# Peripheral Oxidative Stress Blood Markers in Patients With Chronic Back or Neck Pain Treated With High-Velocity, Low-Amplitude Manipulation

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## Abstract

**Objective:** The purpose of this study was to investigate oxidative-stress parameters in individuals with chronic neck or back pain after 5 weeks of treatment with high-velocity, low-amplitude (HVLA) spinal manipulation.

**Methods:** Twenty-three individuals aged 38.2 ± 11.7 years with nonspecific chronic neck or back pain verified by the Brazilian Portuguese version of the Chronic Pain Grade, with a sedentary lifestyle, no comorbidities, and not in adjuvant therapy, underwent treatment with HVLA chiropractic manipulation twice weekly for 5 weeks. Therapeutic procedures were carried out by an experienced chiropractor. Blood samples were assessed before and after treatment to determine the activities of the antioxidant enzymes superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx), and the levels of nitric oxide metabolites and lipid hydroperoxides. These blood markers were analyzed by paired Student *t* test. Differences were considered statistically significant, when $P$ was $<.05$.

**Results:** There was no change in catalase but an increase in SOD (0.35 ± 0.03 U SOD per milligram of protein vs 0.44 ± 0.04 U SOD per milligram of protein; $P < .05$) and GPx (7.91 ± 0.61 nmol/min per milligram of protein vs 14.07 ± 1.07 nmol/min per milligram of protein; $P < .001$) activities after the treatment. The nitric oxide metabolites and the lipid hydroperoxides did not change after treatment.

**Conclusion:** High-velocity, low-amplitude spinal manipulation twice weekly for 5 weeks increases the SOD and GPx activities. Previous studies have shown a relationship between pain and oxidative and nitrosative parameters; thus, it is possible that changes in these enzymes might be related to the analgesic effect of HVLA spinal manipulation. (J Manipulative Physiol Ther 2015;38:119-129)

**Key Indexing Terms:** *Antioxidant Enzymes; Oxidative Stress; Manipulation; Spinal; Manual Therapy; Chiropractic*

The physiologic effects of vertebral manipulation may result from its effect on the flow of information to the central nervous system.[1,2] It is suggested that the spinal biomechanical dysfunction known as vertebral subluxation disturbs the neurologic function by sensitizing paraspinal sensory afferents, especially the proprioceptors and nociceptors in joints and muscles.[3-6] This sensitization possibly leads to plastic changes in cells of the central nervous system.[6] On the other hand, high-velocity, low-amplitude (HVLA) spinal manipulation supposedly alters the central sensory processing by favoring a nociceptive modulation due to low-frequency stimulation of mechanonociceptors of paraspinal tissues, which contributes to analgesia.[4] This central modulation may also influence muscle and visceral reflex responses.[1,2,6]

The peripheral sensitization after activation of nociceptors and the subsequent central sensitization are associated with neuronal excitability and the presence of pronociceptive molecules locally.[7,8] In pain conditions, there is an increase in neural activity due to neuronal excitability, with more utilization of metabolic substrates and increased production of reactive oxygen and nitrogen species (RS).[9,10] RS, which include free radicals and peroxides, are normally formed in the cell respiration process and play an important role in both physiologic and pathologic conditions.[11]

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At moderate concentrations, the RS may act as important physiologic regulators in intracellular signaling pathways.[12,13] In the development of persistent pain states, as occurs in nerve injury or inflammatory insult, the increase in RS seems to be essential not only for the induction but also for the maintenance of central sensitization in the spinal cord.[13-16] It is thought that hydrogen peroxide ($H_2O_2$), nitric oxide (NO), and superoxide are the main RS involved in the central sensitization process.[17]

An overwhelming production of reactive oxygen species can generate oxidative stress, leading to deleterious effects on cellular function.[18] Oxidative stress occurs as a result of an imbalance between increased production and/or reduced degradation of oxygen reactive species. It may lead to damage to lipids, protein, and DNA.[18] To counteract reactive oxygen species–induced cell damage, biological systems have evolved endogenous mechanisms to protect themselves in normal physiologic conditions.[19] The cellular antioxidant mechanisms involve nonenzymatic compounds and enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx).[19,20] Superoxide dismutase is specific to superoxide-radical detoxification. Superoxide dismutase can rapidly dismutate the superoxide radical, yielding $H_2O_2$ and oxygen.[21] Hydrogen peroxide is a diffusible reactive oxygen species that contributes to the development of pathologic pain states, not only by generating harmful reactive species but also by modulating synaptic plasticity.[22] The presence of $H_2O_2$ apparently affects the release of intracellular calcium, leading to neuronal sensitization and pronociceptive patterns in interneurons in the spinal cord dorsal horn.[15] Catalase activity, in turn, converts $H_2O_2$ to water.[23] Furthermore, GPx is an important enzymatic mechanism for the disposal of peroxides, producing water or alcohol and reduced glutathione.[24]

