

# To what extent could bilharzial co-infection aggravate liver damage in chronic hepatitis C virus (HCV) patients?

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**Abstract:** Among its neighboring countries, Egypt had its higher rates of HCV infection. The relation between bilharzial co-infection and the progression of hepatitis, in HCV-infected patients, was studied in this work. 55 liver biopsies from 55 HCV-infected patients, with and without bilharzial co-infection, were used. *Histopathologically*, characteristic but not diagnostic histopathologic features of chronic HCV infection were revealed. 5 hepatocellular carcinoma (HCC) cases, with different grades of differentiation, were detected. Bilharzial co-infection was accompanied by the more progressive changes. *Depending on METAVIR*, 39 cases were classified at stages 0:3 of fibrosis, whereas 11 were at stage 4 (=cirrhosis). Collagen fibers continued to increase with the progression of the disease. *Immunohistochemically*, AFP was diagnostic in 80% of hepatocellular carcinoma (HCC), whereas only three (3/39=7.5%) cases of stages 0:3 of fibrosis were positive. *Using DNA image analysis*, most of cases (44/55=80%) were diploid and the rest (11/55=20%) were aneuploid, with high SPFs. Among the aneuploid HCC cases, one of the 3 well differentiated was tetraploid and the poorly differentiated one was multiploid; presenting a higher frequency of DNA abnormal population. Interestingly, both the two aneuploid cirrhotics and the tetraploid well differentiated HCC were belonged to patients with bilharzial co-infection. [Nature and Science 2010;8(10):62-71]. (ISSN: 1545-0740).

**Key words:** Histopathology, Human liver, Hepatocellular Carcinoma, HCV.

## 1. Introduction

Hepatitis C virus (HCV) is recognized as a major health problem. An estimated 170 million people are infected worldwide WHO, (1997). In the United States and Europe, HCV infection has been detected in 1 to 2% of the general population Alter, (1997). In Egypt, HCV antibodies were detected in 10-30% of the general population Arthur et al., 1997. Furthermore, HCV is less prevalent in countries neighboring Egypt that have similar socio-medical conditions. The importance of HCV infection lies in its ability to cause insidious and progressive liver damage in the majority of patients. 85% of HCV-infected patients will become chronically infected, usually for decades, leading to hepatic fibrosis, cirrhosis, and ultimately hepatocellular carcinoma El-Zayadi et al., 1997. How hepatitis viruses cause HCC is still not fully understood. Several studies have shown that HCC often occur within the setting of cirrhosis and rarely in the absence of it Saadah et al., 2001. The risk of developing cirrhosis appears to be related to the degree of inflammation and varies from less than 25 risky per year in those with mild disease to over 10% in patients with severe inflammation El-Serag, (2002).

Why, then, is Egypt so seriously? Previous studies have suggested that, the Egyptian HCV epidemic results came from the use of unsterile injection equipments, during mass treatment of the general population with parenteral anti-schistosomal therapy (PAT) Frank et al., 2000. The present study aimed at evaluating to what extent bilharzial co-infection could aggravate liver damage in HCV-chronically infected patients.

## 2. Material and Methods

**Patients Selection:** Individuals were interviewed according to a pre-tested questionnaire; regarding age, gender, obesity, socio-demographic data, past history of bilharzial treatment (PAT) and potential risk factors of liver morbidity. Selectively, they were chosen basing on clinical examination, doppler ultrasonography, and pre-studying tests. Clinically, they might be presented with ascities, lower limb edema, or complications associated with chronic hepatitis and/or bilharziasis. They were admitted for splenomegaly, injection of esophageal varices, aspiration of ascitis fluid, or seeking treatment of chronic hepatitis. Ultrasonographically, they may be presented with enlarged portal vein diameter (PVD), perportal fibrosis, and organomegally.

Pre-Studying Tests \*Bilharziasis: Serum

antibodies to bilharzial infection were detected using modified micro-titration technique of indirect hemagglutination test (IHAT), according to Morsy et al., 1978. Additional stool analysis was done to select *S. mansoni* infection cases.

