Halofuginone as An Antifibrotic Therapy in Chronic Schistosomal Liver Fibrosis in Mice

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Liver fibrosis is the principal feature of injury caused by chronic liver disease and determines the major clinical events that lead to liver-related deaths. This study was planned to investigate the possible effect of halofuginone on experimentally induced hepatic schistosomal fibrosis. Ninety, laboratory bred parasite free, Swiss albino mice were used in this study and were divided into five groups (gp.): gp. (1) infected non treated, gp. (2) infected & praziquantel treated, gp. (3) infected & praziquantel and halofuginone treated, gp. (4) infected & halofuginone treated; and gp (5) normal non infected control. The antifibrotic efficacy of halofuginone (stenorol) was evaluated on the basis of liver weight, ova count in liver tissue, biochemical estimation of hepatic hydroxyproline and total collagen content; and histopathological assessment of granuloma size and collagen fiber deposition by H&E and Masson trichrome stains. Most significant reduction in number of ova, granuloma size and hydroxyproline and total collagen contents was noticed in group (3). These results were assured by collagen staining that showed very scanty collagen fibers or even their disappearance especially at 24th week post-infection (p.i.). In gp. (2), in spite of the reduction in the number of ova, collagen fibers appeared in the granulomas as dispersed fibers at 20th week p.i., while at the 24th week p.i., granuloma was replaced by scar of collagen fibers. Gp. (4) that received halofuginone alone showed less reduction in the size of granuloma and hydroxyproline and total collagen contents than in gp. (2). It was concluded that halofuginone seemed effective in controlling the fibrotic process in mice liver infected with *Schistosoma mansoni*. When halofuginone was combined to praziquantel, best results were attained. The mechanism is probably through interruption of collagen type I synthesis and attenuation of the collagen already deposited in the liver. Although, these results are promising in experimental schistosomiasis, further studies are required to study its possible application in human to treat old established hepatic fibrosis.

INTRODUCTION

Hepatic fibrosis is a common response to various insults such as parasitic infection, chronic viral infection, autoimmune diseases, hereditary, toxic damage, etc. [(1)]. Hepatic fibrosis is associated with activation of hepatic stellate cells (HSC), the major source of the extracellular matrix (ECM) proteins. The predominant ECM protein synthesized by the HSC is collagen type I. The progressive accumulation of connective tissue results in destruction of normal tissue architecture and function [(2)].

In schistosomiasis, healing of parasite egg-induced granuloma can lead to periportal fibrosis [(3)] and may lead to obstruction of portal flow, resulting in portal hypertension with all its complications specially bleeding oesophageal varices[(4)]. Fibrogenesis is a dynamic potentially reversible process mediated through the immunological responses to parasite eggs trapped in the liver [(5)]. It is mediated by macrophages, lymphocytes and fibroblasts with certain growth factors as transforming growth factor β (TGFβ) and fibrogenic mediators [(6)]. Both CD4+ lymphocytes and Kuppfer cells produce TGFβ after contact with soluble egg antigen. TGFβ stimulates HSC activation and production of matrix proteins [(7)]. In schistosomiasis, collagen synthesis is quantitatively prominent specially type I, after its cross linking, and it has been a target for therapeutic strategies against fibrosis [(8)].

Halofuginone (Stenorol, Intervet*), the synthetic compound of a natural product from plant *Dichroa febrifuga Lour*, is used mainly as an anticoccidial drug in poultry. Febrifugine (the natural product) had an antimalarial and anticoccidial effect but has a narrow safety margin. Synthetic variations previously made by American Cyanamid led to halofuginone feed additive for prevention of coccidiosis and is licensed for use in broiler chickens [(9)]. It is a well-known inhibitor of collagen type I synthesis [(1)] which has been reported to inhibit the gene expression of collagen type α1 (I) in vitro and in animals. In liver, halofuginone prevented collagen type I gene expression in dimethylnitrosamine- [(10)] and thioacetamide- induced cirrhosis in rats [(2)]. Several authors studied the role of different chemicals, cytokines and herbs to treat or prevent early fibrotic processes due to various causes [(11-16)]. The advanced liver fibrosis and cirrhosis had been regarded irreversible until quite lately. However, experimental and clinical studies confirmed the possibility of stopping or even decreasing the stage of liver fibrosis through causal factor elimination and application of pharmacological preparation of potential antifibrotic activity [(17)]. However, to our knowledge, no literature was reported to treat the well established schistosomal hepatic fibrosis.

The goal of this study was to investigate the possible antifibrotic role of halofuginone on

experimentally induced chronic schistosomal hepatic fibrosis in mice.

MATERIAL AND METHODS

Parasite: *Schistosoma mansoni* cercariae shed from laboratory bred *Biomphlaria alexandrina* snails were purchased from the Schistosome Biological Supply Program, Theodore Bilharz Research Institute, Imbaba, Giza.

