as follows: Infected control (A); infected received grapefruit juice before infection (B); or received grapefruit juice before infection and treated with ART (200 mg/k) at 5,6 and 7 weeks post infection (WPI) (C); or for 3 successive doses at 12 WPI (D); infected treated with ART alone (200 mg/k) at 5,6 and 7 WPI (E); infected received grapefruit juice (0.5 ml) half an hour before treatment with ART (200 mg/k) at 5,6 and 7 WPI (co-administration) (F). All groups were sacrificed 14 WPI. Some parasitological, biochemical and histopathological estimations were done. Results revealed that, the highest percent of worm reduction was observed in-group F (94.2%) compared to group C,E & D (87.3%, 77.6% & 65.9% respectively). The level of ALT, GGT, urea, thiol, albumin and alkaline phosphatase tend to normalize in accordance with the parasitological results. Neither hepatic granuloma nor prominent histopathological changes could be

detected in group F. A minimal number of granulomas (1-3/animal) was observed in group E; meanwhile, the least diameter and collagen content of hepatic *S. haematobium* granulomas were observed in group C (125.6±5.5 & 2.4±0.572 respectively). The results of the present study demonstrated that, co-administration of grapefruit juice just before treatment with ART at 5, 6& 7 WPI enhanced its anti-schistosomal activity and improved the histopathological changes.

Keywords

S. haematobium, Artemether (ART), Grapefruit juice.

Introduction

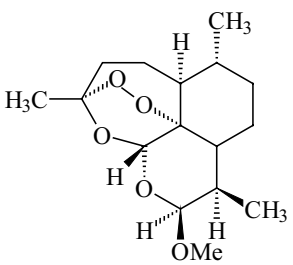
Human schistosomiasis is a chronic and debilitating disease that remains one of the most prevalent parasitic infections in the humid tropics, with estimated 650 million people at risk of infection and 200 million actually infected [1]. *Schistosoma haematobium* causes urinary schistosomiasis in most African countries. Adult *S. haematobium* worms live in the veins of the vesicle plexus around the bladder especially the upper trigone, and along the ureters. Human pathology is caused by those eggs trapped within the urogenital system, and kidneys are involved when reached by adult worms [2]. Also, infection with *S. haematobium* frequently causes mild degrees of periportal fibrosis (grade I) as well as hepatomegaly and splenomegaly as evidenced by both histopathologic examination and by ultrasound [3]. Investigating the pathogenic mechanisms of some disorders of *S. haematobium infection* revealed accumulation into bilharzioma of the liver and granulomatous hepatitis surrounded by bilharzial pigment deposition[4]. Jacobs et al. [5] infer that in vivo, *S. haematobium* can positively modulate *S. mansoni* egg antigen-induced granuloma formation and hepatic fibrosis resulting in more severe liver pathology.

Since there is no vaccine available for treatment of schistosomiasis, chemotherapy is the current strategy for schistosomiasis control [6]. Recently, there has been considerable discussion about the development of tolerance or

resistance to praziquantel being the drug of choice [7]. Oxamniquine and meterifonate, as alternative antischistosomal drugs have become difficult to obtain in most African countries [8]. Therefore, there is a great need for research and development of novel and inexpensive aids against schistosomiasis.

The Chinese herb Artemisia annua L that belongs to Family Asteracea (composite flowers) yield between 0.01 % and 0.5% of artemisinin. Artemether which is a derivative of artemesinin has been already widely used against malaria [9]. It also showed activity against *S. japonicum* [10], *S. mansoni* [11] and *S. haematobium* [12]. Artemether was used in randomized field trials and was found to reduce the incidence of infection by 60-100 %. The juvenile stages of the parasites (schistosomulae) are especially susceptible to artemether [13].

Artemether



The chemical formula of artemether is C16 H26 O5, with relative molecular mass 298.4, its chemical name is decahydro-10- methoxy - 3, 6, 9 - trimethyl - 3, 12- epoxy -12H- pyrano 1,2- benzodioxepin, and pharmacological name is artemetherum [14].