Nitric oxide may react with the superoxide radical and form peroxynitrite, a very deleterious nitrogen species, which may lead to lipid and protein peroxidation and damage.[17] In addition, NO has a modulatory role in pain states at both the central and peripheral levels.[25,26] Nitric oxide has been implicated in synaptic plasticity and multiple mechanisms involving central sensitization.[26] The involvement of NO in peripheral nociception is corroborated by data demonstrating the local release of NO by an inflammatory stimulus.[27] Nitric oxide plays a controversial role in pain modulation, that is, NO can mediate nociception or induce an antinociceptive effect.[26] The modulatory effect of NO may be related to neuronal excitability.[25]

In a previous study, we demonstrated a possible correlation between HVLA manipulation and oxidative-stress parameters. An increase in systemic catalase activity was demonstrated after 6 sessions of HVLA manipulation in 2 weeks of treatment.[28] Glutathione peroxidase activity, in turn, seemed to require a longer period of treatment (>2 weeks) because only a tendency to increased activity was found in the period considered.[29] These results led us to hypothesize that the effects of HVLA spinal manipulation on antioxidant activity may depend on the treatment period. Therefore, to provide more information about this hypothesis, the purpose of this study was to assess RS in blood of patients with nonspecific chronic neck or back pain treated by HVLA spinal manipulation for 5 weeks, twice a week, to analyze the activity of the antioxidant enzymes SOD, catalase and GPx, the levels of NO metabolites, and the formation of lipid hydroperoxides (LOOHs), which are formed by the action of reactive oxygen species on polyunsaturated fatty acids,[30] in the blood of the patients before and after the treatment.

## Methods

### Subjects

This study selected individuals with nonspecific[31] chronic neck or back pain as described by Guzman et al[32] and Lawrence et al,[33] respectively. The criteria for inclusion in the study were symptoms must be present for at least 90 days[34] and have an average intensity greater than 2 of 10 on a visual numerical pain scale.[35] The exclusion criteria were symptoms related to serious pathologies such as malignancy, infection, inflammatory disorder, or fracture. Patients were also excluded, when there were signs of lumbar or cervical spinal cord compromise or radiculopathy and/or a history of neck or back surgery. In addition, subjects were only eligible for trial inclusion if spinal manipulation was an appropriate therapy for their condition. The subjects had to be nonsmokers, not be on adjuvant therapy, and have a sedentary lifestyle because exercise can influence the biochemical parameters studied.[36,37]

### Experimental Procedures

Fifty subjects (18-60 years old) with chronic neck pain or back pain were recruited through advertisements in the local newspapers of Vale dos Sinos (free distribution, 13 000 copies, May-July 2012; Jornal Bem Estar) and advertisements in public agencies (Agência do Sistema Nacional de Emprego e Fundação Gaúcha do Trabalho e Ação Social, June 2011-2012; and City Hall of Novo Hamburgo, August-December 2012). Each volunteer was identified by a code during the selection process; the same code was subsequently used to identify the records and blood samples. An inclusion/exclusion questionnaire was administered, and the history and a physical examination were used to screen the participants. The frequency, intensity, and disability of the symptoms were assessed by the Brazilian Portuguese version of the Chronic Pain Grade (CPG-Br), an adapted and validated version of the original graded chronic pain scale from Von Korff et al.[38,39] The CPG-Br evaluates self-perception of pain. The scale has 7 questions that assess pain intensity and disability. Six questions request numerical scores ranging from 0 to 10, including the pain intensity at the moment, in the worst phase, and the average pain intensity. An additional question

