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Enhancement of antioxidant and anti-inflammatory activities of bioflavonoid rutin by complexation with transition metals

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Abstract

The antioxidant and anti-inflammatory activities of two transition metal complexes of bioflavonoid rutin, Fe(rut)Cl_3 and Cu(rut)Cl_2, were studied. It was found that Cu(rut)Cl_2 was a highly efficient *in vitro* and *ex vivo* free radical scavenger that sharply decreased (by 2–30 times compared to the parent rutin): oxygen radical production by xanthine oxidase, rat liver microsomes, and rat peritoneal macrophages; the formation of thiobarbituric acid-reactive products in microsomal lipid peroxidation; and the generation of oxygen radicals by bronchoalveolar cells from bleomycin-treated rats. The copper–rutin complex was also a superior inhibitor of inflammatory and fibrotic processes (characterized by such parameters as macrophage/neutrophil ratio, wet lung weight, total protein content, and hydroxyproline concentration) in the bleomycin-treated rats. The antioxidant activity of Fe(rut)Cl_3 was much lower and in some cases approached that of rutin. Fe(rut)Cl_3 also stimulated to some degree spontaneous oxygen radical production by macrophages. We suggested that the superior antioxidant and anti-inflammatory activity of the copper–rutin complex is a consequence of its acquiring the additional superoxide-dismuting copper center. The inhibitory activity of Fe(rut)Cl_3 was lower, probably due to the partial reduction into Fe(rut)Cl_2 in the presence of biological reductants; however, similarly to the copper–rutin complex, this complex efficiently suppressed lung edema.

Keywords: Metal–rutin complexes; Free radicals; Bleomycin; Lung pathology

1. Introduction

The harmful intervention of free radicals in normal metabolic processes leading to pathologic changes is a consequence of their interaction with various biological compounds inside and outside cells. To protect biomolecules against the attack of free radicals and/or to suppress the resultant damage, numerous natural and synthetic free radical scavengers and antioxidants have been developed and studied. Among them, flavonoids, natural polyphenolic compounds, have attracted significant interest [1]. Actually, the beneficial clinical and curative effects of flavonoids in the treatment of virus infection, inflammation, diabetes mellitus, headache, etc. in humans have long been shown, but they are "to a large extent based on empirism since this praxis is much older than the science of chemistry" [2]. However, at present, many studies show the importance of the antiradical activity of flavonoids. Thus, it has been shown that flavonoids are the effective inhibitors of lipid peroxidation, oxygen radical overproduction by inflammatory cells, free radical-mediated cytotoxicity, and chromosome damage [3–16].

For several years we have studied the antioxidant activity of rutin, a non-toxic flavonoid glycoside with P vitamin activity, and found that this flavonoid is able to suppress various free radical-mediated processes such as *in vitro* lipid peroxidation [6,14], the mutagenic effect of asbestos fibers on human lymphocytes [9], and the overproduction of free radicals in iron-overloaded rats [16]. We showed that rutin may be successfully applied for the treatment of patients with Fanconi anemia and β-thalassemia (congenital diseases associated with oxygen radical overproduction) [17–19]. Antioxidant effects of rutin were also shown by other

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Abbreviations: BLM, bleomycin; CL, chemiluminescence; HBSS, Hanks' balanced salt solution; and TBA, thiobarbituric acid.

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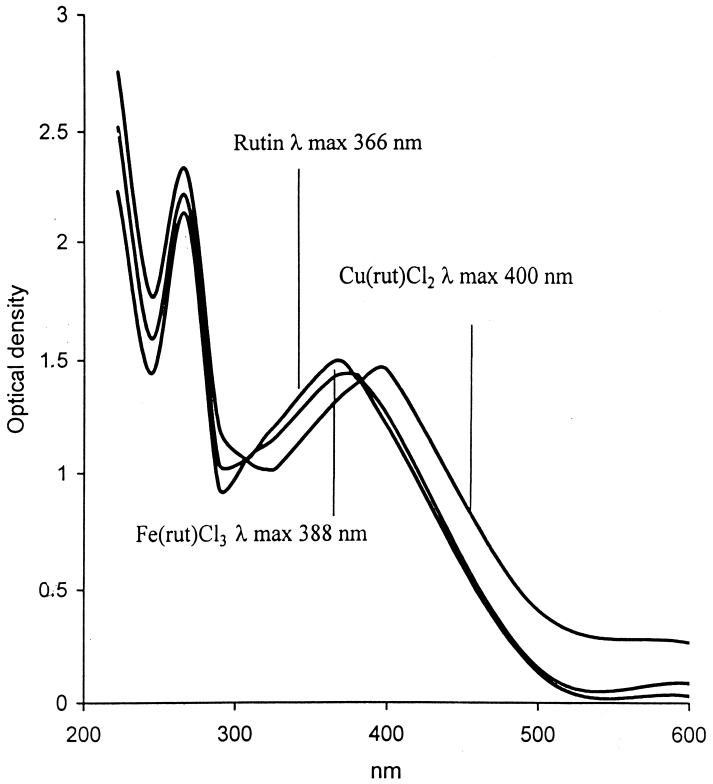

Fig. 1. Optical spectra of rutin, Fe(rut)Cl_3, and Cu(rut)Cl_2.

authors. Thus, Negre-Salvayre *et al*. [8] found that rutin potentiated the antilipoperoxidative capacity of α-tocopherol and ascorbic acid. Grinberg *et al*. [20] showed that rutin suppressed oxygen radical overproduction in thalassemic erythrocytes.

