

# Evaluation of HIF-1 $\alpha$ and iNOS in ischemia/reperfusion gastric model: bioimpedance, histological and immunohistochemical analyses

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**Summary.** Gastrointestinal ischemia/reperfusion (I/R) generates pathological alterations that could lead to death. Early ischemic damage markers could be used to guide therapy and improve outcomes. Aim. To relate hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ) activation and inducible nitric oxide synthase (iNOS) expression to gastric impedance changes due to I/R damage. Methods. Experimental animals were randomly distributed into 3 groups: control, ischemia (30 min) and I/R (60 min). Gastric ischemia was generated by celiac artery clamping for 30 min, and then blood flow was restored for 60 min. Impedance spectra and biopsies of the glandular portion were obtained for histological and immunohistochemical analyses. Immunodetection of both HIF-1 $\alpha$  and iNOS was performed. Results. Under ischemia and I/R conditions, there was an increase ( $p < 0.05$ ) in the impedance parameters. Histologically, under ischemic conditions, edema and necrosis were observed in epithelium and significant vascular congestion. In I/R condition, alterations of the glandular and luminal integrity were found, which generated areas of epithelial erosion. Immunohistochemical analysis of HIF-1 $\alpha$  revealed an increase ( $p < 0.01$ ) in the number of immunoreactive cells in the ischemia ( $35.7 \pm 13.9$ ) and I/R ( $119.9 \pm 18.8$ ) conditions compared to the control

( $0.8 \pm 1.2$ ). Immunodetection of iNOS showed an increase ( $p < 0.01$ ) in the number of cells expressing iNOS under the ischemia ( $5.4 \pm 2.9$ ) and I/R conditions ( $27.4 \pm 11.3$ ) was observed compared to the control ( $0.4 \pm 0.8$ ). Conclusion. Early changes in impedance in response to I/R is related to histopathological changes, the nuclear stabilization and translocation of HIF-1 $\alpha$  as well as expression of iNOS.

**Key words:** Gastric impedance, Hypoxia-inducible factor, Inducible nitric oxide synthase, I/R, Bioimpedance

## Introduction

The state of shock is a pathological condition in which an overall insufficiency of tissue perfusion leads to a distribution of oxygen and nutrients that is insufficient for the needs of tissues (Li et al., 2014). The gastrointestinal mucosa is the first site affected during this condition. Gastrointestinal ischemia/reperfusion (I/R) generates a series of pathological alterations that lead to multiple organ failure (MOF) (Du et al., 2015), which remains the primary cause of death in intensive care units, with a mortality between 30% and 80% (Li et al., 2007). Gastric I/R injury has been extensively studied, however no markers of early ischemic damage have been identified in the gastric mucosa that can be used to guide therapy.

To evaluate the progression of tissue damage under ischemic conditions, impedance spectroscopy has been

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used. During the course of ischemia, the tissue exhibits characteristic changes in impedance spectra; these changes are mainly caused by cellular edema, intercellular junction damage and an accumulation of metabolic products (Gersing, 1998; Casas et al., 1999). A minimally invasive device consisting of a nasogastric/spectrometric probe, a spectrometer, a control and deployment system was designed. The level of gastric mucosal damage caused by ischemia has been continuously identified and monitored in different experimental models using this device (Beltran et al., 2010b, 2013, 2015).

Under hypoxic conditions, hypoxia-inducible factor (HIF) coordinates the cellular response and is composed of two subunits, HIF-1 $\alpha$  and HIF-1 $\beta$ . In situations with low oxygen concentrations, the HIF-1 $\alpha$  subunit is stabilized and translocated into the nucleus by forming a dimer with the HIF-1 $\beta$  subunit, thus recruiting transcriptional coactivators (Semenza et al., 1991; Semenza and Wang, 1992) and inducing the expression of genes containing hypoxia response elements (HREs) (Feinman et al., 2010; Hernansanz-Agustin et al., 2014).

The production of NO, which is generated by a reaction catalyzed by a family of enzymes called nitric oxide synthases (NOSs) with 3 isoforms (neuronal, endothelial and inducible), is 100-1000 times higher under ischemic conditions due to the inducible nitric oxide synthase (iNOS), which is expressed in response to cellular stress, whereas the endothelial (eNOS) and neuronal (nNOS) NOSs are expressed constitutively (Kibbe et al., 1999). It has been reported that the production of NO from iNOS causes damage to the gastric mucosa (Gemici et al., 2010).

In this study, we evaluated molecules associated with ischemic stress, such as HIF-1 $\alpha$  and iNOS, and related them with histological and impedance changes in gastric mucosa subjected to I/R damage.

