“A POTENTIAL NONINVASIVE BIOMARKER OF NONALCOHOLIC STEATOHEPATITIS”

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Abstract:
Background/Aim: Liver biopsy remains the gold standard for characterizing the histology of nonalcoholic fatty liver disease, yet this procedure is invasive. Oxidative stress plays an important role in the pathogenesis of steatohepatitis and erythrocytes are known to exert pro-oxidant activity. We hypothesized that erythrocytes damaged by oxidative stress could represent potential biomarkers capable of distinguishing steatosis from steatohepatitis. Methods: Our sample included 37 obese patients who underwent bariatric surgery and liver biopsy. Patients were classified according to histological diagnosis with respect to steatosis or steatohepatitis. Oxidative stress measurements were performed by chemiluminescence. Erythrocytes obtained from the whole blood of patients were stained to evaluate cell morphology by optical microscopy. Results: Higher levels of oxidative stress were seen in obese patients with steatohepatitis in comparison to those with steatosis (Cohen's d=4.1). Erythrocytes damaged by oxidative stress were found in 81.2% of patients with steatosis and in 95.2% of the steatohepatitis cases (Phi= 0.22). These results were confirmed in an in vitro model. Oxidative stress levels were found to be elevated in 60.6% of the cases in which modified erythrocytes were also present. Conclusions: The results indicated that altered erythrocytes may represent a novel noninvasive biomarker capable of distinguishing steatosis from steatohepatitis.

Keywords: Biomarkers, erythrocytes, nonalcoholic fatty liver disease, nonalcoholic steatohepatitis, oxidative stress.

INTRODUCTION

The spectrum of nonalcoholic fatty liver disease (NAFLD) includes steatosis and steatohepatitis (NASH), the latter of which presents a risk of progression to cirrhosis and hepatocellular carcinoma [1]. Since distinguishing between steatosis and NASH has proven challenging, liver biopsy remains the gold standard for confirmatory diagnosis. Although this invasive method carries a relatively low risk of developing complications, this is not negligible [2,3]. Sampling bias has also been reported in patients with NAFLD, potentially affecting both diagnosis and disease staging [4]. In light of these limitations associated with liver biopsy, the evaluation of serum biomarkers and noninvasive testing to distinguish steatosis from NASH warrants further investigation[5,6].

The pathogenic mechanism underlying NAFLD and the progression from steatosis to NASH has not yet been fully elucidated. Oxidative stress (OxS) is a damaging condition arising from an imbalance in the production of oxidants, mainly provoked by reactive oxygen species (ROS). OxS has been focused on as a common pathogenic mechanism in several hepatic diseases, in addition to the pathogenesis of NAFLD, and ROS is known to be a relevant factor in NAFLD progression [7]. Study conducted in humans and animal models has demonstrated a strong association between OxS levels and the severity of NASH[8].

Red blood cells (RBC) possess antioxidant and pro-oxidant activities[9]. In the microenvironment, it has been suggested that RBCs may produce ROS, leading to oxidative damage affecting these cells’ functional and morphological properties [10].

The present study sought to evaluate whether erythrocytes modified by oxidative stress hold potential as a biomarker to aid in the differential diagnosis between steatosis and NASH.

PATIENTS AND METHODS

Study design:
The present case series included severely obese patients (body mass index-BMI ≥35Kg/m²), seen at the Obesity Surgery and Treatment Center in the city of Salvador, Bahia.
-Brazil who underwent bariatric surgery (BAS) and liver biopsy between September 2013 and April 2014. All cases involving alcohol consumption ≥ 20g/day or other liver diseases were excluded. A group of healthy volunteers who did not meet NAFLD criteria were included from the Professor Edgard Santos University Hospital Complex in the city of Salvador.

This study received approval from the Institutional Review Board for Human Research of the Federal University of Bahia Medical School–UFBA, Bahia-Brazil, and was conducted in accordance with the principles outlined in the Declaration of Helsinki. All participants provided written informed consent prior to enrollment.

**Clinical evaluation:**

All patients completed a questionnaire containing the following variables: gender, age, risk factors for NAFLD, alcohol intake and other factors related to a range of liver and systemic diseases. Patients were then subjected to a full physical examination and submitted to the following additional testing: comprehensive liver panel, lipid and glycemic profiles, serum insulin, hepatitis B surface antigen (HBsAg), anti-hepatitis C virus (HCV), ferritin, transferrin saturation index, and abdominal ultrasound.