Animals: Laboratory bred, parasite free, Swiss albino mice, 4-5 weeks old and 16 -18 gm in weight each, were used in this study. Mice were maintained with free access to commercial diet and water. Eighty mice were submitted to subcutaneous injections with a dose of 80 *Schistosoma mansoni* cercariae / mouse according to **Peters and Warren** [18]. Ten mice were used as a control non infected group.

Mice were divided into 5 groups:

Group (1): comprised 20 mice that received no treatment (infected non treated group).

Group (2): comprised 20 mice that received praziquantel (PZQ) (infected & PZQ treated group).

Group (3): comprised 20 mice that received halofuginone 3 ppm. and praziquantel (infected, PZQ & H. treated group).

Group (4): comprised 20 mice that received halofuginone 3 ppm. alone (infected & H. treated group).

Group (5): comprised 10 mice that were used as a control non infected group.

Drug regimen:

Halofuginone (Stenorol* Intervet) was administered to groups (3 & 4) as a feed additive in a dose of 3 ppm (Stenorol) for three months, which started from the 3rd month post infection (p.i) till the 6th month p. i.

Praziquantel (PZQ) was available as Biltricide (Bayer) 600 mg tablets. PZQ was given in the 12th week p.i. in a dose of 500 mg / kg / dose according to **Ismail *et al.*,** [19]**.** This dose was followed by another dose after 9 days to assure killing of all eggs in the tissues, since PZQ has the capacity to kill only mature ova [20]**.**

Ten mice of groups 1, 2, 3 and 4 were sacrificed at the end of 20th week p.i. The numbers that remained from all groups were sacrificed at the end of the 24th week p.i. The control non infected mice were also sacrificed at one time.

The liver of each mouse was taken, weighed and divided into three portions; one part was used for tissue digestion, the second was preserved in 10% formalin for histopathological examination and the third part was weighed and kept frozen at -70°C for hydroxyproline assay and collagen determination.

1-Parasitological assay:

Egg counting in liver: Fragments of liver tissue were weighed and left in 0.5% potassium hydroxide solution for digestion and counting of *S. mansoni* eggs according **to Cheever *et al.*,** [21]. The results were expressed as the number of eggs / gm liver tissue.

2- Biochemical assay;

Determination of the Collagen content of liver: Tissue hydroxyproline and total collagen contents were determined by the modified method of **Edward's and 0' Brien** [22]**.**

3- Histopathological evaluation: liver biopsies were fixed in 10% formalin and were routinely dehydrated, embedded in paraffin and cut into 4 µm-thick sections. Sections were stained with haematoxylin and eosin (H & E) for routine histopathological examination and with Masson's trichrome for collagen fibers staining. Measurement of the size of granulomas was done in H & E sections by the use of ocular micrometer lens fitted on a light microscope. The mean diameter of each granuloma was obtained by measuring two perpendicular diameters. For each section, ten granulomas were measured and the mean diameter of all lesions was then calculated using the method described by **Jacobs *et al.*,**[23].

Statistical analysis: was done by using SPSS [24] Version 8.0 software. ANOVA test was used to test significance between different treatments at $P \leq 0.05$.

RESULTS

Table (1): the weight of liver, number of egg / gm liver and the size of granuloma among the different groups (mean ± SD):

GROUP	NUMBER OF MICE		WEIGHT OF THE LIVER (GM) (MEAN ± SD)		EGG COUNT / GM LIVER TISSUE.(MEAN ± SD)		SIZE OF GRANULOMA (μM) (MEAN ± SD)	
	20th	24th	20th week	24th week	20th week	24th week	20th week	24th week
1	10	7	1.9 ± 0.4	1.8 ± 0.3	3250.3 ± 413	3365.2 ± 356	642.9 ± 26.54	516.57 ± 17.09
2	9	8	1.3* ±0.12	1.25* ± 0.2	352.13* ± 270	249.3* ± 240	470.56* ± 25.05	390.67* ± 19.05
3	10	7	1.27* ±0.1	1.22* ± 0.15	332.67* ± 270	210.15* ± 280	391.15** ± 21.79	220.533** ± 18.71
4	9	7	1.7 ±0.3	1.6 ± 0.22	2950.5 ±391	2917.23 ±376	567.4 ± 26.54	412.32 ± 17.09
5	10	-	1.2 ±0.14		-	-	-	-