Grapefruit juice (Citrus paradise), family Rutaceae, has been shown to interact with various drugs [15]. These drugs differ in their chemical and pharmacological properties, but are in common extensively metabolized by a form of cytochrome P450, CYP3A4. Although grapefruit juice increases the plasma drug concentration, it scarcely affects the elimination half-lives. These results suggest that grapefruit juice mainly alters the first- pass metabolism,

particularly in the small intestine. In the present study, we aimed to study the effect of co-administration of grapefruit juice with ART. Evaluation of the effect of its administration before infection with *S. haematobium* either alone or in groups treated with ART at different maturation stages of the parasite on the outcome of infection was also assessed.

Experiment

Animals

Golden hamsters, 6-8 weeks old, were bred and maintained at the experimental animal research unit of Schistosome Biological Supply Center (SBSC), Theodor Bilharz Research Institute (TBRI), Guiza, Egypt. They were maintained on a standard commercial pellet diet (El-Kahira Company for Oils and Soap). The animals were given Carrot, Lettuce and milk as a source of vitamins. They were kept in air-conditioned animal house at 20-22◦C. The animal experiments were conducted in accordance with the internationally valid guidelines and maintained at TBRI animal unit under animal ethics guidelines.

Infection of animals

Animals were exposed individually to 300 cercariae of the Egyptian strain of *S. haematobium* (provided by SBPC of TBRI) using ring method [16].

Chemicals

Artemether (CAS-71963-77-4) was the product of Kunming Pharmaceutical Corp. (Kunming, China; Batch No. 010377). Artemether was suspended in 7% Tween-80 and 3% ethanol and given orally in a dose of 200mg/kg body weight**.** Grapefruit was purchased from local Egyptian markets and squeezed by hand.

Experimental design:

Hamsters were randomly allocated into 6 groups (A – F), each of 5-7 animals at the beginning of the experiment:

Group A: Infected control, treated with vehicle only. Group B: Infected animals received grapefruit juice (0.5 ml) half an hour before infection.

Group C: Infected animals received grapefruit juice (0.5 ml) half an hour before infection and treated with ART (200 mg/k) at 5,6 and 7 weeks post infection (WPI).

Group D: Infected animals received grapefruit juice (0.5 ml) half an hour before infection and treated with ART (200 mg/k for 3 successive doses) at 12 WPI.

Group E: Infected animals treated with ART (200 mg/k) at 5,6 and 7 WPI.

Group F: Infected animals received grapefruit juice (0.5 ml) half an hour before treatment with ART (200 mg/k) at 5,6 and 7 WPI (co-administration).

Hamsters were sacrificed 14 WPI.

Parasitological studies

Each Hamster was perfused with cold saline using Automatic pipetting Machine-scientific equipment products (Division of Baltimore Machine and Equipment Inc., Baltimore, Maryland, USA). Worms recovered were counted [17]. Parts from liver were used for estimation of egg load i.e. number of ova/ gram tissue [18], Large intestine was removed and used for oogram pattern i.e. the percentage of different egg developmental stages [19] and for determination of egg load.

Biochemical parameters

Blood was collected and serum was separated by centrifugation at 3000 r.p.m. for 10 min. and analyzed for liver and kidney function tests: Alanine amino transferase, ALT [20], gamma glutamyl transferase, GGT [21], total protein [22], albumin [23], globulin, alkaline phosphatase [24], urea [25], thiol [26] and creatinine [27] using bio-Merieux laboratory reagents and instruments (Marcy-L'Etoile, France).

Histopathological techniques

To study the host response elicited by the different studied groups (Group A-F); liver samples were collected after scarification 14 WPI, fixed in 10% formalin, rinsed in different grades of alcohol to be processed into paraffin blocks. For granuloma measurements, five sections each of 5µm thick and at

a distance of 250µm from the preceding one were prepared and stained with hematoxylin & eosin (Hx&E) and Masson trichrome stains. With an ocular micrometer; measurements were conducted for non-contiguous granulomas, each containing a single egg in its center. The mean diameter of each granuloma was obtained by measuring the diameters of the lesion at right angles to each other and then dividing these values by two [28]. The location of the granulomas, their cellular profile and the state of their eggs were examined in the prepared sections.