**Histological assessment:**

Liver biopsies were performed at the time of BAS and analyzed by a single hepatic pathologist. The histological diagnosis of NAFLD considered the criteria established by Brunt et al. 2011 [11].

The patients were classified in 2 groups according to histological diagnosis: Steatosis (G1) and NASH (G2).

**ROS measurements in whole blood samples:**

ROS production was evaluated in fresh whole blood samples obtained from all patients. Venous blood samples of 2 mL were collected in heparin-coated tubes. Measurements were performed using a sensitive photon counter [12] by real-time chemiluminescence employing L-012 (Wako Pure Chemical Industries, Osaka, Japan), a luminol-based chemiluminescent probe.

Whole blood samples that did not undergo any prior separation were added to Petri dishes and diluted with Hanks’ balanced salt solution at a ratio of 1:1. After performing baseline readings for 200 seconds, 50μM of L-012 was added to each plate and readings were taken after samples reached a plateau of photon emission activated by ROS as measured by L-012.

The effects of ROS were characterized using specific inhibitors: hydralazine and desferroxamine as inhibitors of peroxynitrite, and superoxide dismutase and sodium azide as inhibitors of myeloperoxidase [13].

**Erythrocyte microscopy analyses:**

During the collection of fresh whole blood samples from patients, a drop of native blood was used for smear preparation using Wright’s stain [14]. RBC morphology was visualized by light microscopy.

**In vitro erythrocyte oxidation:**

Fresh whole blood samples collected from healthy individuals were centrifuged at 3000 x g for 10 min. Following plasma and buffy coat removal by aspiration, erythrocytes were washed in phosphate buffered saline (PBS) twice by centrifugation under identical conditions. An in vitro model that exposed normal erythrocytes to ROS was employed to mimic conditions provoked under NAFLD in vivo. To induce controlled oxidative damage to normal erythrocytes under experimental conditions, well-studied oxidizing agents were selected: Hydrogen peroxide (H_2O_2) + Diamide. H_2O_2, a water-soluble oxidant with high cell membrane permeability, is thought to primarily affect cellular cytoplasmic components [15]. Diamide, which functions as a thiol-oxidizing reagent, has been widely used in experimentation to stimulate oxidative stress [16].

At identical concentrations, 100 μM of diamide and 100 μM of H_2O_2 were added to a 1.45 x 10^6 cells/mL suspension of normal erythrocytes, then left to settle for 30 minutes at 37°C in a chamber. Blood smears were fixed and stained with Wright’s stain for viewing under light microscopy as described elsewhere [14]. Untreated erythrocytes were considered as negative controls.

**Statistical analysis:**

The present study employed a non-probabilistic sampling method, in which sample units were selected according to patients’ availability at an outpatient clinic specialized in treating liver disease, which was subject to patient agreement to participation. Due to these conditions, it was not possible to obtain any reliable estimates regarding standard error. Consequently, as our analysis is purely descriptive in nature, it does not require the use of statistical hypothesis testing, nor the calculation of p values, nor confidence intervals [17,18].

Qualitative variables, such as gender, hepatic condition and level of oxidative stress, were described by univariate and bivariate analyses. Due to great variability in the original scale of measurement, the oxidative stress variable was presented through logarithmic function, entailing the calculation of logarithmic mean and standard deviation. This variable was also categorized as high and low levels, considering the median photon reading as a cut-off.

The phi coefficient was used to verify associations among the qualitative variables studied [19]. To evaluate the potential effect of hepatic condition on oxidative stress, differences in standardized logarithmic means were evaluated by Cohen’s d, a statistical measure of effect size [20]. All analyses were performed using the statistical computational package R, version 3.3 [21].

**RESULTS**

The present sample included 37 severe obese patients, whose mean ages in cases of steatosis and NASH were 36.2 (SD=9.8) and 42.1 (SD=11.3) years, respectively. Regarding gender, 87.5% of the patients with steatosis were female, while 76.2% of the patients with NASH were male, presenting a strong degree of association (Phi =0.63) between gender and hepatic condition.
Overall mean BMI in patients was 40.6 (SD= 2.7) kg/m$^2$, while in cases of NASH mean BMI was 42.9 (SD= 5.2) kg/m$^2$ (Cohen’s d = 0.5). No significant differences were seen in BMI between the two groups. Histological diagnosis of steatosis (G1) was observed in 43% (n=16) of the obese patients, while NASH (G2) was diagnosed in 57% (n=21).

