British Journal of Pharmacology (2008) 153, 1288–1295
 0007–1188/08 $30.00
www.brjpharmacol.org

RESEARCH PAPER

Colchicine suppresses neutrophil superoxide production in a murine model of gouty arthritis: a rationale for use of low-dose colchicine

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Background and purpose: When used to treat gouty arthritis, colchicine is believed to work by inhibiting microtubule-dependent cell infiltration. However, *in vitro,* colchicine also reduces monosodium urate (MSU)-induced superoxide production by neutrophils. Our study aimed to compare the effects of colchicine on neutrophil superoxide production and infiltration in an *in vivo* model of acute gouty inflammation.
Experimental approach: *In vitro:* Human and murine peritoneal neutrophils were incubated with MSU with and without colchicine, and superoxide production was measured. *In vivo:* Mice were treated with colchicine followed by an intraperitoneal injection of MSU to induce acute inflammation. After 4h, the peritoneal cells were recovered to measure superoxide production and neutrophil infiltration. Sera were tested for liver and renal toxicity.
Key results: Colchicine dose-dependently inhibited MSU-induced superoxide production by both human and murine neutrophils *in vitro.* Oral colchicine inhibited MSU-induced superoxide production by neutrophils *in vivo* at doses 100 times lower than those required to inhibit neutrophil infiltration and without acute liver or renal toxicity. Neutrophils treated with colchicine *in vivo* still produced superoxide in response to another stimulus, 4-β-phorbol-12-myristate-13-acetate.
Conclusions and implications: These results show a beneficial effect of colchicine for the treatment of MSU-induced superoxide production *in vivo* at sub-toxic doses without compromising superoxide production by other physiological processes. This is the first *in vivo* data to provide a biological rationale that supports the implementation of low dose, non-toxic, colchicine therapy for the treatment of gouty arthritis.
British Journal of Pharmacology (2008) **153,** 1288–1295; doi:10.1038/bjp.2008.20; published online 11 February 2008

Keywords: colchicines; gout; MSU; superoxide; *in vivo*

Abbreviations: MSU, monosodium urate crystals; PMA, 4-β-phorbol-12-myristate-13-acetate; WST, 1-(2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt

Introduction

Gout is a form of arthritis caused by mono-sodium urate (MSU) crystals forming in and around the joints causing an inflammatory reaction that manifests as intense pain, swelling and reddening of the skin. The water-soluble alkaloid colchicine is one of the recommended treatments for acute gout and for prophylaxis against acute attacks during initiation of urate-lowering therapy (Terkeltaub, 2003; British National Formulary, 2006; Zhang *et al*., 2006). Colchicine is believed to reduce inflammation by inhibiting MSU-induced migration of neutrophils and other leukocytes via blockade of microtubule formation (Cronstein *et al*., 1995; Ben-Chetrit and Levy, 1998). This has been shown to occur without affecting local stimulation or the production of the neutrophil chemokine interleukin 8 (Matsukawa *et al*., 1998). It is therefore proposed that colchicine interferes directly with the migration of neutrophils from the blood. However, alternative effects of colchicine contributing to therapeutic efficacy have not been extensively investigated. Microtubule formation is important for a variety of other cell functions, including cell division and intracellular transport (Valiron *et al*., 2001; Caviston and Holzbaur, 2006) Consequently, colchicine has the potential to affect other, microtubule-dependent, inflammatory processes in gout.

Infiltrating neutrophils play a key role in the inflammatory response to MSU in gout with neutrophil superoxide production making a major contribution to the inflammatory response and tissue injury. Colchicine has been shown to suppress MSU-induced superoxide production by human

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Received 5 September 2007; revised 23 November 2007; accepted 13 December 2007; published online 11 February 2008

neutrophils *in vitro* and this inhibitory effect can be blocked by taxol, a known microtubule-stabilizing agent (Roberge *et al*., 1996). Recovery of superoxide production in the presence of taxol indicates that the inhibitory effect of colchicine occurs at the level of microtubule formation. The inhibition of superoxide production by colchicine may result from interference with intracellular transport and therefore assembly of the NADPH oxidase complex responsible for superoxide production.

Superoxide production by neutrophils can be induced by a variety of different stimuli, many of which are not blocked by colchicine. For example, colchicine does not inhibit microtubule-independent superoxide production induced by the peptide formyl-Met-Leu-Phe, opsonized zymosan or the PKC ligand, 4-β-phorbol-12-myristate-13-acetate (PMA) (Minta and Williams, 1986; Gaudry *et al*., 1993; Vrba and Modriansky, 2004). In addition, colchicine has been shown to specifically inhibit MSU-induced tyrosine phosphorylation, an event upstream of superoxide production (Gaudry *et al*., 1993; Roberge *et al*., 1993, 1996). Colchicine, therefore, exhibits some specificity for inhibition of MSU-induced superoxide production and signalling. These findings indicate that, in addition to suppressing leukocyte infiltration, colchicine has the potential to specifically inhibit MSU-induced superoxide production by neutrophils during an attack of gouty inflammation.

