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Human carotid body neuroglobin, vascular endothelial growth factor and inducible nitric oxide synthase expression in heroin addiction

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Summary. Aims: The carotid body (CB) represents the prime site for detecting and responding to hypoxia. Since the role of heroin in respiratory depression with consequent hypoxia is known, the authors were able to investigate morphological and molecular modifications occurring in the CB of heroin addicted subjects compared to subjects who died because of trauma.

Methods and results: CB sampled from six 27 year old subjects, slides were treated with Mallory Trichrome staining or used for immunohistochemical analysis to detect Neuroglobin (NGB), Hypoxia Inducible Factor-1 (HIF-1α), Vascular Endothelial Growth Factor (VEGF), Inducible Nitric Oxide Synthase (i-NOS), Bax and cleaved Caspase-3 proteins. Mallory Trichrome staining shows an increase in the connective tissue in heroin subjects compared to controls and a parallel reduction in parenchymal area. Immunohistochemical analyses in heroin subjects found a decrease in NGB and an increase in HIF-1α and VEGF compared to controls; i-NOS expression was not statistically significant. Bax and cleaved caspase-3 were positive only in the heroin subjects.

Conclusions: These results could confirm the typical hypoxic condition occurring in heroin addicts. Since NGB may function as a reactive oxygen or nitrogen species scavenger and as apoptotic cell death protector, the decrease in its expression may suggest a key role of this globin in human CB impairment due to heroin addiction.

Key words: Carotid body, Chemoreceptor, Heroin addiction, Hypoxia, NGB

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Introduction

As an arterial chemoreceptor, the carotid body (CB) is sensitive to partial blood oxygen pressure, pH reduction, and increases in CO_2 partial pressure. Its stimulation induces an increase in ventilatory frequency and volume. Human CB is an ovoid tissue mass, weighing approximately 18 mg, and is situated at the carotid bifurcation between the external and internal carotid arteries. It is composed of lobules, separated by connective tissue, containing two different populations of cells: chief or type I cells, which are neuron-like cells and primary oxygen sensors in the CB, in turn separated into light, dark, and pyknotic (Verna, 1979; Pallot et al., 1986) and subtentacular or type II cells which have a supporting role (Porzionato et al., 2005; Pardal et al., 2007). Type I cells making synaptic contact with nerve terminal, mediate an increase in chemosensory discharges in the carotid sinus nerve in response to hypoxia, hypercapnia, and acidosis (Iturriaga and Alcayaga, 2004; Varas et al., 2006; Del Rio et al., 2011). Due to these particular functions, and its high blood flow and metabolism, the CB represents an experimental model suitable for studying hypoxia-related processes such as aging and intake of opiates like heroin (Di Giulio et al., 2003; Porzionato et al., 2005).

Endogenous opioid peptides are neurotransmitters able to modulate respiratory rhythm, and produce their effects through activity at three major receptor subtypes: μ, κ, and δ, expressed in respiratory centres where the opioids depress neuronal activity (White and Irvine, 1999). δ opioid receptors, involved in the depression of chemosensory discharge after opioid injection (Kirby and McQueen, 1986), are also localized in the CB.

Aging is characterized by a reduction in homeostatic adaptation to metabolic requirements, a decrease in

oxygen supply to tissues and a reduction of tissue pO_2. The homeostatic processes reduction also includes CB cell adaptation processes affecting oxygen supply to tissues (Di Giulio et al., 2003; Cataldi and Di Giulio, 2009). In fact, CB undergoes several morphological, physiological, and biochemical modifications during heroin-addiction and aging in both humans and rats. All these conditions are characterised by an increase in total connective tissue and a percentage increase in type II cells (Pokorski and Lahiri, 1981; Di Giulio et al., 2003; Porzionato et al., 2005, 2009). The increase in connective compartment and the reduction of glomic tissue may be ascribed to arteriosclerosis of the glomic arteries, as occurs in aging, and interpreted as a sign of early tissue aging (Di Giulio et al., 2003; Porzionato et al., 2005). These morphological modifications observed in heroin addiction, aging, and hypoxia exposure are obviously triggered by molecular events. Among the proteins involved in the occurrence of such responses, Hypoxia Inducible Factor-1 (HIF-1α), a key protein regulating the adaptation to hypoxia, is included. HIF-1 is composed of an oxygen-sensitive α-subunit, HIF-1α, and a constitutively expressed ß-subunit, HIF-1ß (Leiser and Kaeberlein 2010; Zhang et al., 2011) and it controls the induction of several genes involved in erythropoiesis, angiogenesis, and vasodilatation in hypoxic conditions (Cataldi and Di Giulio, 2009). In addition, Neuroglobin (NGB), a member of the vertebrate globin family present in the CB (Di Giulio et al., 2006; Verratti et al., 2009), expressed in both central and peripheral nervous system and in the retina, undergoes expression modifications during hypoxia events since its expression is related to oxygen consumption rates (Sun et al., 2011). NGB can play a pivotal role in several processes, such as NO detoxification, oxygen transport and storage, and probably supplies O_2 to the mitochondrial respiratory chain (Burmester and Hankeln, 2009).