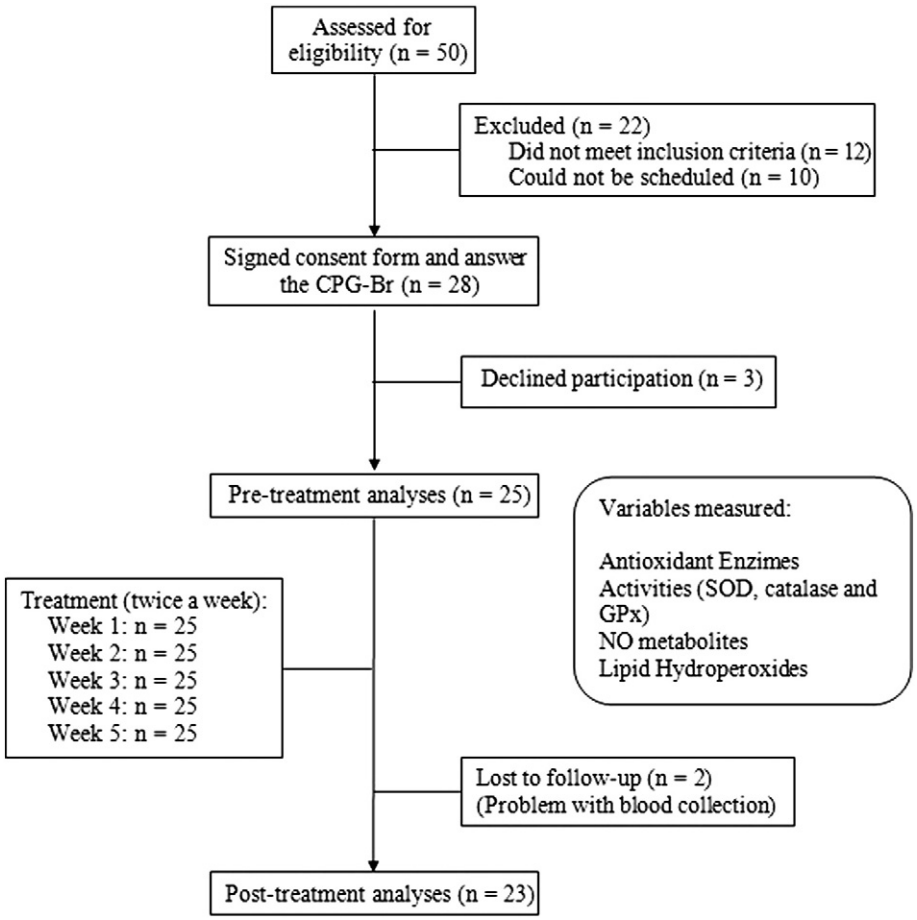


**Fig 1.** *Diagram shows the flow of participants through the course of the study. CPG-Br, Brazilian Portuguese version of the Chronic Pain Grade; GPx, glutathione peroxidase; SOD, superoxide dismutase.*

assesses the number of days on which the person could not pursue their usual activities due to pain.[38] Twenty-five individuals symptomatic for nonspecific chronic neck or back pain were selected for this study (for more details, see Fig 1). All selected subjects signed the required consent form before beginning the study. The participants were subsequently scheduled for the first blood-sample collection, 1 day before the first session of treatment. Then, the subjects underwent 10 sessions of spinal HVLA manipulation (diversified techniques), twice weekly for 5 weeks. High-velocity, low-amplitude procedures were performed on all segments of the spine with restriction of movement as determined by motion palpation. Subjects with neck or back pain were considered as a single group because they were given full spine adjustment. Finally, 1 day after the treatment ended, the subjects gave a final blood sample.

Blood samples were always drawn in the early hours of the morning (from 7 AM to 9 PM). Patients were instructed to fast for 8 hours and reduce their intake of sausages, red meat, fat, chocolate, caffeine, citrus fruits, and whole grains up to 24 hours before giving blood samples.[40,41] The blood samples were drawn from the cubital vein using disposable needles and monovettes (BD Vacutainer, BD, Franklin Lakes, NJ, cod. 367874) with anticoagulant heparin. The first volume of blood (2 mL) was discarded to avoid hemolysate material in the sample.[41] Finally, 7 mL of venous blood was quickly obtained and kept on ice until the preparation of samples for later analysis.

The interview, treatment, and blood sampling were performed in a private chiropractic clinic. The blood samples were analyzed in the laboratories of the Physiology Department of the Federal University of Rio Grande do Sul. The therapeutic procedures were carried out by an experienced doctor of chiropractic (> 7 years of practice); the questionnaires were administered by a second doctor of chiropractic, and the blood samples were drawn by a nurse.

This study was approved by the Ethics Committee of the Federal University of Rio Grande do Sul (no. 2008233). All participants provided signed consent.

### Blood Sample Preparation

Blood samples were centrifuged at 2700*g* for 20 minutes in a refrigerated centrifuge (Sorvall RC 5B–Rotor SM 24; DuPont Instruments, Wilmington, DE). Plasma samples

were aliquoted for later analysis of LOOHs and NO metabolites and stored at −70°C. For analysis of LOOH, the plasma was diluted 1:1 (vol/vol) in butylated hydroxytoluene (2,6-di-tert-butyl-4-methylphenol) (90% in methanol) before being stored.[42] Red blood cells (RBCs) were washed 3 times with cold 0.9 N phosphate-buffered saline. Aliquots of RBCs were stored at −70°C in 1 mmol/L HCl, and 4 mmol/L $MgSO_4$ buffer was diluted 50:500 (vol/vol) for later analyses of enzymatic antioxidant activity.[43]

### Determination of Antioxidant Enzyme Activities

In this study, CuZnSOD activity was determined. The activity of SOD, expressed as units per milligram of protein, was measured based on its action to neutralize the superoxide radicals to prevent oxidation of adrenalin to adrenochrome, a colorful byproduct that can be measured at 480 nm (Spectrophotometer SP22; Bioespectro, Curitiba, Paraná, Brazil). Superoxide dismutase activity was determined by measuring the velocity of inhibition of adrenaline oxidation. The reaction medium contained glycine buffer (50 mmol/L, pH 11.3) and adrenalin (60 mmol/L, pH 2.0).[44]

Glutathione peroxidase activity was measured by after nicotinamide dinucleotide phosphate acid oxidation at 340 nm (Spectrophotometer T60U; PG Instruments Limited, Leicestershire, UK) as described by Flohé and Günzler.[45] Glutathione peroxidase results were expressed as nanomoles of peroxide/hydroperoxide reduced per minute per milligram of protein.