*** Significant compared to group (1& 4).**
**** Significant compared to group (1, 2 & 4).**

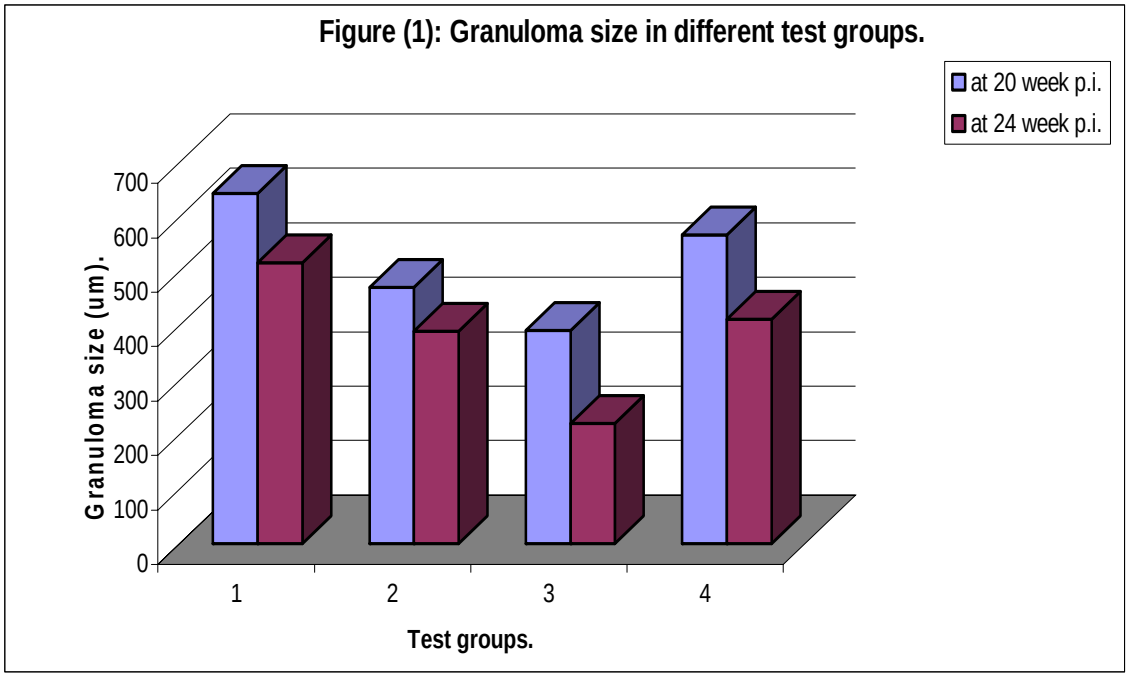
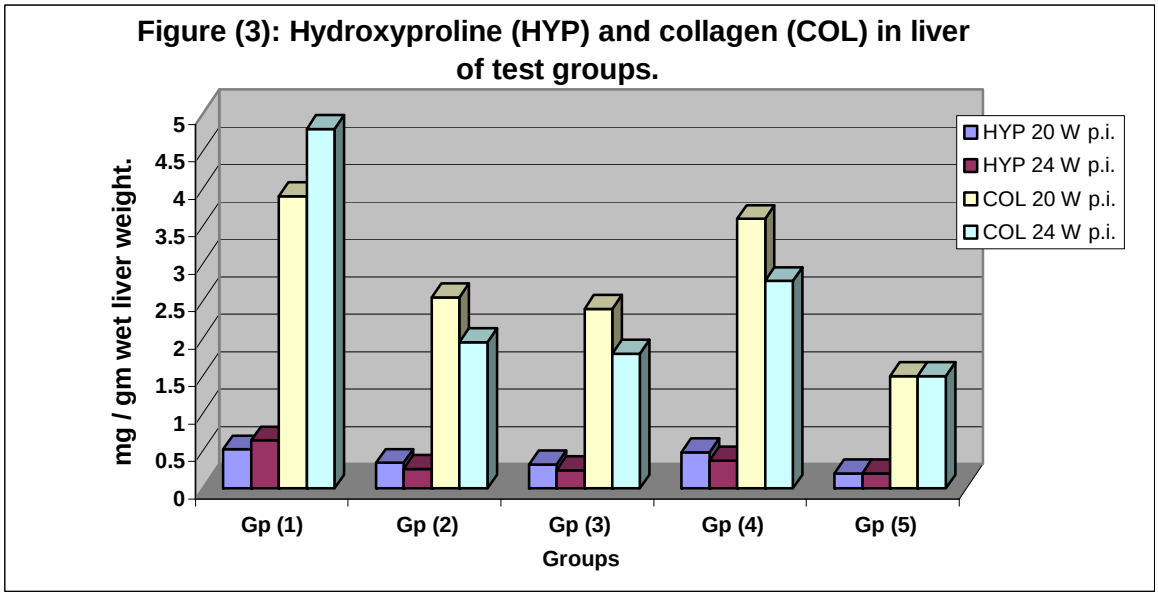


Table (2): Estimation of hydroxyproline and total collagen contents in liver of the studied groups:

Group	Liver hydroxyproline (mg/gm wet liver weight)		Calculated collagen (mg / gm wet liver weight)	
	20th week	24th week	20th week	24th week
1	0.52 ± 0.22	0.64 ± 0.18	3.9 ± 1.65	4.8 ± 1.35
2	0.34 ± 0.17*	0.26 ± 0.13*	2.55 ± 1.27*	1.95 ± 0.975*
3	0.32 ± 0.12*	0.24 ± 0.1*	2.41 ± 0.9*	1.82 ± 0.75*
4	0.48 ± 0.19	0.37 ± 0.16**	3.62 ± 1.425	2.77± 1.2**
5	0.2 ± 0.1	0.2 ± 0.1	1.5 ± 0.75	1.5 ± 0.75

* Significant in relation to groups (1 & 4)
** Significant in relation to group (1). (P ≤0.05).