Moreover, previous studies have also evaluated the expression of Vascular Endothelial Growth Factor (VEGF) and Inducible Nitric Oxide Synthase (i-NOS), involving angiogenesis and vasodilatation, respectively, in the CB of old and young rats (Di Giulio et al., 2005).

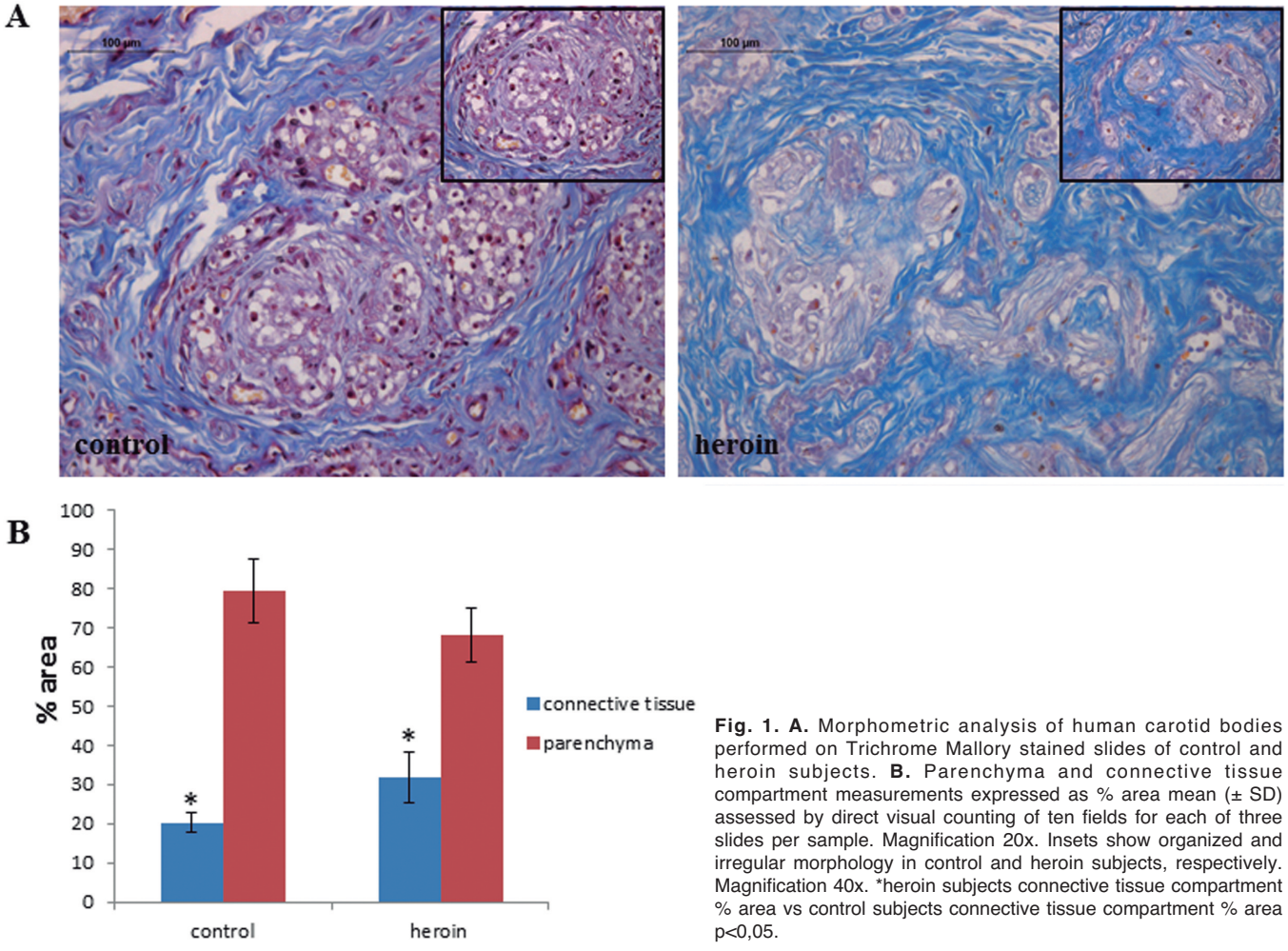


Fig. 1. A. Morphometric analysis of human carotid bodies performed on Trichrome Mallory stained slides of control and heroin subjects. **B.** Parenchyma and connective tissue compartment measurements expressed as % area mean (± SD) assessed by direct visual counting of ten fields for each of three slides per sample. Magnification 20x. Insets show organized and irregular morphology in control and heroin subjects, respectively. Magnification 40x. *heroin subjects connective tissue compartment % area vs control subjects connective tissue compartment % area $p<0,05$.

i-NOS is Ca^{2+} independent and is expressed mainly in pathophysiological conditions such as inflammatory response or hypoxia (Ye et al., 2002).

