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Serum Levels of Glycosaminoglycans and Chondroitin Sulfate/Hyaluronic Acid Disaccharides as Diagnostic Markers for Liver Diseases

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The glycosaminoglycan (GAG) content and composition of human serum are suggested to play roles in chronic diseases and may provide useful diagnostic biomarkers. The current study examined 57 serum samples taken from normal individuals and patients with different liver diseases. Two quantitative methods were applied and compared to analyze serum samples from multiple individuals. Results showed a significant increase in GAG concentration in the serum samples of patients with nonalcoholic fatty liver disease, cirrhosis, and hepatocellular carcinoma. Moreover, the percentages of 4-sulfated chondroitin sulfate disaccharide and hyaluronic acid disaccharide in the sera of patients with cirrhosis and hepatocellular carcinoma were significantly increased, compared with healthy individuals.

**Keywords** Glycosaminoglycan; Chondroitin sulfate; Hyaluronic acid; Biomarker; Liver disease

**INTRODUCTION**

Currently, with the increase of high-fat diets, more people get chronic diseases, including obesity, dyslipidemia, fatty liver, cirrhosis, and cancer. Nonalcoholic fatty liver disease (NAFLD) has become the most common liver disease in the world, and the prevalence of NAFLD has approximately doubled in the past decade. It encompasses a wide spectrum of conditions associated with overaccumulation of fat in the liver, ranging from simple steatosis to nonalcoholic steatohepatitis (NASH) and cirrhosis. NASH, especially, may eventually develop into cirrhosis and hepatocellular carcinoma (HCC). Therefore, accurate diagnosis at earlier stages is crucial for improving therapeutic outcome. Unfortunately, the diagnostic procedures are laborious and not risk-free. Patients with suspected liver damage are initially subjected to liver function tests. If some indexes are abnormal, patients are then subjected to diagnostic imaging, such as ultrasound and computed tomography (CT), and assays to determine the presence of antibodies against hepatitis virus. Finally, liver biopsy is still recommended for most liver diseases. It is an invasive procedure, however, which results in severe complications in about 0.5% of cases. In addition, sampling error is common and the biopsy specimen seems to be poorly reliable when its length is inferior to 25 mm. Moreover, liver fibrosis is evaluated by histologic scores, which have interobserver variability. The noninvasive identification of biomarkers, which can provide reliable differential diagnoses for the characterization of liver diseases, is desirable.

Chondroitin sulfate (CS) is a kind of GAG composed of N-acetylgalactosamine (GalNAc) and glucuronic acid (GlcA), which constitute a disaccharide repeating unit, and it can be sulfated at different positions of GalNAc and/or GlcA residues. It attaches to a core protein to form chondroitin sulfate proteoglycan. CS represents the major GAG in blood circulation. Other serum GAGs include heparin sulfate, keratin sulfate, and hyaluronic acid (HA). HA is composed of alternating N-acetyl-glucosamine (GlcNAc) and GlcA units. In contrast to other GAGs, HA does not contain sulfate groups and is not
covalently attached to a core protein, but it can bind to proteoglycans and other proteins to organize pericellular and extracellular matrix (ECM). Metastases often contain cancer cells with a high level of HA expression and correlate with those of the original tumors, suggesting an active role for HA in the progression of the cancer.[7,8]

Human serum serves as a typical clinical specimen due to its convenient accessibility for long-term monitoring. It has been demonstrated that the low-molecular-weight fraction of human serum or plasma provides a rich source for potential biomarkers of diseases generated through enzymatic cleavage.[9,10] CS and HA have been found in human serum or plasma and carry important biological information.[11] The variations of GAGs in human serum or plasma are also vital for investigating and monitoring disease conditions. For example, the total amount of GAGs in serum was found to be closely related to diabetic pathology.[12]

Investigation of GAGs as biomarkers for cancer and other chronic diseases, including hepatocellular carcinoma,[13–16] hepatic fibrosis,[17] ovarian cancer,[18] prostate cancer,[19] gastric carcinoma,[20,21] and pancreatic carcinoma,[22] has been the focus of many recent studies. In addition, diagnostic methods based on GAGs have typically centered on the analysis of GAG structure and concentration. Elevated levels of HA, a major component of the ECM, was found to be associated with the degree of severity and spread of the disease through the body in several studies.[23–25] Another study found a link between relapse in prostate cancer and undersulfation in chondroitin disaccharides.[19] A further study in hepatic carcinoma revealed that both an increase in the overall level of CS and undersulfation of CS were associated with more aggressive forms of cancer.[16] Therefore, evaluating the variations of CS/HA has great potential for disease diagnosis and prognosis.