## Material and methods

### Ethics statement and animal treatments

Fifteen male Wistar rats weighing 350-450 g were used in this experiment. The animals were randomly distributed into 3 groups: control, ischemia and I/R with 5 rats each. The animals were raised and housed in the vivarium of the Universidad Autonoma Metropolitana - Iztapalapa under controlled environment (22 $\pm$ 2°C, 50-60% relative humidity and 12 h light - dark cycles). The animals were given *ad libitum* access to water and standard food until the time of surgery. The protocol was approved by the ethics committee of the National Center for Research in Imaging and Medical Instrumentation according to the Official Mexican Standard (NOM-062-ZOO-1999) regarding technical specifications for production, care and handling of laboratory animals.

### Gastric I/R model

Gastric I/R was induced according to the method

proposed by Wada et al. (1998), and reported also by Kobata et al. (2007). The rats were anesthetized with 2 mLs of an anesthetic cocktail composed of 0.05 mLs of ketamine (2.5 mg/kg), 0.25 mLs of propionylpromazine (0.2 mg/kg) and 0.1 mLs of xylazine (1.1 mg/kg) administered intraperitoneally. A longitudinal incision was made below xiphoid appendage along ventral midline to the anterior region of the penis. For the ischemia group, the stomach was exposed, celiac artery was identified and clamped for 30 min, completely blocking blood flow. For the reperfusion group, 30 min after ischemia was generated, the clamp was removed, thereby restoring blood flow for 60 min. To measure the gastric impedance, the stomach was opened along the greater curvature, a saline lavage was performed, and the nasogastric/spectrometric probe was introduced. Subsequently, biopsies of the glandular region were taken for histological analysis and were fixed in 10% formaldehyde. Biopsies for histological and immunohistochemical analysis were stored at 4°C until processing. At the end of each procedure, the animals were sacrificed according to the Official Mexican Standard (NOM-062-ZOO-1999).

### Gastric impedance parameters

The impedance spectra were obtained through the nasogastric/spectrometric probe, which had 4 silver electrodes at its distal tip. Through the external electrodes, a current of 1 mA was injected into the gastric tissue, and a sweep was performed at frequencies from 100 Hz to 1 MHz. The device measures the voltage of tissue, and then calculates the impedance (resistance and reactance) measurements. The impedance spectrometer measures the flow of electrons restriction (resistance), and the tissue capacity to store and release energy due to the cellular membranes (reactance), which were taken every 30 s for 10 min for each animal through a software designed specifically for this spectrometer. Subsequently, the spectra (including 23 frequencies) were processed off-line using a mathematical model to calculate the impedance parameters that are characteristic of the gastric mucosa according to the previously described method (Beltran et al., 2010a). Gastric spectra present two dispersion regions, at frequencies below 10 kHz (low frequencies) and above 10 kHz (high frequencies). The low-frequency central resistance ( $R_L$ ) and low-frequency central reactance ( $X_L$ ) were obtained as the main parameters indicative of tissue damage (Beltran et al., 2010b).

### Histological analysis

The biopsies were processed and embedded in paraffin according to a standard histological technique. Cross sections of 5  $\mu$ m were mounted on silane covered glass slides and stained with Hematoxylin-Eosin. Nine sections per specimen were observed on a light field microscope (Axioscope II, Carl Zeiss Microscopy,

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Thornwood, NY, USA). The images were digitized, and qualitative analysis of the gastric mucosa was performed with AxioVision 4.8 software (Carl Zeiss Microscopy, Thornwood, NY, USA).

### Immunohistochemical analysis of HIF-1 $\alpha$ and iNOS

The sections were deparaffinized and subjected to antigenic recovery in a sodium citrate buffer (pH 6.0) for 40 min in an antigen retriever (Retriever 2100, Electron Microscopy Sciences, Hatfield, PA, USA). Endogenous peroxidase was blocked with 3% hydrogen peroxide for 30 min. Blocking of non-specific binding was performed with 4% albumin for 1 hour. The sections were then incubated with the primary anti-HIF-1 $\alpha$  (mouse monoclonal, ab1, Abcam, Cambridge, UK; 1:15 dilution) and anti-iNOS (rabbit polyclonal, SC-649, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; 1:150 dilution) antibodies in a humid chamber at 4 °C overnight after which they were washed in Tris buffer. Samples were then incubated with anti-mouse (SC-2005, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; 1:300 dilution) and anti-rabbit (SC-2004, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; 1:300 dilution) secondary antibodies, respectively, both conjugated to horseradish peroxidase (HRP) within the humid chamber for 2 hours at room temperature. The antigen-antibody complex was developed with a diaminobenzidine solution using an Invitrogen immunodetection kit (ZYMED Laboratories 00-2114, CA, USA). Finally, the samples were counterstained with Hematoxylin and then the slides were washed in tap water. The slides were dried and mounted with a cover slip and mounting media. The obtained samples were observed with a light microscope (Axioscope II, Carl Zeiss Microscopy, Thornwood, NY, USA). The images were digitized and analyzed using AxioVision 4.8 software (Carl Zeiss Microscopy, Thornwood, NY, USA). The number of immunoreactive cells was

quantified in 3 randomly selected fields from 3 sections per specimen (Gomez-Santos et al., 2017). The plugin “Cell Counter” (Image J) was used to count the immunoreactive cells (Brencher et al., 2017).