\*HCV: Anti-HCV testing was done applying the micro-particle enzyme immunoassay (MEIA), using the Imx automated system (Abbott diagnosis, USA), following the manufacturer's instructions. To differentiate samples contained HCV-RNA from those with eradicated HCV viraemia, reverse transcription polymerase chain reaction (RT-PCR) was applied.

Sample Selection: From 55 selected patients; 55 liver biopsies were taken according to Grand and Neuberger (1999). The biopsies were immediately fixed in formal buffered saline and processed, according to Culling (1974), to prepare paraffin blocks. Section thickness and preparation depended, accordingly, on the technique used.

Methods:\* Histopathological and Histochemical Examination: Serial 5  $\mu$ m sections were cut, mounted and stained with H&E. The stained were examined microscopically and evaluated according to METAVIR (1994), for grading and staging of fibrosis. Distribution of collagen fibers were demonstrated by staining paraffin sections with Masson's trichrome stain.

\*Immunohistochemical Staining of Alpha fetoprotein (AFP): According to Osborn and Domagala (1991), peroxidase anti-peroxidase (PAP) methods; using ready to use kit, following the manufacturer's instructions, was applied.

\*DNA Image Analysis: According to Hedley et al., 1993, and corresponding to each of the 55 H&E slides, five 50  $\mu$ m paraffin sections were cut, deparaffinized, rehydrated, and the nuclear suspension was done. According to Schulte and Wittkind (1990), the air-dried prepared slides were treated for 60 minutes in 5N HCL, to hydrolyze nuclear DNA. The slides were directly transferred to freshly prepared Feulgen stain, using CAS DNA staining commercial kit (Qualitative DNA staining kit, cell analysis INC, Elmhurst, IL, USA). Then the slides were dehydrated, mounted, and covered. The stained slides were analysis with Hund CML image analysis and software (Hund H500, Wetzlar, FRG), in order to calculate DNA indices (DI) and coefficient of variation (CV) of measured peaks. For each slide, 20 lymphocytes were used as an internal diploid DNA content. Four hundred, non-overlapping

hepatocyte nuclei were then measured.

### 3. Results

Histopathologic examination of 55 liver biopsies (figures 1-8) revealed that; 50 cases had chronic hepatitis lesions (table 1), whereas the remainder 5 had HCCs, with different grades (table 2). Using METAVIR scoring system (table 2); most of the chronic hepatitis cases (39/50) were at stages 0-3, while the remaining 11 cases were at stage 4 (=cirrhosis). Increased collagen deposition; with minimal amounts in stage 1 reaching to thick fibrous bands in stage 4, was detected (figures 9&10). AFP positivity was detected in three (7.5%) of chronic hepatitis cases, 5 (45%) of the cirrhotics, and four (80%) of the HCCs (figures 11 & 12, tables 4). DNA ploidy correlated with the aggressiveness of the histopathological lesions (figures 13 & 14, table 5); diploidy was detected in 80% whereas aneuploidy presented in 20% of cases (2 cirrhotics and 5 HCCs).

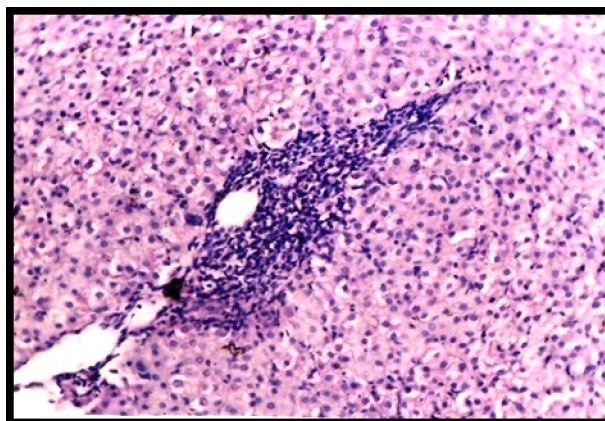


Fig (1): Chronic active hepatitis (H&E X 150)

