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Chloramphenicol-Induced Oxidative Stress in Human Neutrophils

Paulina L. Páez, María C. Becerra and Inés Albesa

Department of Pharmacy, Faculty of Chemical Sciences, National University of Córdoba, Córdoba, Argentina

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Abstract: The aim of this study was to evaluate the *in vitro* effect of chloramphenicol in order to determine its potential toxic effects on human neutrophils, by using assays of reactive oxygen species (ROS) determination, nitrite measurement and antioxidant systems. Chloramphenicol enabled the oxidative stress response of neutrophils and increased the ROS production at 2, 4, 8 and 16 μg/ml, while ROS generation decreased at high concentrations (32 μg/ml). The nitroblue tetrazolium assay shows that neutrophils incubated with chloramphenicol increased the intracellular ROS, with the extracellular production rising with a corresponding increase in antibiotic concentration. Enzymatic activities – superoxide dismutase, catalase and diaphorase enzymes – increased after chloramphenicol treatment, while the glutathione level decreased in neutrophils incubated with antibiotic. The results obtained in the present work suggest that the study of susceptibility to oxidative stress in neutrophils before chloramphenicol treatment could be adequate for *in vitro* toxicity screening.

Oxidative stress has been associated with hepatic and renal toxicity [1], but leucocytes can also be affected in the reactive oxygen species (ROS) production by toxic substances [2]. The regulation of ROS production is particularly important in neutrophils, because these cells perform an important function in host defence against bacterial infections by producing ROS and nitrogen species, hydrolytic and proteolytic enzymes, and antimicrobial polypeptides. Although neutrophils also produce nitric oxide, the levels are low and likely to result from the activity of a constitutive nitric oxide synthase [3]. Deregulation of nitric oxide and increased oxidative and nitrosative stress are implicated in tissue damage [4].

Several chemical agents can alter the cellular functions associated with the oxidative metabolism, thereby stimulating ROS production in neutrophils [5]. Antibiotics have various side effects, and some of them may influence the functions of neutrophils [6]. Free radical reactions have been suggested to be involved in the toxic effects of several antibiotics [7–11]. Idiosyncratic aplastic anaemia can occur in predisposed patients after chloramphenicol, irrespective of the dosage. This is thought to be due to the production by the gut flora of a nitro-reduction derivative of chloramphenicol. This derivative can induce DNA damage in replicating haematopoietic stem cells, resulting in marrow hypocellularity and progressive pancytopaenia [12]. The most common presentation, however, is a reversible, dose-dependent bone marrow suppression, which usually occurs when serum chloramphenicol levels exceed 25 mg/l for prolonged periods of time. This condition is associated with the inhibition of mitochondrial protein synthesis, and is characterized by mild marrow hypocellularity, anaemia, neutropaenia and thrombocytopaenia [13]. As non-idiosyncratic aplastic anaemia and 'grey baby' syndrome are dose-dependent complications of chloramphenicol usage, sufficient absorption of chloramphenicol must occur via the nasolacrimal duct in order to increase the serum concentrations [14]. These serious side effects limit the successful use of certain drugs. Both *in vitro* and *in vivo* results indicate that the toxicity of diverse chemotherapeutic drugs involves an increased production of ROS and oxidative stress with lipid peroxidation and protein oxidation [15,16].

Studies *in vitro* and in animal models have generated data to suggest that macrolides produce inhibitory effect on neutrophil influx and chemotactic activity. Moreover, macrolide antibiotics inhibit the inducible nitric oxide synthase gene expression and nitric oxide release stimulated by the immune complex [17]. In this way, the chemokinesis and chemotaxis of neutrophils are inhibited by erythromycin, oxytetracycline, doxycycline and chloramphenicol; and at higher concentrations also by sulfonamides and gentamycin [18]. Moreover, chloramphenicol, a lipid-soluble drug, concentrated threefold in enucleated human polymorphonuclear leucocytes (PMN cytoplasts), in a similar way to intact PMN [19].