Assessment of collagen content of different granulomas was conducted in 15 µm thick sections stained with Sirius red (Fluka Chemica, Buchs, Switzerland). Measurements were done in five microscopic fields under x 50 magnification using Ziess microscope fitted to Kontron Image Analysis System (software program, KS 400), Germany.

The accompanied histopathologic changes in the hepatic tissue were also studied including cloudy swelling, hydropic degeneration and/or the presence of inflammatory infiltrate [29].

All values are expressed as mean ± standard error of the mean (M±SE). Independent Students t-test was applied to analyze the significance of differences between mean values and a critical p value was considered to be significant at < 0.05.

Results and Discussion

The present study was undertaken to examine the anti-schistosomal effect of artemether (ART) on the liver of *S. haematobium* infected hamsters in both acute (5, 6 & 7 Ws) and Chronic (12 W) stages of infection. The role of Grapefruit juice administration either before infection or before treatment with artemether was also assessed.

Parasitological criteria

Worms recovered after infection with *S. haematobium* were 39.3±0.4 with almost equal total number of male and female worms. The highest percent of worm reduction (94.2%) was observed in group F that received 0.5ml

grapefruit juice half an hour before treatment with ART (200 mg/k) at 5,6 and 7 WPI (co-administration), causing 100% hepatic shift (Table, 1). This reduction in total number of worms was higher than that of Group E treated with ART alone 5,6 and 7 WPI (77.6%). The enhanced effect observed in group F may be attributed to the increased oral bio-availability of ART. For instance, the consumption of grapefruit juice lead to a marked reduction of Cytochrome P450 (CYP 3A4) activity in the small intestine and liver microsomes [30]. Flavonoids that present in grapefruit juice are polyphenolic compounds having anti-oxidant properties [31] that affects drug metabolism [32] and acts as an inhibitor of Cytochrome P 450 enzyme [33]. Administration of 0.5ml grapefruit juice half an hour before infection with *S. haematobium* in groups treated with ART (200mg/K) at 5,6 and 7 WPI (group C) or 12 WPI for three successive doses (group D) reduced the total number of worms by 87.3% & 65.9% and causing hepatic shift of 46.0% & 18.7% respectively. Administration of grapefruit juice alone before infection reduced the total number of worms by about 8.9% (Table, 1). Xiao et al. [13] found that ART reduced the total number of worms (juvenile stages) when the drug administered at immature stages of infection. In other experimental researches concerning schistosomiasis, the efficacy of ART against juvenile stages of *S. mansoni* [34] and *S. japonicim* [35] were recorded. In the study conducted by Xiao et al., [36] treatment with ART in a dose of 300 mg/kg 2, 3 or 4 WPI in *S. heamatobium* infection and followed by 1-4 repeated doses at the same concentration once every 2-4 weeks showed highly significant worm reduction rates ranging between 78 and 100%. It was found that, treatment with artemether in double dose is preferable than in single dose as regards its effect on *S. mansoni* eggs [35]. Repetition of the dose may subject parasites with different ages and maturity to the drug or could damage those recovering the effect of the first dose. Utzinger, et al. [9] tried to explain the cause of death of schistosomes under the influence of artemether, and found that it is due to liberation of free radicals.

Complete absence of all egg developmental stages and the highest reduction in egg load were observed in groups treated with ART (200 mg/k) at

5, 6 and 7 WPI alone (94.5%) or in association with grapefruit juice administered either before infection (99.1%) or before treatment (99.2 %) (Table 2). This may be due to the sterile effect of ART on the female worms explaining their incapability to oviposition. Worms recovered from this group were difficult to trace, being thin and dwarf. Treatment with ART, 12 WPI reduced the tissue egg load by 52.8% with significant increase in the percentage of dead ova (5 folds).

Biochemical parameters

Infection with *S. haematobium* increased the serum level of ALT, GGT reflecting either chronic or acute active liver damage. Damage of the hepatic cells or impaired permeability of cell membranes was previously reported by Ahmed [37]. However, treatment with ART tends to normalize these levels after death and elimination of the parasite [38]. *Schistosoma haematobium* infection increases creatinine level due to immune complex deposition in the glomeruli resulting in a true glomerulonephritis [39]. Improvement of creatinine level after treatment is also due to death of parasite.

