Journal of Medicinal Plants Research Vol. 5(12), pp. 2508-2513, 18 June, 2011
Available online at http://www.academicjournals.org/JMPR
ISSN 1996-0875 ©2011 Academic Journals

Full Length Research Paper

Effect of the route of garlic treatment on modulation of erythrocytes and plasma redox status in rats

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Accepted 8 April, 2011

Garlic is a widely used medicinal plant exhibiting beneficial health effects such as antidiabetic, antioxidant or hypolipidemic. However several controversies persist about the beneficial or toxic effects of garlic according to its mode of administration in rat. We studied the ability of high dosage garlic to modulate erythrocytes and plasma antioxidant status when administered orally (p.o.) or through intraperitoneal (i.p.) route. In erythrocytes, p.o. garlic treatment was found to be antioxidant as it decreased malondialdehyde (MDA), free iron and hydrogen peroxide (H_2O_2) and increased antioxidant enzyme activities as catalase (CAT), peroxidase (POD) and superoxide dismutase (SOD). Garlic i.p treatment showed pro-oxidant activity as it increased MDA, free iron and H_2O_2. However, unexpectedly it increased the antioxidant enzyme activities. It was concluded that in plasma compartment, garlic treatment (p.o.) exhibited antioxidant nature whereas garlic (i.p.) treatment was clearly pro-oxidant. High garlic dosage was found to be relatively safe when administered orally.

Key words: Garlic, erythrocytes, plasma, redox status, administration mode, free iron, hydrogen peroxide.

INTRODUCTION

Garlic (*Allium sativum* L.) is a medicinal plant which has been widely used as a nutritional supplement in acute as well as in long term experiments (Lawson and Bauer, 1998). Garlic was shown to exert a wide range of pharmacological effects including antiviral, antibacterial (Taratinsev et al., 1992), antitumor (Schaffer et al., 1996), antiatherosclerotic (Campbell et al., 2001), antioxidant (Banerjee et al., 2002) and hypolipidemic activity (Yeh and Liu, 2001). However, several reported effects were deviating and conflicting and depended on experimental duration, garlic dosage and mode of administration (Banerjee and Maulik, 2002). Garlic is generally administered orally (p.o.) or by intraperitoneal (i.p.) route. It was interesting to notice that i.p. treatment with garlic was found more effective regarding its hypo-cholesterolemic effect as compared to other modes of treatment (Alnaqueeb et al., 1996). In a recent study, we could establish that garlic high dose oral treatment exhibited profound antianemic, antifatigue and lipid-lowering activity as compared to i.p. route of treatment (Hamlaoui-Gasmi et al., 2011). Keeping in view the evidence of variations and controversies in obtaining the desired effect of garlic treatment, the present study was designed to investigate its antioxidant potential by using two different routes of treatment (p.o. and i.p.). Anti-oxidant effect was evaluated in erythrocytes and plasma erythrocytes by evaluating MDA, free iron, hydrogen peroxide and antioxidant enzyme activities such as CAT, POD and SOD. The data obtained during present study showed garlic oral treatment to possess antioxidant effect. The putative link between antioxidant nature and effect of garlic on free iron overload was studied and the results are presented in current communication.

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MATERIALS AND METHODS

Chemicals

2-thiobarbituric acid (TBA); 2,6,-di-tert-butyl-4-hydroxy-toluene (BHT); trichloroacetic acid (TCA); hydrogen peroxide (H_2O_2); 2-methoxyphenol (gaiacol); bovine catalase and 4-(1-Hydroxy-2-methylamino-ethyl)-benzene-1,2-diol (epinephrine) were obtained from Sigma-Aldrich Co (Germany).

Preparation of garlic extract

Garlic was purchased from local market, peeled and ground with an electric mincer. It was diluted in double distilled water at 2 g/ml on the basis of the weight of the starting material and centrifuged (Beckman J20, 15 min at 10 000 g and 4°C). Supernatant was aliquoted and stored at – 80°C until used.

