Determination of serum fibrosis index in patients with chronic hepatitis and its relationship to histological activity index

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ABSTRACT

الأهداف: لدراسة مدى إمكانية الإعتماد على مصل الهيالويرونيك (HA) كطريقة غير بضعية لتشخيص مرض الإلتهاب الكبدي وعلاقتة بالتغيرات الهستوباثولوجية بالكبد.

الطريقة: شمل البحث 48 مريض يعانون من مشاكل مزمنة في الكبد تم اختيارهم لهذه الدراسة من قسم أمراض الأطفال في كلاً من مستشفى الجدعاني و مستشفى الحياة – جدة – المملكة العربية السعودية، خلال الفترة مابين نوفمبر 2005م وحتى مارس 2008م. بلغ عدد الأطفال المصابين بالتهاب الكبد المزمن (B) واحد وعشرون طفلاً، 17 طفل مصاب بالتهاب الكبد المزمن (C) و 10 أطفال مصابين بالتهاب كبدي مناعي، بالإضافة إلى 25 طفل من الأصحاء كمجموعة ضابطة. تم قياس نسبة حمض الهيالويرونيك (HA) ووظائف الكبد لجميع الأطفال. كما تم إجراء دراسة هستوباثولوجية لعينات أخذت من الكبد لمجموعة الأطفال المرضى.

النتائج: كان هناك ارتفاع ذو دلالة إحصائية في مستوي حمض الهيالويرونيك (HA) في دم لدى مرضي الالتهاب الكبدي المزمن عند مقارنتهم بالأصحاء. كانت مستويات حمض الهيالويرونيك لدى المرضى المصابين بالتهاب الكبد المزمن C و B، التهاب الكبد المناعي ومجموعة التحكم على التوالي:
33.96، 112.30، 113.05، 111.22 mg/L
كما كان هناك ارتفاع ذو دلالة إحصائية في حمض الهيالويرونيك (HA) لدى مرضى الالتهاب الكبدي المزمن المصحوب بتليف من الدرجة (2 و 3) (درجة تليف 0) (p=0.0054, p=0.0013,) (p=0.0029). كما ارتبط مستوى حمض الهيالويرونيك (HA) بشكل إيجابي مع درجة تليف الكبد حيث كان هناك زيادة ذات دلالة إحصائية مع تليف الكبد من الدرجة (3) 157.96mg/L بالمقارنة مع التليف من الدرجة (2) 122.13mg/L (p=0.0013).

خاتمة: يرتفع حمض الهيالويرونيك (HA) في الدم لدى مرضي الالتهاب الكبدي المزمن ويرتبط مع درجة التليف. كما إن مستويات حمض الهيالويرونيك (HA) يمكن أن تستخدم لتشخيص ومتابعة تليف الكبد في مرضى الالتهاب الكبدي المزمن.

Objectives: To study the reliability of serum hyaluronic acid (HA) as a non-invasive method for the diagnosis of liver fibrosis and its relationship to liver biopsy findings.

Methods. In a prospective controlled clinical trial, 48 patients with chronic liver disease were selected from Pediatric Departments, Al-Jedaany and Al-Hayat Hospitals, Jeddah, Kingdom of Saudi Arabia, from November 2005 to March 2008. Twenty-one with chronic hepatitis B infection, 17 with chronic hepatitis C infection, and 10 with autoimmune hepatitis in addition to 25 healthy controls. Serum HA and liver function tests were carried out for the studied cases. The value of HA was correlated with the histopathologic findings of liver biopsy in chronic hepatitis patients.

Results. Serum HA increased significantly in chronic hepatitis cases compared with control. The mean serum HA was 111.22 mg/L for patients with chronic hepatitis B, 113.05 mg/L for hepatitis C, 112.30 mg/L for autoimmune hepatitis, and 33.96 mg/L for control group. Serum HA significantly increased in chronic hepatitis patients with stage 2 (p=0.0029) and stage 3 (p=0.0013) fibrosis compared with stage 0 (p=0.0054) fibrosis. Serum HA positively correlated with the degree of liver fibrosis, it increased significantly with stage 3 fibrosis (157.96 mg/L) compared with stage 2 fibrosis (122.13 mg/L) (p=0.0013).

Conclusion. Serum HA increased in chronic hepatitis, and its level correlates with the degree of fibrosis. Serum HA levels can be used for diagnosis and followed up of liver fibrosis in patients with chronic hepatitis.