Histopathological criteria

Infection with S. haematobium causes marked histopathological changes in the liver in spite of its great association with bladder cancer [40]. In schistosomiasis, granuloma formation around eggs is mediated by CD4 T-helper cells specific for egg antigen [41], and the magnitude of hepatic granuloma depends upon which type of T-helper cell subpopulation contributes to the immuno-pathology [42]. Histopathological studies indicated that artemether was most active against 28-day-old S. heamatobium schistosomula [43]. Damage to the tegument, subtegumental musculature, parenchymal tissue and gastrodermis were reported in juvenile forms of S. mansoni upon treatment with artemether [44].

In this study, treatment with artemether 5, 6 & 7 WPI provides a minimal number (1-3/animal) of portal, cellular granulomas (Fig. 1a) exhibiting a significant lower diameter (200.3±5.0) and moderate collagen content

(3.79±1.32, Fig. 1b) compared to infected control group, that have large (290.5±15), parenchymal, fibrocellular granulomas and a collagen content of 7.291±0.937 (Fig. 1c). Administration of grapefruit juice half an hour before infection (Group B) revealed large parenchymal fibrocellular granulomas (Fig. 1d). Its administration before infection in group C that received ART 5, 6 &7 WPI decreased the granuloma diameter to 125.6±5.5 and collagen content to 2.4±0.572 (Fig. 1e). However, animals of Group D revealed portal, or parenchymal fibrocellular crowded and even adherent large granulomas expressing the highest collagen deposition 9.452±1.134 (Fig. 1f). Complete disappearance of granuloma formation was observed in-Group F where grapefruit juice (0.5 ml) was co-administered half an hour before treatment with ART (200 mg/k) 5, 6 &7 WPI (Table 5), thus producing minimal histopathologic changes in the form of focal hydropic degeneration of hepatocytes 20.5±5.5 and mild inflammatory infiltrate 29.3±2.4 (Table 6). Xiao et al. [45] reported that artemether is activated by haematin, cleaving the endoperoxide bridge of the drug resulting in the generation of free radicals which play a role in the complete resolution of eggs and granulomas and yet an immunomodulatory role can not be ruled out.

In conclusion, artemether is prominently efficient as an anti-schistosomal drug when administered at immature stages of *S. haematobium* infection (5, 6 &7 WPI) compared to mature stages (12 WPI). Grapefruit co-administration with ART enhances its anti-schistosomal effect proved by both parasitological and histopathological assessments. Its administration before infection gives less promising results

Table 1. ***Effect of treatment with grapefruit (G.F) (0.5 ml) alone or combined with artemether (ART) (200 mg/kg) at different time intervals on worm distribution and load in hamsters infected with 300 S. haematobium cercariae. (Values given are Means±SE).***

Animal groups	Worm distribution					
	Total No. of Males	Total No. of Females	Total No. of Couples	Total No. of worms	% Worm reduction	% Hepatic shift
A	19.8±1.4	19.5±1.4	19.2±1..5	39.3±0.4	0	10.9
B	17.0±3.6	12.6±2.8	12.6±2.8	35.8±1.1	8.9	11.7
C	4.5±1.6	0.5±0.3	0.5±0.3	5.0±1.4	87.3*	46.0*
D	9.0±0.6	5.3±0.5	5.3±0.5	13.4±0.5	65.9*	18.7
E	0.6±0.6	2.8±0.3	2.8±0.3	8.8±0.8	77.6*	26.1*
F	1.8±1.2	0.5±0.3	0.3±0.3	2.3±1.3	94.2*	100*

A= Infected control group. B= Group infected and treated with (G.F) 1/2hr before infection. C= Group infected and treated with (G.F) 1/2hr before infection and (ART) 5, 6, 7 WPI. D= Group infected and treated with (G.F) 1/2hr before infection and (ART), for 3 successive doses 12 WPI. E= Group infected and treated with (ART) only at 5, 6, 7 WPI. F= Group infected and treated with (G.F) 1/2hr before treatment with (ART) 5, 6, 7 WPI.