Catalase activity was determined after the decrease in absorption at 240 nm (Spectrophotometer T60U; PG Instruments Limited) in a reaction medium containing 50 mmol/L phosphate buffer (pH 7.2) and 10 mmol/L $H_2O_2$[46-48] and expressed as picomoles of $H_2O_2$ reduced per minute per milligram protein.

### Protein Measurement

Protein was measured by the method of Lowry et al,[49] using bovine serum albumin as standard.

### Determination of NO Metabolites

Pure plasma samples without previous preparation or dilution were analyzed for NO metabolites. To measure NO metabolites, nitrites ($NO_2$) were determined using the Griess reagent, in which a chromophore with a strong absorbance at 540 nm is formed by reaction of $NO_2$ with a mixture of 0.1% naphthylethylenediamine and 1% sulfanilamide. Nitrates ($NO_3$) were determined as total $NO_2$ (initial $NO_2$ plus $NO_2$ reduced from $NO_3$) after their reduction using $NO_3$ reductase from *Aspergillus* species in the presence of nicotinamide dinucleotide phosphate acid. A standard curve was established with a set of serial dilutions ($10^{-8}$ to $10^{-3}$ mol/L) of sodium $NO_2$. Absorbance at 540 nm was obtained (spectrophotometer, Zenyth 200; Anthos, Eugendorf, Austria). Results were expressed as micromoles of $NO_2$ plus $NO_3$ per liter.[50]

### Determination of LOOHs

The method is based on oxidation of iron ferrous ($Fe^{2+}$) to the ferric form ($Fe^{3+}$) in the presence of LOOHs and formation of complexes of $Fe^{3+}$ with xylenol orange.[30,51] According to the technique adapted from Sodergren et al,[42] for each sample, a blank reduced with triphenylphosphine (TPP) 10 mmol/L in absolute methanol was used.

At the time of the test, a working reagent was prepared with 81% (vol/vol) of 90% methanol, 2 mmol/L xylenol orange (o-Cresolsulfonphthalein-3′-3″-bis [methyliminodiacetic acid sodium salt]) to a final concentration of 100 μmol/L, 1 mol/L sulfuric acid to a final concentration of 25 mmol/L, 40 mmol/L 2,6-di-tert-butyl-4-methylphenol to a final concentration of 4 mmol/L, and 10 mmol/L ferrous sulfate to a final concentration of 250 μmol. Samples were prediluted 1:10 in Milli-Q (Direct-Q3, Millipore SAS, Molsheim, France) water before the test. Then, they were divided into 2 tubes with 90 μL of sample in each. The samples were incubated for 30 minutes with 10 μL of 90% methanol or 10 μL of 1 mmol/L TPP. After incubation, the samples were pipetted in duplicate in the microplate and incubated with working reagent (1:9) with stirring for 1 hour at room temperature. Absorbance at 560 nm was obtained (spectrophotometer, Zenyth 200; Anthos), and the absorbance values of the duplicates obtained with TPP were subtracted from the values for the duplicates without TPP. Results are expressed in nanomoles of LOOHs per milligram of protein.

### Statistical Analysis

For statistical analysis, subjects with neck or back pain were considered as a single group. The comparison was made before and after treatment, independently of the pain location and for each blood marker assessed. Data were analyzed with the Sigma State package version 3.1 (Systat Software Inc, Chicago, IL) for Windows. Statistical differences were analyzed by paired Student *t* test. Differences were considered statistically significant, when $P$ was $<.05$.

## Results

Initially, 28 subjects were eligible for the study but 3 declined to participate, giving an initial sample of 25 patients. No patients were lost during the course of the treatment, but at the final blood collection, it was not possible to draw enough blood for the analyses from 2 patients. Thus, the final sample size involved 23 subjects (Fig 1). Determinations of the antioxidant enzymes SOD and catalase were obtained from all patients. The GPx activity was determined for 21 patients (2 missing samples). Because of the presence of hemolysate in the plasma sample, determinations of NO metabolites and LOOHs were obtained from 21 and 19 patients, respectively. No severe adverse effects were reported by any participant during the treatment; a few patients reported mild soreness at the site of the vertebral segment manipulated.

**Table 1.** *Demographic and Clinical Characteristics of Subjects*

| | n = 23 |
|---|---|
| Mean age ± SD, y | 38.2 ± 11.7 |
| Female/male | 18/5 |
| Mean body mass index ± SD, kilograms per square meter | 24.9 ± 4.1 |
| Patient complaints [a] | |
| Neck pain | 45% |
| Low back pain | 14% |
| Neck pain and low back pain associated | 41% |

[a] Distribution of patients according to the location of painful complaints.

**Table 2.** *Classification on CPG-Br*

| | n = 23 |
|---|---|
| Grade 0 (no pain) | 0% |
| Grade I (low intensity) | 5% |
| Grade II (high intensity) | 45% |
| Grade III (moderately limiting) | 30% |
| Grade IV (severely limiting) | 20% |
| Pain disability | |
| Low disability (grade I and II) | 50% |
| High disability (grade III and IV) | 50% |
| Pain intensity [a] (mean ± SD) | 6.9 ± 1.3 |

Distribution of patients according to the classification on CPG-Br.