Since NGB, HIF-1α, VEGF, and i-NOS involvement in hypoxia events has been demonstrated and heroin addiction, leading to respiratory depression, can induce a hypoxia condition (White and Irvine, 1999), the aim of our study was to investigate molecular modifications of such proteins in an experimental model suitable for studying hypoxia-related processes, such as CB of opiate addicts who died due to heroin intoxication.

Materials and methods

The present study investigates, by immunohistochemical analysis, the expression and the localization of NGB, HIF-1α, VEGF, i-NOS, Bax and cleaved caspase-3 in human carotid bodies, sampled at autopsy from six 27±2 year old male subjects three died of trauma and were clinically diagnosed without chronic pulmonary or cardiovascular diseases, three died of heroin intoxication. In these cases, there was a clinical history of at least 4 years of heroin addiction. Autopsies were performed between 18 and 78 h after death. Specimens were taken of the right carotid bifurcation, including 20 mm of the common carotid and 20 mm of the internal and external carotid arteries. The study has been approved by the local Ethical Committee and was performed according to the Italian laws on autopsied human tissues (Porzionato et al., 2005).

Light microscopy analysis and immunohistochemistry

Tissues were fixed in 10% phosphate-buffered

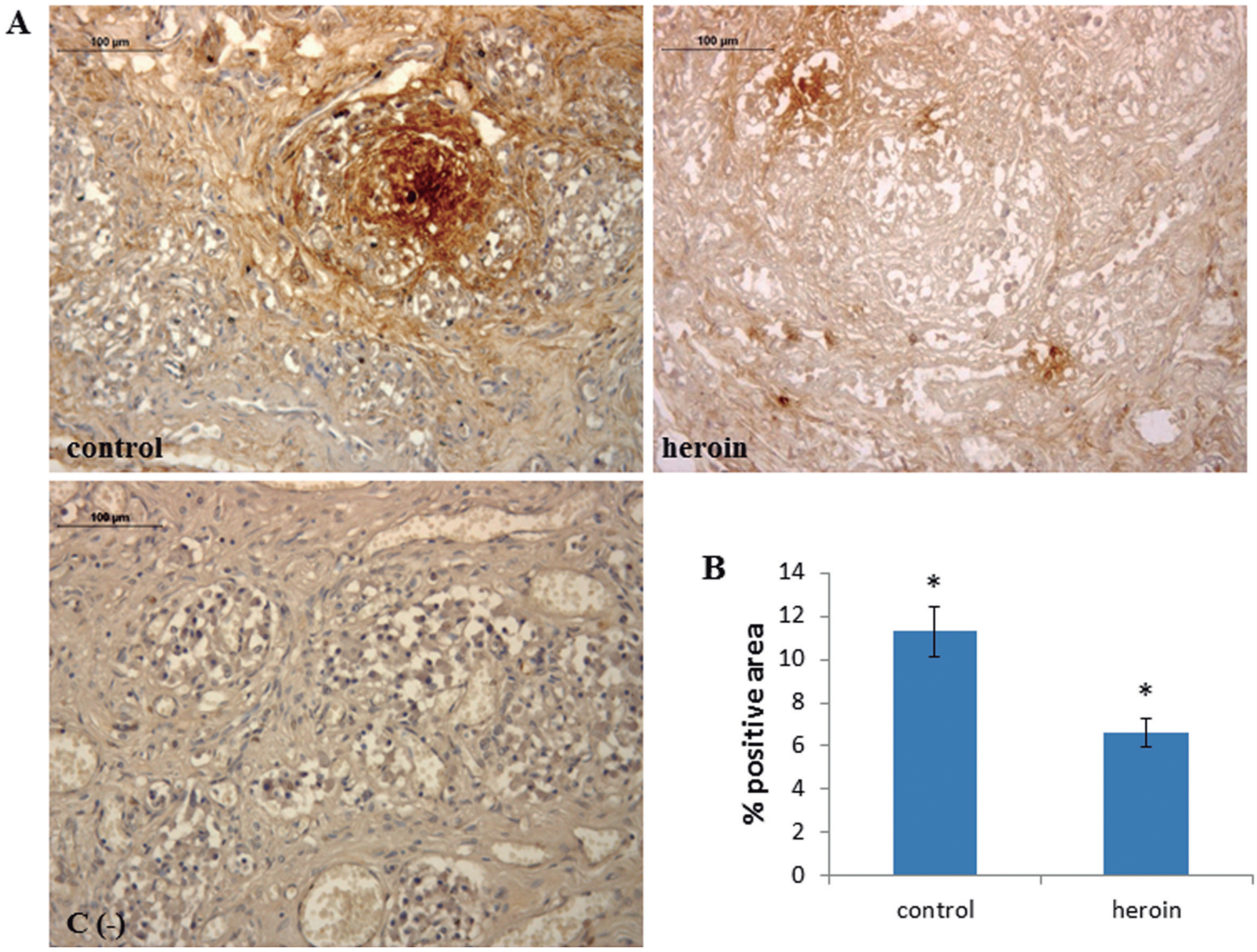


Fig. 2. A. Immunohistochemical detection of NGB expression in human carotid bodies of control and heroin subjects; C(-) negative control. **B.** Graphic representation of NGB percentage positive area (± SD) densitometric analysis determined by direct visual counting of ten fields (mean values) for each of three slides per sample at 40x magnification. *: heroin subjects NGB vs control subjects NGB $p<0,05$.