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Patients with chronic liver disease are often asymptomatic with few clinical signs. Recognition of the fibrosis or cirrhosis is difficult without liver biopsy. Also, accurate detection of stage of the disease is important and may need multiple liver biopsies.[1] As a response to injuries, an extracellular matrix (ECM) represents a group of macromolecules, including collagens, non-collagen glycoproteins, matrix bound growth factors, glycosaminoglycans, proteoglycans, and matrix proteins are synthesized,[2] followed by remodeling and ultimately fibrosis. Although, fibrosis in the liver may be a progressive process leading to cirrhosis, one may speculate that fibrosis is also a potentially reversible process in its early stages.[3]

The assessment of fibrosis in hepatic diseases can be used, not only for the diagnosis, but also for selecting the therapeutic options and follow up of the cases.[4] Hyaluronic acid (HA), a high molecular weight glycosaminoglycan, is present in most tissues as a component of the ECM. Circulating HA is rapidly eliminated, mainly in hepatic sinusoidal, endothelial cells by way of HA receptors. The half-life of HA in circulation is normally 2-5 minutes. Therefore, the concentration of HA in the serum could mirror both the change in tissue outflow and impairment in HA catabolism in the liver. Both content and the synthesis of HA, are increased in fibrotic liver.[5]

Non-invasive evaluation of liver fibrosis is of great clinical interest. Many parameters for non-invasive diagnosis of liver fibrosis have been studied extensively earlier, but none have yet replaced liver biopsy as the gold standard.[6] The present study was conducted to investigate the reliability of serum HA as a non-invasive method for the diagnosis of liver fibrosis, through its correlation with the fibrosis score of liver biopsies in patients with chronic hepatitis.

Methods. Forty-eight children with chronic hepatitis were selected from Pediatric Departments, Al-Jedaany and Al-Hayat Hospitals, Jeddah, Kingdom of Saudi Arabia from November 2005 to March 2008. In addition, 25 healthy children of the same age and gender served as controls. The age of children ranged from 4-15 years. Children who had acute hepatitis or other causes of chronic liver diseases were excluded from the study. The patients were classified into 3 groups: Group I, included 21 cases with chronic hepatitis B infection (positive for HBsAg); Group II, included 17 cases with chronic hepatitis C infection (positive for HCV Ab), and Group III, included 10 cases with chronic autoimmune hepatitis (positive for ANA and ↑ γ globulin). They were aged and gender-matched with 25 healthy control children.

Our study was approved by the clinical research committee of the hospital and performed according to ethical procedures. A written consent was obtained from parents for the participation in our study. All cases were subjected to full history taking, and thorough clinical examination. Serum HA, bilirubin, alanine transferase (ALT), aspartate transaminase (AST), serum albumin, and prothrombin time were carried out for both patients and control groups. Also, liver biopsies was carried out for studied patients.

Needle liver biopsy was carried out. The patients were classified according to the presence of fibrosis and we used Knodell's histological activity index for staging the liver biopsy[7] (**Table 1**). For fibrosis staging, we included an adequately sized biopsy and an impeccable connective tissue stain as recommended.[8] The size of the sample was 20 mm long, 14 mm wide, and containing 11-15 completed portal tract (the concept of minimum number of complete portal tracts).[9]

The biopsies were scored in blinded way using the same scoring system as outlined above for the prediction. The histopathologist was unaware of the clinician's prediction. The histopathologist had an access to the slides stained by Hematoxylin and Eosin and Masson's trichrome for fibrosis. Regarding the light microscopic study, liver specimens were fixed in neutral buffered formaline solution, then washed and dehydrated in ascending grades of alcohol. Clearance with xylene was made. A 6 µm thick paraffine sections were made, and stained by Hematoxylin-Eosin staining.[10] Other sections were stained with Masson's trichrome staining for collagen.[11]

Transmission Electron Microscopic Study.[12] The small specimens of liver were fixed in a mixture of 2,5% glutaraldehyde in (0.2 M) cacodylate buffer (pH. 7.4) for 24 hours, then washed in 2 changes cacodylate buffer, then post fixed for 2 hours in osmium tetroxide; specimens were dehydrated in ascending grade of ethyl alcohol and embedded in Epon 812. The semithin section (1 um) was cut by ultramicrotome and stained with toluidine blue and examined by light microscope to show the tissue and for good selection and localization of the needed part to be examined in thin section. The ultrathin sections (100 mm) were prepared and stained

Table 1 - Knodell's histological activity index for staging.[7]

Knodell's scoring	Components
Stage 0	No fibrosis
Stage 1	Periportal fibrous expansion
Stage 2	Porto-portal fibrosis
Stage 3	Porto-central fibrosis (Bridging with distortion)
Stage 4	Cirrhosis

with uranyl acetate and lead citrate and examined by JEOL model 100 CX transmission electron microscope

(Jeol model 100 CX Electron microscope, Ltd Company, Tokyo, Japan).