Although the above data show the efficiency of rutin as an antioxidant and free radical scavenger and suggest the clinical relevance of rutin in the management of "free radical" pathologies, this compound is not the most active antioxidative flavonoid, especially in comparison with flavonoid aglycones such as quercetin or kamferol [1]. Unfortunately, the use of aglycones as pharmaceutical agents is restricted due to their mutagenic and cytotoxic activity [1]. Therefore, it is desirable to develop a method to enhance the antioxidant and free radical-scavenging potential of nontoxic flavonoids such as rutin without radically altering their structure. We suggested that this can be achieved by the preparation of transition metal–rutin complexes, in which the flavonoid molecule acquires an additional superoxide-dismuting center without the formation of new covalent bonds.

In the present work, the antioxidant and free radical-scavenging properties of two iron– and copper–rutin complexes were studied. Furthermore, the inhibitory effects of these complexes on lung inflammation and interstitial fibrosis in rats induced by anticancer antibiotic bleomycin were investigated. It was found that the iron– and copper–rutin complexes manifested enhanced antioxidant activities and significantly suppressed inflammation and lung edema in BLM-treated rats.

2. Materials and methods

2.1. Chemicals

Rutin, bleomycin, xanthine, cytochrome *c*, TBA, the chemiluminescent probes luminol and lucigenin, the media HBSS and minimal essential medium (MEM), and the enzymes bovine erythrocyte CuZnSOD (copper, zinc superoxide dismutase, EC 1.15.1.1) and xanthine oxidase (EC 1.1.3.22) were purchased from Sigma Chemical Co. The other salts, solvents, and acids were of the greatest available chemical purity.

2.2. Preparation of metal–rutin complexes

The metal–rutin complexes Fe(rut)Cl_3 and Cu(rut)Cl_2 were prepared by mixing rutin with iron or copper salts in Tris–HCl or phosphate buffers at pH 7.4. The formation of complexes was characterized by the shift of a long-wave rutin maximum of 366 nm to the right, the maximum shift being achieved at 1:1 stoichiometry (Fig. 1).

2.3. Animals

Male Wistar rats weighing 150–200 g were maintained under standard laboratory conditions with chow diet and water *ad lib*.

2.4. In vitro *experiments*

2.4.1. Xanthine–xanthine oxidase system

Xanthine oxidase (1.0 U/mL), catalase (500 U/mL), cytochrome *c* (25 μM), and rutin or metal–rutin complexes (1–50 μM) were incubated in 0.1 M phosphate buffer (7.8 pH) at 25°. After starting the reaction with xanthine (100 μM), the reduction of cytochrome *c* was monitored spectrophotometrically at 550 nm on an Ultraspec spectrophotometer (LKB).

2.4.2. Preparation of liver microsomes

The rat liver was perfused with 0.9% NaCl solution and homogenized with 1.12% KCl solution (1:3 v/v). The homogenate was centrifuged at 10,000 $\times$ *g* for 20 min, then the supernatant was separated and centrifuged at 105,000 $\times$ *g* for 60 min. After protein analysis by the Lowry method [21], microsomes were immediately used in the experiments.

2.4.3. Lipid peroxidation of rat liver microsomes

Microsomes (0.5 mg protein/mL) were incubated with $FeCl_3$ (50 μM) and ADP (the iron chelator [22], 800 μM) with or without rutin and metal–rutin complexes in 0.1 M phosphate buffer (pH 7.4) at 37°. The reaction was started by adding 40 μL (7.5 mM) NADPH in phosphate buffer. The total volume of the incubation mixture was 1 mL. After a 30-min incubation, lipid peroxidation was terminated by adding 15% trichloroacetic acid (1 mL) and a 10-mM ethanol solution of β-naphthol (0.1 mL). Then, 0.375% TBA solution (1 mL) was added, and the reaction mixture was heated at 100° for 15 min. After centrifugation of precipitated proteins, the content of TBA-reactive products was determined by measuring the absorbance at 535 nm.

2.4.4. Measurement of lucigenin-amplified chemiluminescence in microsomes

CL measurements were monitored at 37° and during continuous mixing on a 1251 Luminometer (LKB) with a programmed IBM computer. Lucigenin (40 μM) was added to the incubation mixture containing microsomes (0.5 mg protein/mL), $FeCl_3$ (50 μM), ADP (800 μM), and rutin or metal–rutin complexes at corresponding concentrations in 0.1 M phosphate buffer (pH 7.4) at 37°. At the beginning, the basal CL level was registered for 2 min, then the reaction was started by adding 40 μL (7.5 mM) NADPH in phosphate buffer, and the CL amplitude was measured.