formalin for 72 hours and dehydrated through ascending alcohols and xylene and then paraffin embedded. Samples were then de-waxed (xylene and alcohol progressively at lower concentrations) and the slides, 5 μm thick, were processed for Trichrome Mallory staining (Tricromica kit) (Bio Optica, Milano, Italy), as suggested by the data sheet, to distinguish connective compartment from parenchyma, and for immunohistochemical analysis. In order to detect NGB, HIF-1α, VEGF, i-NOS, Bax and cleaved caspase-3 proteins immunohistochemical analysis was performed by means of Ultravision LP Detection System HRP Polymer & DAB Plus Chromogen (Lab Vision Thermo, CA, USA). Slides have been incubated in the presence of mouse monoclonal anti-NGB (primary antibody dilution 1:500) (Biovendor, Heidelberg, Germany, Cat. No. RD182043100), mouse monoclonal anti-HIF-1α and anti-Bax, rabbit polyclonal anti-VEGF and anti-i-NOS antibodies (primary antibodies dilution 1:100) (Santa Cruz Biotechnology, CA, USA, Cat. No. sc-53546; sc-7480; sc-152; sc-651, respectively), rabbit monoclonal anti cleaved caspase-3 (primary antibody dilution 1:200) (Cell Signalling Technology, Danvers, MA, Cat. No. 9604) and then in the presence of specific HRP-conjugated secondary antibodies. Peroxidase was developed using diaminobenzidin chromogen (DAB) and nuclei were hematoxylin counterstained. Negative controls were performed by omitting the primary antibody. Samples were observed by means of light microscopy Leica DM 4000 (Leica Cambridge Ltd, Cambridge, UK) equipped with a Leica DFC 320 camera (Leica Cambridge Ltd, Cambridge, UK) for