In this study, for the purpose of discovering new biomarkers for noninvasive human liver disease diagnosis, we comprehensively analyzed the serum CS/HA disaccharides in a total of 57 samples from patients with three types of liver diseases and one type of chronic illness and healthy individuals by means of fluorescence-assisted reverse-phase high-performance liquid chromatography (RP-HPLC) and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) approaches. Quantitative measurement of disaccharides was used to analyze the difference of CS/HA fine structures in various samples. LC-MS/MS with the multiple reaction monitoring (MRM) mode could quantify target markers in less than 5 minutes, while the RP-HPLC method yielded more compositional information. We found increased levels of 4SCS in the majority of the liver diseases. In addition, a significantly elevated level of 0SHA was also observed in cirrhosis and HCC samples. This is the first reported analysis aiming to uncover quantity and compositional differences in CS/HA of various liver diseases and to enrich our understanding of potential biomarkers in the serum.
RESULTS AND DISCUSSION

In the present work, we investigated the modifications of CS in the serum samples from patients with liver diseases, including hyperlipidemia, NAFLD, liver fibrosis, cirrhosis, and HCC. The goal of this study was to examine the difference in terms of the structures and amounts of GAGs in blood between controls and patients and whether or not the possible differences could be used as a diagnostic biomarker.

Serum Total GAG Levels

Serum total GAG levels in healthy controls and patients with hyperlipidemia, NAFLD, cirrhosis, and HCC (Fig. 1) are shown. There was a progressive increase in serum GAGs from healthy controls to patients with NAFLD to patients with cirrhosis and HCC. Serum GAGs in patients with cirrhosis and HCC were significantly higher (77.0%, $P < 0.001$; 81.9%, $P < 0.001$) than that in controls. Serum GAGs in the NAFLD group were also significantly higher than healthy controls (21.9%, $P < 0.05$). Moreover, serum GAGs in patients with cirrhosis and HCC were also remarkably higher than those of patients with NAFLD (45.1%, $P < 0.05$; 49.2%, $P < 0.05$), indicating the level of serum GAGs may be used to represent the severity of chronic liver diseases. However, there was no significant difference of GAGs between the control group and patients with hyperlipidemia, and no significant difference between cirrhosis and HCC groups, suggesting the level of serum GAGs could not be used to differentiate the two serious liver diseases.
Figure 2: Representative RP-HPLC chromatograms showing (A) standard CS/DS and HA disaccharides and CS/HA disaccharides from (B) healthy, (C) hyperlipidemia, (D) NAFLD, (E) cirrhosis, and (F) HCC serum samples. The peaks are as follows: 1, Tri SCS; 2, SCS; 3, AMAC; 4, SECS; 5, SDCS; 6, 4SCS; 7, 2SCS; 8, 6SCS; 9, 0SHA; 10, 0SCS.

GAGs were then digested with a mixture of chondroitin lyase ABC and chondroitin lyase ACII. The generated CS/HA disaccharides were labeled with 2-aminoacridone and analyzed by RP-HPLC. The chromatograms are shown in Figure 2. Disaccharide peaks were identified by comparison of the elution profiles with nine disaccharide standards.