2.4.5. Isolation of peritoneal macrophages

Rats were given ethyl ether narcosis, and then the cells of peritoneal excudate were obtained by lavage of the peritoneal cavity with 5 mL HBSS. The lavage fluid was filtered and centrifuged at 300 $\times$ *g* for 10 min. Cell pellets were washed twice and finally resuspended in 1 mL MEM (minimal essential medium) supplied with 5% fetal bovine serum. Cell preparations were examined microscopically using Wright–Giemza staining (the differential cell count) or vital trypan blue staining for cell viability determination. Peritoneal cell suspensions contained more than 90% macrophages, 95% of which did not include trypan blue.

2.4.6. Measurement of lucigenin- or luminol-amplified CL produced by peritoneal macrophages

Macrophage suspension (5.0×10^5 cells), lucigenin (500 μM) or luminol (500 μM), and rutin or metal–rutin complexes were incubated with or without BLM (10 μg/mL) in HBSS (pH 7.4) at 37° in the CL unit of a luminometer for 3 min. After measurement of background for 2 min, CL was activated by adding opsonized zymosan (200 μg/mL), and the CL amplitude was measured.

2.5. Animal experiments

Rats were divided into 5 groups. Rats in the control group (Group 1, 16 animals) were subjected to sham intratracheal injection with 1 mL saline followed by daily intraperitoneal injections with the same saline solution (1 mL) for 27 days. Rats in the experimental groups were injected with 1 mL of BLM solution (1 mg/mL) intratracheally followed by daily intraperitoneal injections of 1 mL saline solution (Group 2, 16 animals), 1 mL (0.1 mM) rutin solution (Group 3, 15 animals), 1 mL (0.1 mM) Fe(rut)Cl_3 solution (Group 4, 15 animals), or 1 mL (0.1 mM) Cu(rut)Cl_2 solution (Group 5, 16 animals) for 27 days. The animals were killed under ethyl ether narcosis on the 3rd, 7th, and 28th days. Broncho-alveolar cells were isolated using the lung lavage procedure with 10 mL of preheated HBSS. The cell smears were prepared, fixed with methanol, and stained by the Wright–Giemza method [23] for microscopic differential cell counting. The intensity of lung inflammation was assessed on the basis of the macrophage/neutrophil ratio in broncho-alveolar cell suspension, the water leakage in the lung, and the protein content in cell-free lavage fluid. The protein content was measured by the Lowry method [21]. The removed lung tissue was analyzed by the Stegemann method [24] to determine the hydroxyproline content as a biochemical marker of lung fibrosis. Oxygen radical production by broncho-alveolar cells was measured by luminol- and lucigenin-amplified CL as described above for the *in vitro* experiments.

2.6. Statistics

All results are from experiments carried out in duplicate. Each point was a mean of 3 or 4 independent measurements. All data are presented as means $\pm$ SD. Student's *t*-test was used. $P < 0.05$ was considered to be significant.

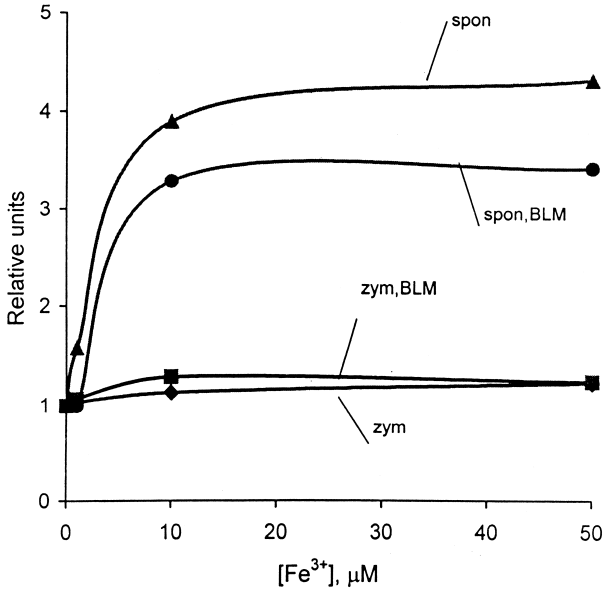

Fig. 2. Effects of ferric ions on spontaneous (spon) and zymosan (zym)-stimulated luminol-amplified CL of rat peritoneal macrophages in the presence and absence of BLM. All CL values were calculated relative to the control values obtained in the absence of ferric chloride. Macrophage suspension (5.0×10^5 cells), luminol (500 μM), and ferric chloride (10–50 μM) were incubated with or without BLM (10 μg/mL) in HBSS (pH 7.4) at 37°. After measurement of background CL intensity for 2 min, opsonized zymosan (200 μg/mL) was added, and the CL amplitude was measured.