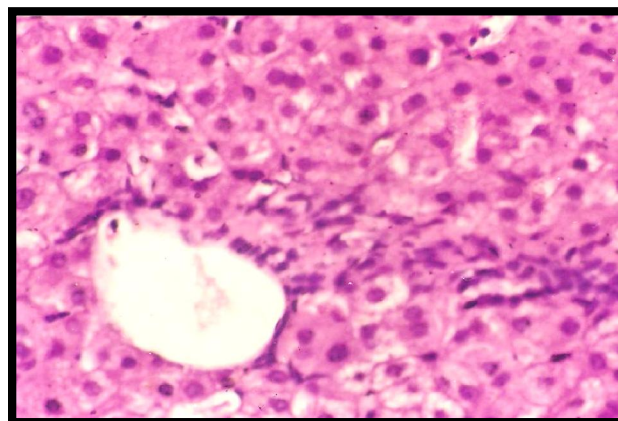
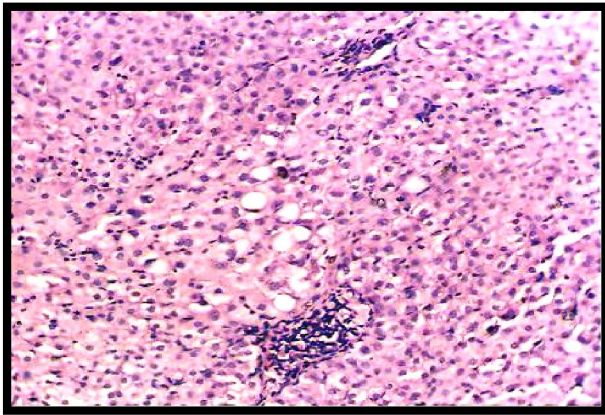
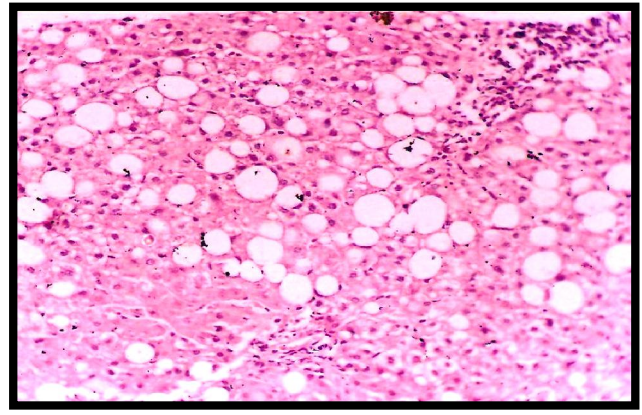


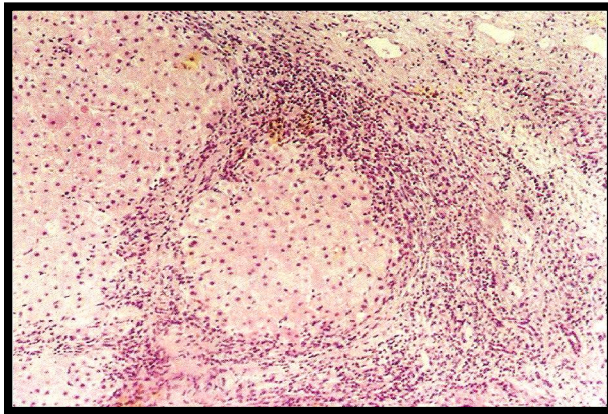
Fig (2): Double nucleation and ballooning degeneration (H&E X 400)