To date, the ability of colchicine to inhibit MSU-induced superoxide production by neutrophils has only been illustrated *in vitro* in single-dose experiments and it is not known whether this effect occurs *in vivo* or is relevant in ameliorating inflammation in gout. Using a murine model of MSU-induced inflammation, we have shown here that, in addition to inhibiting neutrophil infiltration, colchicine can dose-dependently inhibit MSU-induced superoxide production by *in vitro*- and *in vivo*-activated neutrophils. We also show that colchicine inhibits MSU-induced superoxide production *in vivo* at doses lower than those required for inhibition of neutrophil infiltration and without acute toxicity. These findings indicate that a major effect of low doses of colchicine used to treat gout may be the inhibition of superoxide production by neutrophils. Confirmation of this inhibitory effect *in vivo* provides evidence to support the use of lower doses of colchicine in treating gout, thus avoiding the gastrointestinal toxicity associated with widely used regimens.

Methods

Animals

All animal and experimental procedures were approved by the Victoria University of Wellington (Wellington, New Zealand) Animal Ethics Committee and carried out in accordance with their guidelines for the care of animals. C57BL/J6 male mice were bred and housed in a conventional animal facility at the Malaghan Institute of Medical Research (20 °C, 14/10 light/dark cycle, food/water on demand). All animals used for the experiments were aged between 8 and 12 weeks.

Blood leukocyte preparations

Whole-blood was collected with informed consent from healthy volunteers into vacutainers containing EDTA. Human white blood cells were prepared by centrifuging whole blood (850 *g*) for 10 min to isolate the buffy coat layer. Total cell numbers were counted using a haemocytometer and an aliquot (100 μl) was fixed onto slides using a cytospin. The fixed cells were stained with Diff-Quick and the percentages of different cell types were determined microscopically using standard histological criteria. Cell suspensions (70% neutrophils) were then adjusted to contain 1×10^6 neutrophils per ml for *in vitro* testing.

Human neutrophils and peripheral blood mononuclear cells were isolated from whole blood using Polymorphprep, as described by the manufacturer. Cells were counted using a haemocytometer and plated out at 1×10^6 cells ml^{-1} for *in vitro* testing. Neutrophil and monocyte preparations were >98 and >80% pure, respectively, and 100% viable as determined by Trypan blue exclusion.

Measurement of superoxide production in vitro

Cell preparations were incubated with colchicine for 10 min at room temperature then incubated at 37 °C for 20 min with MSU (3 mg ml^{-1}) in the presence of WST-1 (1-(2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt) (0.35 mM). Samples were centrifuged to remove the MSU crystals and the supernatants were transferred to a flat bottom plate. The supernatant absorbance was read at 450 nm (endpoint) using a Versamax plate reader. Superoxide production is presented relative to superoxide produced by neutrophils + MSU alone (100%).

Monosodium urate crystal-induced murine peritonitis

C57Bl/J6 mice (five per group) were injected with 3 mg of MSU (0.5 ml phosphate-buffered saline, intraperitoneous (i.p.)) (Getting *et al*., 1997; Chen *et al*., 2006). Colchicine (in phosphate-buffered saline) was administered by subcutaneous (s.c.) (50 μl) or i.p. (50 μl) injection, or by oral gavage (250 μl), at the time points indicated. A 4 h after MSU treatment, the mice were killed (CO_2 followed by cervical dislocation) and the peritoneal cavities were lavaged (3 ml phosphate-buffered saline). The peritoneal cells were washed with HBSS and processed as described for human WBCs. Cell viability was >95% as determined by Trypan blue exclusion.

Superoxide production by murine peritoneal neutrophils ex vivo

Peritoneal cells containing between 52 and 68% neutrophils were plated out at 1×10^6 neutrophils per ml, WST-1 (0.35 mM) was added to the cell suspension in the presence or absence of PMA (0.32 μM) and the sample absorbance read at 450 nm over 20 min (Tan and Berridge, 2000). Superoxide production is presented relative to superoxide produced by neutrophils + MSU alone (100%).

Serum liver enzymes and creatinine

Blood was collected from mice immediately after death (CO_2 asphyxiation followed by cervical dislocation) by cardiac puncture and serum separated after clotting. The serum samples were then sent away for testing (Gribbles Veterinary, Hamilton, New Zealand) to determine the levels of the liver enzymes aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, lactate dehydrogenase and the renal marker creatinine. Levels are presented as the fold increase above naïve controls (1.0).

Statistical analysis

Statistical analyses were performed using one-way analysis of variance followed by post-test Tukey (if overall $P<0.05$). A P-value <0.05 was considered significant.