Measurement of serum HA concentration. The blood sample was drawn from the patients after an overnight fasting (fluid and sugar are allowed), and the serum was stored at -20°C until assayed. The serum was thawed before starting assay procedure. The dye solution was prepared by dissolving Alcan blue dye (8 GX purchased from Sigma,Sigma-Aldrich, St. Louis, USA), in 0.5 mol/L sodium acetate to produce a final concentration of 1.4 mg/ml. The dye solution was prepared shortly before it was uses. Sodium acetate was chosen after testing a variety of solvents, which permit the solubilization of both dye, and dye H complexes as well as causes no interference in complex formation. A volume of 0.1 ml of the sample and of standard 125.5 mg of HA was pipette into one of series of test tubes. In each tube, 1.2 ml of the dye solution was added and contents were thoroughly mixed. A tube of 0.1 ml of water was added, serves as a blank. After 10 minutes of incubation at room temperature, the absorbent at 480 nm of each tube was measured against the blank in cuvettes with 10 mm light patch, and a capacity of 1 ml with a spectrophotometer (RA-50 Bayer, Germany). The absorbencies of the standards were plotted against their concentrations, and the HA content of each sample tube was then estimated by comparison to the resulting standard curve. The results were calculated and expressed as mg/L.

Liver function tests was carried out on serum samples using multilayered analytical slides by (Kodak Ektachem DT60 II analyzer, New York, USA).

Descriptive statistical tests were presented as means ± standard deviations and correlation coefficient. T test was also used when appropriate. Statistical significance was defined as a p value <0.05.[13]

Results. The present study was carried out in 48 children with chronic hepatitis [27 males (56%) and 21 females (44%)]. Histopathological findings of liver biopsy using Knodell's fibrosis scoring system revealed the following: 10 patients with no fibrosis (stage 0 fibrosis), 15 patients with stage 2 fibrosis, and 23 patients with stage 3 fibrosis.

Hematoxylin-Eosin staining. A liver section of chronic hepatitis patient showing some hepatocytes was swollen and cleared (hydropic degeneration), and few were shrunken and dark with nuclear disintegration (apoptotic cells) (**Figure 1**). **Figure 2** showed marked

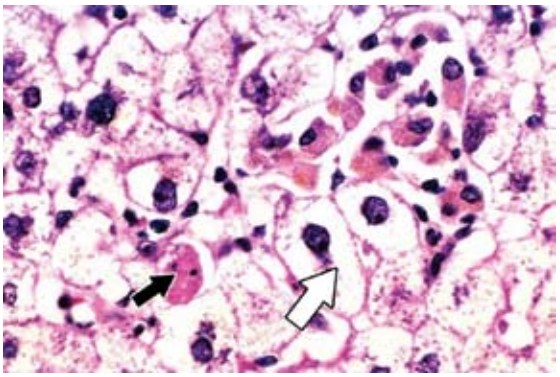

Figure 1 - A photomicrograph of a section of liver in chronic hepatitis patient showing some hepatocytes were swollen and clear (white arrow) and few were shrunken and dark with nuclear disintegration (black arrow) (hematoxylin and eosin stain x 1000)

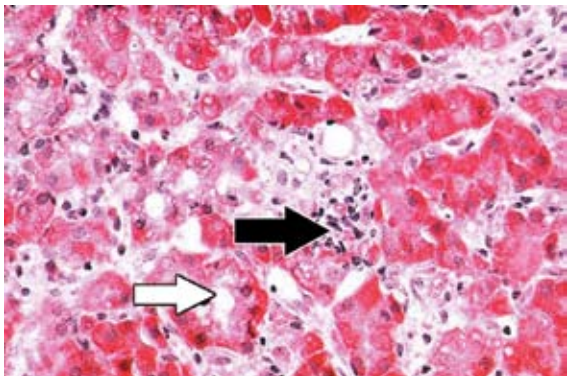

Figure 2 - A photomicrograph of a section of liver in chronic hepatitis patient showing marked degeneration and necrosis of hepatocytes in many regions of lobule (white arrow) with lymphocytes infiltration (black arrow) (hematoxylin and eosin stain x 400).