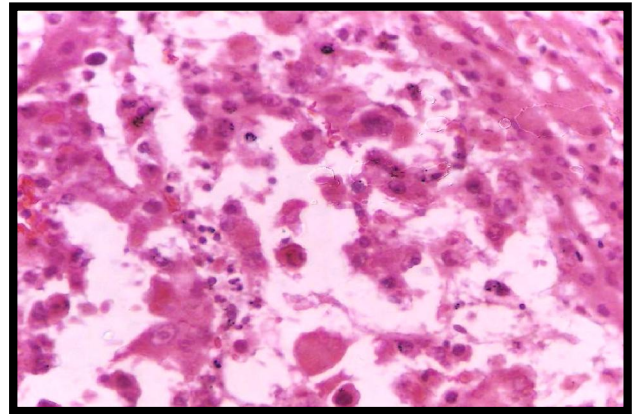
**Fig (3):** Mild steatosis (H&E X 150)



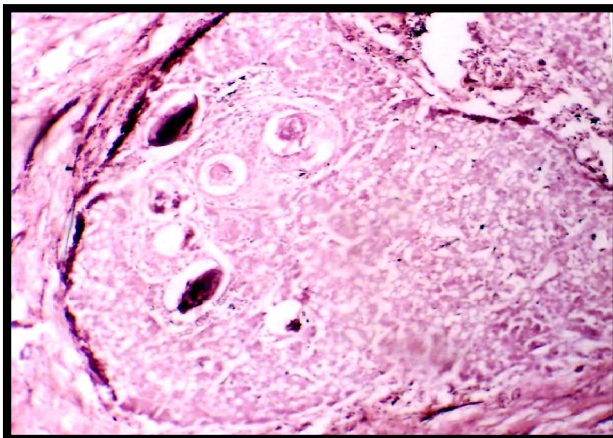
**Fig (4):** Marked steatosis (H&E X150)



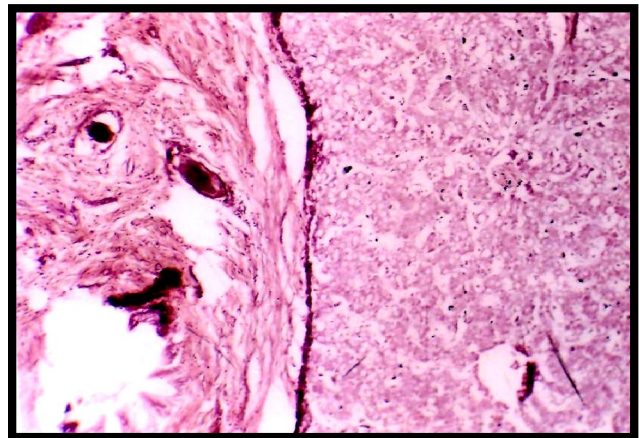
**Fig (5):** Active cirrhosis (H&E X 150)



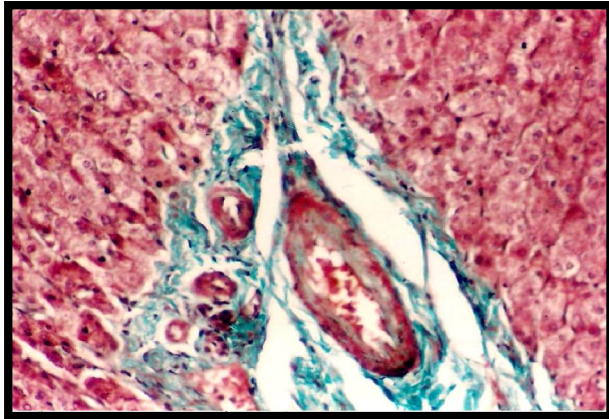
**Fig(6):** Mod. diff. HCC (H&E X 150)



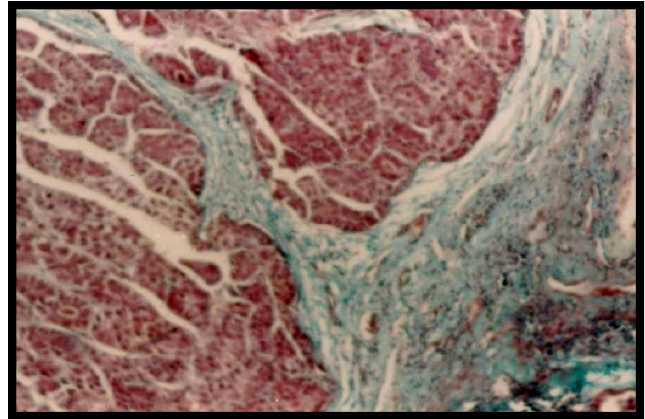
**Fig (7):** Bilharzial granuloma (H&E X 150)