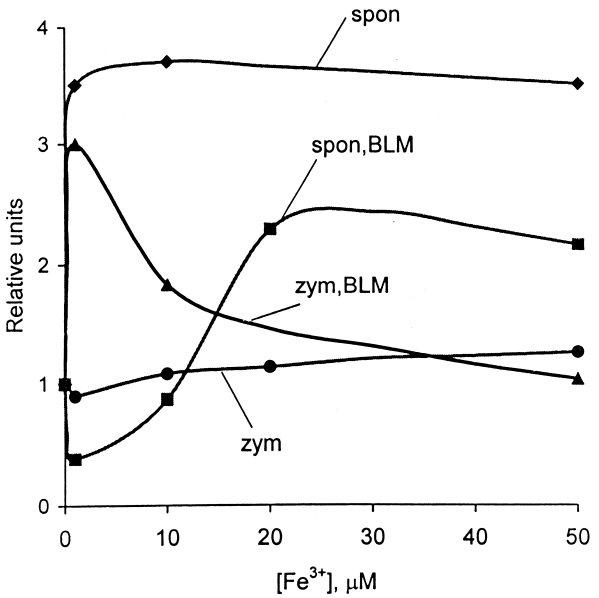

Fig. 3. Effects of ferric ions on spontaneous (spon) and zymosan (zym)-stimulated lucigenin-amplified CL of rat peritoneal macrophages in the presence and absence of BLM. All CL values were calculated relative to the control values obtained in the absence of ferric chloride. Experimental conditions were the same as in Fig. 2.

3. Results

3.1. Effects of iron ions, copper ions, and rutin and metal–rutin complexes on oxygen radical production by rat peritoneal macrophages

Ferric ions sharply enhanced spontaneous luminol-amplified CL produced by rat peritoneal macrophages in the presence and absence of BLM and had no effect on zymosan-stimulated CL (Fig. 2). The effect of ferric ions on lucigenin-amplified CL was more complicated (Fig. 3), while cupric ions were always inhibitory (Fig. 4). Rutin inhibited all kinds of luminol- and lucigenin-amplified CL by macrophages. $Cu(rut)Cl_2$ was a much more efficient inhibitor (by 2–30 times) of oxygen radical production than rutin. The inhibitory effect of $Fe(rut)Cl_3$ on zymosan-activated CL was 2–8 times higher than that of rutin, but this complex was stimulatory in the case of spontaneous CL (Figs. 5 and 6).

3.2. Inhibition by rutin and the iron–rutin complex of free radical production by xanthine oxidase and rat liver microsomes

Rutin and $Fe(rut)Cl_3$ inhibited superoxide production by xanthine oxidase and rat liver microsomes (measured via cytochrome *c* reduction and lucigenin-amplified CL, respectively) and the formation of thiobarbituric acid-reactive products in microsomal lipid peroxidation in a concentration-dependent manner. Corresponding I_{50} values are cited

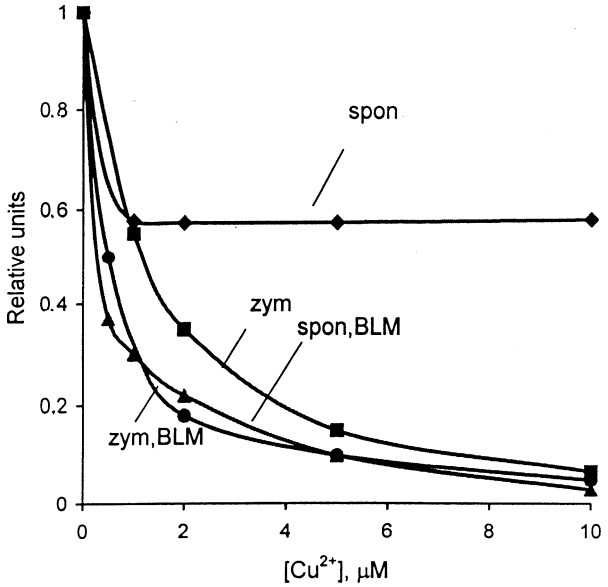

Fig. 4. Effects of cupric ions on spontaneous (spon) and zymosan (zym)-stimulated luminol-amplified CL of rat peritoneal macrophages in the presence and absence of BLM. All CL values were calculated relative to the control values obtained in the absence of cupric chloride. Macrophage suspension (5.0×10^5 cells), luminol (500 μM), and cupric chloride (1–10 μM) were incubated with or without BLM (10 μg/mL) in HBSS (pH 7.4) at 37°. After measurement of background CL intensity for 2 min, opsonized zymosan (200 μg/mL) was added, and the CL amplitude was measured.