Reagents and chemicals

Polymorphprep (Axis-Shield, Oslo, Norway) was obtained through Medica Pacifica Ltd (Auckland, New Zealand). WST-1 was from Dojindo Laboratories (Kumamoto, Japan). Diff-Quick Staining Kit was from Dade Behring (Newark, NJ, USA). All other reagents were obtained through Sigma-Aldrich (Auckland, New Zealand).

Results

Colchicine inhibits MSU-induced superoxide production by human neutrophils in vitro *in a concentration-dependent manner*

Human blood contains up to 70% neutrophils making it a reliable source of neutrophils for *in vitro* study. To establish that the blood-derived neutrophils were the main source of superoxide production following MSU exposure, we compared superoxide production among WBCs, purified neutrophils and peripheral blood mononuclear cells. MSU administration induced comparable levels of superoxide production by both the purified neutrophils and WBCs (Figure 1a), whereas isolated peripheral blood mononuclear cells failed to produce superoxide following exposure to MSU (Figure 1b). Colchicine inhibited MSU-induced superoxide production by both human WBCs and isolated human peripheral blood neutrophils stimulated by MSU *in vitro* (Figure 1a) in a concentration-dependent manner. Purification of the neutrophils appeared to initiate a high background of superoxide production that was increased further by the addition of MSU. This background superoxide production was also inhibited by colchicine, indicating that background and MSU-induced activation of NADPH oxidase may occur via the same microtubule-dependent pathway.

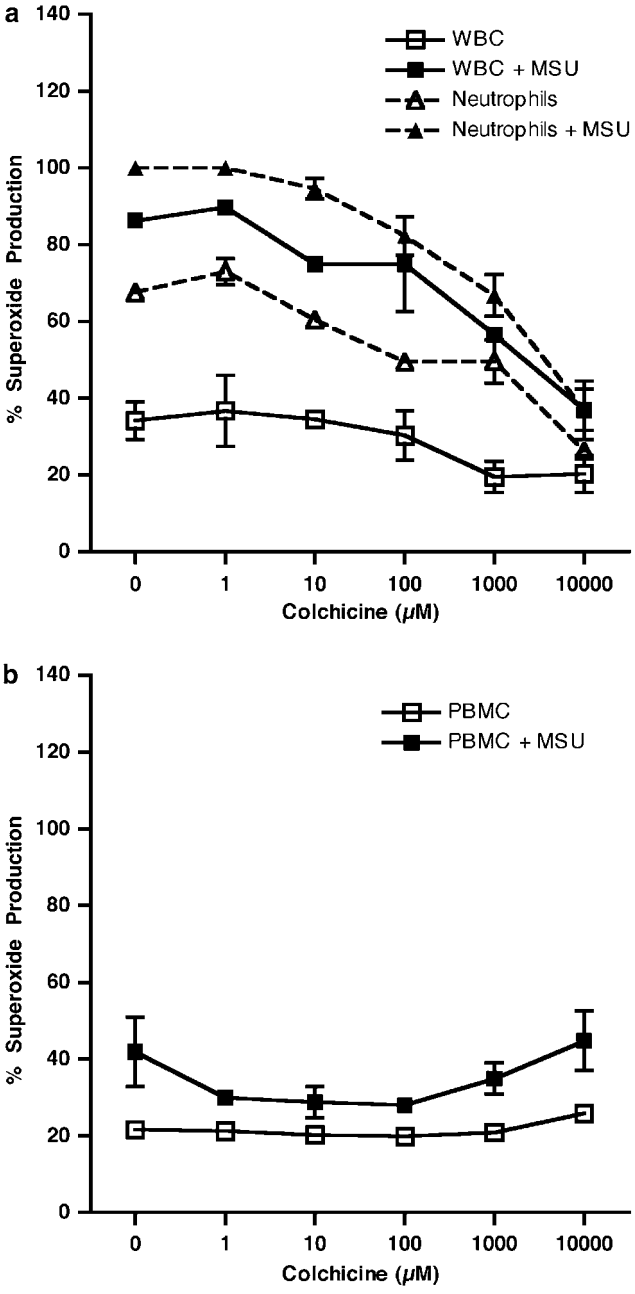

Figure 1 Colchicine inhibits MSU-induced superoxide production by human blood neutrophils *in vitro* in a concentration-dependent manner. (**a**) WBCs, neutrophils and (**b**) peripheral blood mononuclear cell were isolated from human blood and treated with MSU (3 mg ml^{-1}) *in vitro* in the presence of colchicine and tested for superoxide production. Superoxide production was normalized to that produced by neutrophils after MSU alone (A_{450} endpoint = 0.25 = 100%). Data are representative of triplicate experiments. MSU, monosodium urate.