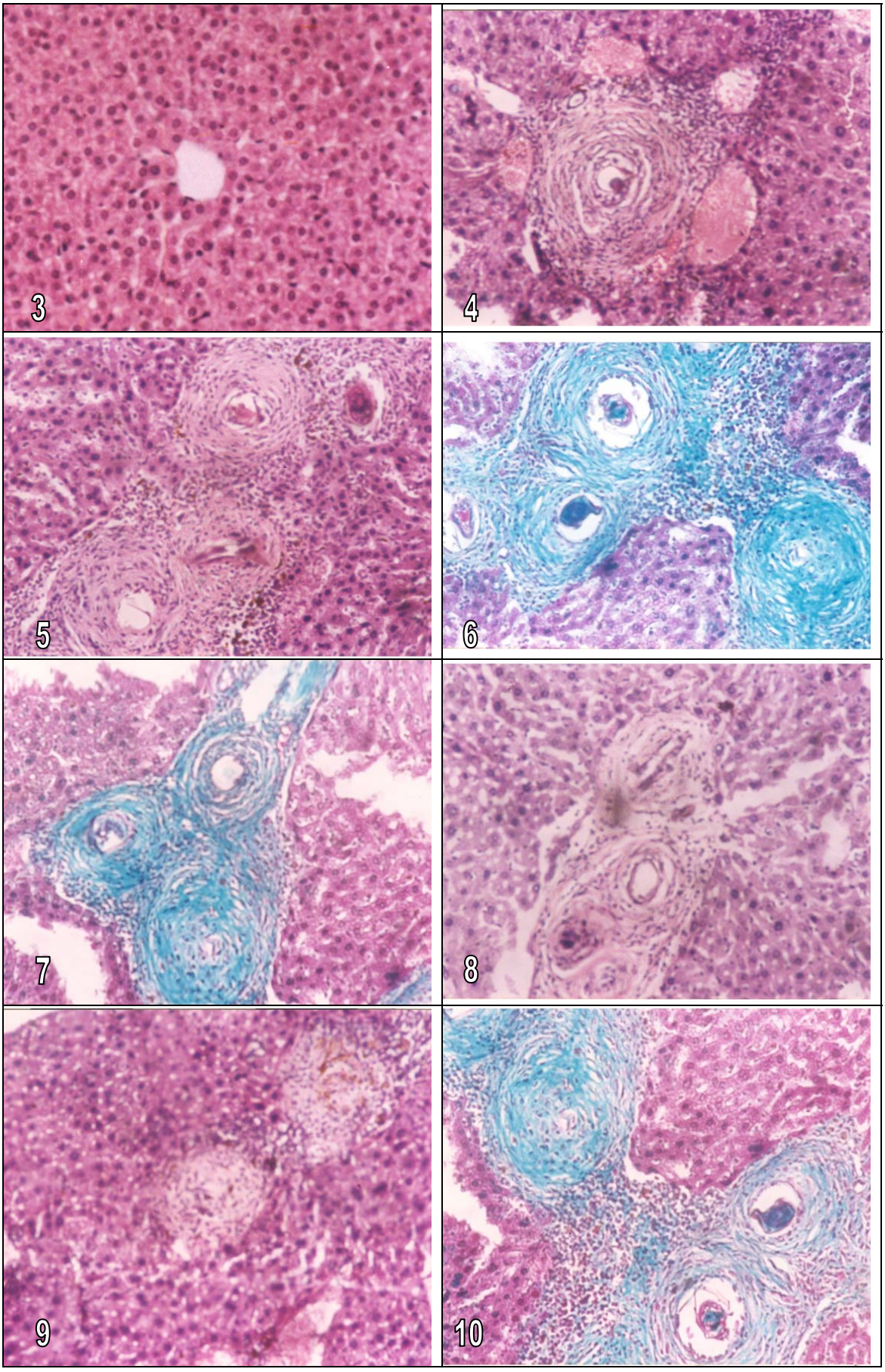
The results of the parasitological assay of antifibrotic efficacy of *halofuginone* including liver weight and egg count /gm liver tissue were presented in table (1). The size of granulomas was presented in table (1) and figure (1). The results of biochemical assay of hepatic hydroxyproline and collagen contents were presented in table (2) and figure (2).