Animals and treatment

Male wistar rats (180 to 200 g) from Pasteur Institute (Tunis) were maintained in animal facility for one week at room temperature 22 ± 1°C and a 12/12 h dark/light cycle. They were supplied with standard chow diet and tap water ad libitum. Procedures with laboratory animals and their care were conducted in conformity with institutional guidelines of Tunis University of Medical Sciences and in accordance with the NIH guidelines. Animals were randomly divided into four groups of 10 animals each:

Group I received standard diet (control). Group II received standard diet supplemented with aqueous extract of garlic (5 g/kg bw). Group III was injected (i.p.) with 9‰ NaCl (control). Group IV was injected (i.p.) with garlic (5 g/kg bw). Animals were treated daily for 30 days and checked for weight gain or loss. At the end of treatment duration, blood samples were collected following standard procedure and processed for studies on antioxidant status parameters.

Blood processing

Whole blood was obtained by cardiac puncture and collected into heparinized tubes. Erythrocytes were isolated from plasma by centrifugation at 1000 g for 10 min at 4°C and homogenized using a hypotonic buffer Tris-HCl 10 mM pH 7.5, $MgCl_2$ 5 mM, NaCl 10 mM.

Lipid peroxidation determination

Lipid peroxidation was determined by MDA measurement according to the double heating method (Draper and Hadley, 1990). Briefly, aliquots from erythrocyte homogenates or plasma were mixed with BHT-TCA solution containing 1% BHT (w/v) dissolved in 20% TCA (w/v) and centrifuged at 1000 g for 5 min at 4°C. Supernatant was blended with 0.5 N HCl, 120 mM TBA in 26 mM Tris and then heated at 80°C for 10 min. After cooling, absorbance of the resulting chromophore was determined at 532 nm using a Bio-Rad UV-visible spectrophotometer. MDA levels were determined by using an extinction coefficient for MDA-TBA complex of 1.56 10^5 $M^{-1}cm^{-1}$.

Protein determination

Total soluble proteins were determined according to biuret method(Ohnishi, 1978). Briefly, at acidic pH soluble proteins constituted with copper a colorful complex measurable at 546 nm.

Analysis of antioxidant enzyme activities

All spectrophotometric analyses were performed with a Beckman DU 640B spectrophotometer.

Catalase (CAT) activity was assayed by measuring the initial rate of H_2O_2 disappearance at 240 nm. The reaction mixture contained 33 mM H_2O_2 in 50 mM phosphate buffer pH 7.0 and CAT activity was calculated using the extinction coefficient of 40 mM-1cm-1 for H_2O_2 (Aebi, 1984).

Peroxidase (POD) activity was measured at 25°C using gaiacol as hydrogen donor.

The reaction mixture contained 9 mM gaiacol, 19 mM H_2O_2 in 50 mM phosphate buffer pH 7 and 50 µl of enzyme extract in 1 ml final volume. The reaction was initiated by the addition of H_2O_2 and monitored by measuring the increase in absorbance at 470 nm. Peroxidase activity was expressed in nmol of gaiacol oxidized per min with a molecular extinction coefficient of 26.2 mM-1 for calculation (Chance and Maehly, 1955).

Superoxide dismutase (SOD) activity was determined by using modified epinephrine assay (Misra and Fridovich, 1972). At alkaline pH, superoxide anion O_2^- causes the autoxidation of epinephrine to adenochrome; while competing with this reaction, SOD decreased the adenochrome formation. One unit of SOD is defined as the amount of extract that inhibits the rate of adenochrome formation by 50%. Enzyme extract was added in 2 ml reaction mixture containing 10 µl bovine catalase (0.4 U/µl), 20 µl epinephrine (5 mg/ml) and 62.5 mM sodium carbonate/sodium bicarbonate buffer pH 10.2.

Changes in absorbance were recorded at 480 nm. Characterization of SOD isoforms was performed using KCN (3 mM) which inhibited Cu/Zn-SOD or H_2O_2 (5 mM) affecting both Cu/Zn-SOD and Fe-SOD. Mn-SOD was insensitive to both inhibitors.