Superoxide production by in vivo*-activated murine neutrophils is inhibited by colchicine* ex vivo *in a concentration-dependent manner*

To establish that murine neutrophils also produce superoxide in response to MSU, we assessed the effect of colchicine on murine neutrophils *ex vivo*. Neutrophils are found only in small numbers in the mice blood (up to 10%), making it a limited source of neutrophils for study. As a result, we chose to investigate the effect of colchicine on neutrophils that had migrated into the peritoneum (up to 70% of infiltrating leukocytes) following i.p. injection of MSU. Consistent with *in vivo* activation by MSU, cells from the peritoneal lavage produced high levels of superoxide without requiring restimulation *ex vivo* and superoxide

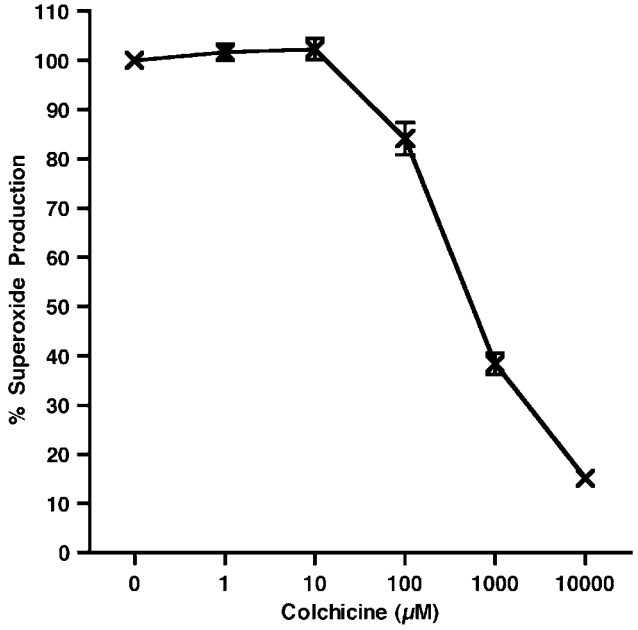

Figure 2 Colchicine inhibits MSU-induced superoxide production by mouse peritoneal neutrophils *ex vivo* in a concentration-dependent manner. C57Bl/J6 mice ($n=6$) were treated with 3 mg ml^{-1} of MSU intrapertoneally. Peritoneal cells were isolated 4 h after administration of MSU, treated with colchicine *ex vivo* and analysed for superoxide production. Superoxide production was normalized that produced by neutrophils after MSU alone (A_{450} endpoint = 0.39 = 100%). Results are representative of duplicate experiments. MSU, monosodium urate.

production was also inhibited by colchicine *ex vivo* in a concentration-dependent manner (Figure 2).

Colchicine inhibits MSU-induced superoxide production by murine neutrophils in vivo

To test the efficacy of colchicine *in vivo*, we first investigated the effect of 5 μmol kg^{-1} colchicine administered by different routes concurrently with MSU (i.p.). At 4 h after MSU administration, s.c. and i.p. treatment with colchicine inhibited both superoxide production (Figure 3a) and neutrophil infiltration (Figure 3b), whereas oral administration had no effect. Oral colchicine only inhibited superoxide production and neutrophil infiltration when administered at least 3 h prior to MSU administration (Figures 4a and b). Oral colchicine administered 24 h prior to MSU failed to block neutrophil infiltration, but still inhibited superoxide production *in vivo* (Figures 4a and b).

Low-dose colchicine inhibits MSU-induced superoxide production by neutrophils in vivo

Treatment with lower doses of colchicine 4 h before administration of MSU inhibited superoxide production without inhibiting cellular infiltration (Figures 5a and b). As colchicine therapy can cause acute tissue toxicity, serum levels of liver enzymes and creatinine from naive and colchicine-treated mice were measured as surrogates for liver