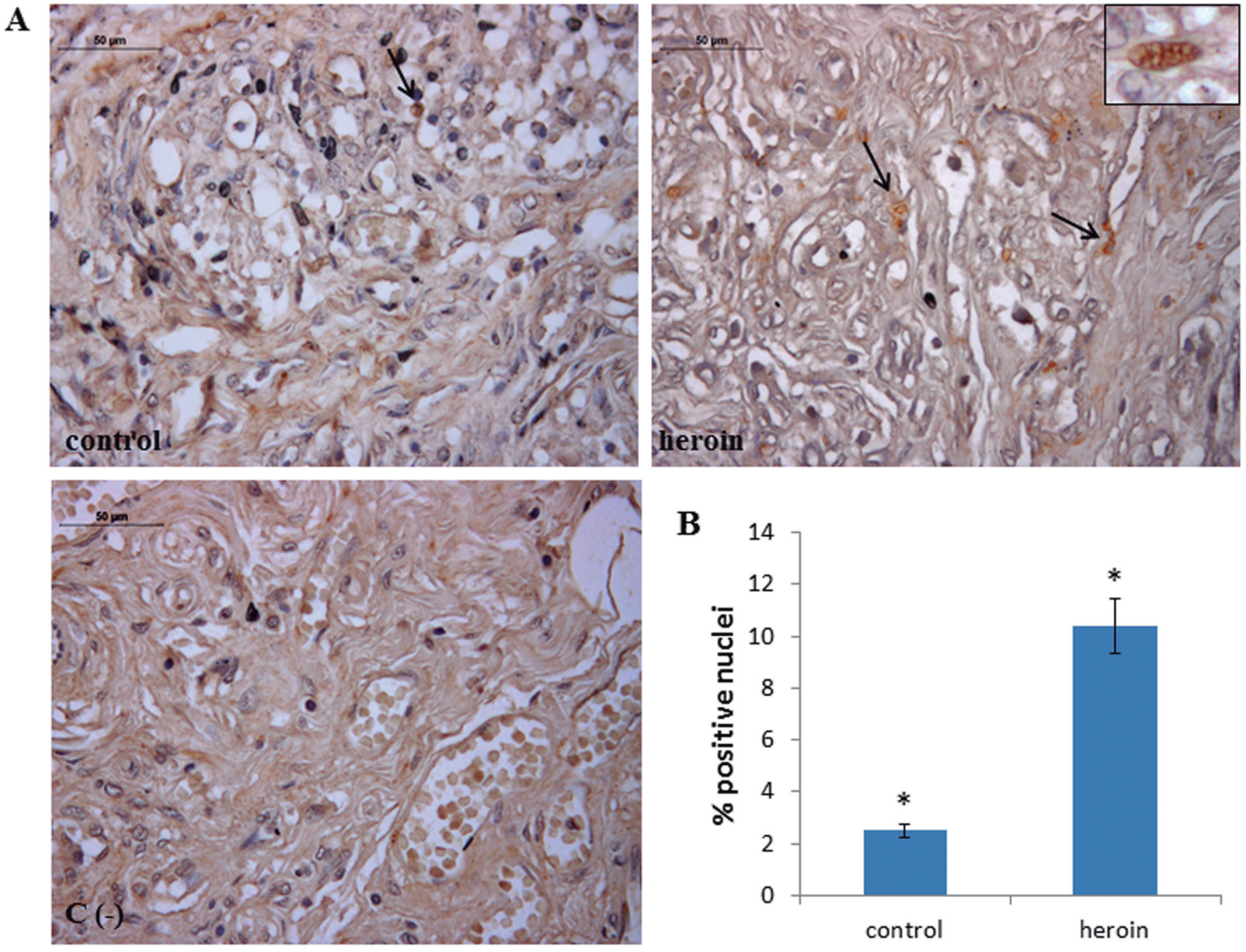


Fig. 3. A. Immunohistochemical detection of HIF-1α expression in human carotid bodies of control and heroin subjects; C(-) negative control. **B.** Graphic representation of HIF-1α positive nuclei percentage (± SD) densitometric analysis determined by direct visual counting of ten fields (mean values) for each of three slides per sample at 40x magnification. *: heroin subjects HIF-1α vs control subjects HIF-1α $p<0,05$.

computerized images.

Computerized morphometry measurements and image analysis

After digitizing the images deriving from Trichrome Mallory and immunohistochemical stained sections, QWin Plus 3.5 software (Leica Cambridge Ltd, Cambridge, UK) was used to evaluate connective compartment and parenchyma area percentage, NGB, HIF-1α, VEGF, i-NOS, Bax and cleaved caspase-3 expression. Image analysis of protein expression was performed through the quantification of threshold area for immunohistochemical brown colour per ten fields of light microscope observation. Parenchyma and connective tissue compartment measurements are expressed as % area mean (± SD) assessed by direct visual counting of ten fields for each of three slides per sample. Densitometric analysis of immunohistochemistry was determined by direct visual counting of ten fields (mean values) for each of three slides per sample at 40x magnification. QWin Plus 3.5 assessments were logged to Microsoft Excel and processed for Standard Deviations and Histograms. The statistical significance of the results was evaluated using the T-Test and the Linear Regression Test, with p=0.05.

Results

Mallory Trichrome staining was performed in order to evaluate the distribution of the CB parenchyma and connective tissue in different experimental conditions. A