[a] Arithmetic mean of the reported pain at the moment, the worst pain, and the middle pain, according to CPG-Br.

### Characterization of Pain Symptoms

The demographic characteristics of the subjects are shown in Table 1. The characteristics of the pain of the neck or back were reported by the patients using the CPG-Br and are shown in Table 2. According to the CPG-Br, half of the patients had disabling pain, and of these, one-third had severely limiting symptoms and the others only a moderately limiting condition. Almost all of the remaining subjects had pain of high intensity but slightly limiting, and only 1 patient had pain of low intensity and not limiting. Overall, mean pain intensity was greater than 50% on a 10-point visual numerical pain scale, wherein the characteristics of pain at the moment, in the worst phase, and the average pain intensity were considered.

### Antioxidant Enzyme Activities

The SOD and GPx activities were significantly higher after 10 sessions of HVLA spinal manipulation for 5 weeks (SOD: pretreatment, 0.35 ± 0.03 U SOD per milligram of protein, posttreatment, 0.44 ± 0.04 U SOD per milligram of protein; $P = .005$) (GPx: pretreatment, 7.91 ± 0.61 nmol/min per milligram of protein, posttreatment, 14.07 ± 1.07 nmol/min per milligram of protein; $P < .001$; mean ± SEM) (Fig 2). Considering the general average of the SOD and GPx activities of patients, the increase after the treatment was around 27% for SOD (Fig 2A) and more than 77% for GPx (Fig 2B) activities. The catalase activity, in these experimental conditions, did not show any significant change after the treatment (pretreatment, 3.72 ± 0.19 pmol/min per milligram of protein, posttreatment, 4.25 ± 0.35 pmol/min per milligram of protein; $P > .05$) (Fig 2C).

### Nitric Oxide Metabolites

The NO metabolites ($NO_2 + NO_3$) showed no significant change in the plasma from patients with nonspecific chronic neck or back pain treated by HVLA spinal manipulation (Fig 3A). The level of NO metabolites was 78.32 ± 6.73 $\mu$mol/L before treatment, whereas in posttreatment was 81.81 ± 7.12 $\mu$mol/L.

### Lipid Hydroperoxides

Similarly to NO metabolites, no significant difference was found in the LOOHs levels of the plasma from patients with nonspecific chronic neck or back pain treated by HVLA spinal manipulation (Fig 3B). The total amount of plasmatic LOOHs was 4.33 ± 3.4 nmol/mg of protein in pretreatment and 5.89 ± 5.0 nmol/mg in posttreatment.

## Discussion

The present study demonstrated that treatment twice weekly for 5 weeks of individuals with nonspecific chronic neck or back pain by HVLA spinal manipulation increased SOD and GPx activities, with no significant changes in catalase activity, LOOHs, or NO metabolites in the systemic blood. In our previous study, in contrast, catalase activity increased after 6 interventions of HVLA spinal manipulation in patients with neck pain, whereas no significant change was observed in SOD and GPx activities. However, GPx activity showed a tendency to increase with a shorter period of treatment.[28,29] These results suggest that the effect of HVLA spinal manipulation on antioxidant enzyme activities may be affected by the length of time and the frequency of treatment.

Before discussing our results, it is necessary to note some considerations. In the present study, no control group (a group without pain) was used. The absence of this group is because our pretreatment values for the SOD, catalase and GPx activities, and NO metabolites showed a similar range to that described in the literature for healthy individuals.[52-54] The choice not to include a control group also took into account previous studies that assessed oxidative parameters in blood, without a healthy control group.[55,56]

Our result showed no changes in the LOOH levels. It is possible that the lack of change in LOOHs may be due to the increase in antioxidant enzyme activities and the availability of NO metabolites. The presence of LOOHs causes oxidative stress-mediated damage by the action of peroxynitrites and hydroperoxides on polyunsaturated fatty acids.[30] The rise in SOD activity reduces the availability of the superoxide

