cadmium strongly inhibited the coverting enzyme which converts the active decapeptide angiotensin I to active pressure peptide angiotensin II. This leads to an increase in feedback of PRA. However, the PRA did not change suggesting that feedback upregulation is not invariable associated with an increase in PRA. Takemore *et al.* [9] and Benctas *et al.* [10] using different ACE inhibitors in rats was well as in essential hypertensive patients did not observe a significant increase in PRA.

Cadmium-induced hypertensive response could be due to the activation of the aminergic system in rats. We have reported previously that cadmium treatment of rats produced increased serum levels of catecholamines [3]. The present observation is supported by Fadloun and Leach [4] who reported the induction of dopamine-$\beta$-hydroxylase activity by cadmium in rats. Dazu and Pratt [11] reported that angiotensin II activates the target organs resulting in the release of norepinephrine. Cadmium-induced increase in the blood pressure of rats is independent of the PRA. However, the possible effects of vasopressin or some specific peptide produced by cadmium need to be elucidated. In conclusion, cadmium produced a hypertensive response and significantly inhibited serum ACE activity without effecting the PRA.

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# Heparin: does it act as an antioxidant *in vivo*?

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**Abstract**—Previous studies have shown that heparin antagonizes oxygen radical-mediated injury to endothelial cells, suggesting an antioxidant role of the drug. In the present investigation, the hypothesis that heparin exerts direct antioxidant effects was tested in several experimental models. We have found that 1, 3, 5, 10, 20, 40 and 80 U/mL of heparin do not scavenge superoxide anion, hydrogen peroxide, hydroxyl radical or the stable free radical 1,1-diphenyl-2-pycrylhydrazyl. Moreover, the drug is ineffective towards iron-driven linolenic acid peroxidation, autooxidation of brain homogenate and linolenic acid peroxidation mediated by human internal mammary artery homogenate. Specific studies on the potential iron-binding–inactivating capacity of heparin prove the drug to be totally ineffective. Finally, the loss of protein sulphydryls from human plasma induced by hypoxanthine–xanthine oxidase-generated oxygen radicals is not prevented by heparin. In conclusion, heparin, even at concentrations far higher than those usually used therapeutically, has no direct antioxidant properties. Thus, other mechanisms not strictly antioxidant-type must be involved in heparin-mediated cell protection against toxic oxygen metabolites.

Heparin, a glycosoaminoglycan, is employed clinically as an antithrombotic for both preventive and therapeutic purposes, and its use as an antiatherogenic agent has also been stressed [1, 2]. Recent studies have shown that heparin pre-incubation protects cultured endothelial cells from damage by toxic oxygen metabolites, suggesting that the drug has antioxidant properties [3]. Moreover, Hladovec [4] demonstrated a decrease in circulating endothelial cells

when heparin was injected 5 min prior to hydrogen peroxide infusion in the rat, and a binding effect of the drug on peroxidation-catalysing transition metals has also been hypothesized [5].

In spite of this limited experimental evidence, to the best of our knowledge no research has been devoted to investigate more directly potential effects of heparin. In this paper, we present a series of studies to test the hypothesis that heparin exerts an antioxidant action *in vivo*.

*Methods and Results*

1,1-Diphenyl-2-pycrylhydrazyl (DPH*), cytochrome *c* (type VI, from horse heart), xanthine, hypoxanthine, hydrogen peroxide, guaiacol, horseradish peroxidase (type VI), ascorbic acid, 2-deoxyribose, linolenic acid, butylated hydroxytoluene (BHT), thiobarbituric acid (TBA), EDTA, ferrozine, and 5,5'-dithiobis(2-nitrobenzoic acid) were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Xanthine oxidase (sp. act. 1 U/mg) was from Boehringer Mannheim GmbH (Germany). All other chemicals were of the highest reagent grade commercially available. Procedures were carried out in plastic or acid-washed glassware, and solutions were prepared in Chelex 100 resin-treated doubly distilled water.

Bovine lung heparin sodium (152 USP U/mg) was purchased from Sigma and used at concentrations of 1, 3, 5, 10, 20, 40 and 80 U/mL, corresponding to 6.57, 19.7, 32.9, 65.8, 131.57, 263.16 and 526.3 μg/mL, respectively. In this regard, it must be noted that 1 U/mL of whole blood prevents blood clotting, and that infusion of 10,000 U of heparin results in a human plasma concentration of 3 U/mL [1].

For each biochemical test, data were calculated as the means ± SD of five different experiments (performed in duplicate and differing from each other by less than 10%) for both controls and the various heparin concentrations. Dose-dependent effects of heparin were studied by one-way analysis of variance, followed by the Bonferroni and Student–Newman–Keuls tests to detect significantly different means [6]. $P < 0.05$ was considered as statistically significant.