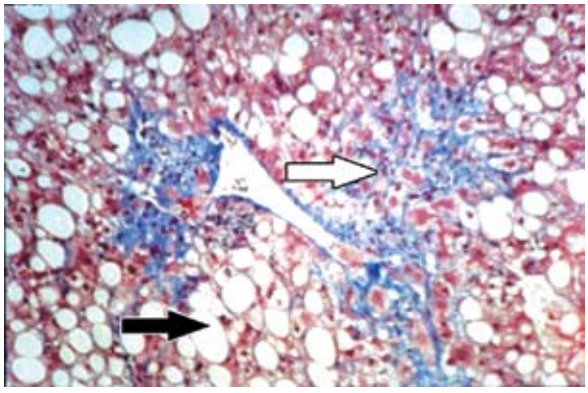

Figure 3 - A photomicrograph of a section of liver in chronic hepatitis patient with stage 0 fibrosis showing some hepatocytes were swollen and clear (black arrow) with normal amount of collagenous fibers within liver lobule that lay outside the sinusoids (white arrow) (Masson's trichrome stain X 400)

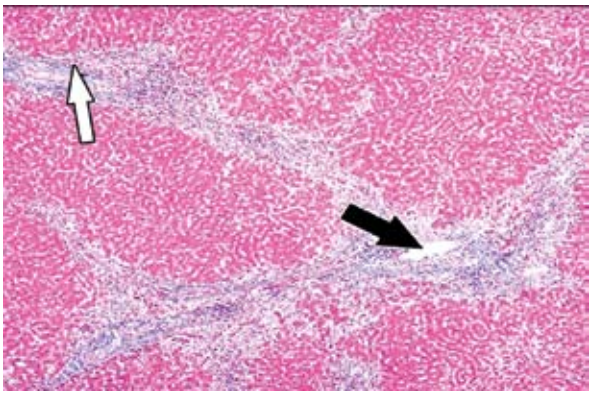

Figure 4 - A photomicrograph of a section of liver in chronic hepatitis patient with stage 2 fibrosis showing fibrous expansion of portal vein (black arrow) and increase collagenous fibers those extend from one to other portal tract (white arrow) (porto portal bridging fibrosis) (Masson's trichrome stain X 200)

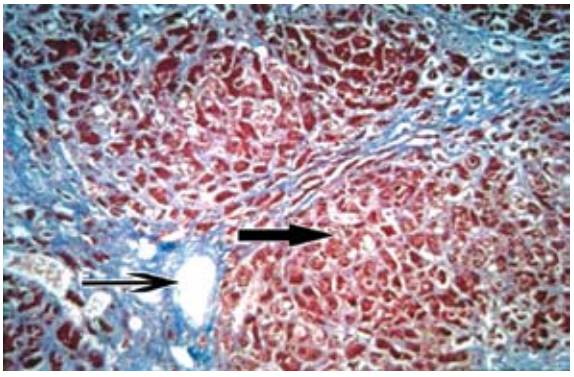

Figure 5 - A photomicrograph of a section of liver in chronic hepatitis patient with stage 3 fibrosis showing marked increase of collagenous fibers around blood vessels in portal tract and central vein (thin arrow) (porto central bridging fibrosis) and the hepatocytes showed more vacuolation in their cytoplasm and their nuclei were lysis (big arrow) (bridging necrosis) (Masson's trichrome stain x 200).

degeneration and necrosis of hepatocytes in many regions of lobule (confluences of focal necrosis) with lymphocytes infiltration.

Masson's trichrome staining. A liver section of chronic hepatitis patient with stage 0 fibrosis are shown in **Figure 3**. **Figure 4** shows a section of liver in chronic hepatitis patient with stage 2 fibrosis. **Figure 5** shows a section of liver in chronic hepatitis patient with stage 3 fibrosis

Transmission electron microscopic results. A liver section of chronic hepatitis patient showing marked dilation of cisternae of endoplasmic reticulum with large nuclei and eccentric prominent nucleoli and near the center of the nucleus, there was a large nuclear body containing electron light vesicles and massive aggregation of virus like particles, which caused marked