* Significant difference versus infected control ($p < 0.05$).

Table 2. ***Effect of treatment with grapefruit (G.F) (0.5 ml) alone or combined with artemether (200 mg/kg) at different time intervals on percent of egg developmental stages and tissue egg load in hamsters infected with 300 S. haematobium cercariae. (Values given are Means±SE).***

Animal Groups	% egg developmental stages (Oogram Pattern)			Tissue egg load (No. of ova / gm tissue x103)		
	% Immature	% Mature	% Dead	Liver	Intestine	Total
A	36.70±2.11	53.00±3.80	10.30±2.53	10.535±0.959	44.54±54.85	54.854±2.670
B	53.50±8.21	36.25±7.47	10.25±2.78	10.29±1.56	41.61±2.94	51.99±4.22
C	0±0*	0±0*	0±0*	0.37±0.14*	0.11±0.07*	0.48±0.16*
D	23.75±3.75*	17.5±4.3*	58.75±7.73*	8.32±0.47*	17.55±3.069*	25.87±3.19*
E	0±0*	0±0*	0±0*	0.45±0.15*	0.16±0.16*	0.61±0.29*
F	0±0*	0±0*	0±0*	0.13±0.08*	0.30±0.28*	0.43±0.35*

* Significant difference versus infected control ($p < 0.05$).

Table 3. ***Effect of treatment with grapefruit (G.F) (0.5 ml) alone or combined with artemether (ART) (200 mg/kg) at different time intervals on serum ALT, GGT. Total protein, albumin, globuline and A/G ratio in hamsters infected with 300 S. haematobium cercariae. (Values given are Means±SE).***

Animal groups	ALT (Unit/ml)	GGT (Unit/ml)	Total protein (gm/100ml)	Albumin (gm/100ml)	Globulin (gm/100ml)	A/G ratio
NC	92.5±7.5	2.1±0.09	6.6±0.4	3.6±0.3	3.0±0.1	1.2
A	159.4±8.2	5.4±0.17	6.7±0.5	3.0±0.2	3.9±0.2	0.72
B	150.0±9.1	5.1±0.21	6.5±0.2	3.4±0.2	3.5±0.2	0.86
C	112.3±9.4*	2.9±0.12*	6.3±0.3	3.3±0.3	3.0±0.5	1.13
D	123.4±8.1	3.2±0.17	6.3±0.4	3.2±0.4	3.0±0.2	1.1
E	110.3±7.4*	2.8±0.18*	6.4±0.5	3.5±0.3	3.2±0.3	1.0
F	100.1±1.3*	2.5±0.12*	6.4±0.2	3.6±0.3	2.9±0.1	1.21

NC= Normal control group. * Significant difference versus infected control ($p< 0.05$).

Table 4. Effect of treatment with grapefruit (G.F) (0.5 ml) alone or combined with artemether (200 mg/kg) at different time intervals on serum creatinine, urea, thiol and alkaline phosphatase in hamsters infected with 300 S. haematobium cercariae. (Values given are Means±SE).

Animal groups	Urea (Unit/ml)	Creatinine (Unit/ml)	Thiol (Umol/L)	ALK. Ph (Kind& King units/ml)
NC	61.3±2.5	0.66±0.09	851.3±55.6	21.4±1.15
A	83.1±3.2	2.01±0.13	652.3±45.3	33.01±.95
B	84.2±4.1	2.1±0.11	649.4±43.2	32.2±1.10
C	65.5±3.2*	0.83±0.12*	811.3±49.3*	25.4±0.82*
D	70.2±4.2*	1.01±0.07*	792.4±51.1	26.3±0.99*
E	65.3±4.4*	0.78±0.08*	803.4±50.2*	25.9±0.92*
F	64.3±2.1*	0.70±0.03*	832.5±41.2*	24.3±1.0*

NC= Normal control group

* Significant difference versus infected control ($p< 0.05$).

Table 5. Assessment of Hepatic granulomas & Parasite Eggs in S. haematobium infected hamsters after treatment with grapefruit (G.F) (0.5 ml) and/or artemether (200 mg/kg) at different time intervals.