### Statistical analysis

Statistical analysis was performed with a parametric ANOVA test followed by Tukey’s *post hoc* test. Data were analyzed using the IBM SPSS 21 (IBM Corporation, New York, USA) and GraphPad Prism 6 (GraphPad software, San Diego, CA, USA) programs and are expressed as the mean value  $\pm$  SD. The level of significance was set at  $p < 0.05$ .

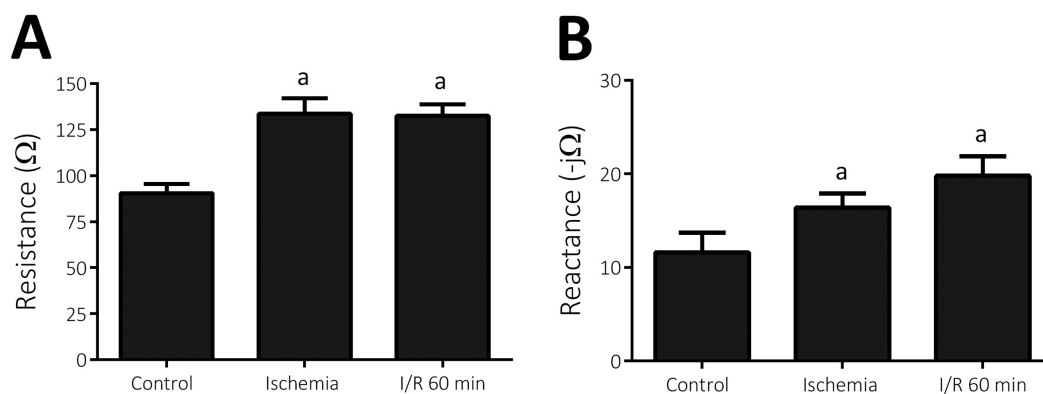
## Results

### Impedance spectrometry

The gastric impedance parameters, resistance ( $R_L$ ) and reactance ( $X_L$ ), were obtained at low frequencies for each of the groups.  $R_L$  was approximately 50% ( $p < 0.05$ ) higher in the ischemia ( $133.7 \pm 8.3 \Omega$ ) and I/R ( $132.5 \pm 6.3 \Omega$ ) groups than in the control ( $90.5 \pm 4.9 \Omega$ ) group.  $X_L$  increased in the ischemia ( $16.4 \pm 1.5 -j\Omega$ ) and I/R ( $19.8 \pm 2.1 -j\Omega$ ) groups relative to the control ( $11.6 \pm 2.1 -j\Omega$ ) group ( $p < 0.05$ ) (Fig. 1).

### Histological analysis

Histological analysis of the control group, revealed that the cells of the glandular epithelium and blood vessels of the lamina propria were found without pathological signs (Fig. 2A). In the ischemia group, cells of the glandular epithelium were observed with an increase in eosinophilia, suggesting cellular edema; cells with pyknotic or karyolytic nuclei were also found. In some glands, the volume of epithelial cells decreased, thus increasing the area of the glandular lumen. At the



**Fig. 1.** Quantitative analysis of impedance parameters at low frequencies under conditions: Control, Ischemia and I/R. There is a significant increase in resistance (**A**) and reactance (**B**) at low frequencies in the ischemia and I/R 60 min groups with respect to the control. The data are expressed as the Mean  $\pm$  SD. <sup>a</sup>:  $p < 0.05$  vs. Control.

level of the lamina propria, dilated blood vessels with abundant erythrocytes were observed, suggesting an early inflammatory process (Fig. 2B). In the I/R group, dilated blood vessels with abundant erythrocytes were observed, cells with decreased cell volume were found as well as cells in the process of necrosis, which compromised the integrity of the glandular architecture, generating areas of erosion in the gastric mucosa (Fig. 2C-D).