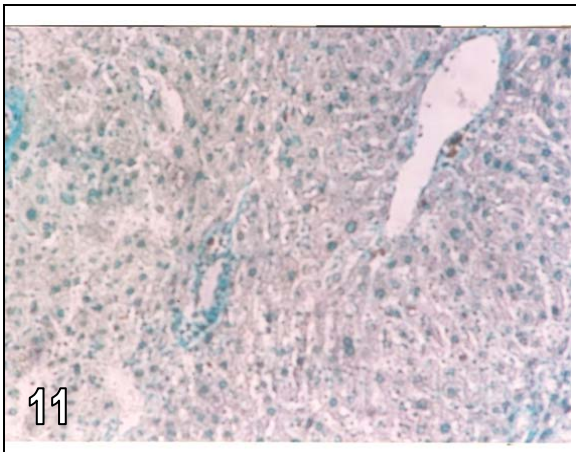
Histopathological results:

As regards to the histopathological findings, liver sections of the control non infected mice showed normal histological architecture (fig 3). In group (1) (control infected), mice sacrificed at the 20th week p.i. showed numerous eggs surrounded by granulomas composed of lymphocytes, macrophages, neutrophils, eosinophils and fibroblasts (Fig 4). In mice sacrificed at the 24th week p.i., the granulomas decreased in size and became less cellular (Fig 5) and the collagen fibers appeared as abundant fibers oriented in various directions in some granulomas, while in others showed progressive accumulation and concentric orientation (fig 6 & 7).

In group (2) (infected and PZQ treated), at the 20th week p.i., liver sections showed few eggs surrounded by granulomas formed of lymphocytes, macrophages, neutrophils, eosinophils and fibroblasts, the demarcation between the granuloma periphery and the liver parenchyma was sharp (Fig 8). While, at 24th week p.i. the granulomas decreased in size with poor demarcation between them and the liver parenchyma (fig 9). The collagen fibers in the granulomas appeared fragmented and dispersed into a loose amorphous and abundant matrix (Fig 10 & 11).

LIST OF FIGURES:

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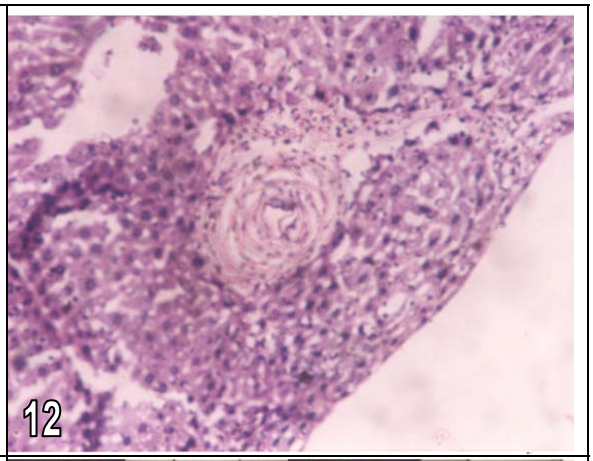
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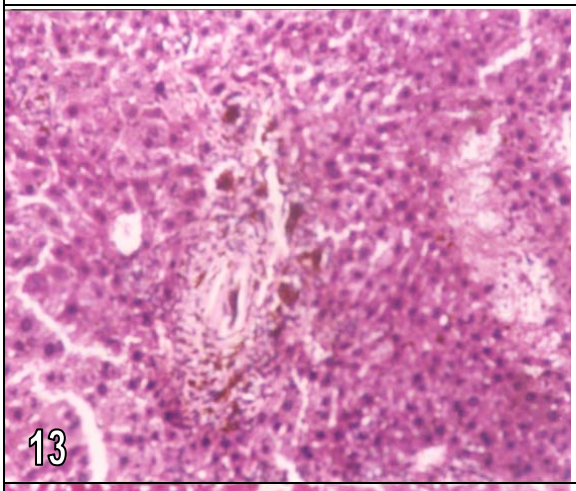
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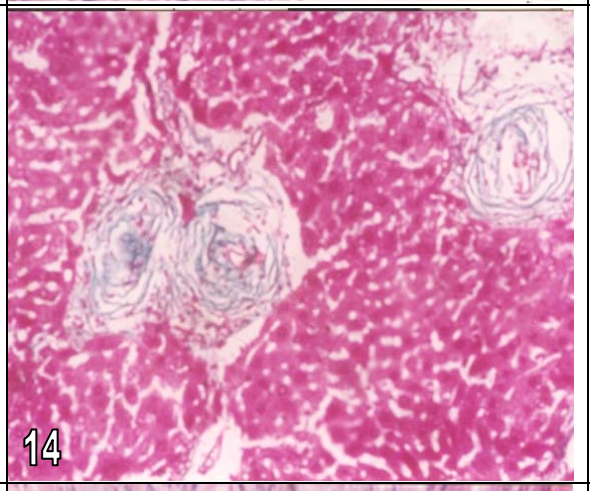
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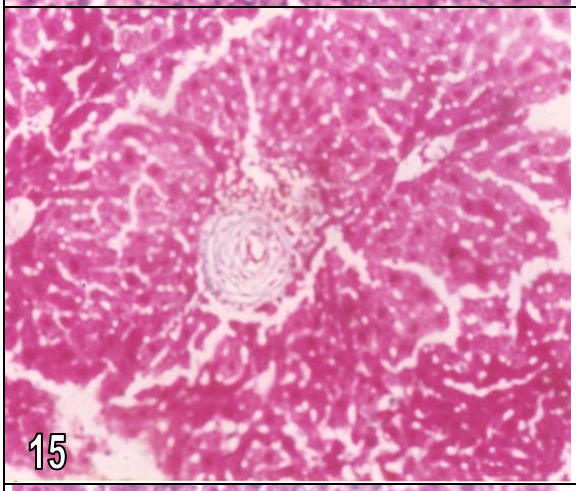
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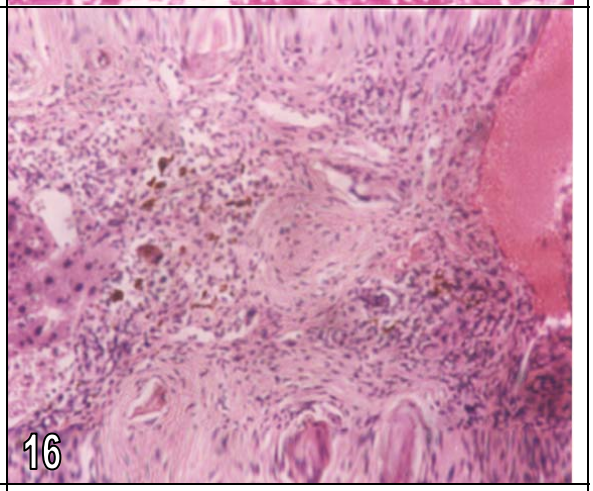
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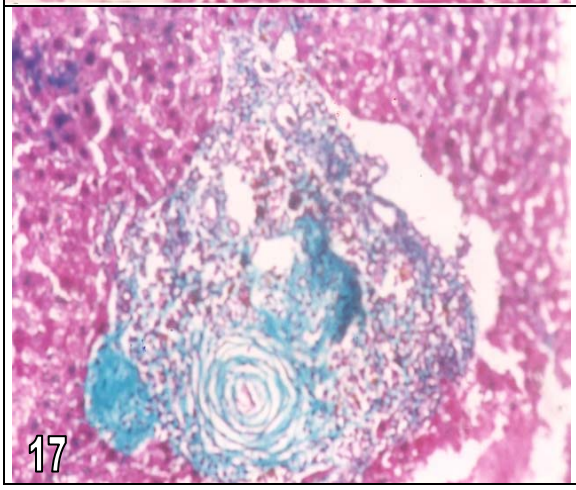
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FIG.3: Liver section of a control non infected mouse (group 5) showing normal hepatic architecture (H&E, X63).
FIG.4: Liver section of a group (1) mouse at the 20th week p.i., showing schistosomal granuloma formed of ova surrounded by lymphocytes, macrophages, neutrophils, eosinophils and fibroblasts (H&E, X63).
FIG 5: Liver section of a group (1) mouse at the 24th week p.i., showing multiple fibrocellular granulomas around ova (H&E, X63)
FIG 6: Liver section of a group (1) mouse at the 20th week p.i., showing abundant collagen fibres with concentric orientation around ova (Masson's trichrome, X63).
FIG 7: Liver section of a group (1) mouse at the 24th week p.i., showing multiple granulomas with less abundant collagen fibres with concentric orientation around ova (Masson's trichrome, X63).
FIG 8: Liver section of a group (2) mouse at the 20th week p.i., showing few ill developed granulomas (H&E, X63).
FIG 9: Liver section of a group (2) mouse at the 24th week p.i., showing small sized and degenerating granulomas with mild mononuclear cell infiltration (H&E, X63).
FIG 10: Liver section of a group (2) mouse at the 20th week p.i., showing moderate amount of dispersed collagen fibres around ova (Masson's trichrome, X63).
FIG 11: Liver section of a group (2) mouse at the 24th week p.i., showing replacement of the granuloma by focally fragmented collagen fibres (Masson's trichrome, X63).
FIG 12: Liver section of a mouse in group (3) at 20th week p.i., showing one small granuloma surrounded with mononuclear leucocytic infiltration (H&E, X63).
FIG 13: liver section of a mouse in group (3) at 24th week p.i., showing small regressing granulomas with bilharzial pigment around (H&E, X63).
FIG 14: Liver section of a mouse in group (3) at 20th week p.i., showing small granulomas with scanty, fragmented collagen fibers (Masson's trichrome, X63).
FIG 15: Liver section of a mouse in group (3) at 24th week p.i., showing very small granuloma with very scanty collagen fibers (Masson's trichrome, X63).
FIG 16: Liver section of a mouse in group (4) at 20th week p.i., showing multiple granulomas surrounded by collagen fibers (H&E, X63).
FIG 17: Liver section of a mouse in group (4) at 24th week p.i., showing moderate amount of collagen fibres (Masson's trichrome, X63).