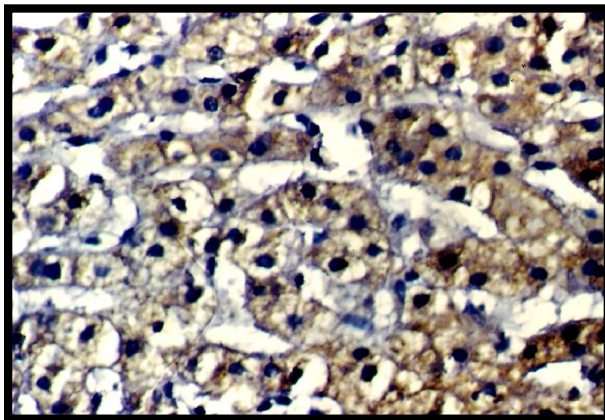
**Fig (8):** Periportal fibrosis (H&E X 150)



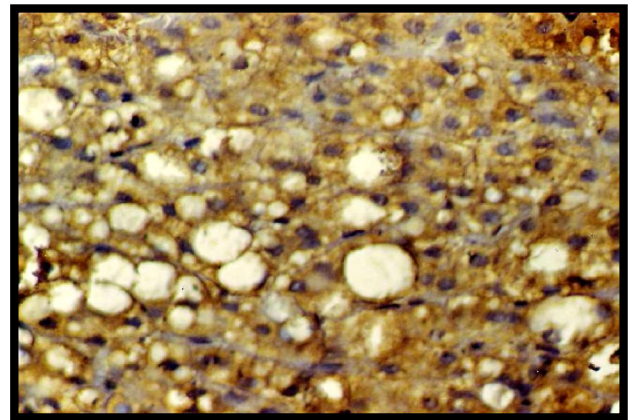
**Fig (9):** Stage 2 fibrosis (Masson's trichrome stain X 300)



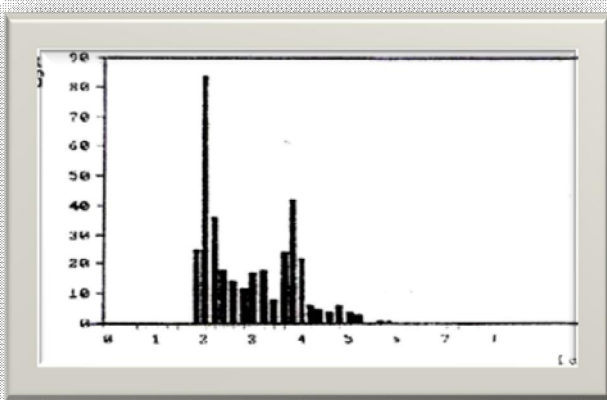
**Fig (10):** Cirrhosis (Masson's trichrome stain X 150)



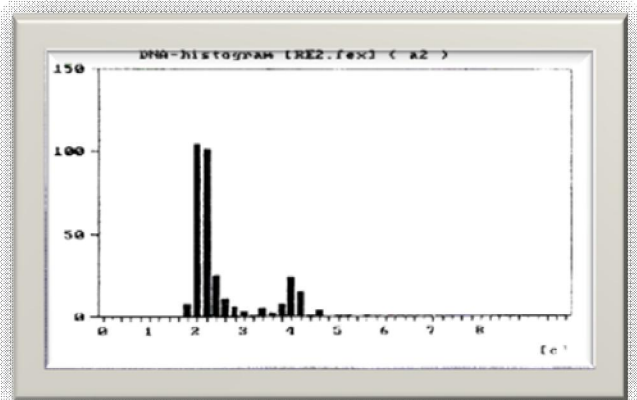
**Fig (11):** AFP Mild positive (PAP X 400)