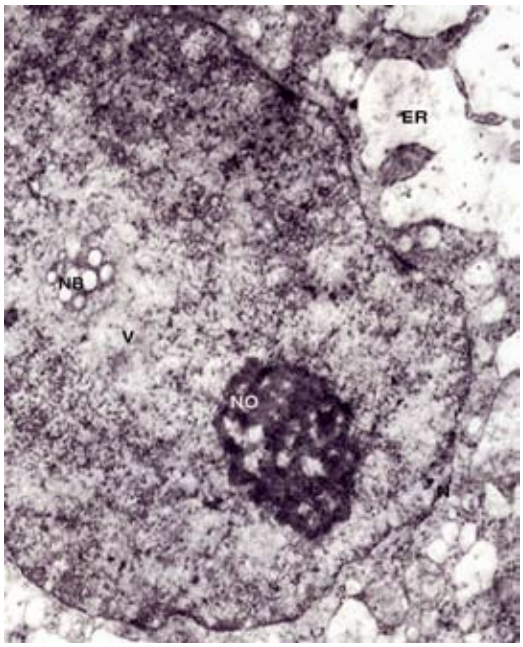

Figure 6 - Transmission electron micrograph of a section of liver in chronic hepatitis patient showing marked dilation of cisternae of endoplasmic reticulum (ER) with large nuclei (N) and eccentric prominent nucleoli (NO) and near the center of the nucleus there was a large nuclear body containing electron light vesicles (NB) and massive aggregation of virus like particles (V) which caused marked refraction of nucleoplasm especially around nuclear body x 13,500)

refraction of nucleoplasm especially around nuclear body (**Figure 6**). A large number of virus like particles in the cytoplasm, with electron dense subunits can be seen in the particles, lightly stained glycogen rosettes, and there was a mitochondrial swelling and damage (**Figure 7**). There is an increase in number and size of membrane-bound dense bodies probably lysosomes and microbodies (**Figure 8**). Serum HA was significantly increased in chronic hepatitis patients (groups 1, 2, and 3) compared with control group (p<0.001) (**Table 2**). No significant difference was noted in serum HA among the patient groups (t=1.28). **Table 3** shows the serum levels of HA among the studied groups according to the stage of fibrosis. Serum HA was significantly increased in chronic hepatitis patients with stage 2 and 3 fibrosis compared with chronic hepatitis without fibrosis (stage 0 fibrosis); p<0.001. Also, patients with stage 3 fibrosis has significantly increased serum HA compared with patients with stage 2 fibrosis; p<0.001 (**Table 3**). **Table 4** shows the mean and standard deviation of serum bilirubin, ALT, AST, albumin, and prothrombin time according to the stage of fibrosis. For all parameters there were no significant differences between stage 0 and stage 2 fibrosis, but there were significantly lower levels in stage 3 compared to stage 0 and stage 2, and

these differences were highly significant statistically. Prothrombin time was significantly prolonged in stage 3 compared with stage 2 fibrosis, but no difference was noted between stage 0 and 2 fibrosis. It is shown in **Table 5** that there was a positive correlation between serums HA and liver function tests in stage 0 and stage 2 fibrosis, but this relation becomes insignificant in stage 3 fibrosis (p>0.05).

Discussion. Chronic liver disease has been defined as a continuing inflammatory lesion of the liver, which may lead to liver cell damage and progressive accumulation of intrahepatic fibrous tissue. This may cause impairment of liver cell function, distortion of the hepatic architecture and ultimately, cirrhosis, and all its sequels[14] or continue unchanged or to subside spontaneously or with treatment.[15] In the fibrotic liver, the total collagen content in the liver increases from 3-10 fold.[16] Liver biopsy was the only way to evaluate fibrosis in the liver.[17] However, the accuracy of liver biopsy in assessing fibrosis has also been questioned to sampling errors and intra- and inter- observer variability that may

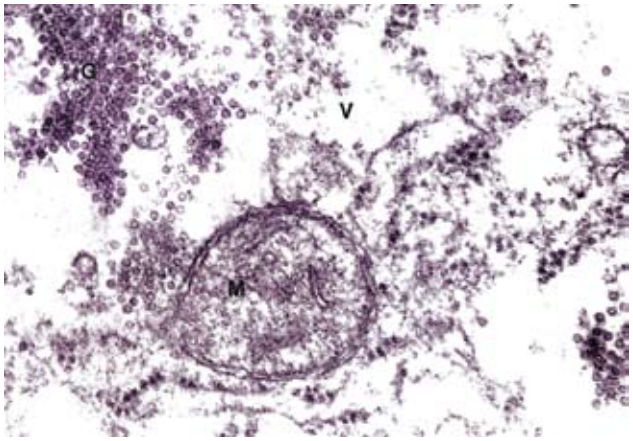

Figure 7 - Transmission electron micrograph of a section of liver in chronic hepatitis patient showing large number of virus like particles (V) in the cytoplasm, with electron dense subunits can be seen in the particles, lightly stained glycogen rossttes (G), and mitochondrial swelling and damage (M) (x 73.000)