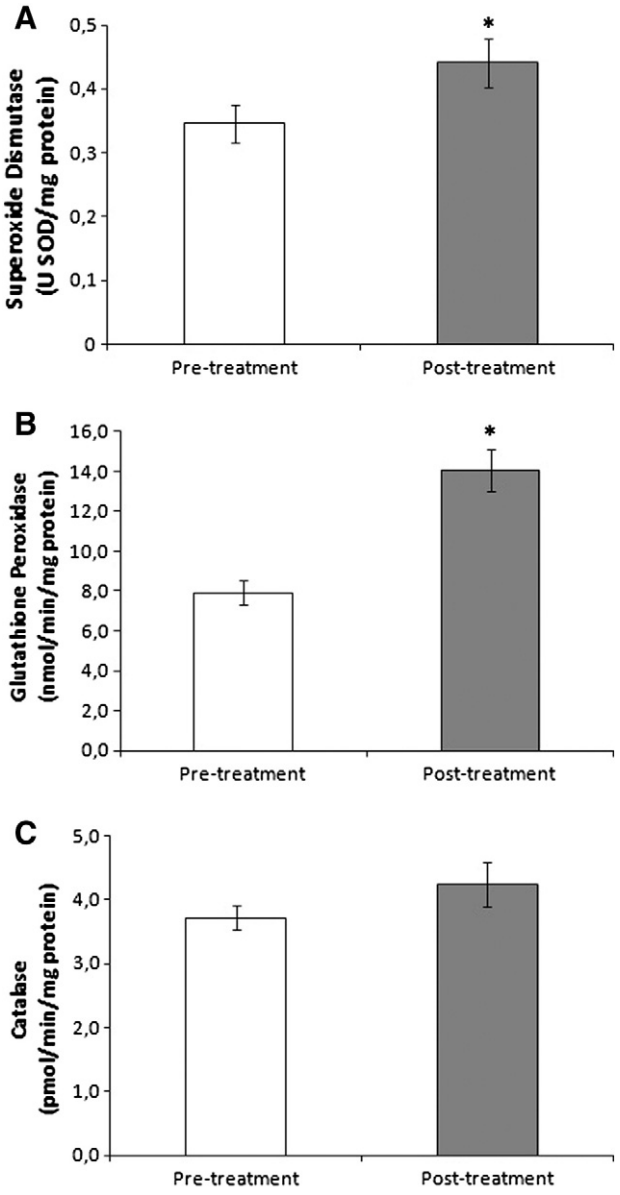


**Fig 2.** *Antioxidant activity of SOD (expressed units of SOD per milligram of protein) (n = 23) (A), GPx activity (expressed as nanomoles per minute per milligram of protein) (n = 21) (B), and catalase (expressed as picomoles per minute per milligram protein) (n = 23) (C) in RBCs from patients with nonspecific chronic neck or back pain. Data are represented as mean ± SEM.* $^{*}P < .05$ *(paired Student t test).*

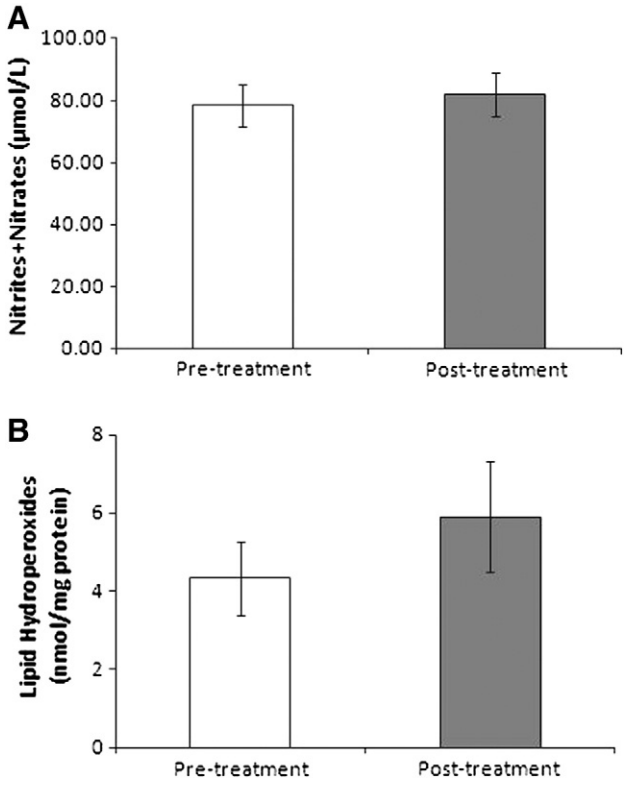


**Fig 3.** *Determination of NO metabolites ($NO_2$+$NO_3$) (expressed as micromoles per liter) (n = 21) (A) and LOOHs (expressed as nanomoles per milligram of protein) (n = 19) (B) in plasma from patients with nonspecific chronic neck or back pain. Data are represented as mean ± SEM. There was no significant difference after treatment (paired Student t test). SOD, superoxide dismutase.*

radical. This radical can react with NO, forming peroxynitrite, a harmful oxidant agent.[17,57] Glutathione peroxidase catalyzes the reduction of $H_2O_2$ and organic hydroperoxides at the expense of glutathione. It also functions as a defense against peroxynitrite-mediated oxidations.[58] Thus, the increase in the SOD and GPx activities may be contributing to prevent the increase of peroxynitrite. Furthermore, it is known that in pain processing or in injury to neural tissue, there is involvement of reactive species including superoxide, NO, and peroxynitrite.[15,16,57,59] Thus, the increase in SOD activity, which decreased the availability of superoxide, may be one contributing factor to the analgesic effect of the HVLA spinal manipulation treatment because no change was found in NO metabolites in our study.