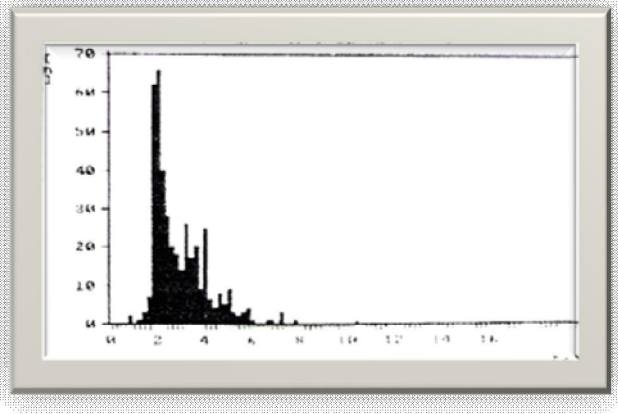
**Fig (12):** AFP Marked positive (PAP X 400)



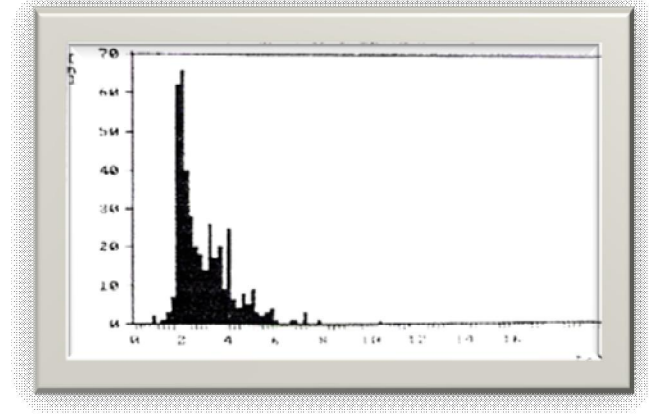
**Fig (13):** Chronic hepatitis, with diploidy (DI 1= 0.99, DI 2= 1.97, SPF= 7.11) (DNA histogram)



**Fig (14):** Marked cirrhosis, with diploidy (DI 1= 1.03, DI 2= 2.07, SPF= 10.28) (DNA histogram)



**Fig (15):** Well diff. HCC, with aneuploidy (DI 1= 1.04, DI 2= 1.61,SPF= 24.68) (DNA histogram)



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Histopathological Findings	No.	%
• Portal Inflammation	50	100
• Fatty Changes (Steatosis)	40	80
• Bile Duct Damage	12	24
• Lobular Inflammation	10	20
• Interface Activity	41	82
• Focal (Spotty) Necrosis	15	30
• Confluent Necrosis	3	6
• Bridging Necrosis	10	20
• Fibrosis, With Expansion Of The Portal Tract	13	26
• Portal Fibrosis, Without Cirrhosis	20	40
• Architectural Distribution, With Cirrhosis	11	22
• Pericellular Fibrosis	13	26
• Bilharzial Pigments	5	10
• Bilharzial Granuloma	10	20

Fibrosis (METAVIR)		No	%
Score	Description		
0	• No Fibrosis	0	0
1	• Stellate Enlargement of Portal Tract, Without Septa Formation.	6	12
2	• Enlargement of Portal Tract, With Rare Septa Formation.	13	26
3	• Numerous Septa, Without Cirrhosis.	20	40
4	• Cirrhosis.	11	22
<b>Total</b>		<b>50</b>	<b>100</b>

Grades of Differentiation		No	%
• Well Differentiated HCC.		3	60
• Moderately Different. HCC.		1	20
• Poorly Differentiated HCC.		1	20
• Total		5	100

AFP	Histopathological Changes						Total	
	Chronic Hepatitis		Cirrhosis		HCC		No	%
	No	%	No	%	No	%		
-	36	92.3	6	54.5	1	20	43	78.2
+	2	5.12	3	27.3	1	20	6	10.9
++	1	2.56	2	18.2	1	20	4	7.27
+++	-	-	-	-	2	40	2	3.63

-= no reaction,  
 += mild reaction,  
 ++ = moderate reaction, and  
 +++ = strong reaction.