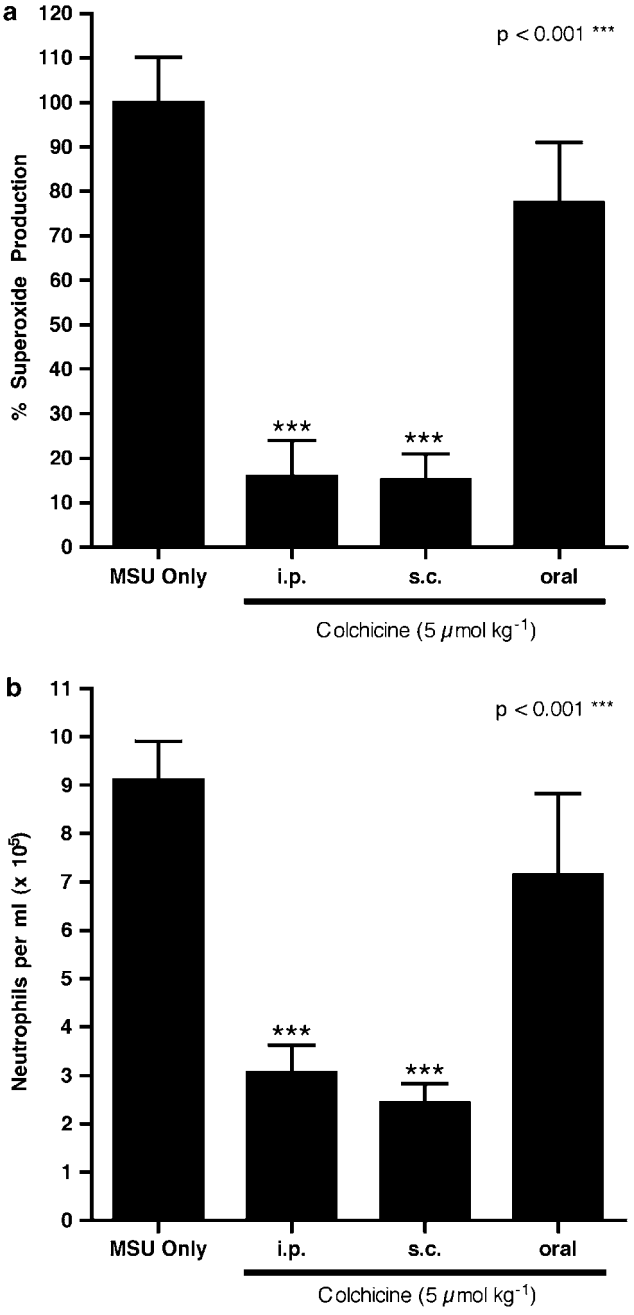

Figure 3 Intraperitoneal and subcutaneous administration of colchicine inhibits MSU-induced superoxide production and neutrophil infiltration *in vivo*. C57Bl/J6 mice ($n=5$) were treated with colchicine intraperitoneally, subcutaneously or by oral gavage 0 h before i.p. administration of 3 mg ml^{-1} of MSU. Peritoneal cells were isolated 4 h after administration of MSU and analysed for (**a**) superoxide production and (**b**) neutrophil infiltration. Superoxide production was normalized to that produced by neutrophils after MSU alone (A_{450} kinetic = 2.18 = 100%). Statistical analysis was carried out by one-way analysis of variance followed by post-test Tukey. Results are representative of duplicate experiments. MSU, monosodium urate.

and renal toxicity. Doses of colchicine that inhibited both neutrophil infiltration and superoxide production caused a striking increase in serum aspartate aminotransferase,