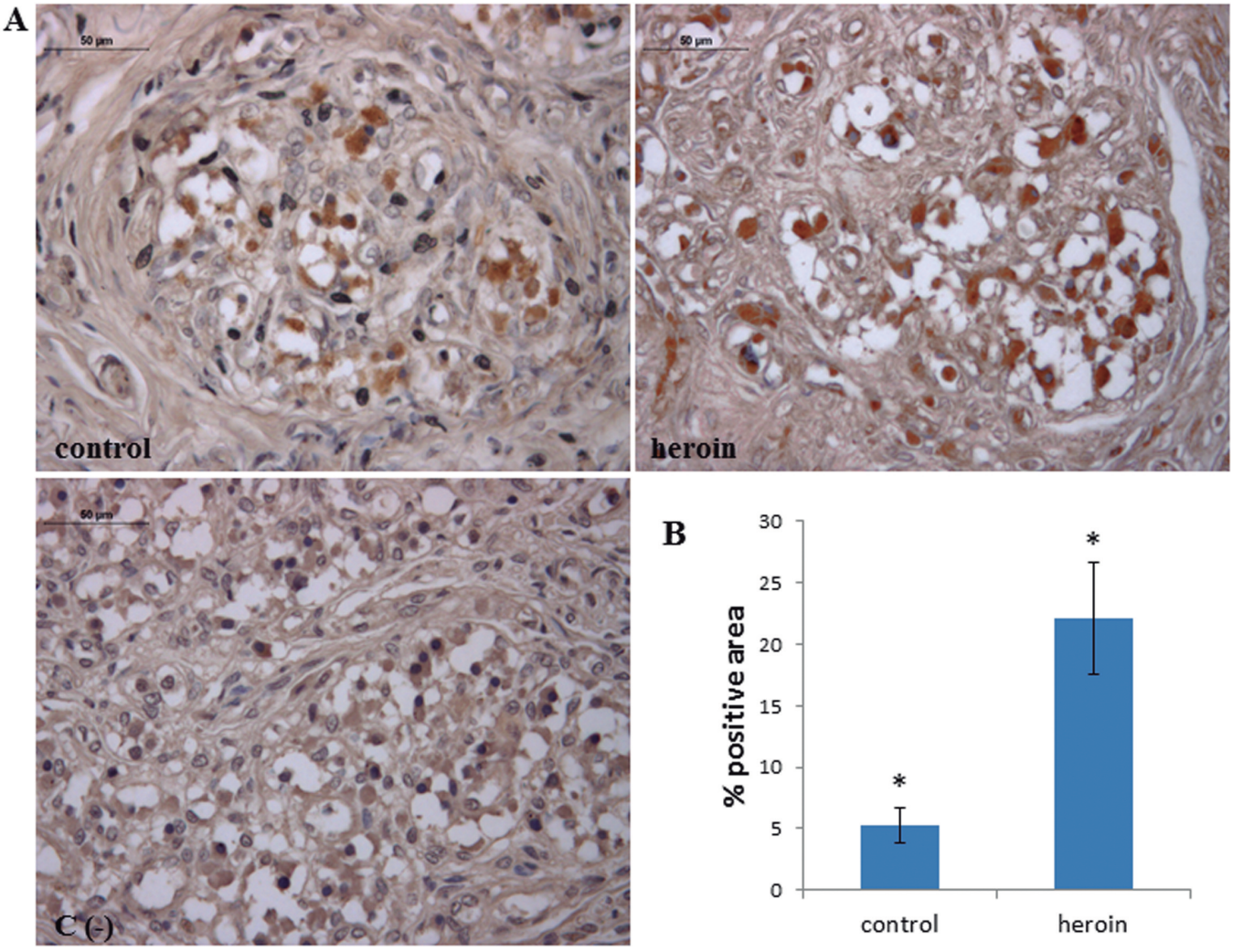


Fig. 4. A. Immunohistochemical detection of VEGF expression in human carotid bodies of control and heroin subjects; C(-) negative control. **B.** Graphic representation of VEGF percentage positive area (± SD) densitometric analysis determined by direct visual counting of ten fields (mean values) for each of three slides per sample at 40x magnification. *: heroin subjects VEGF vs control subjects VEGF p<0,01.

significant increase in connective tissue area percentage (31.9±2.4%) was found in heroin subjects compared to controls (20.4±2.4%), along with a parallel reduction in parenchyma area percentage (68.1±5.0% and 79.5±6.2%, respectively) (Fig. 1). Following this, immunohistochemical analysis was used to investigate NGB, HIF-1α, VEGF, iNOS, Bax, and Cleaved Caspase 3 expression and localization in human carotid bodies. A decrease of NGB expression was seen in heroin subjects (6.63±0.5%) compared to controls (11.3±0.9%) (Fig. 2). A larger number of HIF-1α positive nuclei was detected in heroin subjects compared to controls (10.04±1.0% vs 2.5±0.3%, respectively) (Fig. 3). VEGF expression was evaluated revealing a significantly higher positive area in heroin subjects (22.1±1.4%) compared to controls (5.2±4.6%) (Fig. 4). i-NOS expression was also investigated, being higher in heroin subjects (6.1±2.7%) compared to controls (3.7±1.0%), although this difference is not statistically significant (Fig. 5). Bax and cleaved caspase-3, proapoptotic molecules were then evaluated in order to estimate apoptotic event occurrence. Immunohistochemistry analyses for both of the tested molecules were positive in the heroin subjects (3.4±1.16% and 8.94±0.9%, respectively), while control subjects did not show any positive labelling (Fig. 6).

Discussion

The mammalian CB is a small, neural crest derivative neuroendocrine organ that behaves like an arterial chemoreceptor monitoring the partial pressure of oxygen and carbon dioxide, as well as pH, osmolarity,