DNA& Cell Cycle	Chronic Hepatitis (n = 39)	Cirrhosis (n = 11)	HCC (n = 5)	P
<u>S-Phase:</u> Range $X \pm SD$	2.20 – 10.75 7.12 $\pm$ 3.67	5.85 – 17.9 12.23 $\pm$ 5.18	21.0– 39.3 26.46 $\pm$ 9.1	0.001 S
<u>DI 1:</u> Range $X \pm SD$	0.91 – 1.08 0.99 $\pm$ 0.08	0.95 – 1.07 1.03 $\pm$ 0.03	0.99-1.08 1.035 $\pm$ 0.4	0.093 NS
<u>DI 2:</u> Range $X \pm SD$	1.91 – 2.08 1.98 $\pm$ 0.07	1.67 – 2.09 2.01 $\pm$ 0.11	1.23-2.32 1.59 $\pm$ 0.29	0.0001 S
<u>Proliferation:</u> Range $X \pm SD$	17.9 – 33.80 24.37 $\pm$ 4.36	27.5 – 46.6 36.14 $\pm$ 6.28	53.5-74.9 64.23 $\pm$ 7.1	0.0001 S

DNA:	No	%	No	%	No	%	0.004 S
	Diploidy	39	100	9	81.8	0	
Aneuploidy	0	0	9	18.2	5	100	

NS = Non significant,

S = Significant, and

HS = Highly significant.

#### 4. Discussion

In parallel with the advance of hepatitis, wide range of inflammatory and degenerative changes, was presented in various patterns; either confined to portal tracts, forming lymphoid aggregates around the damaged bile ducts and encroached on the portal blood vessels, or involving progression of fibrosis and the response to antiviral therapy *Oben and Paulon, (2007)*. Spotty necrosis and apoptosis were observed in all cases with activity, whereas confluent and bridging necrosis were observed in 6% and 20% of cases, respectively. These considered worrisome for progression piecemeal necrosis (interface hepatitis). Lobular necrosis and / or lobular inflammation were observed in some cases. This agreed with *Jarmay et al., 1998*. The high percentage (80%) and haphazard distribution of steatosis agreed with *Wong et al., 1996* and *Jarmay et al., 1998*. In contrast, it was observed only in 9.2% of Chinese cases *Ren et al., 2000*. The presence of steatosis in chronic hepatitis HCV infected patients appeared to modulate the of chronic hepatitis; supporting the relationship between HCV and hepatic inflammation and damage, in agreement with *Poynard et al., 1997*, *Brunt (2000)*, and *Mangoud et al., 2004*.

As considered as the end result of the ongoing liver injury and cell death *Saadah and Younosis, (2001)*, fibrosis was existed in different degrees. Portal fibrosis and periportal fibrosis with fibrous tissue septa, without cirrhosis, were observed in 40% of cases. The fibrous tissue extended into adjacent liver parenchyma and connected portal tracts with central vein (in various arrangements) causing architectural distortion, with cirrhosis, in 26% of cases. In other words, and according to METAVIR, few cases were in stage 1 fibrosis, while most of patients were in stage 2, 3, and 4. These agreed with *Pybus et al., 2003* and *El-Zayadi (2004)*, and disagreed with *Shiha and Zalata (2002)*. These lesions of chronic persistent hepatitis were not necessarily benign lesions, as individuals with an initial biopsy can

progress to cirrhosis *Yano et al., 1996*. For bilharzial cases, bilharzial pigments and ova were observed in 5 cases, while the granulomatous reactions were seen in 10 cases. Any other characteristic histopathological changes of bilharziasis were thought to be masked by bridging fibrosis, distributed architecture and the starling cirrhotic regenerating nodules, in agreement with *Mangoud et al., 2004*.