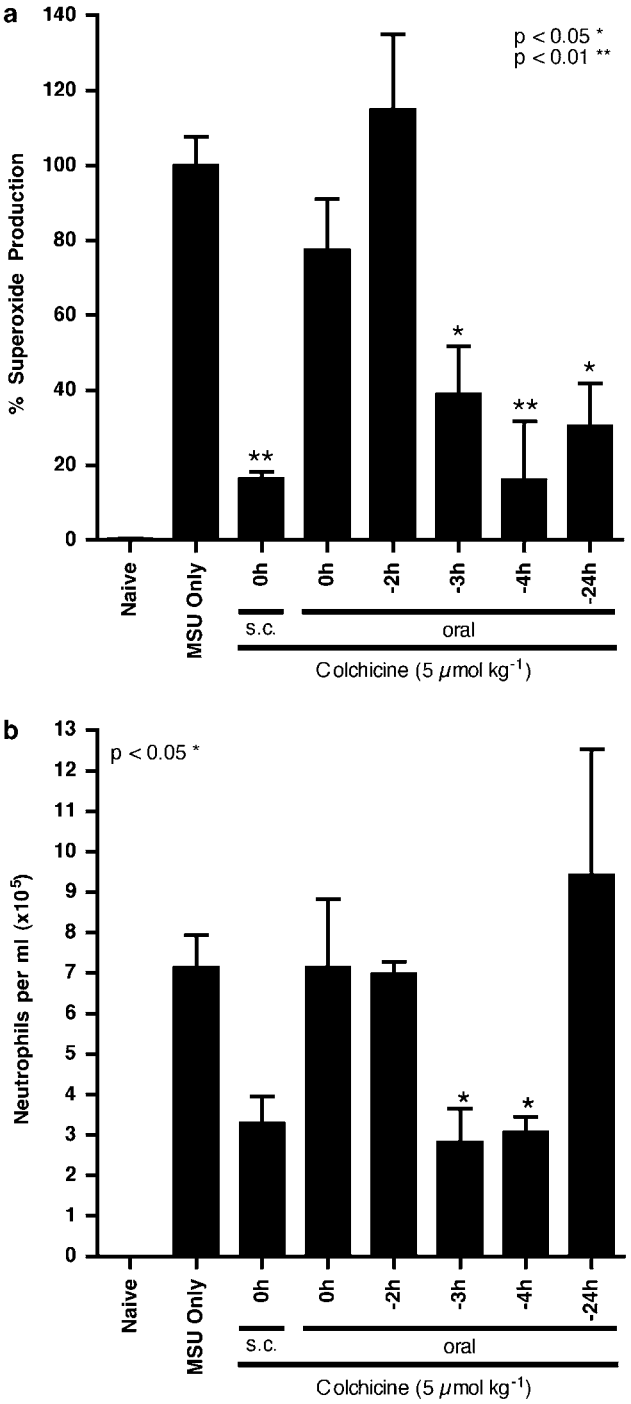

Figure 4 Oral colchicine exhibits a delayed inhibitory effect on MSU-induced superoxide production and neutrophil infiltration *in vivo*. C57Bl/J6 mice ($n=5$) were treated with colchicine by oral gavage 0, 2, 3, 4 or 24 h, or by s.c. injection 0 h, before administration of 3 mg ml^{-1} of MSU i.p. Peritoneal cells were isolated 4 h after administration of MSU and tested for (**a**) superoxide production and (**b**) neutrophil infiltration. The rate of superoxide production was normalized to that produced by neutrophils after MSU alone (A_{450} kinetic = 2.96 = 100%). Statistical analysis was carried out by one-way analysis of variance followed by post-test Tukey and Student's *t*-test. Results are representative of duplicate experiments. MSU, monosodium urate.

alanine aminotransferase and lactate dehydrogenase, whereas lower doses that only inhibited neutrophil superoxide production had liver enzyme levels comparable with the disease control (Figure 5c).

In vivo *treatment with colchicine does not inhibit PMA-induced superoxide production by MSU-activated peritoneal neutrophils*
To determine whether neutrophils from MSU-treated and MSU/colchicine-treated mice were still capable of producing superoxide in response to alternative stimuli, peritoneal cells were restimulated with PMA *ex vivo*. PMA exposure increased superoxide production in all of the treatment groups (Figures 6a and b).

Discussion

This study demonstrates that in a murine model of MSU-induced inflammation, colchicine inhibits and prevents MSU-induced superoxide production *in vivo* at significantly lower concentrations than those required for inhibition of neutrophil infiltration, without inducing acute toxicity.

In our model, the inhibitory effects of oral colchicine first appeared 3 h after administration of colchicine, compared with the immediate effect observed following parenteral delivery. This short delay is consistent with the absorption required after oral administration where, in humans, peak plasma levels of colchicine are reached between 1 and 3 h after an oral dose (Thomas *et al*., 1989; Ferron *et al*., 1996; Ben-Chetrit and Levy, 1998).

The inhibition of microtubule formation by colchicine is believed to play a role in both leukocyte infiltration in gout and MSU-induced superoxide production by neutrophils *in vitro*. Therefore, colchicine could be expected to exhibit similar inhibitory effects on these inflammatory processes in MSU-induced inflammation *in vivo*. In fact colchicine inhibited neutrophil superoxide production at doses 10- to 100-fold lower than those required to inhibit neutrophil migration with no indication of acute liver toxicity. Colchicine has been reported to accumulate preferentially in leukocytes and granulocytes in particular (Chappey *et al*., 1993). This accumulation of colchicine may account for the low-dose effects on neutrophils without damaging hepatocytes, but it is unclear as to why such accumulation would not equally affect neutrophil migration and superoxide production. Based on the premise that microtubule destabilization is the mechanism by which colchicine inhibits MSU-induced superoxide *in vitro*, it appears that MSU-induced formation of the NADPH oxidase complex is significantly more sensitive than cell migration, to microtubule disruption by colchicine *in vivo*.

Colchicine also exhibited a longer term inhibitory effect on superoxide production than on neutrophil infiltration *in vivo*. Following a single dose of colchicine, only superoxide production was still inhibited after 24 h. Colchicine is reported to readily distribute into the tissues of both mice and humans (Back and Walaszek, 1953; Ben-Chetrit *et al*., 1994). In humans, low concentrations of colchicine can be