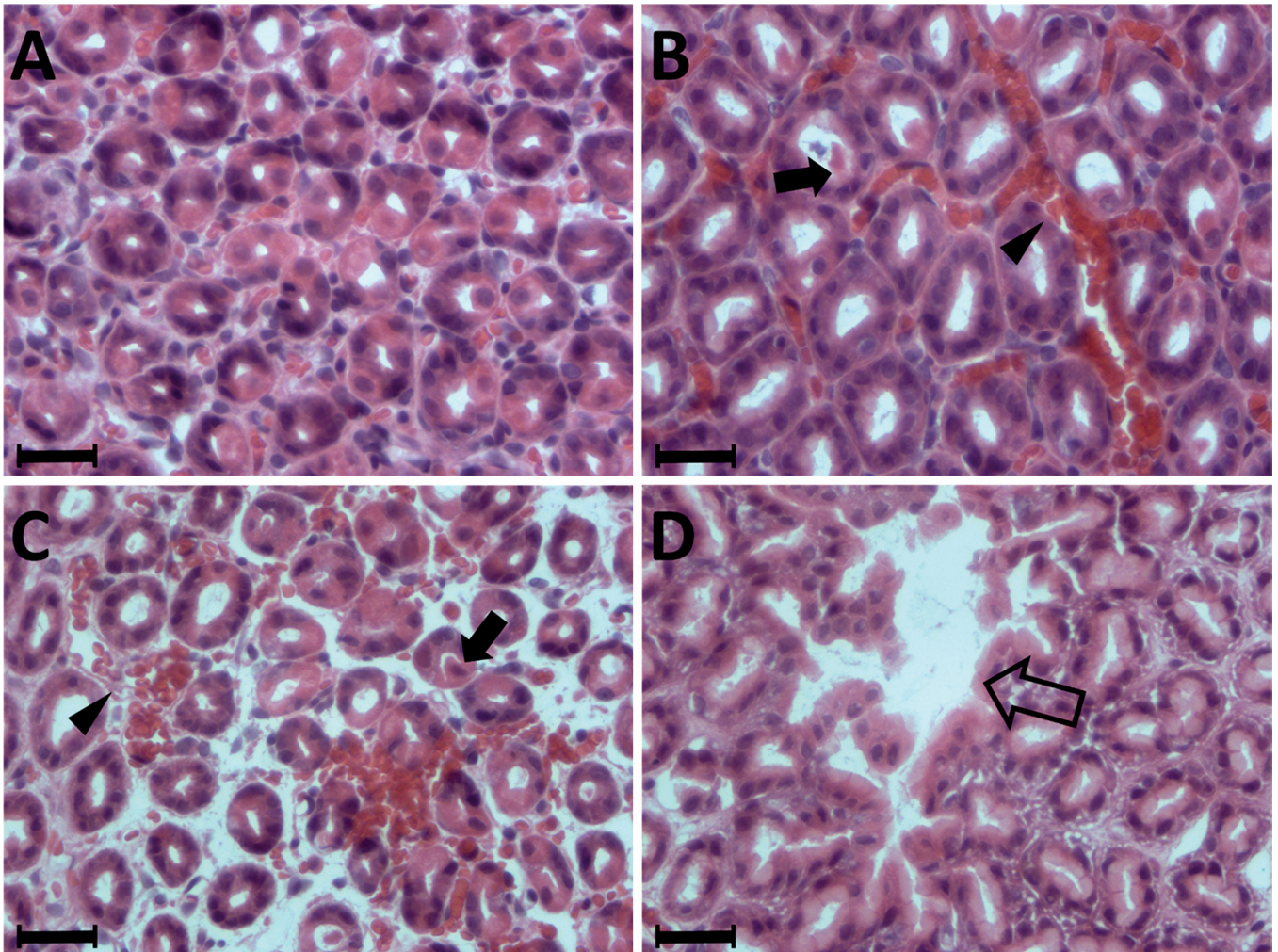
#### *Immunohistochemical detection of HIF-1 $\alpha$*

Immunohistochemical analysis of HIF-1 $\alpha$  revealed immunoreactivity in macrophages in the ischemia group (Fig. 3B). In the reperfusion condition, glandular epithelial cells were found with intense immunoreactivity, mainly in the nucleus (Fig. 3C).

These results indicate that under I/R conditions, HIF-1 $\alpha$  is stabilized and translocates to the nucleus. Quantitative analysis showed a greater ( $p < 0.01$ ) number of immunoreactive cells in the ischemia ( $35.7 \pm 13.9$ ) and I/R ( $119.9 \pm 18.8$ ) groups than in the control group ( $0.8 \pm 1.2$ ) (Fig. 3D).

#### *Immunohistochemical detection of iNOS*

Immunohistochemical analysis of iNOS showed cytoplasmic immunoreactivity that was found primarily in macrophages attached to the glandular epithelium in the ischemia group (Fig. 4B). In the reperfusion condition, we found that iNOS mainly localized in cells of the glandular epithelium and macrophages (Fig. 4C). Quantitative analysis showed a greater ( $p < 0.01$ ) number of immunoreactive cells in the ischemia ( $5.4 \pm 2.9$ ) and



**Fig. 2.** Representative photomicrographs displaying gastric mucosal damage generated by ischemia and I/R for 60 min. **A.** Normal gastric mucosa in a rat control. **B.** Gastric mucosa in ischemic conditions for 30 min. Vascular congestion (arrowhead) and some necrotic cells (black arrow) are observed in **C** and **D.** Gastric mucosa in conditions of ischemia/reperfusion. Necrotic cells (black arrow) and areas of erosion of the glandular epithelium (hollow arrow) are observed. H&E. Scale bars: 50  $\mu$ m.