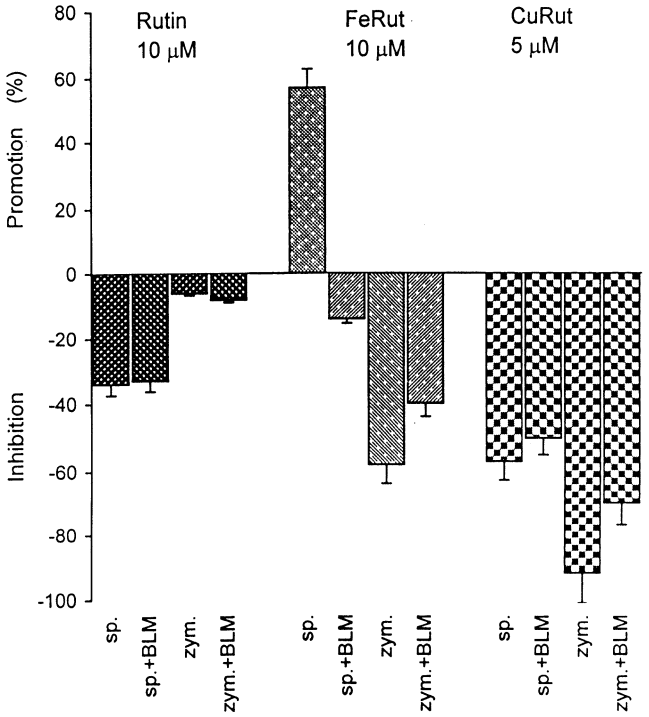

Fig. 5. The effects of rutin and metal–rutin complexes on spontaneous (sp.) and zymosan (zym.)-stimulated luminol-amplified CL of rat peritoneal macrophages. All CL values were calculated relative to the control values obtained in the absence of inhibitors. The data are presented as means ± SD. Macrophage suspension (5.0×10^5 cells), luminol (500 μM), and rutin or metal–rutin complexes were incubated in HBSS (pH 7.4) at 37°. After adding opsonized zymosan (200 μg/mL), the CL amplitude was measured.

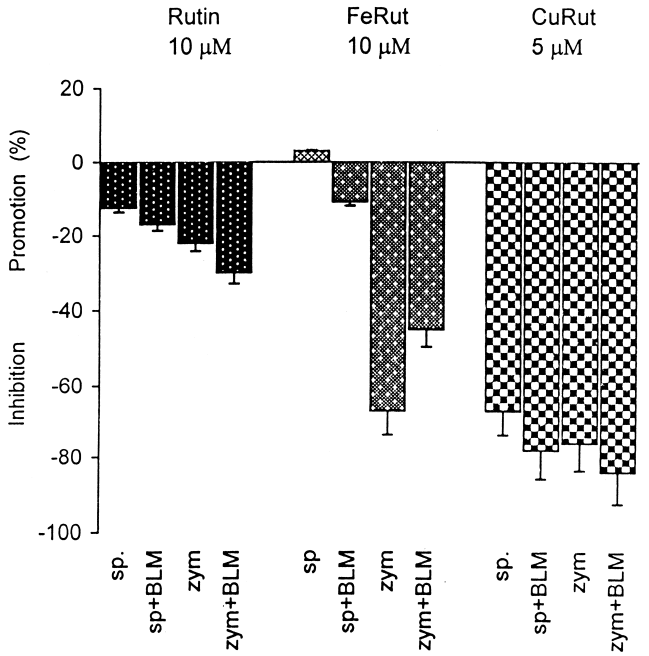

Fig. 6. The effects of rutin and metal–rutin complexes on spontaneous (sp.) and zymosan (zym.)-stimulated lucigenin-amplified CL of rat peritoneal macrophages. All CL values were calculated relative to the control values obtained in the absence of inhibitors. The data are presented as means ± SD. Experimental conditions were the same as in Fig. 5.

in Table 1 together with the data obtained earlier for $Cu(rut)Cl_2$ [25]. The effects of all compounds on copper, zinc superoxide dismutase (SOD)-inhibitable (>90%) cytochrome *c* reduction by xanthine oxidase depended exclusively on their interaction with superoxide ion, because they did not affect the xanthine oxidase activity measured via the formation of uric acid over the whole concentration range studied (from 1 to 50 μM, data not shown).

3.3. The effects of rutin, Fe(rut)Cl$_3$, and Cu(rut)Cl$_2$ on oxygen radical production by broncho-alveolar cells under ex vivo *conditions*

The intensities of luminol- and lucigenin-amplified CL produced by zymosan-stimulated broncho-alveolar cells isolated from bleomycin-treated rats (Group 2) were 1.7 ± 0.05 and 2.3 ± 0.2 times higher than the values for the control animals of Group 1. The administration of $Cu(rut)Cl_2$ to BLM-treated animals significantly ($P < 0.01$) diminished both luminol- and lucigenin-amplified CL, with values approaching those for control animals of Group 1 at the end of experiment. (Fig. 7). The effects of rutin and $Fe(rut)Cl_3$ were not so favorable. Rutin strongly inhibited luminol-amplified CL on the 3rd day, but had no effect on the 7th and 28th days. $Fe(rut)Cl_3$ even enhanced luminol CL at the beginning of the experiment, although the CL intensity fell to the control value by the end (Fig. 7A). Rutin and $Fe(rut)Cl_3$ had no effect on lucigenin-amplified CL (Fig. 7B).