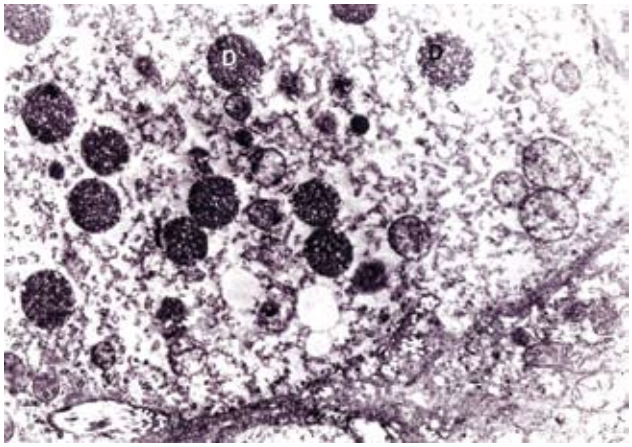

Figure 8 - Transmission electron micrograph of a section of liver in chronic hepatitis patient showing increase number and size of membrane bound dense bodies probably lysosomes and microbodies (D) (x 16,500)

Table 2 - Seum hyaluronic acid (HA) among the studied groups according to causes.

Group	N	HA (mg/L)	Test of significance	
		Mean ± SD	T	*P* value
Group I - Hepatitis B	21	111.22 ± 58.73	t1 = 5.47	0.0024*
Group II - Hepatitis C	17	113.05 ± 56.85	t2 = 6.36	0.0017†
Group III - Autoimmune hepatitis	10	112.30 ± 45.99	t3 = 4.45	0.0031‡
Group IV - Control	25	33.96 ± 13.94		
Test of significance		One way analysis of variance F"=15.27; p<0.001		

*Group I versus control, †Group II versus control, ‡Group III versus control

Table 3 - Serum Hyaluronic acid (HA) among the studied groups according to stages of fibrosis.

Stages of fibrosis		N	HA (mg/L)	Test of significance	
			Mean ± SD	T	*P* value
1	Stage 0 [no fibrosis]	10	40.44 ± 24.85	t1 = 15.84	0.0029*
2	Stage 2	15	122.13 ± 11.97	t2 = 19.54	0.0013†
3	Stage 3	23	157.96 ± 21.09	t3 = 6.83	0.0054‡
Test of significance			One way Anova F"=15.27 p<0.001		

*stage 0 versus stage 2, †stage 2 versus stage 3, ‡stage 0 versus stage 3

Table 4 - Serum bilirubin, alanine transaminase and aspartate transaminase among the studied groups according to stages of fibrosis.

Liver function tests	Stage 0 (n=10)	Stage 2 (n=15)	Stage 3 (n=23)
Serum bilirubin (mg/dl)			
Mean ± SD	1.59 ± 2.75	2.04 ± 2.88	0.71 ± 0.15
T-test	0.92	4.26	3.02
P-value	>0.05 (S0 versus S2)	<0.01 (S2 versus S3)	<0.001 (S0 versus S3)
Alanine transaminase (u/l)			
Mean ± SD	67.31 ± 86.18	98.75 ± 123.91	5.13 ± 3.88
T-test	0.22	5.09	4.26
P-value	>0.05 (S0 versus S2)	<0.01 (S2 versus S3)	<0.001 (S0 versus S3)
Aspartate transaminas (u/l)			
Mean ± SD	124.16 ± 140.56	164.00 ± 176.06	17.63 ± 8.05
T-test	0.81	24.47	33.34
P-value	>0.05 (S0 versus S2)	<0.01 (S2 versus S3)	<0.001 (S0 versus S3)
Albumin(g/l)			
Mean ± SD	3.01 ± 1.05	2.35 ± 0.81	2.0 ± 0.70
T-test	0.86	4.71	3.35
P-value	>0.05 (S0 versus S2)	<0.01 (S2 versus S3)	<0.001 (S0 versus S2)
Prothrombin time (sec)			
Mean ± SD	14.82 ± 2.51	16.9 ± 0.85	18.3 ± 2.1
T-test	0.74	6.74	3.01
P-value	>0.05 (S0 versus S2)	<0.01 (S2 versus S3)	<0.001 (S0 versus S2)

S - stage

Table 5 - Correlation of serum Hyaluronic acid (HA)with liver function tests according to stage of fibrosis.