I/R ( $27.4 \pm 11.3$ ) groups than in the control group ( $0.4 \pm 0.8$ ) (Fig. 4D).

*Relationship between gastric impedance parameters and gastric mucosal damage and stabilization of HIF-1 $\alpha$  and iNOS expression*

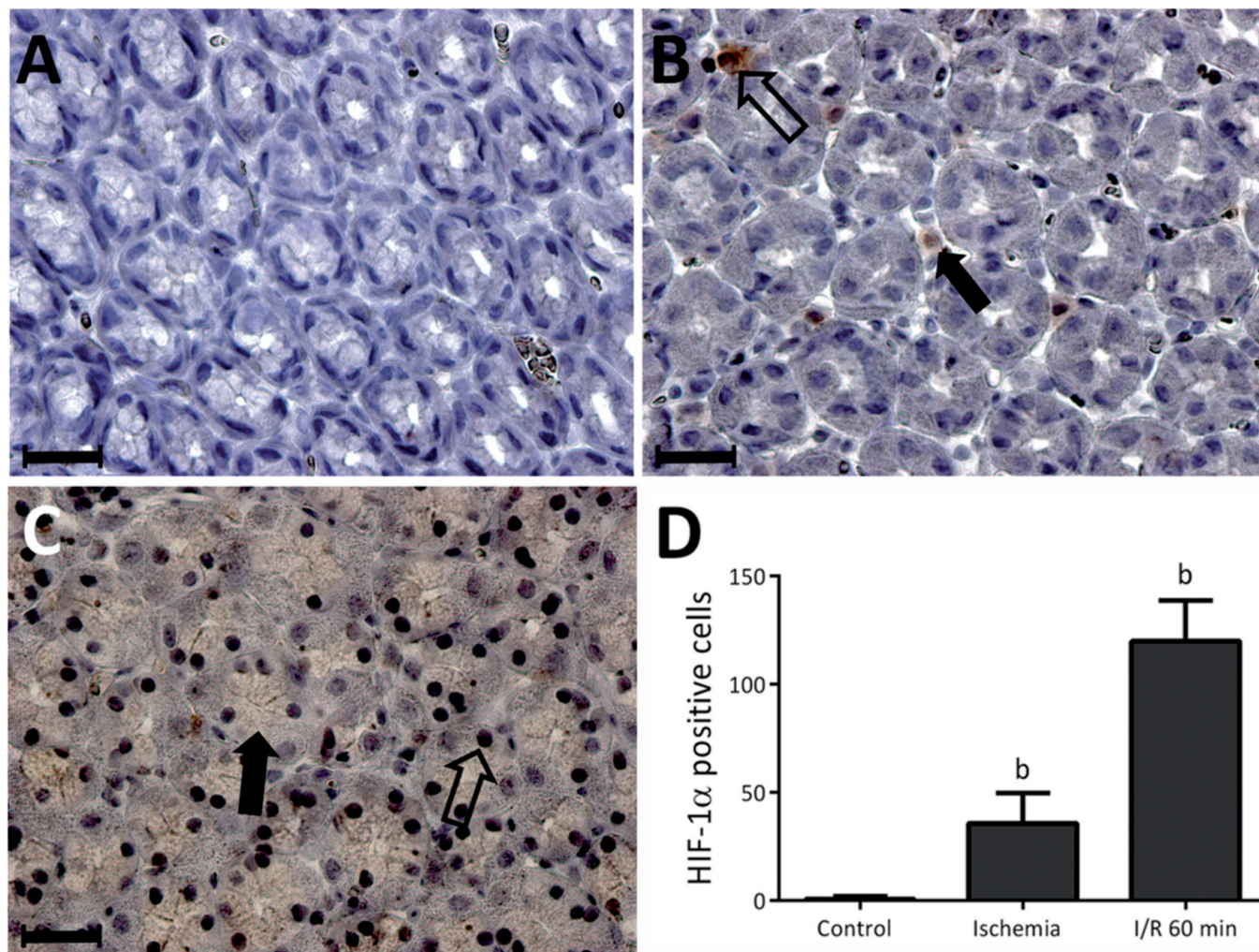
Taken together, the results described in Table 1 show that under reperfusion condition,  $X_L$  is the impedance parameter with a greater change. The increase in  $X_L$  may be related to the cell damage found histologically, in addition to the HIF-1 $\alpha$  translocation and iNOS expression under reperfusion condition. Correlation analysis was made between impedance parameters  $R_L$  and  $X_L$  and the biomarkers proposed HIF-1 $\alpha$  and iNOS (Fig. 5). Reactance is more related with HIF-1 $\alpha$  ( $r=0.7$ ) and iNOS ( $r=0.6$ ) expression than resistance.