Since all heparin concentrations were ineffective in the various tests performed, only the results obtained with the highest concentration (i.e. 80 U/mL) are given.

*Antioxidant determination using DPH.* It is known that substances which can act as antioxidants by virtue of their reducing potential and capacity to react with oxidant radicals, including alkoxyl- and peroxyl-radicals, may reduce the stable free radical DPH to 1,1-diphenyl-2-picrylhydrazine [7, 8]. The reaction system (3 mL final volume) contained 0.06 mM DPH (previously dissolved in ethanol) and 50 mM Tris–HCl buffer, pH 7, with or without heparin. Each tube was incubated for 30 min at 37° and absorbance values were recorded spectrophotometrically at 517 nm against appropriate blanks.

Heparin had no effect on the 517 nm absorbance values of DPH (0.163 ± 0.019 vs 0.161 ± 0.017 in control vs heparin-containing samples, not significant).

*Superoxide anion ($O_2^-$) scavenging.* The potential scavenging effect of heparin on $O_2^-$ was studied in an assay system containing 0.1 mM xanthine, cytochrome *c* (0.129 mg of protein/mL of reaction mixture) and 0.05 M potassium phosphate buffer, pH 7.5, with 1 mM EDTA [9]. The reaction was started by adding xanthine oxidase (0.015 mg of protein/mL of reaction system) and the $O_2^-$-mediated increase in absorbance values at 550 nm [9] was recorded during a 10-min incubation at 25° with a double beam Varian DMS 200 spectrophotometer. This incubation time was selected in the light of previous experiments demonstrating a decrease in absorbance values at 550 nm after 10 min incubation of the reaction system.

Uric acid formation was also followed at 292 nm, to exclude possible xanthine oxidase–heparin interaction. Moreover, absorbance values were recorded beforehand with heparin but without xanthine oxidase, and without either heparin or xanthine oxidase. Results were expressed as nmoles cytochrome *c* reduced per millilitre per 10 min, using a molar extinction coefficient of $2.1 \times 10^4$ mol$^{-1}$ cm$^{-1}$ [10].

Heparin showed no effect on $O_2^-$ (4.7 ± 0.7 vs 4.3 ± 0.5 nmol cytochrome *c* reduced/mL/10 min in control vs heparin-containing samples, not significant). Moreover, the drug did not directly influence cytochrome *c* reduction or xanthine oxidase activity.

*Hydrogen peroxide ($H_2O_2$) scavenging.* The effect of heparin on $H_2O_2$ was first studied in 1-mL quartz cuvettes containing 2 mM $H_2O_2$ in 0.1 M phosphate buffered saline, pH 7.4. Absorbance values at 240 nm due to $H_2O_2$ were recorded spectrophotometrically during a 30-min incubation at 37° with or without various heparin concentrations. Blanks consisted of heparin and buffer. In order to study the effect of heparin on lower and more physiological $H_2O_2$ concentrations (50 μM), a guaiacol–peroxidase system was then used [11]. $H_2O_2$ was assayed via the formation of a 436 nm-absorbing brown chromogen in 1-mL reaction mixtures containing 0.15 M $KH_2PO_4$/KOH buffer, pH 7.4, 50 μL of guaiacol solution (50 μL pure guaiacol in 25 mL of water) and 10 μL of horseradish peroxidase (5 mg/mL in buffer).

Absorbance values at 240 nm (UV study) and 436 nm (guaiacol–peroxidase system) were 0.094 ± 0.007 vs 0.089 ± 0.005 and 0.202.2 ± 0.007 vs 0.197 ± 0.01 for control vs heparin-containing samples, respectively (not significant).

*Hydroxyl radical (OH·) scavenging.* We used the deoxyribose test to detect possible OH· scavenging properties of heparin [11–13]. Moreover, this test performed without EDTA may give information regarding the iron-binding properties of various substances [11–13]. Reaction mixtures contained, in a final volume of 1 mL, the following reagents at the final concentrations stated: 10 mM $KH_2PO_4$-KOH buffer, pH 7.4, 1.42 mM $H_2O_2$, 2.8 nM deoxyribose, 20 μM $FeCl_3$ (premixed or not with 100 μM EDTA before addition to the reaction system). Ascorbic acid (0.1 mM) was added to start the reaction and the tubes were incubated at 37° for 60 min with or without various heparin concentrations. One millilitre of 0.6% aqueous solution of TBA, 1 mL of 2.8% trichloroacetic acid and 0.04 mL of 5% BHT in ethanol were then added to each millilitre of reaction mixture, followed by 30 min heating at 95°. After cooling, the pink chromogen was read at 532 nm against appropriate blanks. Results were expressed as nmoles of TBA reactants (TBAR) per millilitre per 60 min, using a molar extinction coefficient of $1.53 \times 10^5$ mol$^{-1}$ cm$^{-1}$ [14].