Free iron determination

Erythrocyte and plasma free iron level weres determined according to Leardi et al. (1998) using a commercially available kit from Biomaghreb, Tunisia. Briefly, at acidic pH 4.8 all Fe^{3+} is released from transferrine. Ascorbic acid reduced Fe^{3+} to Fe^{2+} which constituted with ferrozine a colorful complex measurable at 560 nm.

H_2O_2 determination

H_2O_2 was determined enzymatically according to Chance et al. (1979) using a commercially available kit from Biomaghreb (Tunisia).

Statistical analysis

All data were expressed by mean values ± SEM. Statistical analysis was carried out using student's t-test and one way analysis of variance (ANOVA test). Statistical p value less than 0.05 was considered significant.

RESULTS

Differential effect of garlic (p.o. and i.p.) treatment on erythrocytes and plasma lipoperoxidation

The results presented in Figure 1 show the data of garlic dosage administered either by p.o. or i.p. way on erythrocytes (Figure 1A) and plasma (Figure 1B)

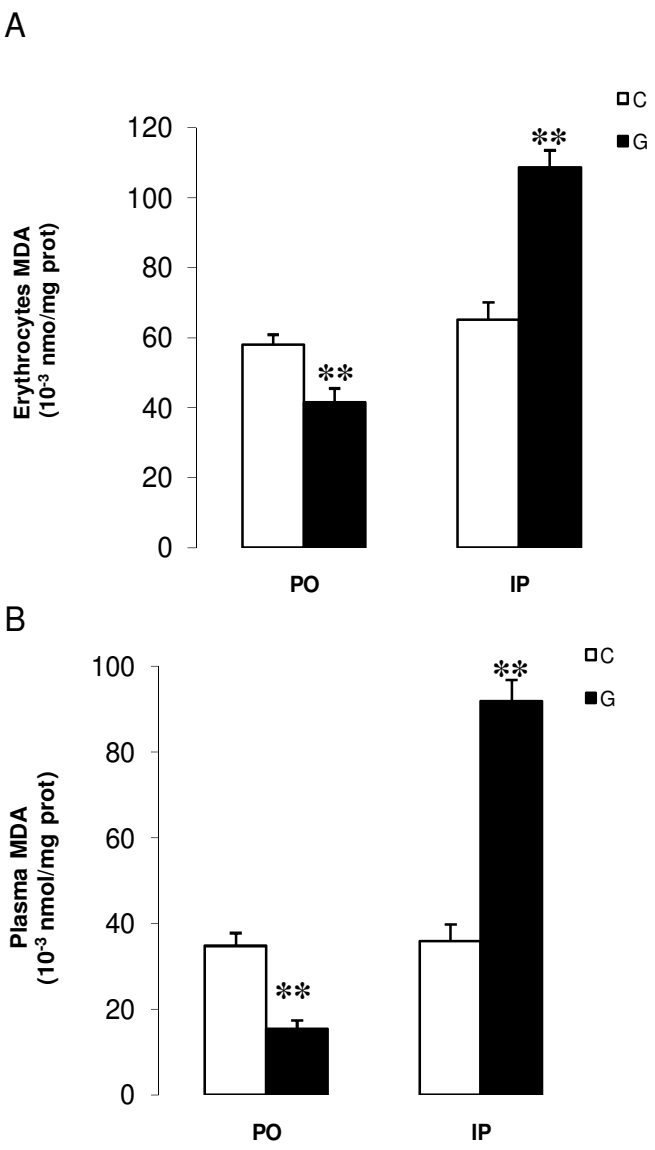

Figure 1. Effect of garlic way of administration on erythrocytes and plasma lipoperoxidation. NaCl 9‰ (C □) or garlic (G ■) were p.o. or i.p. administered to rats during one month and erythrocytes (Figure A) and plasma (Figure B) MDA determined. Results are expressed by mean ± SEM of 10 rats per group and assays done in triplicate. indicated $p < 0.01$.

lipoperoxidation. When administered by p.o. route, garlic decreased erythrocytes (- 46%) and plasma (-56%) MDA. However, the i.p. administered garlic increased MDA in erythrocytes (+56%) and in plasma (+135%).