The analysis of CS/HA disaccharides in various sera manifested that 0SCS, 0SHA, and 4SCS were the major disaccharides in both the control and the patient samples (Fig. 3). Similar results were reported elsewhere for normal plasma fractions.[26,27] Tri SCS was not observed in any of the serum samples, which was also consistent with a previous report.[28] Moreover, the contents
Figure 3: Measured levels of CS/HA disaccharides in serum samples. \(0S_{\text{HA}}\) is presented relative to the total CS-GAGs found in each sample. Degree of sulfation is the average number of sulfate groups/disaccharide calculated from the disaccharide analysis. The values are given as percentages of total disaccharides; significant differences (\(P < 0.05\)) were observed in both 4S\(_{\text{CS}}\) and 0S\(_{\text{HA}}\) disaccharides.

of 4S\(_{\text{CS}}\) (peak 6 in Fig. 2) and 0S\(_{\text{HA}}\) (peak 9 in Fig. 2) are notably higher in the HCC group than in the healthy control. Statistical analysis (Fig. 4) indicated that 0S\(_{\text{HA}}\) was significantly higher in the groups of cirrhosis (34.8%, \(P < 0.05\)) and HCC (36.3%, \(P < 0.05\)) patients than the healthy control, which is consistent with the previously reported studies.\(^{[29–31]}\) The level of 4S\(_{\text{CS}}\) was significantly higher in patients with cirrhosis (6%, \(P < 0.05\)) and HCC (7%, \(P < 0.05\)) as compared to the control.

The levels of 4S\(_{\text{CS}}\) and 0S\(_{\text{HA}}\) in the HCC patient group are slightly higher than those of the cirrhosis patient group, but the difference was not statistically significant. The 6S\(_{\text{CS}}\) and 2S\(_{\text{CS}}\) contents of the healthy control and the hyperlipidemia, NAFLD, cirrhosis, and HCC patient groups are decreasing. It is also noteworthy that the degree of sulfation among the tested groups was elevated, but these differences were not significant. No apparent trends or major differences were observed concerning 0S\(_{\text{CS}}\) and disulfated CS disaccharides.

Furthermore, LC-MS was employed to quickly analyze the disaccharides based on their degree of sulfation. A tandem quadrupole mass spectrometer was used to achieve highly sensitive detection. The optimized conditions for MS detection are shown in Table 2. The total ion chromatography (TIC) of the CS/HA-derived disaccharides from the serum samples analyzed by LC-MS/MS is displayed in Figure 4. The elution position of HA overlapped with that of 0S\(_{\text{CS}}\) in the TIC of the disaccharide standards (Fig. 4A), and they could not be distinguished by the extracted ion chromatography for the same cleavage rules either (data not shown). Therefore, the amount of HA was acquired based on the AMAC-RP-HPLC method as shown above. Nonsulfated disaccharides (0S\(_{\text{CS}}\) and 0S\(_{\text{HA}}\)) were eluted first, whereas trisulfated disaccharide was eluted at last. Disaccharide peaks were identified by the mass-to-charge ratio (\(m/z\))
<table>
<thead>
<tr>
<th>Sugar type</th>
<th>Sulfation pattern</th>
<th>Known amount (M1, ng)</th>
<th>Calculated amount (ng)</th>
<th>Known amount (M2, ng)</th>
<th>Calculated amount (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0S_HA</td>
<td>None</td>
<td>10</td>
<td>9.0 ± 1.2</td>
<td>14.4 ± 1.1</td>
<td>15</td>
</tr>
<tr>
<td>0S_CS</td>
<td>5</td>
<td>5.2 ± 0.6</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>6S_CS</td>
<td>Single</td>
<td>10</td>
<td>10.3 ± 0.6</td>
<td>5</td>
<td>5.7 ± 0.5</td>
</tr>
<tr>
<td>4S_CS</td>
<td>10</td>
<td>11.5 ± 1.4</td>
<td>26.0 ± 1.5</td>
<td>5</td>
<td>14.1 ± 1.3</td>
</tr>
<tr>
<td>2S_CS</td>
<td>5</td>
<td>4.4 ± 0.9</td>
<td>15</td>
<td>15</td>
<td>19.3 ± 0.7</td>
</tr>
<tr>
<td>SE_CS</td>
<td>Double</td>
<td>5</td>
<td>4.8 ± 0.8</td>
<td>20</td>
<td>14.5 ± 0.6</td>
</tr>
<tr>
<td>SB_CS</td>
<td>10</td>
<td>9.6 ± 0.4</td>
<td>28.2 ± 0.7</td>
<td>15</td>
<td>4.7 ± 0.3</td>
</tr>
<tr>
<td>5D_CS</td>
<td>15</td>
<td>13.9 ± 0.7</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tri S_CS</td>
<td>Triple</td>
<td>10</td>
<td>9.0 ± 0.9</td>
<td>15</td>
<td>15.4 ± 0.6</td>
</tr>
</tbody>
</table>
Figure 4: Total ion chromatograms of (A) disaccharide standards, (B) healthy serum sample, and (C) hyperlipidemia, (D) NAFLD, (E) cirrhosis, and (F) HCC patient samples. The most abundant ions found in each peak are shown. Peak 1 contains nonsulfated disaccharides (m/z 378.1). Peak 2 contains monosulfated disaccharides (m/z 458.1). Peak 3 contains disulfated disaccharides (m/z 538.1). Peak 4 contains trisulfated disaccharides (m/z 747.1 = 618.1 + dibutylamine).