## Discussion

Gastrointestinal I/R damage is a condition that occurs in some critically ill patients (Li et al., 2007) due

**Table 1.** Relationship between the gastric impedance parameters and stabilization of HIF-1 $\alpha$  as well as expression of iNOS under the following conditions: Control, Ischemia and I/R. Data are expressed as Mean  $\pm$  SD. Resistance at low frequencies ( $R_L$ ), reactance at low frequencies ( $X_L$ ) and number of immunoreactive cells (IRC).

	Control	Ischemia	I/R	p
$R_L$ ( $\Omega$ )	90.5 $\pm$ 4.9	133.7 $\pm$ 8.3	132.5 $\pm$ 6.3	<0.05
$X_L$ ( $-j\Omega$ )	11.6 $\pm$ 2.1	16.4 $\pm$ 1.5	19.8 $\pm$ 2.1	<0.05
IRC. HIF-1 $\alpha$	0.8 $\pm$ 1.2	35.7 $\pm$ 13.9	119.9 $\pm$ 18.8	<0.01
IRC. iNOS	0.4 $\pm$ 0.8	5.4 $\pm$ 2.9	27.4 $\pm$ 11.3	<0.01

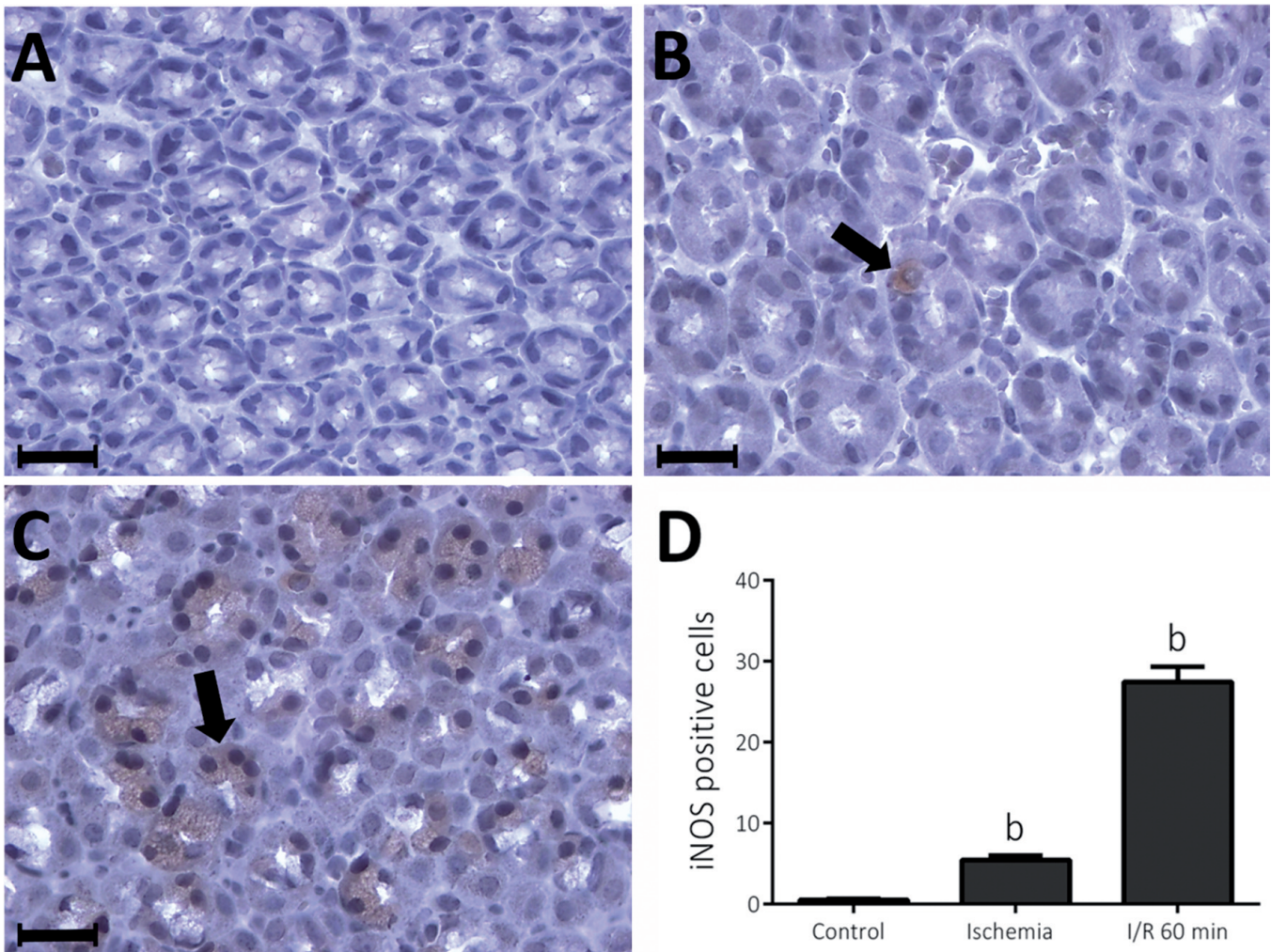


**Fig. 3.** Immunodetection of HIF-1 $\alpha$  in control (A), ischemia (B) and I/R (C) conditions. In ischemic condition, cytoplasmic positive immunoreactivity in macrophages was identified, while in I/R condition, glandular epithelium cells showed intense cytoplasmic (black arrow) and nuclear (hollow arrow) immunoreactivity. D. There is a significant increase in the number of immunoreactive cells in the ischemia and I/R groups with respect to the control. The data are expressed as the Mean  $\pm$  SD. <sup>b</sup>:  $p < 0.01$  vs. control. Scale bars: 50  $\mu$ m.

to diverse pathological conditions such as shock, burns, trauma, surgeries, among others. Thus, it is important to have tools that can identify this damage at an early stage as well as reverse or minimize the damage. Minimally invasive tools such as the one proposed in this study, which is based on impedance spectroscopy, could early help clinicians to identify ischemic gastrointestinal problems that trigger a series of pathophysiological alterations which may culminate in MOF, the principal cause of morbidity and mortality in Intensive Care Unit (ICU) patients (Kannan et al., 2011). If impedance changes are related with molecules associated with ischemic stress, such as HIF-1 $\alpha$  and iNOS, a guide therapy should be proposed to improve ICU outcomes.