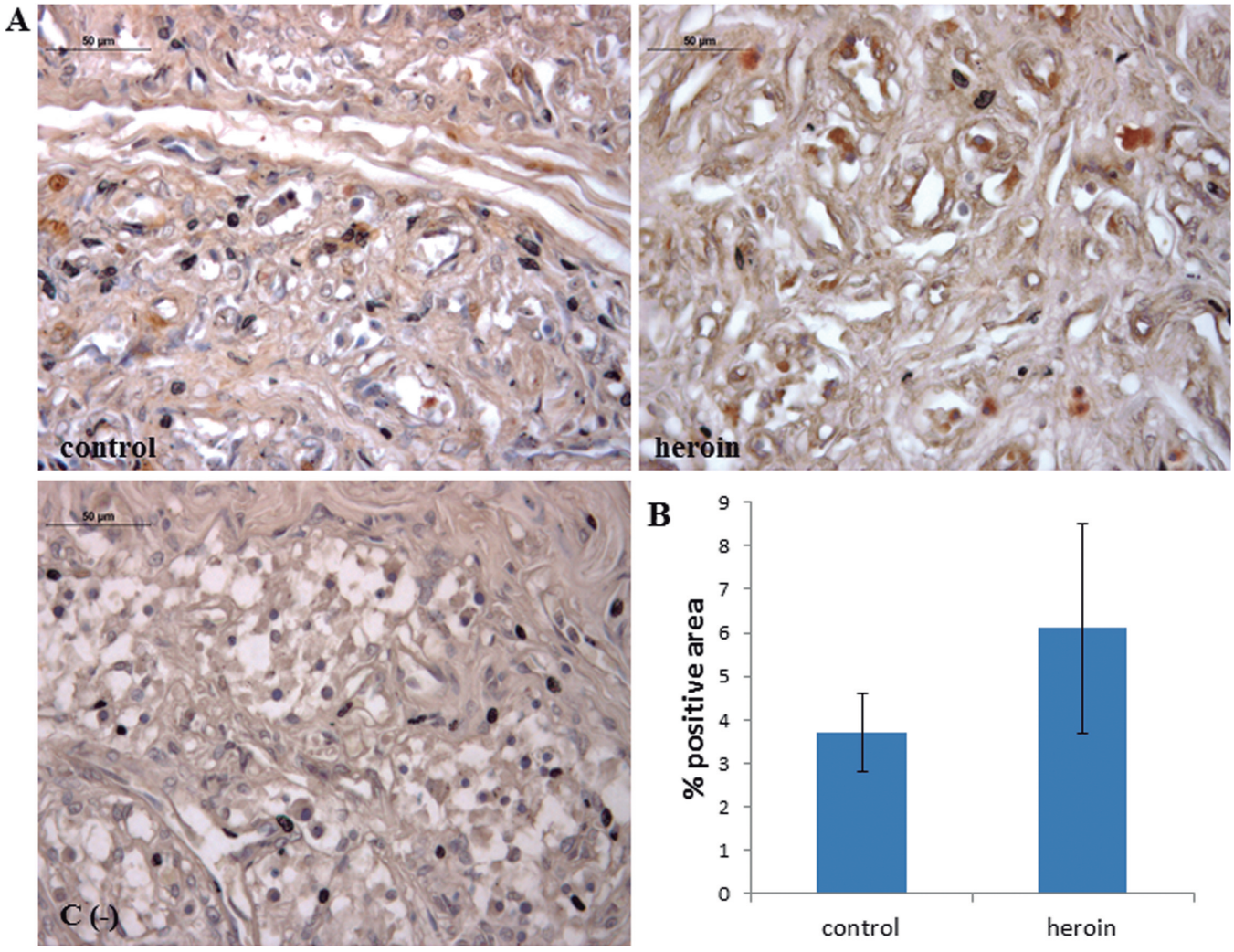
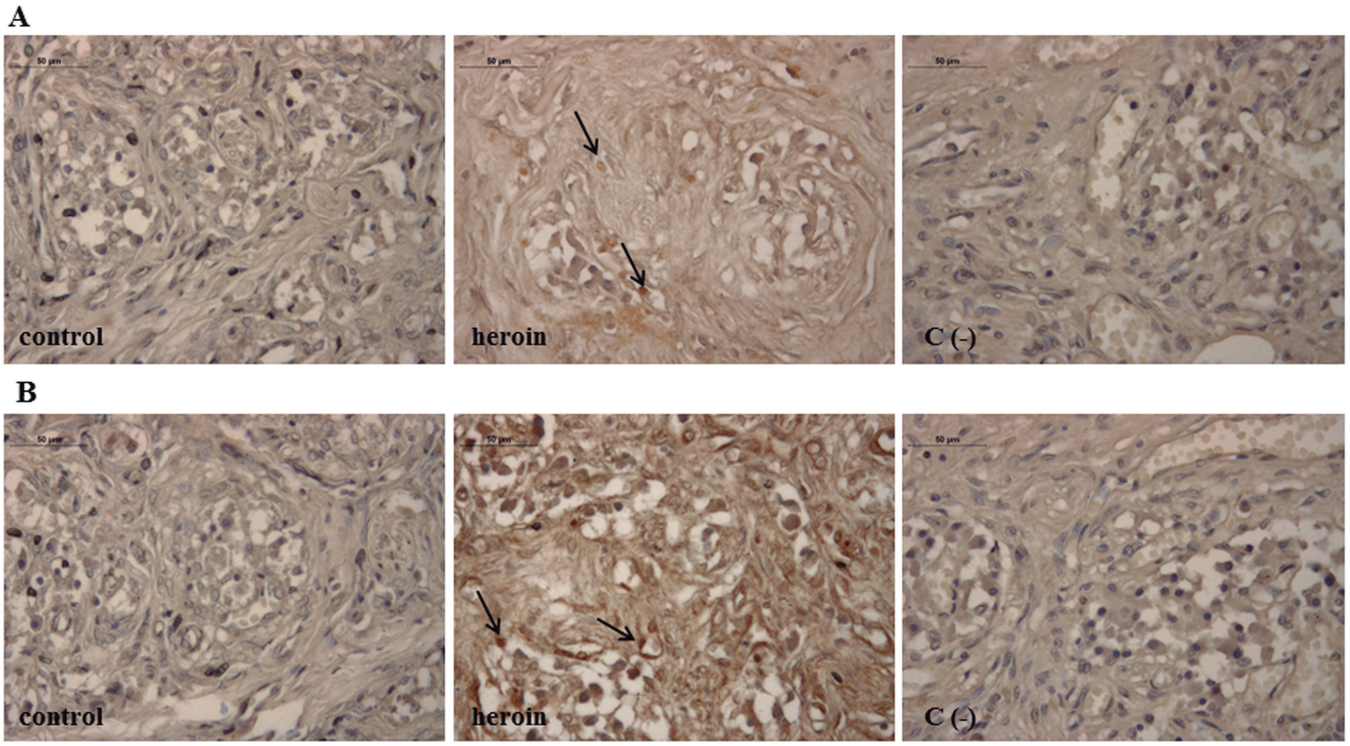