according to disaccharide standards. It was noted that nonsulfated disaccharides and monosulfated disaccharides, which could be eluted in less than 5 minutes, could be utilized as disease prediagnosis markers. However, it was difficult to separate disaccharide isomers by this method, which prompted us to employ RP-HPLC as an alternative.

Comparison of LC-MS/MS and RP-HPLC approaches was performed to test the accuracy and reproducibility of the results. Two mixtures of known disaccharide standards, M1 (10, 5, 10, 5, 10, 10, 5, 10, 15, and 10 ng of 0SHA, 0SCH, 6SCH, 4SCH, 2SCH, SETH, SBCH, SDCH, and Tri SCH, respectively) and M2 (15, 10, 5, 5, 15, 20, 15, 5, and 15 ng of 0SHA, 0SCH, 6SCH, 4SCH, 2SCH, SETH, SBCH, SDCH, and Tri SCH, respectively) were analyzed by both methods. The results (Table 1) showed that the calculated amounts were consistent with the known
Table 2: Parameters for determination of disaccharides by LC-MS/MS

<table>
<thead>
<tr>
<th>Disaccharides</th>
<th>Retention time (min)</th>
<th>MRM transitions (m/z)</th>
<th>Fragmentor (V)</th>
<th>Collision energy (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔUA-GlcNAc/ΔUA-GalNAc</td>
<td>1.65</td>
<td>378.1 &gt; 175.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>90</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>378.1 &gt; 157.1</td>
<td>90</td>
<td>9</td>
</tr>
<tr>
<td>ΔUA-GalNAc + S1</td>
<td>3.22</td>
<td>458.1 &gt; 300.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>150</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>458.1 &gt; 342.1</td>
<td>150</td>
<td>15</td>
</tr>
<tr>
<td>ΔUA-GalNAc + S2</td>
<td>8.02</td>
<td>538.1 &gt; 300.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>110</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>538.1 &gt; 97.1</td>
<td>110</td>
<td>35</td>
</tr>
<tr>
<td>ΔUA-GalNAc + S3</td>
<td>9.06</td>
<td>747.1 &gt; 667.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>747.1 &gt; 458.1</td>
<td>100</td>
<td>25</td>
</tr>
</tbody>
</table>

<sup>a</sup>Quantifying ion.

amounts. Therefore, the LC-ESI-QQQ MS method is a fast, sensitive, and reliable method for qualitative and quantitative analysis of CS/HA disaccharides.

It was found that the monosulfated disaccharides of serum chondroitin sulfate (Fig. 5) increased from 45.5 ± 8.6 ng in the normal control to 119.5 ± 29.8 ng in HCC patients, an increase of 263%. In patients with cirrhosis, monosulfated disaccharides also increased to 105.6 ± 13.9 ng, an increase of 232%. These data suggested that monosulfated disaccharides of serum chondroitin sulfate may be valid biomarkers for cirrhosis and HCC diagnosis. The contents of disulfated disaccharides and trisulfated disaccharides were too low to observe significant differences.