Table 1
Inhibitory effects of metal–rutin complexes and rutin on *in vitro* free radical processes (I_{50} values, μM)

	Cyt. *c* reduction[a] by xanthine oxidase	Formation of[b] TBA-reactive products	Lucigenin-[c] amplified microsomal CL
Rutin	35 ± 0.3	90 ± 10	20 ± 3
$Fe(rut)Cl_3$	22 ± 2	>500	23 ± 3
$Cu(rut)Cl_2$ [25]	2.5 ± 0.2	10 ± 3	3 ± 1

[a] Xanthine oxidase (1.0 U/mL), catalase (500 U/mL), cytochrome *c* (25 μM), and rutin or metal–rutin complexes (1–50 μM) were incubated in 0.1 M phosphate buffer (7.8 pH) at 25°. After starting the reaction with xanthine (100 μM), the reduction of cytochrome *c* was monitored spectrophotometrically at 550 nm.

[b] Microsomes (0.5 mg protein/mL) were incubated with $FeCl_3$ (50 μM) and ADP (800 μM) with or without rutin and metal–rutin complexes in 0.1 M phosphate buffer (pH 7.4) at 37°. The reaction was started by adding 40 μL (7.5 mM) NADPH in phosphate buffer and was terminated after 30 min. The content of thiobarbituric acid-reactive products was determined by measuring the absorbance at 535 nm.

[c] Lucigenin (40 μM) was added to an incubation mixture containing microsomes (0.5 mg protein/mL), $FeCl_3$ (50 μM), ADP (800 μM), and rutin or metal–rutin complexes at corresponding concentrations in 0.1 M phosphate buffer (pH 7.4) at 37°. The reaction was started by adding 40 μL (7.5 mM) NADPH in phosphate buffer, and the CL amplitude was measured.

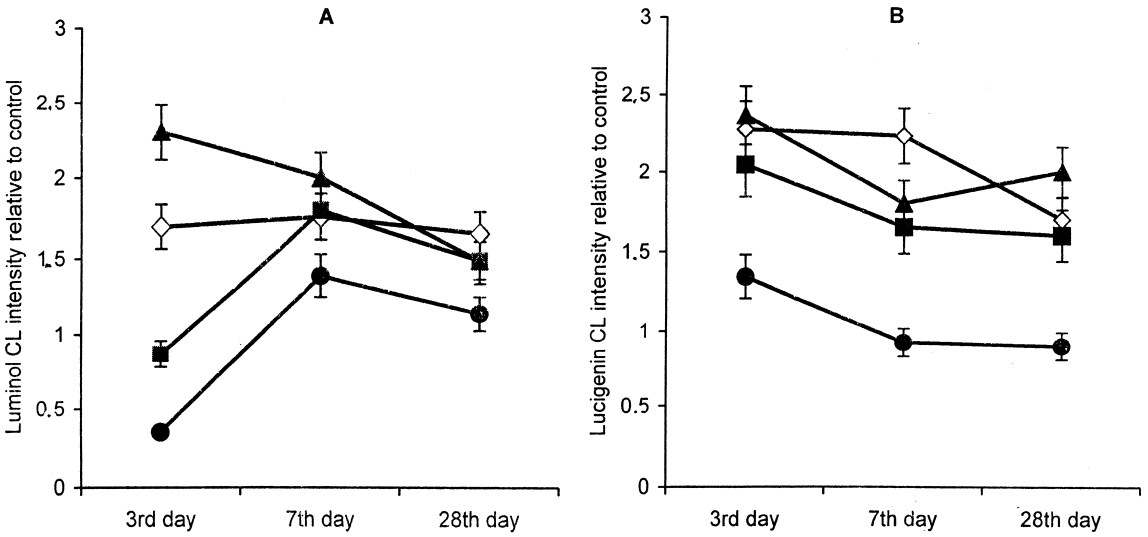

Fig. 7. CL intensities produced by zymosan-stimulated broncho-alveolar cells isolated from bleomycin-treated rats calculated relative to CL values for the animals of control Group 1. All data are presented as means ± SD. ◇ Group 2, BLM; ■ Group 3, rutin; ▲ Group 4, Fe(rut)Cl_3; ● Group 5, Cu(rut)Cl_2. (A) Luminol-amplified CL. (B) Lucigenin-amplified CL.