The parameters of gastric impedance at low frequencies ( $R_L$  and  $X_L$ ) in normal and ischemic

conditions have been previously reported by Beltran et al., 2013. Under ischemic conditions, an increase in both parameters were identified, which is related to the histopathological alterations identified in these conditions (edema and cell damage). Those changes are mainly produced because cells are not able to generate enough energy to operate ion pumps and extracellular water penetrates into the cell (Szopinski et al., 2011). As a consequence, the cells swell; thus, the characteristics of the lateral domain are modified, which causes greater resistance to the flow of electric current (Malbrain et al., 2014). Under reperfusion conditions, an increase in  $X_L$  is related to the cellular damage of the glandular epithelium, which generates zones of erosion in the gastric mucosa. This type of injury induced by I/R conditions has been reported in the same model of



**Fig. 4.** Immunodetection of iNOS in control (A), ischemia (B) and I/R (C) conditions. In ischemic conditions, positive immunoreactivity in macrophages (black arrow) was identified, while in I/R conditions, glandular epithelium cells showed cytoplasmic positive immunoreactivity (black arrow). **D.** There is a significant increase in the number of immunoreactive cells in the ischemia and I/R groups with respect to the control. The data are expressed as the Mean  $\pm$  SD. <sup>b</sup>:  $p < 0.01$  vs. control. Scale bars: 50  $\mu$ m.

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occlusion of the celiac artery for 30 min and reperfusion of 1 hour (Kobata et al., 2007).