In general, the cytotoxic effects of therapeutic drugs include diverse metabolic changes that affect the host cell's normal functioning, with oxidative stress being one of the alterations provoked. Similarly, some antibiotics seem to affect the oxidative state of cellular components; for example, the action of chloramphenicol on cytochrome P450. This is related to enzymatic oxidation, via an increase in ROS, while the coadministration of antioxidant vitamins may attenuate its toxic action [20].

Research into the toxicology of chloramphenicol indicates that its propensity to cause damage to the blood-forming

Author for correspondence: Inés Albesa, Department of Pharmacy, Faculty of Chemical Sciences, National University of Córdoba, Haya de la Torre y Medina Allende, University City, 5000 Córdoba, Argentina (fax +54 351 4334127, e-mail inesalbesa@fcq.unc.edu.ar).

organs might be related to its potential for nitro-reduction and subsequent production of nitric oxide. These observations suggest that the para-nitro group of chloramphenicol could be the cause of haematotoxicity and hypotension in susceptible individuals [21].

Recently, it has been described that chloramphenicol-induced keratinocyte apoptosis was associated with the activation of caspases and increased production of ROS [22].

The objective of the present investigation was established taking into consideration work done on the haematotoxic effects of chloramphenicol and its ability to induce oxidative alterations in hepatic cells [20]. On the basis of these previous studies, the present work was carried out with the purpose of evaluating both the capacity of chloramphenicol to generate reactive species in human neutrophils, and also the antioxidant response by superoxide dismutase (SOD), diaphorase, catalase (CAT) and reduced glutathione (GSH).

Materials and Methods

Chemicals and reagents. Ficoll-Hypaque (Histopaque-1077), dextran, luminol (4-amino-2,3-dihydro-1,4-phthalazine-dione), nitroblue tetrazolium (NBT), riboflavin, methionine, nicotinamide adenine dinucleotide (NADH), glutathione reductase, NADPH and N-(1-naphthyl)ethylenediamine dihydrochloride were all obtained from Sigma-Aldrich (St. Louis, MO, USA). 5,5′-Dithiobis-2-nitrobenzoic acid was obtained from Fluka Biochemical (St. Louis, MO, USA).

Other chemicals used were chloramphenicol (Pharafarm, Buenos Aires, Argentina), dimethyl sulfoxide (Anedra, Buenos Aires, Argentina), hydrogen peroxide (Panreac, Barcelona, Spain) and sulfanilamide (Merck, Darmstadt, Germany).

Neutrophils preparation from human blood. Neutrophils and monocytes were isolated by a combined dextran/Ficoll-Hypaque sedimentation procedure. Sedimentation in dextran (6% solution) was performed before gradient centrifugation. A mixture of Ficoll-Hypaque (Histopaque-1077) was then used to isolate the mononuclear cells from the remaining haematic cells. After sedimentation, hypotonic lysis of the erythrocytes was carried out. The neutrophil layer was washed twice and suspended in Hanks' balanced salt solution (HBSS). Cell preparations were adjusted to 10^6 cells/ml for the assay. The viability of cells was estimated by trypan blue dye exclusion [23] and was greater than 95%.

Determination of ROS by chemiluminescence assay. Chemiluminescence was measured using a luminometer (Bio-Orbit 1253, Turku, Finland) with disposable polypropylene tubes. The basal value of the neutrophil chemiluminescence was measured in the presence of luminol (4-amino-2,3-dihydro-1,4-phthalazine-dione). The 10^6/ml neutrophils were incubated with 0.1 ml of 3.4 μM luminol, 0.1 ml of phosphate buffer pH 7.2 and 0.1 ml of chloramphenicol (2, 4, 8, 16 and 32 μg/ml). The chemiluminiscence background of each vial was checked before being used. Chemiluminescence was measured at room temperature in a Bio-Orbit luminometer. The light emission was measured for 120 sec., at 5-sec. intervals. Results were expressed as relative light unities (RLU) at different times, with subtraction of the background [24].