In agreement with those of *Zaki et al., 2003*, *Mahrous et al., 2004*, and *Mangoud et al., 2000* when they studied similar Egyptian population, the present study supported the rapid progression course of HCV infection in the presence of bilharzial infection. As a result, cirrhosis develops rapidly with aggressive course. In contrast, *Blanton et al., 2006* found that a minority of individuals, infected with bilharziasis, developed hepatic fibrosis. *Habib et al., 2001* stated that the association between HCV and bilharzial infections may be due to prior anti-schistosomal injection treatment and not a previous or recent schistosomal infection. *Kamel et al., 1994* could not find an association between anti-HCV positivity and the presence and number of *S mansoni* ova in the stools or with ultrasound-defined schistosomal hepatic fibrosis in a rural community, highly endemic for *S mansoni*.

Several lines of evidence indicated a strong causal association between HCV and HCC *Beretta, (2008)*. In addition, *DeMetri et al., 1995* found the role of prolonged liver necrosis and regeneration leading to cirrhosis seemed to be the most important condition for HCC development in HCV infected patients. In the present study, 5 cases were diagnosed as HCC, in agreement with *Colombo et al., 198*), *Kalamani et al., 1991*, and *Tanaka et al., 2008*. Consequently, *Kamel et al., 2000* concluded that, in Egypt, HCV patients with bilharzial co-infection were characterized with more advanced liver diseases and higher incidence of cirrhosis and HCC.

*PAP reaction against AFP:* In phase of liver cell regeneration (as observed after hepatitis) AFP production was slightly enhanced. The increase was correlated with growth and cell multiplication of hepatocytes *Tsai et al., 1994*. In this study, AFP was

useful in differentiating between the benign and malignant liver biopsies, in agreement with *Furui et al., 1995* and. It was absent (i.e. negative) in most of the chronic HCV cases (84%) in comparison with one (20%) of the HCC cases. The poorly and moderately differentiated HCCs revealed high expression than the well differentiated did. In accordance with study of *Tsuji et al., 1999*, AFP was not useful in differentiating between atypical hyperplasia and well differentiated HCC, while it was well expressed in moderately and poorly differentiated HCCs.

**DNA Image Analysis :** In normal liver, most hepatocytes are in the resting (G0), but in setting of chronic hepatitis, cirrhosis and HCC, there was an increase in hepatocellular proliferation *Look et al., 1995*. Several factors; including respond to cell injury, loss of intact reticulin framework, altered intra-hepatic microvasculature, and abnormalities in intercellular communications, were thought be causative agent for this proliferation *El-Sobky et al., 2004* and *Akkiz, (2008)*.

However, to our knowledge, only few reports have been published on hepatocyte DNA content and their analysis in schistosomiasis. When comparing the percentages of SPF in biopsies taken from those with only HCV infection and those with concomitant bilharziasis, without separation between cancerous and non-cancerous, it was  $14.6 \pm 3.95\%$  and  $22.72 \pm 7.3\%$ , respectively, with highly significant difference. These results agreed with those reported by *Schmid et al., 1995* and *Topi et al., 1991* as the cirrhotic liver cells showed impaired balance between factors of proliferation and inhibition activity. Comparatively, and as an adjunctive tool for the evolution of liver biopsy material, that potentially difficult to interpret, image analysis was useful. The latter authors concluded that; foci of morphological atypical hepatocytes found in liver with cirrhosis may contain cells with a distinct DNA aneuploid peak detectable by image cytometry. Foci with higher grade dysplasia had a higher frequency of aneuploidy compared with foci of low grade dysplasia, although this tendency was not statistically significantly. These findings supported the recognition of liver cell dysplasia as a morphologic entity; containing an aneuploid subpopulation. Such this lesion may be a precursor for the development of HCC *Bakaa et al., 2002* and *Ann et al., 1997*.

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