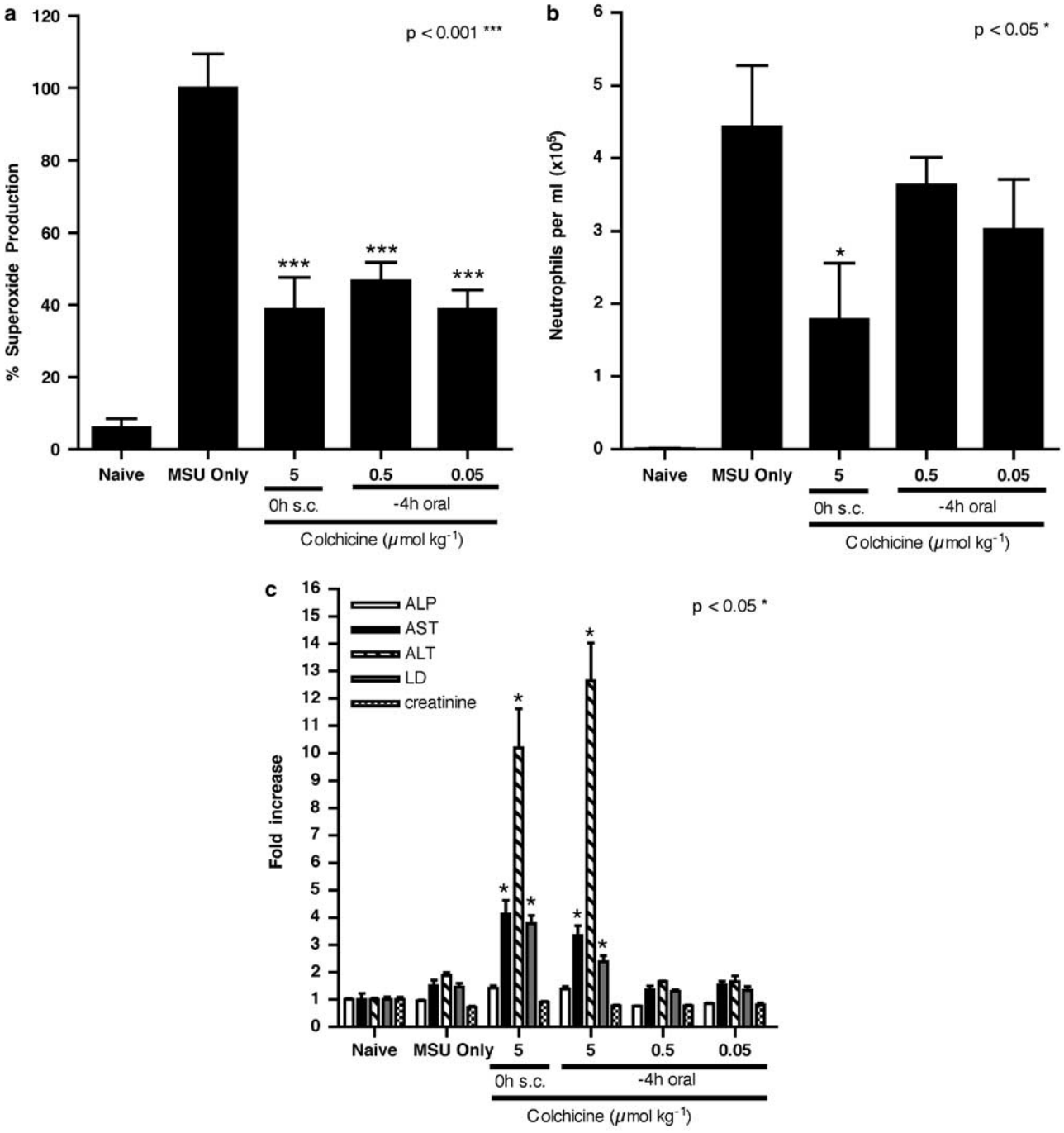

Figure 5 Low-dose colchicine inhibits MSU-induced superoxide production without causing acute toxicity in MSU-treated mice. C57Bl/J6 mice ($n = 5$) were treated with colchicine by oral gavage 4 h, or by s.c. injection 0 h, before administration of 3 mg ml^{-1} of MSU i.p. Peritoneal cells were isolated 4 h after administration of MSU and tested for (**a**) superoxide production and (**b**) neutrophil infiltration. (**c**) Blood was collected 4 h after i.p. administration of MSU and the serum levels of liver AST, ALT, ALP, LDH and creatinine measured. The rate of superoxide production was normalized to that produced by neutrophils after MSU alone (A_{450} kinetic = 2.77 = 100%). Statistical analysis was carried out by one-way analysis of variance followed by post-test Tukey. Results are representative of duplicate experiments. ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase; MSU, monosodium urate.

detected in the serum for several days after administration, as it is slowly released from the tissues. In the mouse model, the longer lasting effect of colchicine on superoxide production may result from a similar slow release of the drug from tissue depots thereby maintaining the drug at levels that only suppress neutrophil superoxide production. Our data therefore indicate that low-dose colchicine may have a prolonged beneficial effect in gout treatment by continued suppression of superoxide production at doses that avoid the undesirable side effects, including vomiting