Erythrocytes antioxidant enzyme activities

The data outcome shown in Figure 2 dealt with the effect of garlic mode of administration on erythrocyte anti-oxidant enzyme activities. Oral garlic treatment increased CAT (+31%) (Figure 2A), POD (+88%) (Figure 2B) and SOD (+60%) (Figure 2C) activities; in the later case, Fe-SOD was strongly up-regulated (+88%) when compared to Cu/Zn isoform (+37%). Garlic (i.p.) treatment also up-regulated CAT (+116%), POD (+55%) and SOD (+86%); the two isoforms that is Fe and Cu/Zn were increased to the same extent respectively by + 100 and + 90%.

Plasma antioxidant enzyme activities

The effect of garlic mode of administration (p.o. and i.p.) on plasma antioxidant enzyme activities is presented in Figure 3 p.o. garlic increased all three enzyme activities CAT (+27%) (Figure 3A), POD (+90%) (Figure 3B) and SOD (+26%) (Figure 3C). However, garlic i.p treatment decreased CAT (- 50%) and POD (-38%) but increased SOD activity (+ 58%) especially the Cu/Zn isoform.

Effect of garlic mode of administration on erythrocytes free iron level

We further looked at erythrocytes free iron level (Figure 4) and data showed that p.o. garlic treatment significantly decreased erythrocytes free iron (-25%) whereas it increased free iron (+14%) when i.p. route of treatment was used.

Effect of garlic mode of administration on erythrocytes and plasma hydrogen peroxide level

In the present study, the effect of garlic treatment by using two modes of administration (p.o. and i.p.) on erythrocytes and plasma hydrogen peroxide levels were investigated and the results are presented in Figure 5. As expected garlic p.o. treatment decreased H_2O_2 level in erythrocytes (Figure 5A) (-76%) and plasma (Figure 5B) (-15%), however, i.p. garlic treatment increased the levels to (+19%) and (+25%) respectively.

DISCUSSION

In a prior study we demonstrated that high dosage garlic exhibited dual effects in rat, that is, antioxidant or prooxidant depending on the mode of administration. Oral garlic treatment exerted antianemic and lipid-lowering effect whereas garlic i.p. treatment, induced anemia and hepatotoxicity as assessed by elevation in plasma transaminases. The main conclusion drawn was the putative effect of garlic on free iron compartmentalization as well as the relationship between garlic induced free iron deposition and toxic side effects (Hamlaoui-Gasmi et al., 2011). In the present study, garlic oral treatment in erythrocytes was found to induce a significant decrease