The impact of serum GAG contents, disaccharide contents, degree of sulfation, and GAG sequence on signaling has not been illustrated clearly. But many studies reported the role of GAG sulfation and their importance. A

![Figure 5: Scatter diagram of CS monosulfated disaccharides in 57 samples. *: Significantly different from the control, with P < 0.01.](image-url)
reduced sulfation of CS was observed in kidneys of diabetic db/db mice.\cite{32}

Moreover, the difference of CS/HA sulfation may lead to different binding affinities for human transforming growth factor-β1.\cite{33} Sulfation of GAGs led to a significant inhibition of osteoclast differentiation and resorption, which was largely dependent on the degree of sulfation of GAGs rather than the monosaccharide composition.\cite{34}

**CONCLUSION**

This study validated a rapid LC-MS/MS method and a sensitive RP-HPLC method for serum GAG quantification among various liver disease samples. The LC-MS/MS method analyzed disaccharides based on their degree of sulfation, which could be accomplished in 10 minutes, and the RP-HPLC method is a good complement, as it could be used to effectively separate disaccharide isomers. In conclusion, the content of serum GAGs is a potent biomarker to measure the extent of liver impairment. Furthermore, this study confirmed the feasibility of using the RP-HPLC method to identify 4S\textsubscript{CS} and 0S\textsubscript{HA} as potential effective biomarkers for the early detection of high-risk populations about chronic liver diseases. There is still more work to be done, such as the changes in other GAGs, the changes in GAG molecular weights, and the changes in GAG sequences, to confirm and support the current conclusion and apply the study to the therapy of liver diseases.

**EXPERIMENTAL**

**Materials and Methods**

The nine unsaturated CS/HA disaccharides standards 0S\textsubscript{CS}, ΔUA-GalNAc (where ΔUA is Δ-deoxy-L-threo-hex-4-enopyranosyl uronic acid); 4S\textsubscript{CS}, ΔUA-GalNAc4S; 6S\textsubscript{CS}, ΔUA-GalNAc6S; 2S\textsubscript{CS}, ΔUA2SGalNAc; SB\textsubscript{CS}, ΔUA2SGalNAc4S; SD\textsubscript{CS}, ΔUA2S-GalNAc6S; SE\textsubscript{CS}, ΔUA-GalNAc4S6S; Tri S\textsubscript{CS}, ΔUA2S-GalNAc4S6S; and 0S\textsubscript{HA}, ΔUA-GlcNAc were purchased from Iduron Co. (Manchester, UK). Chondroitin sulfate A (sodium salt, isolated from bovine trachea), chondroitin lyase ABC from *Proteus vulgaris*, and chondroitin lyase ACII from *Arthrobacter aurescens*, dibutylamine, 2-aminoacridone (AMAC), and NaCNBH\textsubscript{3} were purchased from Sigma–Aldrich (St. Louis, MO, USA). HPLC-grade solvents were purchased from Merck (Darmstadt, Germany). Ultrapure grade LC water was obtained by purification of distilled water through a Milli-Q gradient system (Millipore, Bedford, MA, USA).
Blood Serum Samples

A total of 57 subjects, 20 healthy individuals and 37 patients, including 8 hyperlipidemia patients, 12 NAFLD patients, 9 cirrhosis patients, and 8 HCC patients from Qingdao Fifth People's Hospital, were enrolled for this study from February 2013 to August 2013. Blood samples were collected from each of them. The healthy controls, who visited the hospital for routine physical examination, were confirmed to have normal liver functions and no viral hepatitis, alcohol or nonalcohol fatty liver, or other diseases. These patients were diagnosed by clear histological examination and imaging evidence such as blood chemistry, α-fetoprotein assay, CT, and hepatic angiography.

Informed consent was obtained from each patient included in the study. The research protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a prior approval by the Hospital Ethics Committee. Fasting blood samples were collected from all subjects and subsequently divided into two portions. The first portion was allowed to coagulate and centrifuged to obtain serum for immediate determination of routine liver function tests. The second portion was kept deeply frozen at –80°C for the investigation of GAGs.