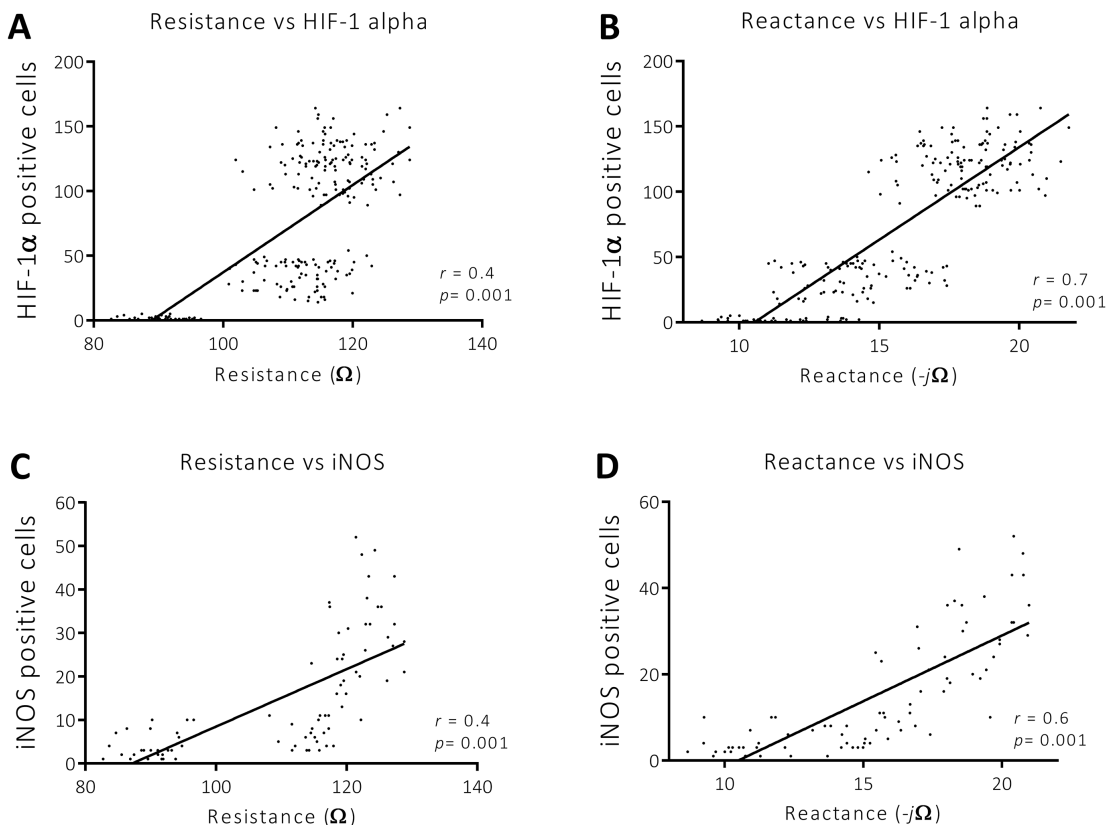
Immunohistochemical analysis for the immunodetection of HIF-1 $\alpha$  allowed us to identify the stabilization and subsequent translocation of HIF-1 $\alpha$  in macrophages and glandular epithelial cells under conditions of ischemia and I/R, respectively. The stabilization of HIF-1 $\alpha$  is due to the inhibition of the hydroxylation of HIF-1 $\alpha$ , which allows its translocation to the nucleus. Hydroxylation is prevented by the limited supply of oxygen that serves as a substrate for prolyl-hydroxylases (Wheaton and Chandel, 2011). Once in the nucleus, HIF-1 $\alpha$  induces the expression of genes that are related to the regulation of angiogenesis, cell survival, energy metabolism and apoptotic and proliferative response; these changes all occur through HREs (Howell and Tennant, 2014).

Under reperfusion conditions, HIF-1 $\alpha$  immunoreactivity was observed in the nucleus. The stabilization of HIF-1 $\alpha$  in the gastric mucosa under I/R conditions has seldom been reported. Wang et al. (2011) identified the stabilization of HIF-1 $\alpha$  in the gastric mucosa in an I/R model by occlusion of lower limb arteries in rats, identifying cells with cytoplasmic and nuclear

immunoreactivity, which is attributed to the stabilization of HIF-1 $\alpha$  due to the generation of reactive oxygen species (ROS) under reperfusion conditions.

A direct relationship between HIF-1 $\alpha$  activation and intestinal I/R injury has been reported. An increase in iNOS protein levels was observed in response to mesenteric occlusion in WT mice (Kannan et al., 2011).

The expression of iNOS in macrophages under ischemic conditions has been reported by Riemann et al., 2016. It has been shown that the expression of iNOS is related to the activation of HIF-1 $\alpha$  because the iNOS gene has HRE in the promoter region (Melillo et al., 1995). This interaction has been studied in models of cerebral ischemia, which showed that the protein levels increase in a time-dependent manner (Matrone et al., 2004; Li et al., 2012). Under reperfusion conditions, iNOS expression is related to the increase in tissue damage that generates areas of epithelial erosion because iNOS generates a greater amount of NO than its constitutive isoforms (eNOS and nNOS) (Kibbe et al., 1999). Peroxynitrite generated by the interaction between NO with ROS causes lipoperoxidation in the membranes, which leads to cell death (Derin et al., 2004).



**Fig. 5.** Correlation between HIF-1 $\alpha$  (A-B) and iNOS (C-D) positive cells with impedance parameters at low frequencies (Resistance and Reactance).

Histopathological alterations, the activation of HIF-1 $\alpha$  and the expression of iNOS could be related primarily to  $X_L$  since this parameter has been reported to be most sensitive to cell damage (Beltran et al., 2010b, 2013). The correlation analysis showed that HIF-1 $\alpha$  is more related with  $X_L$  changes than iNOS expression.

### Conclusion

The results of this study demonstrate that the early changes in gastric impedance parameters at low frequencies generated by I/R can be related to histopathological changes, the stabilization and nuclear translocation of HIF as well as expression of iNOS.

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