In group (3) (infected and PZQ & *halofuginone* treated), at the 20th week p.i., liver sections showed few granulomas which were small sized (fig 12). While mice sacrificed at 24th week p.i. showed limited number of eggs surrounded by very small sized granulomas and the liver parenchyma appeared quite normal (fig 13). As regards the collagen staining in group (3), the collagen fibers were decreased and fragmented at 20th week p.i (fig 14) and appeared very scanty in small granulomas at 24th week p.i. (fig 15).

In group (4) (infected and *halofuginone* treated), multiple eggs were present surrounded by granulomas with little number of inflammatory cells (Fig 16). The collagen fibers present in the central area of granulomas appeared fragmented and moderate in amount (Fig 17).

DISCUSSION

Prolonged liver injury results in hepatocyte damage, which triggers activation of hepatic stellate cells (HSC) and recruitment of inflammatory cells into the liver. The HSC play a critical role in fibrogenesis. They produce collagen type I and secrete pro-fibrogenic cytokines and inhibitors of matrix-degrading enzymes (tissue inhibitor of matrix metalloproteinase (MMP)), causing the production of extracellular matrix deposition over degradation. Hepatic fibrosis was historically thought to be a passive and irreversible process. However, many clinical and experimental studies suggest that this process can be reversed, including the apoptosis of activated HSC. Thus, HSC represent an appealing target for antifibrotic therapy [25].

Many lines of therapy were tried to reduce liver collagen formation, however, non was satisfactory. Steroids were used as inhibitors to prolyl hydroxylase and anti-inflammatory [26]. Praziquantel has been used as an antifibrotic drug by removing the source of antigens, but it interrupts immune modulation, hence causing poor scar absorption in the liver [20]. Colchicine is a microtubular disruptive drug but owing to its side effects, its use as a long term therapy was not widely approved [27]. Lathyrogenic agents, as β-aminopropionitrile (BAPN) or Safironil [28], which is a monoclonal antibody against transforming growth factor β, were tried with varying degrees of success.

Halofuginone has an antifibrotic effect on hepatic stellate cells (HSC) [29]. Halofuginone was found to inhibit collagen α1 (I) gene expression and collagen synthesis in animal models characterized by excessive deposition of collagen [1].

Halofuginone decreased skin collagen in a chronic graft-versus-host disease patient. In liver, halofuginone prevented collagen type I gene

expression in dimethylnitrosamine- [10] and thioacetamide- induced cirrhosis in rats [2]. When halofuginone is given to rats with established fibrosis, it caused a significant reduction in α smooth muscle actin, tissue inhibitor of metalloproteinases-2 (TIMP-2), collagen type I gene expression and collagen deposition [30]. Submicromolar concentrations of halofuginone inhibit HSC proliferation and migration and up-regulate their expression of fibrolytic matrix metalloproteinase (MMP-3 and -13) via activation of p38 mitogen-activated protein kinase (p38 MAPK) and nuclear factor kappa B (NFkappa B) [31]. However, halofuginone worsened liver fibrosis in bile duct obstructed rats [32].

The present study was planned to investigate the role of halofuginone on chronic hepatic fibrosis in *S. mansoni* infected mice.

In the current study *halofuginone* had been administered combined with PZQ and given at the 12th week p. i. As regards the parasitological assay, there was a marked reduction in liver weight in gp. (3) (*Halofuginone* + PZQ) compared with gp. (1) (infected non treated) and may reach the average normal weight six months p. i. There was a highly significant reduction in egg count in liver tissue in gp. (2) treated with PZQ which came in accordance with that of several workers who tested the effect of PZQ on murine schistosomiasis [33, 34]**.** However no reduction in egg count was noticed in gp. (4).

Concerning histopathological examination of liver sections in different groups, there was a significant reduction in granuloma size with decreased cellularity in different test groups examined at 24th week p. i., as compared with the groups examined at 20th week p. i. The decrease in granuloma size in group (1) can be explained by the natural immunomodulation of granuloma [35]. A marked reduction in granuloma size was noticed in the PZQ treated groups (PZQ alone and PZQ + *halofuginone*) which can be explained by the findings of **Cameron and Ganguli,** [36], who stated that once a mouse is cured from *Schistosoma,* granulomatous lesions in the liver shrink progressively afterwards leaving few pigmented scars. This also agrees with **Andrade and Grimaud,**[37] who found that curing schistosomal infection prevents new eggs from reaching the liver, and those that are already there remain active for a maximum of 15 days **.**

In this study, the combining anti schistosomal drug and *halofuginone* (gp. 3*)* led to the most profound reduction in granuloma size; while in Masson's trichrome stained sections, there was resolution of collagen in mice treated and sacrificed early at the 20th week and the very scanty appearance of collagen fibers in mice treated and sacrificed late at the 24th week. These results agreed with **Giboda and Smith**, [28] who stated that the combination of antischistosomal treatment and antifibrotic drug resulted in marked changes in granuloma matrix and cellular infiltrate. This also agreed with **Andrade and Grimaud,** [38] who found that both types of collagen (type I and III) could be found in granuloma making it possible to resolve with effective therapy whether early or late. In group (4) (infected and *halofuginone* treated), there was a significant decrease in the size of granuloma compared to group (1).