3.4. The effects of the administration of rutin and complexes on the development of bleomycin-induced inflammation and lung fibrosis in rats

The administration of rutin, Fe(rut)Cl_3, and Cu(rut)Cl_2 to BLM-treated rats affected both inflammatory and fibrotic processes in the lung. Cu(rut)Cl_2 exhibited a remarkable effect on the macrophage/neutrophil ratio, enhancing it to the normal value of Group 1 ($P < 0.01$). The effects of rutin and Fe(rut)Cl_3 were much smaller (Fig. 8A). Cu(rut)Cl_2 and Fe(rut)Cl_3 decreased the level of lung edema nearly twofold ($P < 0.01$). At the beginning of the experiment, the effect of rutin was insignificant, but it approached a normal value on the 28th day (Fig. 8B). Cu(rut)Cl_2 significantly decreased (by about twofold, $P < 0.05$) the total protein content in the broncho-alveolar liquid of BLM-treated rats, while rutin had practically no effect (Fig. 8C). Both Cu(rut)Cl_2 and rutin significantly lowered the level of hydroxyproline ($P < 0.05$) (Fig. 8D).

4. Discussion

As mentioned above, a main goal of this work was to study the antiradical, antioxidant activity of iron and copper complexes of rutin, which acquire additional superoxide-dismuting centers and thereby may be more effective free radical scavengers compared to the parent rutin. We have already shown [25,26] that the transition metal complexes of rutin are indeed the effective scavengers of active free radicals produced by xanthine oxidase or are formed during lipid peroxidation of liposomes and microsomes. However, it is well known that transition metals might exhibit both anti- and pro-oxidant effects by dismuting superoxide ion or catalyzing the decomposition of peroxides. Therefore, in an attempt to evaluate the antioxidant and possible pro-oxidant effects of iron– and copper–rutin complexes in different experimental models, we studied their effects on free radical generation under *in vitro* and *ex vivo* conditions and in animal experiments with BLM-treated rats and compared them with the effects of free iron and copper ions.

As can be seen in Figs. 2 and 3, ferric ions exhibited a strong stimulatory effect on oxygen radical production by macrophages in the presence and absence of BLM. In contrast, copper ions were always inhibitory (Fig. 4). The complexation of ferric ions by rutin mostly converted them from pro-oxidants into antioxidants, although Fe(rut)Cl_3 still promoted the spontaneous production of oxygen radicals by macrophages (Figs. 5 and 6). Earlier, we found that the iron–rutin complex was a stronger inhibitor of liposomal lipid peroxidation than the copper–rutin complex, but the opposite effects were observed in microsomal peroxidation [26]. The present findings entirely confirm these data. A possible explanation may reside in the fact that in contrast to liposomes where any strong reductants are absent, microsomes and macrophages contain numerous endogenous reductants, which are able to reduce the antioxidant Fe(rut)Cl_3 into pro-oxidant Fe(rut)Cl_2.

Comparison of *in vitro* findings (Figs. 5 and 6, Table 1) with the *ex vivo* data (Fig. 7, A and B) for BLM-treated rats shows that the inhibitory antiradical activity of rutin, Fe(rut)Cl_3, and Cu(rut)Cl_2 increased in the order: Fe(rut)Cl_3 $\cong$ rutin $\ll$ Cu(rut)Cl_2. Thus, the copper–rutin complex is the most efficient scavenger of oxygen radicals under both *in vitro* and *ex vivo* conditions. It should be noted that Cu(rut)Cl_2 was equally effective in the inhibition of cytochrome *c* reduction by xanthine oxidase and in lucigenin-amplified CL production by rat liver microsomes, two dif-