Fig. 5. A. Immunohistochemical detection of i-NOS expression in human carotid bodies of control and heroin subjects; C(-) negative control; **B.** Graphic representation of i-NOS percentage positive area (± SD) densitometric analysis determined by direct visual counting of ten fields (mean values) for each of three slides per sample at 40x magnification

and temperature of arterial blood (López-Barneo et al., 2009). It has four principal components: cell clusters, including type I (glomus cells) and type II cells (subtentacular cells), blood vessels, connective tissue, and nerve fibres. Moreover, since it is characterised by extreme vascularity and blood flow rate for its size (Kameda, 2005) and is the main oxygen chemoreceptor in arterial blood, it is the first device to detect and respond to transient, acute, and chronic hypoxic events (García-Fernández et al., 2005). The role of endogenous and exogenous opiate in basal suppression of the ventilator effects of peripheral chemoreceptors has already been demonstrated (Pokorski and Lahiri, 1991) and supported by enhanced carotid chemoreceptor response to hypoxia after intravenous injection of naloxone, the first pure opiate receptor antagonist (Fechir et al., 2012). On the basis of the above reported evidence, the aim of the study presented here was to investigate morphological and molecular modifications occurring in the CB of subjects who died of heroin intoxication compared with control subjects who died of trauma. An increase of both HIF-1α and VEGF is found in opiate subjects, which seems to confirm the typical tissutal-hypoxic condition present in drug addicts, a consequence of respiratory depression (White and Irvine, 1999). In addition to this, Ye et al. have reported a high level of endogenous NO production by increased expression of NOS enzymes in the rat CB during chronic hypoxia, so the higher i-NOS expression level in the CB of drug addicts could confirm the hypothesis that the increase of hypoxia-induced NO generation enhances the inhibition of carotid chemoreceptor activity consequently blunting hypoxic chemo-sensitivity in the CB during chronic hypoxia (Ye et al., 2002). Moreover, heroin addiction, which resembles chronic hypoxia exposure, could reduce the availability of oxygen to tissues and induces apoptosis, as confirmed by the increase in the expression of pro-apoptotic factors such as Bax and cleaved Caspase-3. Considering that NGB concentration in the brain is ~ 100-400 fold lower than



C

	Bax	cleaved caspase 3
control	-	-
heroin	3,4 ± 1,1	8,94 ± 0,7

Fig. 6. A. Immunohistochemical detection of Bax and cleaved Caspase-3 expression (**A and B**, respectively) in human carotid bodies of control and heroin subjects; C(-) negative control; **C.** Densitometric measurements Bax and cleaved Caspase-3 percentage positive areas (± SD) determined by direct visual counting of ten fields (mean values) for each of three slides per sample at 40x magnification

that in red muscle, NGB does not seem to act as an O_2 transporter, but more likely as an NO scavenger in the brain (Brunori and Vallone, 2007). NGB is also present in fish and amphibians (Awenius et al., 2001), and NGB shares as little as 25 % of the amino acids with vertebrate globins (Burmester et al., 2000), an observation in line with the phylogenetic analyses. The mechanism by which NGB protects cells from apoptotic cell death seems to be its ability to bind and then reduce cytochrome c released from mitochondria during the activation of apoptosis (Brittain et al., 2010a) and so inhibit apoptosome assembly. NGB seems to compete with Apaf-1 in this interaction (Brittain et al., 2010b), Apaf-1 being the major cytosolic protein involved in apoptosome formation (Yu et al., 2001), responsible for the mitochondrial apoptotic pathway, as reported in another experimental model (Zara et al., 2010).

Considering that NGB may function as a scavenger of reactive oxygen or nitrogen species (Herold et al., 2004) and probably intervenes in the apoptotic pathway to prevent cell death (Fago et al., 2008), the decrease of its expression, although small, could suggest a role of this globin in human CB impairment due to heroin addiction, as it would affect the O_2-CO_2 balance, allowing a decrease of CB oxygen sensitivity. Moreover, NGB reduction reduces CB type I cell protection from the oxidative-stress that occurs during heroin addiction, and NGB neuroprotective action loss increases the susceptibility to age-related changes in CB, resembling aging modifications.

The hypoxic state resulting from taking opiates is similar in some respects to that observed during the aging process, with the exception of NGB expression, which as demonstrated in previous work by the authors (Zara et al., 2012) increases in elderly subjects, probably to prevent oxygen desaturation.

It is not known if NGB acts as an oxygen sensor, as the great affinity of oxygen with NGB could lead to hypothesize that NGB protects carotid body type I cells from the oxidative stress that occurs during acute or chronic hypoxia or heroin addiction. CB oxygen consumption is high, and NGB could enhance cell type I survival during aging and stress. Sun et al. (2005) demonstrated a loss of NGB during aging and loss of the neuroprotective action with an increase of susceptibility to age-related neurological disorders. While further study is needed to clarify the role of NGB in chemoreception, the results presented in this paper suggest that NGB could play a role in heroin addiction by trying to compensate for the negative effects of heroin.

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