In the present study, biochemical estimation of total hepatic collagen content of the control infected group (gp.1), showed a significant increase in livers examined 20th week p.i than those examined 24th week p.i. In gp. (4) that received *halofuginone* alone, there was a significant decrease in hepatic collagen content as compared with gp. (1). These results were in agreement with the work done by Bruck *et al.,* [2]. In PZQ treated groups, there was a significant decrease in hepatic collagen with the marked reduction achieved in gp. (3). These results coincided with their respective parasitological and histopathological findings. **Takahashi and Koda,** [39] reported that collagen synthesis was increased in the first weeks of schistosomal infection. Some investigators reported that the highest level of total hepatic collagen was at the 8th week p. i. [40].

In conclusion, *halofuginone* seems to be effective in the control of fibrotic process in mice liver infected with *Schistosoma mansoni.* Best results were attained once combined to praziquantel. The mechanism is probably through inhibition of collagen type I synthesis by inhibiting the gene expression of collagen type α1 (I). However, these results are still experimental; and there may be a new hope for chronic liver fibrosis patients if a human standardized formula is developed. Therefore additional work is required to find whether this can be applied to human without serious adverse effects.

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الملخص العربي

تأثير إعطاء الهالوفيوجينون لعلاج التليف الكبدى فى البلهارسيا المزمنه فى الفئران الصغيره (مايس)

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أقسام الطفيليات[1] وطب المناطق الحاره[2] والباثولوجى[1] ، كلية الطب، جامعتى طنطا[1،2] و المنوفية[3]

يعتبر تليف الكبد الناجم عن البلهارسيا أهم مضاعفات العدوى بالبلهارسيا. ويتم إفراز الكولاجين عن طريق الخلايا الكبديه النجميه تحت تأثير عوامل مختلفه،ويتم تكوين الكولاجين داخل هذه الخلايا فى عدة مراحل. كانت هذه النقطة بالذات هدف الاستراتيجيات العلاجية للتليف، وقد تم إستخدام الهالوفيوجينون بنجاح فى علاج التليف الكبدى الناتج عن إعطاء رباعى كلوريد الكربون فى الفئران الكبيره وأيضا لمنع الإلتصاقات الناتجة بعد العمليات الجراحيه، لذلك فقد اجرينا البحث عن الدور المحتمل للهالوفيوجينون فى التليف الكبدي الناشىء عن البلهارسيا في حيوانات التجارب.

تم إجراء الدراسة على الفئران الصغيرة البيضاء التي تم عدواها بسركاريا البلهارسيا المعوية. وتم استخدام عشرة فئران سليمة كمجموعة قياسية غير مريضة. تم تقسيم الفئران المعديه إلى أربع مجموعات هي (المجموعة القياسية المعديه - البرازيكوانتيل - الهالوفيوجينون - البرازيكوانتيل مع الهالوفيوجينون). تم ذبح بعض الفئران عند الأسبوع العشرون من العدوى، وبعضها عند الأسبوع الرابع والعشرون. تم إجراء بعض الاختبارات النسيجية والطفيلية والكيميائية، مثل عدد البويضات في الكبد ووزن الكبد وحجم الورم الحبيبي ونسبة الكولاجين في الكبد. تبين أن أكثر هبوط في حجم الورم الحبيبي وعدد البيض ونسبة الكولاجين ظهر في المجموعة التي أخذت البرازيكوانتيل مع الهالوفيوجينون ، على حين أدى الهالوفيوجينون إلى نقص في حجم الورم الحبيبي ونسبة الهيدروكسيبرولين. ويبدو من هذا أن الهالوفيوجينون قد يكون ذا فائدة في السيطرة على عملية التليف في كبد الفئران المصابة بالبلهارسيا. وأفضل النتائج كانت عند استعماله مع برازيكوانتيل. وربما كان ذلك من خلال تأثيره على عملية تكوين الكولاجين I ، وأدى أيضا إلى ضمور التليف الموجود وذلك لوجود خلل فى عملية تكوين وامتصاص الكولاجين ، إلا أن هذه النتائج هي نتيجة دراسة حيوانات التجارب، وما زال أمامها المزيد من البحث المدقق لمعرفة ما إذا كان يمكن تطبيقها على البشر.