Liver function test	Correlation (r) and *p*-value (p) of serum HA	Stage 0 (n=10)	Stage 2 (n=15)	Stage 3 n=23
Serum bilirubin (mg/dl)	r	0.77	0.82	0.36
	p	<0.01	<0.001	>0.05
Aspartate transaminase (u/l)	r	0.80	0.79	0.39
	p	<0.01	<0.001	>0.05
Alanine transaminase (u/l)	r	0.78	0.81	0.35
	p	<0.01	<0.001	>0.05
Albumin (g/l)	r	0.75	0.82	0.41
	p	<0.01	<0.01	>0.05
Prothrombin time(s)	r	0.78	0.83	0.37
	p	<0.01	<0.01	>0.05

lead to over- or under- staging.[18] Up to 20% error rate in disease staging has been reported.[19] These findings emphasize the need for accurate non-invasive methods to measure the degree of liver fibrosis. Ideal markers of fibrosis should have a higher degree of sensitivity and specificity, be easily measured and reproducible, readily available, inexpensive, and useful in accurately following the disease progression. The diagnostic panel in its current form simply provided a binary distinction between no or mild and advanced fibrosis that could provide additional and potentially important prognostic information. Reducing the requirement of liver biopsy before initiating treatment would be an important factor in improving the cost-effectiveness and risked-benefit ratio in the management of chronic hepatitis B and C patients.[20]

Hyaluronic acid, a glycosaminoglycan, is synthesized in the plasma membranes of fibroblasts and other cells.[21] The HA is catabolized locally or is carried by the lymph-to-lymph nodes, which have the capacity for its uptake and degradation. Some of the HA enters the general circulation. Hyaluronic acid in the blood is cleared predominantly by the liver, with a small proportion being cleared by the kidney and the spleen.[21] In the liver, the circulating HA is rapidly eliminated, mainly in the hepatic sinusoidal endothelial cells, by way of HA receptors. The half-life of HA in the circulation is normally 2-5 minutes. Therefore, the concentration of HA in the serum could mirror both a change in tissue outflow and an impairment in HA catabolism in the liver. Both content and the synthesis of HA are increased in the fibrotic liver.[5,22]

The present study was conducted to investigate the reliability of serum HA as a non-invasive method for the diagnosis and follow up of liver fibrosis, through its correlation with the fibrosis stage in the histological findings of liver biopsies of the studied patients. Histopathological findings in our study also revealed the presence of inflammatory cells mainly lymphocytes in the portal tract and within the parenchyma, degeneration, and necrosis of hepatocytes, which either focal or confluent according to progression of chronic hepatitis. These histopathological findings also demonstrated by David,[23] McMahon et al,[24] and Theise[25] who defined chronic liver disease as continuing inflammatory lesion of the liver and production of more inflammatory cytokines. Histopathological findings in our study showed that, liver section of patients with chronic hepatitis showed expansion of portal vein, and increased collagenous fibers that extend from one to other portal tract (Porto-portal bridging fibrosis)[stage 2 fibrosis]. Another marked increase in collagenous fibers around blood vessels in portal tract, and central vein (Porto-central bridging necrosis) [stage 3 fibrosis]. Liver fibrosis can result from any chronic liver disease lead to accumulation of ECM components in the liver due to imbalance in their production, deposition, and breakdown.[26] A total collagen content in liver increases 3-10 folds as reported by Schuppan et al.[16] The hepatic ECM components are produced by hepatic stellate cell (Ito cell) (HSC), which is responsible for both the stability of liver architecture and hepatic function.[27] The HSC activation is the main event leading to hepatic fibrosis and it implies 2 steps: initiation and perpetuation.[28] Electron microscopic finding in our study revealed that the nuclei of affected liver cells showed increase in its size with eccentric prominent nucleoli and large nuclear body, which containing electron light vesicles and massive aggregation of virus like particles caused marked rarefaction of nucleoplasm especially around nuclear body. Meuleman et al[29] reported that long term infected hepatocytes showed replication and increased in virus like particles in the nucleus and cytoplasm and accumulation of viral protein, which causes intracellular changes, damage, and massive dysfunction of hepatocytes.

Our electron microscopic findings also revealed that a large number of virus like particles in the cytoplasm, with electron dense subunits can be seen in the particles, mitochondria swelling with loss of cristae, and its granules with osmophilic lipid like substance in matrix as well as dilation of endoplasmic reticulum and increase in the number and size of membrane bound dense bodies probably lysosomes and microbodies. Mitochondrial changes were attributed to fluid accumulation in the mitochondrial matrix, so, the cristae became shortened and interrupted. Impaired mitochondrial function results in reduced oxidative phosphorylation and ATP production. This substance forms the main source of energy for cellular metabolism, many cells functions are impaired.[30] Mitochondria act as one of the favorite organelle for invading viruses and many mitochondrial protein targeted by the virus and lead to pathogenesis of the disease.[31] Hepatitis virus protein localize to mitochondria and bind to voltage dependent anion carrier (VDAC) at the permeability transition pore (PTP) of mitochondria, and leads to its opening with increased permeability of inner membrane to ions, and diffusion of solutes, then osmotic water reflux, passive swelling, and outer membrane rupture.[31] Popper[32] explained dilation of endoplasmic reticulum to cholestasis. Jaatinen et al[33] stated that the dilation of endoplasmic reticulum was due to shift of ions and water into cytoplasm. Lyosomes are concerned with storage and intracellular digestion of exogenous, endogenous materials, and release of lysosomes enzymes, which play a role during autolysis and segregation of damage portion of cytoplasm, preventing spread of damaging effect, and increase in its number and its size in viral hepatitis.[34-35]