OxS results were presented via logarithmic function and this variable was categorized in terms of a high or low level of oxidative stress, with 9382 photons considered the cutoff. Obese patients with NASH presented higher levels of ROS when compared to those with steatosis. The logarithmic mean and standard deviation in cases of steatosis and NASH were 7.9 (SD=0.5) and 9.6 (SD=0.4) respectively, demonstrating the pronounced effect of hepatic condition on OxS levels (Cohen’s d=4.1) (Figure 1).

Accordingly, all patients with steatosis presented low levels of OxS, while 95% with NASH presented high levels (Phi =0.95) (Table 1).

Table 1. Association between the oxidative stress variable, categorized as high or low levels of ROS, in addition to liver condition of patients with steatosis or steatohepatitis.

<table>
<thead>
<tr>
<th>Hepatic condition</th>
<th>Oxidative stress</th>
<th>Steatosis n (%)</th>
<th>Steatohepatitis n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>16 (100)</td>
<td>1 (4.8)</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>0 (0)</td>
<td>20 (95.2)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>16 (43.2)</td>
<td>21 (56.8)</td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as absolute frequency (n) and relative frequency (%). The phi coefficient (Phi =0.95) to verify association among the qualitative variables was used.

Figures 2a and 2b illustrate erythrocyte alterations due to OxS in the peripheral blood of patients with steatosis and NASH, respectively, with the notable appearance of echinocytes, acanthocytes and “cupshaped” stomatocytes cells. Our experimental in vitro model provided confirmation of these erythrocyte forms (Figure 2c). The morphological changes seen in erythrocytes appear to be caused by OxS. Normal erythrocyte morphology was observed in all healthy volunteers (Figure 2d).
Figure 2. Optical microscopy images of erythrocytes in blood smears. (A) and (B) Modified erythrocytes forms in an obese patient with steatosis and steatohepatitis, respectively, with the notable predominance of echinocytes. (C) Intact erythrocytes from a healthy volunteer. (D) Oxidatively modified erythrocytes in vitro treated with H$_2$O$_2$ + diamide.
Erythrocytes modified by OxS were found in 81.2% of the patients with steatosis and in 95.2% of the NASH cases (Φ=0.22) (Table 2).

Table 2. Associations between the presence of erythrocytes modified by oxidative stress and the liver condition of patients with steatosis or steatohepatitis.

<table>
<thead>
<tr>
<th>Hepatic condition</th>
<th>Echinocytes</th>
<th>Steatosis (%)</th>
<th>Steatohepatitis n (%)</th>
<th>Total n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absence</td>
<td>3 (18.8)</td>
<td>1 (4.8)</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>Presence</td>
<td>13 (81.2)</td>
<td>20 (95.2)</td>
<td></td>
<td>33</td>
</tr>
<tr>
<td>Total</td>
<td>16 (43.2)</td>
<td>21 (56.8)</td>
<td></td>
<td>37</td>
</tr>
</tbody>
</table>
Data are presented as absolute frequency (n) and relative frequency (%). The phi coefficient (Phi = 0.22) to verify association among the qualitative variables was used.

Table 3 delineates that in 60.6% of the cases presenting modified erythrocytes, OxS levels were classified as high. This stands in contrast to the low OxS levels seen in 100% of the cases with no modified erythrocytes (Phi = 0.22).

Table 3. Association between the presence and absence of erythrocytes modified by OxS and the OxS variable categorized as high or low levels of ROS.

<table>
<thead>
<tr>
<th>Oxidative Stress</th>
<th>Low n(%)</th>
<th>High n(%)</th>
<th>Total n(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absence</td>
<td>4 (100)</td>
<td>0 (0)</td>
<td>4 (10.8)</td>
</tr>
<tr>
<td>Presence</td>
<td>13 (39.4)</td>
<td>20 (60.6)</td>
<td>33 (89.2)</td>
</tr>
<tr>
<td>Total</td>
<td>17</td>
<td>20</td>
<td>37</td>
</tr>
</tbody>
</table>

CONCLUSIONS

The evaluation of modified erythrocytes could present an easy and inexpensive alternative as a novel noninvasive biomarker to distinguish steatosis from steatohepatitis in severely obese patients, which may improve diagnostic capability in these patients prior to being submitted to bariatric surgery.

ACKNOWLEDGEMENTS

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REFERENCES