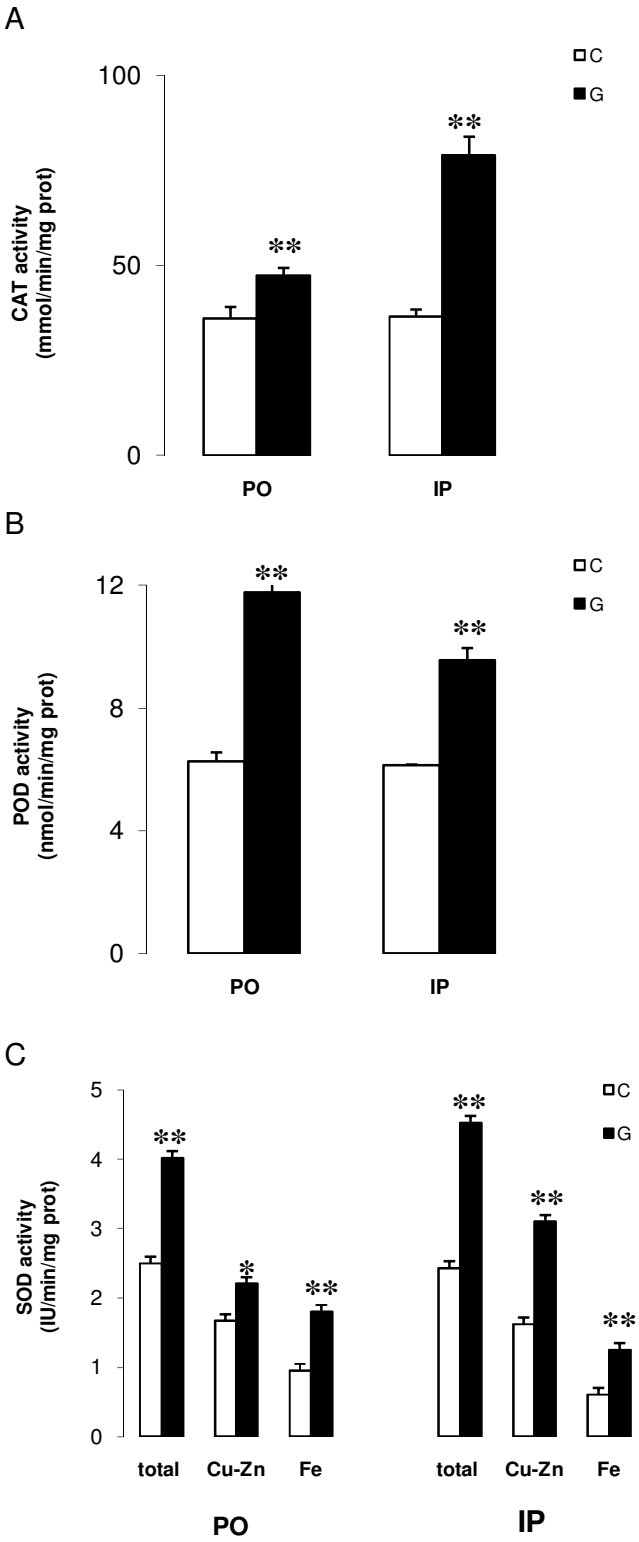

Figure 2. Effect of garlic way of administration on erythrocytes antioxidant enzymes activities status. NaCl 9‰ (C □) or garlic (G ■) were p.o. or i.p. administered to rats during one month and erythrocytes CAT (Figure A), POD (Figure B) and SOD (Figure C) activities determined. Results are expressed by mean ± SEM of 10 rats per group and assays done in triplicate. * indicated $p < 0.05$. ** indicated $p < 0.01$.