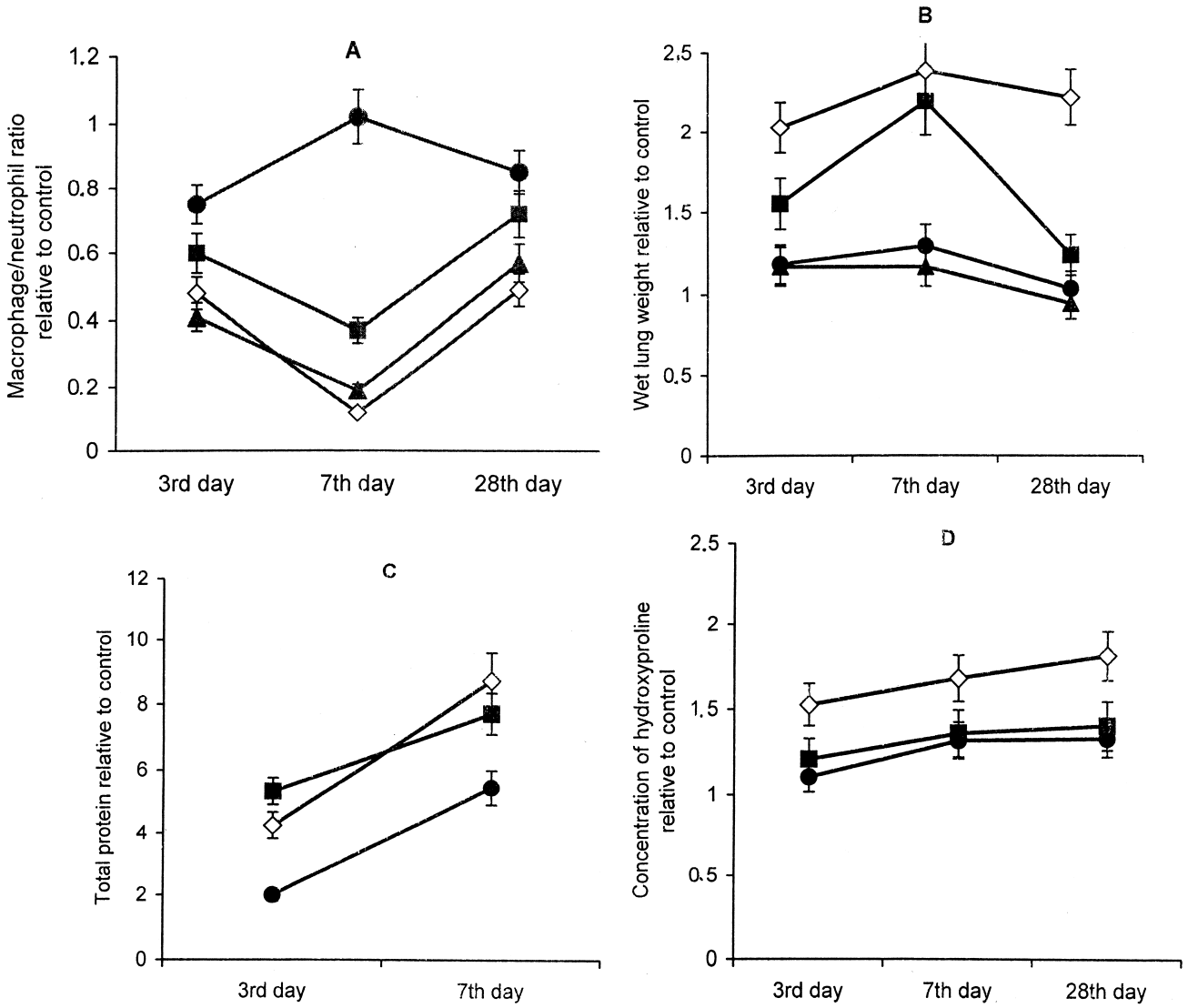

Fig. 8. Effects of administration of rutin, $Fe(rut)Cl_3$, and $Cu(rut)Cl_2$ on inflammatory and fibrotic parameters of bleomycin-treated rats calculated relative to the values for animals of control Group 1. All data are presented as means ± SD. (A) Macrophage/neutrophil ratio. (B) Wet lung weight. (C) The content of total protein. (D) Concentration of hydroxyproline. ◇ Group 2, BLM; ■ Group 3, rutin; ▲ Group 4, $Fe(rut)Cl_3$; ● Group 5, $Cu(rut)Cl_2$.

ferent assays of superoxide detection. These findings confirm the importance of the appearance of a new superoxide-dismuting center in the copper–rutin complex.

In the second part of this work, we studied the inhibitory effects of iron– and copper–rutin complexes on BLM-induced lung inflammation and interstitial fibrosis in rats. We supposed that free radical scavengers and antioxidants may influence the BLM-induced inflammatory processes, which may, at least in part, depend on free radical overproduction. Although both processes are not usually directly connected with free radical overproduction, our findings suggest the importance of free radical-mediated contributions and consequently the possibility of regulation of both fibrosis and inflammation by antioxidants and free radical scavengers. As in the *in vitro* and *ex vivo* experiments, $Cu(rut)Cl_2$ was the most effective inhibitor of inflammatory and fibrotic processes, sharply increasing the macrophage/neutrophil ratio (Fig. 8A), decreasing the wet lung weight to normal value (Fig. 8B), lowering by about twofold the content of total protein (Fig. 8C), and decreasing hydroxyproline concentration virtually to its normal level (Fig. 8D). The *in vitro* and *ex vivo* data showed that $Fe(rut)Cl_3$ in some cases exhibited a certain pro-oxidant activity. Unfortunately, we were unable to study the effect of this complex in all animal experiments, although we did find that, similarly to $Cu(rut)Cl_2$, $Fe(rut)Cl_3$ was very efficient in suppressing lung edema (Fig. 8B). Thus, we believe that despite its weak *in vitro* pro-oxidant activity, the iron–rutin complex might be a good *in vivo* antioxidant and therefore worthy of further investigation.

In conclusion, our findings suggest that the iron– and copper–rutin complexes studied not only retained the antioxidant properties of rutin, but in many cases exhibited enhanced free radical-scavenging activity. This was espe-

cially true for Cu(rut)Cl_2, which is a superior inhibitor of free radical production in *in vitro* model systems and an excellent suppressor of free radical-mediated pathological disorders. We are hopeful that the present data will be considered as a basis for subsequent investigations into the pharmaceutical activity of metal–flavonoid complexes.

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