Animal Groups	Site of granuloma	Granuloma Parameters				State of *S. haematobium* eggs		Collagen content
		Diameter (μm) M±SE	% Cellular	% Fibrocellular	% fibrous	% Deg.	% Intact	Mean±SE
A	Mostly Parenchymal	290.50±15.00	19	72	9	15	85	7.29±0.94
B	Parenchymal	280.60±3.20	27	69	4	20	80	5.18±0.51
C	Portal	125.60±5.50*	69	29	2	100	0	2.40±0.57*
D	Portal + Parenchymal	240.80±10.00	11	81	8	50	50	9.45±1.13
E	Portal	200.30±5.0*	100	0	0	100	0	3.79±1.32*
F	No granuloma formation							

Granuloma parameters are based on examination of 5-7 hamsters with all lesions measured. * Significant difference versus infected control ($p < 0.05$).

Table 6. Associated histopathological changes in the hepatic tissue of the different studied group.

Animal Groups	Cloudy swelling	Hydropic degeneration	Inflammatory cells
A	31.9±4.5	30.0±3.9	86.7±1.7
B	29.7±6.2	29.0±2.5	75.8±2.2
C	0.0±0.0	25.2±1.7	38.7±1.8*
D	13.0±2.2*	66.5±2.2*	59.0±4.6
E	0.0±0.0	26.3±1.5	49.5±3.8*
F	0.0±0.0	20.5±5.5	29.3±2.4*

- Significant difference versus infected control ($p < 0.05$).

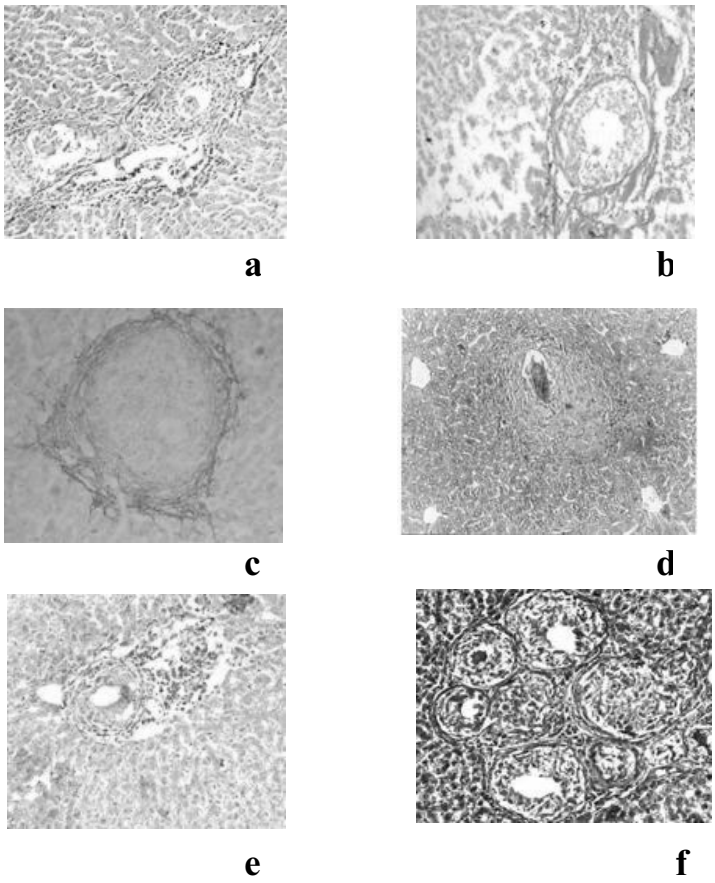


Fig. 1: (a) Portal, cellular granuloma of Group E (Hx.&E.X 200); (b) Portal, cellular granuloma of Group E showing moderate collagen content (Sirius redX 200); (c) Parenchymal, fibro-cellular granuloma of infected control Group A (Sirius redX 200); (d) Parenchymal, fibro-cellular granuloma of Group B (Masson trichrome.X 200); (e) Small portal, cellular granuloma of Group C (Hx.&E.X 200); (f) Adherent large parenchymal, fibro-cellular granulomas of Group D (Masson trichrome.X 200).

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