In this study, the mean serum level of HA were 111.22±58.73 mg/L in hepatitis B group, 113.05±56.85 mg/L in hepatitis C group, 112.30±45.99 mg/L in autoimmune hepatitis group, and 33.96±13.94 mg/L in the control group. There were a highly statistical significant difference in the level of HA between the patient groups and the control, but there was no difference in the level of HA between the different causes of chronic liver diseases. This result is in agreement with the results of other studies carried out on patients with chronic liver disease, which also demonstrated that there was no difference between HA level and the etiology of chronic hepatitis.[36,37] In our study, patients were classified according to the presence of fibrosis and its stage in liver biopsy using Knodell's scoring system The previous results indicated that, Serum HA was significantly increased in chronic hepatitis patients with stage 2 and 3 fibrosis compared with chronic hepatitis without fibrosis (stage 0 fibrosis); $p<0.001$. Also, patients with stage 3 fibrosis have significantly increased serum HA level compared with patients with stage 2 fibrosis; $p<0.001$. This denotes that serum HA level increases with the progression of chronic liver disease. This finding is in accordance with by the studies carried out by many authors.[38-41] They demonstrated that serum HA was increased in patients with chronic hepatitis and that measuring serum HA is potentially useful for assessing the grading of necroinflammation, and staging of fibrosis in patients with chronic liver disease. These findings are also concordant with the Balistreri and Reg,[42] who stated that laminin (a large mosaic protein), hyaluronate, and fibronectin have been suggested as

markers of fibrosis; of these, serum levels of hyaluronate appear to have the most significant correlation with the histological stage of the disease.

Liver function tests in our study were decreased in stage 3 fibrosis. In chronic liver diseases, serum ALT and AST are usually raised, but may fluctuate spontaneously to normal levels, and do not always correlate with the clinical or histological severity and that the previously elevated aminotransferase concentrations typically decrease to the normal as hepatic parynchemal mass shrinks and biosynthetic rate is reduced.[22,42,43] Thus, their rise in stage 0 and 2 fibrosis is due to the process of chronic hepatitis is active while it seems that patients in stage 3 fibrosis have inactive hepatitis. In our study, there was significant positive correlation between serums HA and liver function tests in stage 0 and 2 fibrosis, but this relation was not significant in stage 3 fibrosis.

This result denotes that we can use serum HA level as a useful marker of liver fibrosis in patients with chronic hepatitis, and that liver function tests values were not correlated well with the stage of fibrosis as mentioned before. The explanation of presence of high content of HA in the circulation; first, due to impairment of sinusoidal endothelial cell function that occurs in chronic liver disease; second, is due to reduced functional hepatic mass. The reduction of liver mass is accompanied by reduced functional sinusoidal mass and the transformation of sinusoidal endothelial cells to vascular type endothelium, which advances together with the progression of chronic liver disease, then results in the loss of HA receptors.[44] It is known that Ito cells of the liver are responsible for HA synthesis in the liver. In liver injury, Ito cells proliferate and are transformed into myelofibroblasts. A fraction of newly synthesized HA produced in excess by increased number of transformed Ito cells in the fibrotic liver could easily escape into the blood stream through the fenestrations of the endothelial cells and through the rough junctions between endothelial cells. Therefore, this mechanism as well as impaired catabolism in the diseased liver described before, contribute to the elevation of serum HA in chronic liver disease.[45] It is known that physiological factors, such as exercise or eating increase the input of HA into the circulation by influencing the mobility of the HA pool from the muscle and the gut.[46] In our study, collection of blood samples were carried out after an overnight fasting early in the morning, while patients were resting in bed. So, these potentially confounding factors were unlikely to influence the result of our study.

In conclusion, we found that serum HA levels were increased with the progress of chronic liver disease. Also, these results suggest that serum HA can be a useful marker of liver fibrosis in patients with chronic liver diseases. The present study could be criticized for its small number of participants; however, our findings were significant even with this small number and can be confirmed in larger trials.

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