Heparin was ineffective as a OH· scavenger (10.4 ± 1.9 vs 10.2 ± 1.4 nmol TBAR/mL/60 min in control vs heparin-containing samples, not significant). When the deoxyribose test was performed without EDTA, heparin also exerted no action on the peroxidative sugar degradation (3.6 ± 0.6 vs 3.55 ± 0.4 nmol TBAR/mL/60 min in control vs heparin-containing samples, not significant), indicating lack of an iron binding–inactivating effect [11–13]. Moreover, the drug did not produce a 532 nm-detectable pink colour in the presence of deoxyribose test prooxidants but without sugar addition.

*Heparin–iron interaction.* Besides the deoxyribose test performed without EDTA [11–13], the ferrozine test [8] was used to study iron binding effects of heparin. The reaction mixture contained 150 μM ferrozine in 0.05 M phosphate buffer, pH 7.4, with or without various heparin concentrations. The reaction was started by 15 μM $FeCl_2$

* Abbreviations: DPH, 1,1-diphenyl-2-pycrylhydrazyl; BHT, butylated hydroxytoluene; TBA, thiobarbituric acid; TBAR, TBA reactants.

and absorbance values at 562 nm were read after 4 min. Moreover, the potential inhibitory effect of heparin on $Fe^{2+}$ oxidation (which can result in the generation of reduced forms of oxygen and iron-related radicals [14, 15]) was investigated in phosphate buffer, due to the capacity of phosphates to favour a rapid $Fe^{2+}$ autooxidative process, which reaches completion within a few minutes [16]. The reaction system (final volume 1 mL in quartz cuvette) contained 0.05 M phosphate buffer, pH 7.6, with or without various heparin concentrations, and 0.25 mM $FeSO_4$, which was added last. The increase in absorbance at 310 nm due to $Fe^{3+}$ formation [17] during oxidation was recorded continuously for 2 min with a double beam Varian DMS 200 spectrophotometer.

Absorbance values at 562 nm due to $Fe^{2+}$–ferrozine complex formation were $0.160 \pm 0.013$ and $0.157 \pm 0.016$ in control and heparin-treated samples, respectively (not significant). Moreover, heparin was ineffective towards $Fe^{2+}$ autooxidation in phosphate buffer ($0.625 \pm 0.023$ vs $0.656 \pm 0.027$ absorbance units at 310 nm after 2 min of incubation for control vs heparin-treated samples, not significant).

*Peroxidation of linolenic acid emulsion.* Linolenic acid emulsion was prepared as reported previously [18], obtaining a 0.01 M final fatty acid concentration in 0.1 M phosphate-buffered saline, pH 7.4, with or without heparin. The peroxidative reaction was induced by 0.3 mM $FeCl_2$, allowing 30 min of incubation at 37°. Then, 1 mL of 0.6% aqueous solution of TBA, 1 mL of 25% HCl and 0.04 mL of 5% BHT in ethanol were added to each millilitre of reaction mixture. After heating at 95° for 30 min and cooling with tap water, the chromogen was extracted with *n*-butanol, followed by brief centrifugation and read at 532 nm against TBA-reacted appropriate blanks. Values were expressed as nmoles TBAR per millilitre per 30 min.

Heparin was ineffective towards iron-driven lipoperoxidation ($5.9 \pm 0.8$ vs $5.1 \pm 0.7$ nmol TBAR/mL/30 min with heparin vs without heparin, not significant).

*Autooxidation of brain homogenates.* Brain homogenate lipids are known to undergo spontaneous iron-dependent autooxidation when exposed to air at 37° [19–21]. Guinea pig brain homogenate was prepared as reported previously [19–21] and stored at −70° until use. After thawing, 0.5-mL aliquots were diluted with 1.5 mL of 0.1 M phosphate buffered saline, pH 7.4, and incubated at 37° for 90 min with or without various heparin concentrations. The reaction mixtures were then subjected to the TBA test, as described previously [22]. The difference in absorbance at 532 nm between paired samples incubated at 37° or stored at 2° was used to calculate TBAR formed during the 90-min incubation [21].

After 90 min incubation at 37°, $67 \pm 9.6$ nmol/mL of TBAR were detected. The presence of heparin did not affect this value, resulting in the formation of $66.6 \pm 7.4$ nmol TBAR/mL/90 min (not significant).