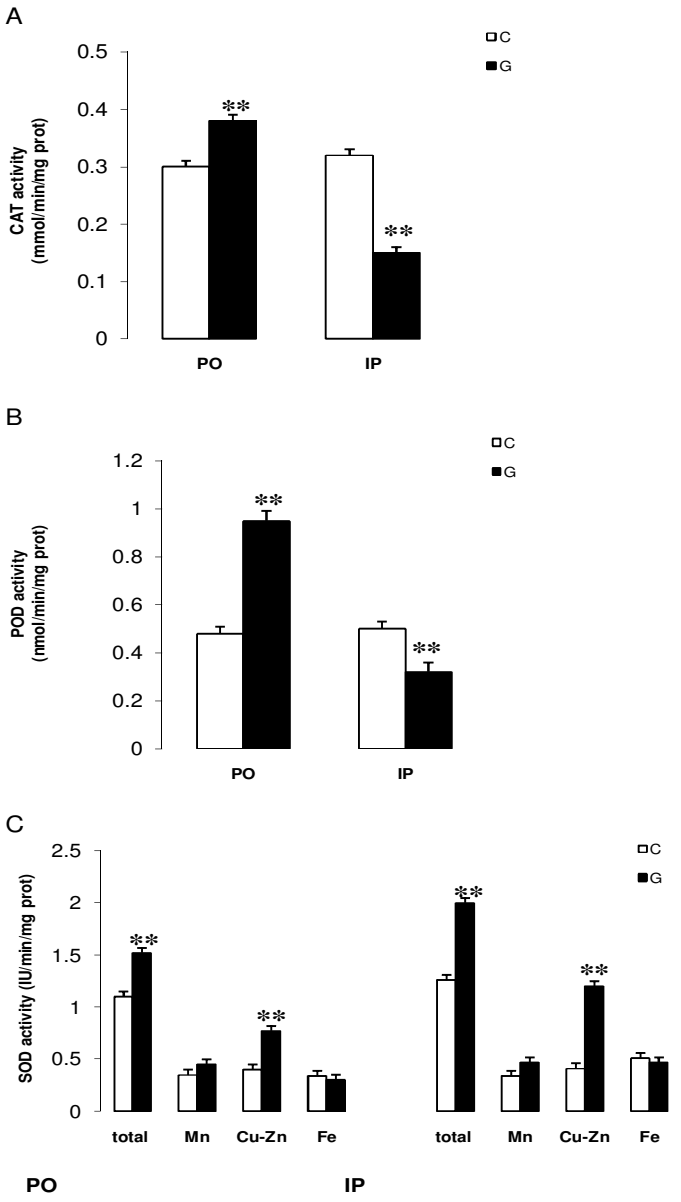

Figure 3. Effect of garlic way of administration on plasma antioxidant enzymes activities status. NaCl 9‰ (C □) or garlic (G ■) were p.o. or i.p. administered to rats during one month and plasma CAT (Figure A), POD (Figure B) and SOD (Figure C) activities determined. Results are expressed by mean ± SEM of 10 rats per group and assays done in triplicate. indicated $p < 0.01$.

in MDA, free iron and hydrogen peroxide. Interestingly, an opposite effect on all the studied parameters was observed when the treatment was given by i.p. route. Our present results are full in agreement with the earlier reports (Hamlaoui-Gasmi et al., 2011). In addition, to substantiate the earlier findings some new observations on the relationship between garlic mode of administration (p.o. and i.p.) and extent of free iron overloading were made. It was well documented that excess free divalent

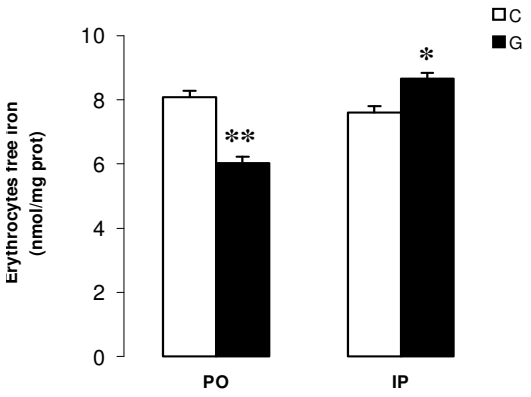

Figure 4. Effect of garlic treatment way of administration on erythrocytes free iron level. NaCl 9‰ (C □) or garlic (G ■) were p.o. or i.p. administered to rats during one month and erythrocytes free iron level determined. Results are expressed by mean ± SEM of 10 rats per group and assays done in triplicate. *indicated $p < 0.05$. **indicated $p < 0.01$.