Sample Preparation

Isolation and purification of GAGs from human serum followed the reported procedures with some modification.[35,36] Briefly, 1 mL of serum was freeze-dried and then proteolyzed. GAGs were purified using a strong anion exchange spin column (Vivapure Q Mini H spin columns were from Sartorius Stedim Biotech), desalted (Microcon Centrifugal Filter Units YM-10, 10k MWCO), and alcohol precipitated. The recovered GAGs were next completely depolymerized using chondroitin lyases. Chondroitin lyase ABC (5 m-units) and chondroitin lyase ACII (2 m-units) in 10 μL of 0.1% BSA were added to the GAG sample in 20 μL of distilled water and incubated at 37°C for 10 h. After boiling to inactivate the chondroitinase at 100°C for 2 min and cooling to rt, the digests were lyophilized.

Quantification of GAGs by Carbazole Assay

Lyophilized GAG samples were reconstituted in 40 μL water, and 10 μL of the solution was subjected to carbazole assay[37] to quantify the amount of GAGs in each sample. GlcA was used as an external standard, and the standard curve was derived by plotting absorbance against the concentration of GlcA.
Separation of AMAC-Labeled Disaccharides by RP-HPLC

CS/HA disaccharides were labeled with 2-aminoacridone and separated and detected by RP-HPLC as reported in the literature\(^{38}\) with some modification. Briefly, a sample containing CS/HA disaccharides or disaccharide standards was added to 5 \(\mu\)L of 0.1 M AMAC solution in acetic acid (AcOH)/dimethyl sulfoxide (DMSO) (3:17, \(v/v\)) and mixed by vortexing for 1 min. Next, 5 \(\mu\)L of 1 M NaBH\(_3\)CN was added in the reaction mixture and incubated at 45\(^\circ\)C for 4 h. Finally, the AMAC-tagged disaccharide mixtures were diluted to 100 \(\mu\)L and were applied to RP-HPLC analysis. HPLC separation was performed on an X-Bridge Shield column (4.6 mm \(\times\) 100 mm, 3.5 \(\mu\)m) from Waters (Milford, MA, USA). Eluent A was 60 mM ammonium acetate (pH 5.6) in water, and eluent B was acetonitrile. After 5 \(\mu\)L of a sample was injected, the gradient stepped from 2\% to 4\% acetonitrile in 2 min. Thereafter, the separation gradient stepped from 4\% to 15\% acetonitrile in 25 min, followed by washing with 60\% acetonitrile for 8 min and then equilibration for 3 min for the next run. The flow rate was 1.5 mL/min. Disaccharides were detected by fluorescence detection with the exiting wavelength at 442 nm and detecting wavelength at 520 nm.

Liquid Chromatography-Mass Spectrometry

HPLC separations were performed on a Zorbax 300SB C18 column (2.1 mm \(\times\) 100 mm, 3.5 \(\mu\)m, Agilent) using a binary solvent system composed of 5\% methanol (eluent A) and 90\% methanol in water (eluent B), both containing 3.5 mM of dibutylamine and adjusted to pH 5.5 with HOAc solution. Samples (5 \(\mu\)L) were injected by a direct injection autosampler. The linear gradient of eluent was 0\% to 45\% of solution B in 10 min. After each run, the column was washed with 100\% B for 5 min and then equilibrated with 100\% A for 5 min. The flow rate was 245 \(\mu\)L/min, and absorbance at 232 nm was monitored during each run. The HPLC was directly coupled to the electrospray ionization (ESI) triple quadrupole (QQQ) mass spectrometer.

Ionization and fragmentation settings were optimized by direct injection of disaccharide standard solutions. MS/MS was performed in the MRM using ESI in negative mode. For each compound, two characteristic product ions were monitored. The most abundant one was used for quantification, while the second one was used as a qualifier. Collision energy and fragmentor voltage were optimized and shown in Table 2. Nitrogen was used as the collision, nebulizing, and desolvation gas. Optimal ESI conditions were capillary voltage 4000 V, nebulizer 40 psi, source temperature 350\(^\circ\)C, and gas flow 9 L/min. The data were processed using a MassHunter Workstation Software for qualitative and quantitative analysis.
Statistical Analysis

All statistical calculations were done using IBM SPSS Statistics 21. Data are expressed as the mean ± SEM. Differences between groups were assessed by one-way ANOVA followed by least significances difference (LSD) post hoc comparison to assess statistical differences between groups. Threshold of significance was defined at $P < 0.05$.

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