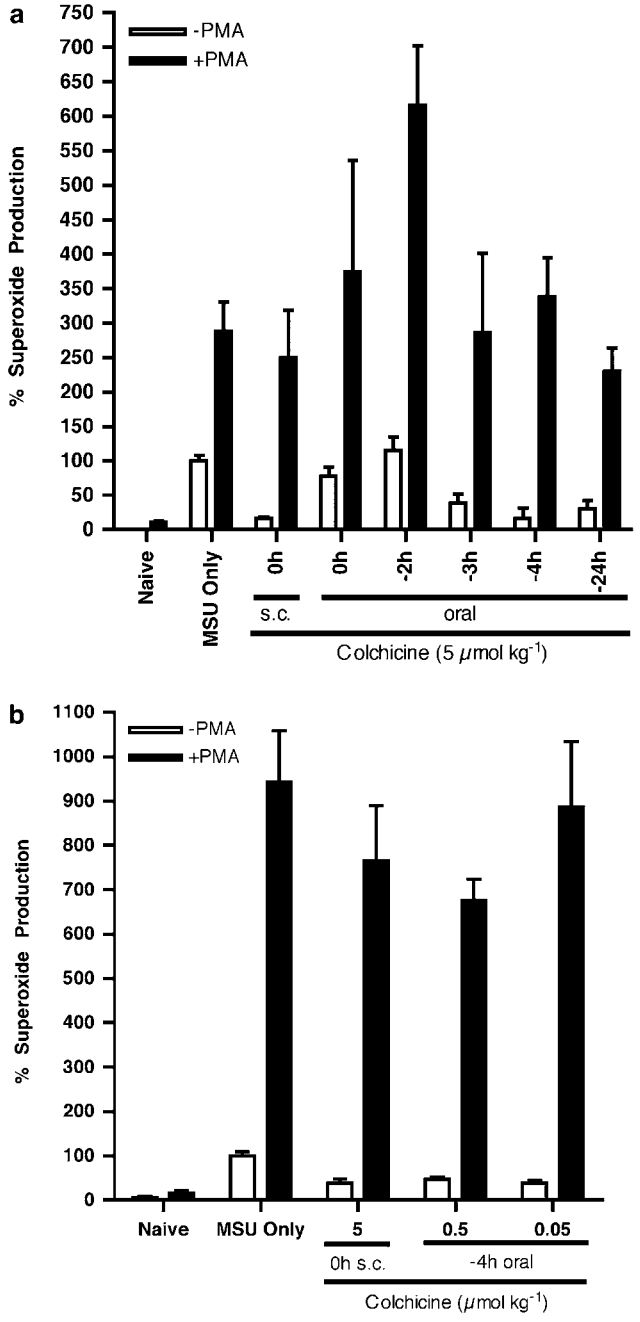

Figure 6 *In vivo* colchicine does not block neutrophil superoxide production induced by PMA *ex vivo*. C57Bl6 mice ($n = 5$) were given colchicine by (**a**) oral gavage 0, 2, 3, 4 or 24 h or (**b**) by oral gavage 4 h before administration of 3 mg ml^{-1} of MSU i.p. Peritoneal cells were isolated 4 h after administration of MSU, treated with PMA (0.32 μM) *ex vivo* and tested for superoxide production. The rate of superoxide production was normalized to that produced by neutrophils after MSU alone, as described for Figures 4 and 5. MSU, monosodium urate.

and diarrhoea, caused by the higher levels of colchicine that are required to block nonspecific leukocyte infiltration.

Colchicine is reported to have differential effects on superoxide production by neutrophils *in vitro* depending on the stimulatory agent. This also appears to be true for neutrophils activated with MSU and suppressed with colchicine *in vivo*. Upon restimulation with PMA, these cells were still capable of extensive superoxide production *ex vivo*. Low-dose colchicine therapy may be a way to specifically inhibit the production of damage-causing superoxide by neutrophils in gout without compromising other superoxide-producing pathways utilized in alternative physiological processes.

In humans, the optimal dose of oral colchicine in acute gout is not well defined. One regimen recommends dosing until relief of pain, vomiting or diarrhoea occurs, up to a maximum dose of 6–8 mg (Emmerson, 1996; British National Formulary, 2006). In the only randomized controlled trial, assessing the efficacy of this dosing regimen, all participants treated with colchicine experienced significant gastrointestinal side effects with only 66% reporting a 50% reduction in pain and clinical symptoms (Ahern *et al*., 1987).

Lower dosages of colchicine, which may retain efficacy with reduced toxicity, have been recommended for the treatment of gout (Paulus *et al*., 1974; Morris *et al*., 2003; Terkeltaub, 2003; Borstad *et al*., 2004; Zhang *et al*., 2006); however, the efficacy and side-effect profiles have not yet been rigorously evaluated. Our study provides a biological rationale for the use of low dose in gout therapy by modifying the inflammatory response to MSU through the inhibition of superoxide without toxic side effects. This beneficial effect may also be translated to other neutrophil-driven inflammatory diseases where superoxide production is susceptible to changes in microtubule formation.

Acknowledgements

This work was supported by the Foundation of Research Science and Technology, New Zealand.

Conflict of interest

The authors state no conflict of interest.

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