However, the dismutation of superoxide radicals results in $H_2O_2$ formation.[21] Hydrogen peroxide has a high damage potential and also contributes to central sensitization.[16] It appears to release intracellular stored calcium, and in this way affects synaptic activity in dorsal horn interneurons, leading to sensitization of these neurons and pronociceptive patterns.[22] However, $H_2O_2$ does not have only deleterious effects. At moderate concentrations, $H_2O_2$ can act as an intracellular messenger that modulates neurotransmitter systems.[58] Thus, it is important to maintain the intracellular concentrations within physiologic levels, restricting somewhat the increase of this molecule that would occur with the increase in antioxidant enzyme activity.

The enzymes catalase and GPx are important tools for the detoxification of $H_2O_2$.[60] An increase in the activity of these enzymes in any conditions indicates a decrease in $H_2O_2$ levels. Our previous results demonstrated that catalase activity increased after the third session of HVLA spinal manipulation and was higher after 6 sessions.[28] However, we described only a tendency to an increase in GPx activity.[29] In the present study, HVLA spinal manipulation for 5 weeks did not induce a significant change in catalase but did increase GPx activity. These changes can regulate the concentrations of $H_2O_2$ in erythrocytes. At moderate concentrations, $H_2O_2$ maintains its beneficial intracellular functions.[58] The change in the activity of catalase and GPx in different periods after treatment by HVLA spinal manipulation appears to indicate that this treatment, directly or indirectly, induced these changes, although they appear to depend on the period of intervention.

It is possible that the effects on RS of the length of time and the frequency of the treatment by HVLA spinal manipulation may be related to the response of the immune system. Studies have demonstrated a close relationship between the oxidative stress balance and the immune system. Cytokines may induce SOD expression, which appears to protect the normal tissue from damage.[61] Some cytokines, such as tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β) and some reactive-oxygen intermediates are involved in the process of signal transduction that leads to activation of the SOD gene.[61,62] Interleukin 6 (IL-6) may also indirectly favor SOD expression by enhancing IL 1 and TNF-α, which magnifies the effect of these cytokines on SOD expression.[61] The relationship between cytokines and SOD activity must be interpreted with caution because the gene expression of SOD and its enzymatic activity may be regulated by different factors and independently of each other.[63]

Some studies suggest an increase in proinflammatory cytokines in the blood of patients with neck or lower back pain of mechanical origin.[64,65] In turn, the treatment with spinal manipulation appears to decrease the proinflammatory cytokines.[65-68] Subjects treated with a single spinal manipulation therapy showed a time-dependent attenuation of lipopolysaccharide-induced production of the inflammatory cytokines TNF and IL-1β.[68] Chiropractic manipulation (activator method) for 2 weeks induced a tendency to reduction in IL-6 levels.[65] Interestingly, comparison between this and our previous results shows that the reduction of IL-6 coincided with the tendency to reduction in SOD activity.[29] Thus, it is possible that the lower SOD activity after the patients with neck pain who were treated by HVLA spinal manipulation for 2 weeks may be due to the reduction in proinflammatory cytokines, probably IL-6. It would be interesting to examine the relationship between proinflammatory cytokines and RS parameters in patients treated by HVLA spinal manipulation.

In tissue inflammation, not only the reactive oxygen species was increased but also NO production.[17] Nitric oxide is a gaseous free radical that is synthesized from L-arginine and may act as an important regulator of vasoregulation, neuronal transmission, immune response, and cell apoptosis.[69] Excessive production of NO after induction of calcium-independent inducible NO synthase has been proposed as a major factor involved in tissue damage.[69] Interestingly, Mazzetti et al[70] found high levels of NO metabolites in chondrocytes stimulated with IL-1β and TNF-α, and these levels were higher in chondrocytes from patients with osteoarthritis than in those patients with rheumatoid arthritis. An animal model of joint immobilization showed reversible neurodegenerative changes, which were related, in part, to NO-mediated oxidative stress.[71] In addition, levels of NO metabolites were higher in individuals with mechanical neck pain compared with healthy controls, which are probably related to the activation of proinflammatory mediators.[64]

Despite the wider variation in values of the NO metabolites in our study, the values were similar to those found by Teodorczyk-Injeyan et al.[64] This study found levels from 12 to 106 μmol/L in patients with pain, whereas our results ranged from 47 to 161 μmol/L. However, we determined $NO_2$ plus $NO_3$ levels, whereas they determined only $NO_2$ levels. According to these authors, high levels of NO combined with neuronal sensitization could contribute to the genesis of the pain state. The vertebral manipulation response, in turn, could down-regulate inflammatory mediators.[66-68] Our data are consistent with the assumption that NO contributes to pain symptoms. However, it is interesting that no significant change was observed in NO metabolites after the HVLA spinal manipulation. In agreement with our findings, another study with HVLA spinal manipulation showed no alteration in plasma levels of NO in healthy subjects, either immediately or 2 hours after the intervention.[72] The same authors found only a correlation with the pain sensitivity at the pressure-pain threshold. These authors suggested that NO can be involved in pain sensitization but may not be a key element in the analgesic effects of spinal manipulation. Our results reinforce this hypothesis.