*Oxidation of linolenic acid emulsion by homogenates of human arterial wall.* Linolenic acid emulsion undergoes oxidation when incubated with tissular homogenates, reflecting the prooxidant potential of the investigated tissue [18]. Human, atherosclerosis-free internal mammary artery specimens were obtained during coronary bypass surgery performed at Cattedra di Cardiochirurgia, University of Chieti, Italy. The specimens were homogenized in 1.15% KCl (1 : 10, w/v) and TBAR assessed under basal conditions according to the method of Uchiyama and Mihara [23]. After heating at 95° for 45 min in the presence of 0.04 mL of 5% BHT in ethanol as antioxidant and cooling, absorbance values of the *n*-buthanol extracted chromogen were recorded at 532 against an appropriate blank. Another suitable amount of homogenate was incubated for 60 min at 37° with 1 mL of 0.01 M linolenic acid in 0.1 M phosphate buffered saline, pH 7.4 [18], with or without various heparin concentrations. Reaction mixtures were then subjected to Uchiyama and Mihara's procedure [23], using appropriate controls. Results were expressed as nmoles TBAR per gram wet tissue per 60 min.

Human internal mammary arteries contain $36 \pm 5.9$ nmol TBAR/g wet tissue, resulting in the generation of $728 \pm 70$ and $760 \pm 86$ nmol TBAR/g wet tissue/60 min when incubated with 0.01 M linolenic acid in the presence and absence of heparin, respectively (not significant).

*Radical-mediated loss of protein thiols of human plasma.* When human plasma is challenged with an oxygen radical-generating hypoxanthine–xanthine oxidase system, a loss of total protein thiols occurs, even though significant changes in lipid peroxidation parameters are not detectable [24]. Plasma was obtained from fresh EDTA-treated blood after venipuncture of healthy adults (22–32 years old). Red cells and buffy coat were removed by centrifugation at 300 *g* for 10 min [24]. Ninety per cent human plasma was incubated for 60 min at 37° with 0.5 mM hypoxanthine and 5 U/mL xanthine oxidase in 0.1 M phosphate buffer, pH 7.4 [24], in the presence or absence of heparin. When required, the reaction was stopped with 33 $\mu$M allopurinol and 100 $\mu$L of plasma were subjected to the 5,5′-dithiobis-2-nitrobenzoic acid)-dependent Ellman's reaction [25], as reported previously [24]. Absorbance values were read at 412 nm against appropriate blanks, and the results were expressed as nmol R-SH/mL plasma, using for calculation a molar extinction coefficient of $13.6 \pm 10^3$ $mol^{-1}$ $cm^{-1}$ [24].

Under these experimental conditions, about 19% of plasma protein sulfhydryls were lost (from $460 \pm 42$ to $372 \pm 39$ nmol R-SH/mL, $P < 0.0001$). The presence of heparin resulted in $369 \pm 44$ nmol R-SH/mL (not significant).

## *Discussion*

The present study demonstrates that heparin exerts no antioxidant effect in several experimental models even at concentrations of 80 U/mL, a value about 25-fold higher than that found in human blood after 10,000 U infusion [1].

In particular, the drug is ineffective as a scavenger of $O_2^-$, $H_2O_2$ and OH· and does not influence significantly the 517 nm absorbance values of the stable free radical DPH. Moreover, heparin does not inhibit linolenic acid peroxidation driven by iron, suggesting that it has no scavenging action on peroxyl and iron-related radicals, and no iron binding–inactivating capacity [14, 15]. These results are confirmed by the deoxyribose test performed with and without EDTA, which may give reliable information about the OH·–scavenging and iron binding action of various substances [11–13]. Heparin is also ineffective in inhibiting the autooxidation of brain homogenates, which is mediated by transition metals such as iron [19–21]. In this context, the drug does not inhibit $Fe^{2+}$–ferrozine complex formation [8] and does not prevent the increase in absorbance at 310 nm due to $Fe^{2+} \rightarrow Fe^{3+}$ conversion in phosphate buffer, indicating a lack of influence on $Fe^{2+}$ autooxidation and related radical generation [14, 15]. Finally, heparin cannot prevent the loss of protein sulfhydryls of human plasma induced by oxygen radicals and shows no effect on the linolenic acid peroxidation mediated by human artery tissue, which suggests that the potential antiatherogenic action of the drug [2] should not be related to the inhibition of polyunsaturated fatty acids and lipoprotein oxidation by vascular parietal cells [26].

In conclusion, this study provides evidence that heparin, even at concentrations far higher than those usually used therapeutically, does not possess antioxidant effects in several *in vitro* models. A direct antioxidant effect of the drug, therefore, should not occur *in vivo* at either low or high blood concentrations. The protective effect reported previously on the oxygen radical-mediated injury to endothelial cells [3, 4] does not apparently involve direct antioxidant-type mechanisms. However, heparin, besides

its known antithrombotic properties [1], has been shown to release the endothelial cell-associated "extracellular superoxide dismutase" [27], decrease abnormal vascular calcium accumulation [28] and inhibit the formation of platelet endoperoxide metabolites [29], resulting potentially in decreased superoxide concentration [27] and radical generation related to calcium-activated phospholipases and prostaglandin biosynthesis [14, 30]. These aspects may deserve further, specific studies using *in vitro* and *in vivo* experimental models.

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