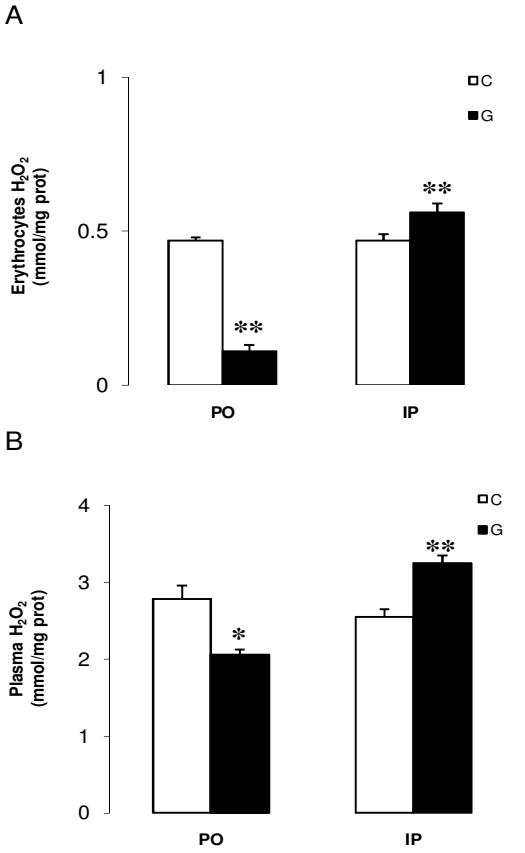

Figure 5. Effect of garlic treatment way of administration on erythrocytes and plasma hydrogen peroxide level. NaCl 9‰ (C □) or garlic (G ■) were p.o. or i.p. administered to rats during one month and erythrocytes (Figure A) and plasma (Figure B) H2O2 level activities determined. Results are expressed by mean ± SEM of 10 rats per group and assays done in triplicate. *indicated $p < 0.05$. **indicated $p < 0.01$.

iron could be highly toxic, mainly via Fenton reaction producing hydroxyl radicals leading to cellular dysfunction (Andrews, 1999). Garlic mode of action might be occurring through iron shuttling proteins such as hepcidin (Papanikolaou et al., 2005) or lipocalin 2 (Deviredly et al., 2005). Earlier, hepcidin was described in various tissues such as liver (Parck et al., 2001), heart (Merle et al. 2007) and pancreas (Kulaksiz et al., 2008), and lipocalin 2 in the liver (Sunil et al., 2007). Hepcidin was reported to play an important role in the pathogenesis of iron overload (Papanikolaou et al., 2005), it was implicated as a key regulator of anemia and inflammation (Ganz, 2003). Furthermore, high levels of hepcidin were established to cause intracellular iron sequestration and decreased its level in the plasma (Pigeon et al., 2001). Thus one can speculate about oral garlic treatment inducing up-regulation of hepcidin and decreasing iron deposition into erythrocytes as observed in the present study.

Conversely, garlic (i.p. treatment) would induce down-regulation of hepcidin and iron overload. Further work on garlic effect on hepcidin level is warranted. In the present study on erythrocytes, we also found that garlic treatment via any mode of administration, could stimulate all antioxidant enzyme measured. Nevertheless, garlic oral treatment increased POD activity to a higher extent as compared to i.p. route of administration. It is worth mentioning that garlic (i.p.) treatment increased more strikingly CAT and SOD activities. This last data is reminiscent of the earlier described pro-oxidant effect of catalase (Heck et al., 1998). It should also be interpreted in the light of oxidative stress-induced ROS (reactive oxygen species) activation of non receptor tyrosine kinases associated with CAT and GPx phosphorylation and their subsequent activity stimulation (Borchi et al., 2010) or with SOD phosphorylation (Csar et al., 2001). In the plasma compartment, orally administered garlic was antioxidant while i.p. garlic pro-oxidant. Orally administered garlic-induced antioxidant effect which was further confirmed by the positive role it exerted on antioxidant enzymes CAT, POD and SOD. However, garlic i.p. administration exerted strong prooxidant effect, which attenuated CAT and POD activities, but unexpectedly increased SOD especially the Cu/Zn isoform. First, this isoform could correspond to the secreted form of the enzyme (Marklund, 1982). Second this apparent discrepancy could be interpreted in the light of deleterious increase in ROS production exerted by Cu-Zn/SOD (Goldstein et al., 2008) especially in such a free iron overload setting (Hamlaoui-Gasmi et al., 2011). Anyway one should also keep in mind that a positive influence of garlic i.p. treatment on plasma SOD activity was insufficient to overcome the elevation of ROS (and consequently MDA) linked to down-regulation of both CAT and POD activities.

In conclusion, we showed that garlic could modulate oxidative stress in rats depending on its route of

administration. The underlying mechanism seems to involve disturbances in iron homeostasis and likely iron shuttling proteins.

ACKNOWLEDGEMENT

This work was financially supported by the Tunisian Ministry of Research.

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