However, it is necessary to use caution in analyzing the relationship between NO and HVLA spinal manipulation. Our study showed that this treatment increases GPx activity. This enzyme catalyzes the reduction of $H_2O_2$ and organic hydroperoxides at the expense of glutathione.[24] Some authors have considered glutathione as an additional cofactor required for maximum activity of NO synthase, the enzyme responsible for synthesis of NO.[73] An increase in GPx activity induces greater consumption of glutathione. Thus, it can be hypothesized that the relationship between NO, glutathione, and GPx has some role in the lack of changes in NO after treatment by HVLA spinal manipulation.

The maintenance of NO levels may explain in part why the increase in SOD activity was not as pronounced as the

increase in GPx because it has been demonstrated that SOD activity is inhibited in the presence of increases in NO formation.[74] Further studies demonstrating the relationship between NO formation and SOD expression will be necessary to clarify the relationship between these molecules in patients with pain treated by HVLA spinal manipulation.

High-velocity, low-amplitude manipulation may cause changes not only in neuronal excitability but also in other physiologic parameters.[2,4-6] We demonstrated again that the HVLA spinal manipulation affects the oxidative status by increasing the activity of the antioxidant enzymes SOD and GPx. In contrast to our previous studies that showed an increase only in catalase activity, the activity of this enzyme did not change in the experimental conditions used here. In addition, we observed no significant changes in LOOHs and NO metabolites. Because the main difference between the studies was the length of time and the frequency of interventions by HVLA spinal manipulation, our results reinforce our hypothesis that the length of time and the frequency of the HVLA treatment are the determinants for these changes in the antioxidant activity in patients with nonspecific chronic neck or back pain. It is possible that the combination of factors such as neuronal excitability, inflammatory response, and oxidative status may be responsible for the entire picture and possibly make some difference in pain attenuation. The most important aspect is that HVLA spinal manipulation increases the activity of antioxidant enzymes. Because the literature shows a relationship between pain and oxidative and nitrosative parameters, changes in these enzymes might be related to the analgesic effect of HVLA spinal manipulation.

### Limitations

This study was performed with HVLA spinal manipulation treatment for 5 weeks, twice a week, in subjects with nonspecific neck or back pain, without a control group. It would be more enlightening to have a healthy control group under the same experimental procedures and also to have a control group of a different therapeutic modality or sham manipulation. It is necessary to consider that this study was not blind, and some placebo effect may have occurred. In addition, it is important to consider the time of the blood collection. In this study, blood was drawn 1 day after the end of treatment. We suggest that future studies assess oxidative and nitrosative parameters in blood collected at different times, including during the interval of treatment, for example, after 1 session of HVLA spinal manipulation. It is also necessary to analyze the oxidative and nitrosative parameters in the serum. The determinations should also include cytokines. Data obtained for women and men require caution in the interpretation because of the systemic influence of female hormones on pain sensitivity. Finally, in future studies, the number of patients should be increased.

## Conclusion

Our study showed that SOD and GPx activity but not catalase activity of the RBCs of patients with nonspecific chronic neck or back pain increased after 10 sessions of HVLA spinal manipulation. This treatment did not induce significant changes in the LOOHs and NO metabolites in the plasma of these patients. Although it is too early to speculate on the role of oxidative and nitrosative species in HVLA treatment, the results of the present study support the hypothesis that the effects of HVLA spinal manipulation on oxidative and nitrosative species depend on the length of time and the frequency of treatment. Because previous studies have shown a relationship between pain and oxidative and nitrosative parameters, changes in these enzymes might be related to the analgesic effect of HVLA spinal manipulation. However, further studies on this topic are crucial for better understanding of the temporal relationship between oxidative and nitrosative parameters and HVLA treatment. This study provides preliminary data for a randomized control trial.

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## Contributorship Information

Concept development (provided idea for the research): C.K., W.A.P.
Design (planned the methods to generate the results): C.K., W.A.P.
Supervision (provided oversight, responsible for organization and implementation, writing of the manuscript): C.K., W.A.P.
Data collection/processing (responsible for experiments, patient management, organization, or reporting data): C.K., A.K., A.H., M.S.M., F.C.K.D., A.P.K.R., T.S., A.K.
Analysis/interpretation (responsible for statistical analysis, evaluation, and presentation of the results): C.K., A.H., A.P.K.R., A.K.
Literature search (performed the literature search): C.K.
Writing (responsible for writing a substantive part of the manuscript): C.K., W.A.P.
Critical review (revised manuscript for intellectual content, this does not relate to spelling and grammar checking): W.A.P.

**Practical Applications**

- This study found that HVLA spinal manipulation therapy results in increase of enzymatic antioxidant activities depending on the length of time and frequency of treatment.
- Once there is a relationship between pain and oxidative and nitrosative parameters, changes in the antioxidant enzymes might be related to the analgesic effect of HVLA spinal manipulation.
- Larger study with a control group should be performed to assess the antioxidative profile and to relate these findings with the analgesic effect of spinal manipulation therapy.

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