Intracellular and extracellular ROS production. Reactive oxygen species production by neutrophils was detected by means of NBT reduction. The 10^6/ml neutrophils suspended in HBSS pH 7 were incubated with 0.1 ml of chloramphenicol (2, 4, 8 and 32 μg/ml) and 0.5 ml of 1 mg/ml NBT at 37° for 30 min. 0.1 ml of 0.1 M HCl was added and the tubes were centrifuged at 10,000 ×g for 10 min. The supernatant was separated (extracellular ROS) and the sediment (intracellular ROS) was treated with 0.4 ml of dimethyl sulfoxide and 0.8 ml of HBSS. The optical density was measured at 575 nm both in supernatant and suspended neutrophils [25].

Nitrite determination. Nitric oxide is rapidly converted to nitrite in aqueous solutions and, therefore, the total nitrite can be used as an indicator of nitric oxide concentration. This was measured using a colorimetric non-enzymatic assay which provided the chemical reduction of nitrite by granulated cadmium. Spectrophotometric analysis of the total nitrite was performed by using Griess's reagent according to the methodology described by Kobayashi *et al*. [26–28]. The 10^6/ml neutrophils were incubated with 0.3 ml of chloramphenicol (4 and 32 μg/ml) for 4 hr at 37°. Samples were added to a tube containing 0.25 ml of 50 mM carbonate buffer (pH 9) and 0.15 g of granulated cadmium for 30 min. at room temperature. The reaction was stopped with 40 μl of 3.5 M NaOH. Samples were deproteinized with 0.4 ml of 120 mM $ZnSO_4$ and centrifuged at 10,000 ×g for 10 min. 0.1 ml of the supernatant was mixed with 50 μl of 2% sulfanilamide in 5% (v/v) HCl and 50 μl of 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride aqueous solution. The formation of the azo dye was measured 15 min. later by spectrophotometry at 540 nm. The absorbance was directly proportional to the nitrite content of the standard solution.

Superoxide dismutase activity. The reaction mixture was obtained by mixing 0.3 ml of 13 mM methionine, 0.1 ml of 75 μM NBT, 0.3 ml of 0.1 nM ethylenediaminetetraacetic acid, and 0.3 ml of 2 μM riboflavin in HBSS. The 10^6/ml neutrophils were incubated with 0.1 ml of chloramphenicol (4 and 32 μg/ml) or HBSS for 2 hr, and with 1.3 ml of the reaction mixture. The absorbance was determined at 560 nm. A unit of SOD was defined as the quantity of enzyme required to produce a 50% inhibition of NBT reduction under the specified conditions indicated above. Riboflavin loses one electron in the presence of light and triggers the generation of O_2^-, which reduces the NBT to blue formazan [29,30].

Determination of diaphorase activity. The 10^6/ml neutrophils were incubated in the presence of 10 μl of chloramphenicol (0.5, 4 and 32 μg/ml) or potassium phosphate buffer with 40 μl of 7 μmol/ml NADH in 20 mM Tris-HCl buffer (pH 7.5), 0.2 ml of 0.2 M NaCl in Tris-HCl buffer and 1.650 ml of Tris-HCl buffer. The diaphorase activity of the neutrophils was assayed spectrophotometrically at 340 nm by following NADH oxidation [31].

Catalase activity. The CAT activity of 10^6 neutrophils in the presence of 0.8 ml of chloramphenicol (4 and 32 μg/ml) or potassium phosphate buffer, and 0.4 ml of hydrogen peroxide solution was measured by monitoring the disappearance of hydrogen peroxide at 240 nm. The stock hydrogen peroxide solution employed was prepared with 85 μl of 30% (v/v) in potassium phosphate buffer, pH 7.0. One unit of CAT was defined as the amount of enzyme able to decompose 90% of the hydrogen peroxide [31].

Assay of GSH. The neutrophil glutathione level was determined colorimetrically on the basis of the absorbance of the reaction product of GSH and 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) [32]. The 10^6/ml neutrophils treated without (in control system) or with chloramphenicol (4 and 32 μg/ml) for 10 min. were incubated with 20 μl of glutathione reductase (6 U/ml), 50 μl of NADPH (4 mg/ml), and 20 μl of DTNB 1.5 mg/ml at 37°. The absorbance was determined at 415 nm. GSH levels were expressed per milligram of protein (μM/mg). The plate reader used for the assay was Bio-Rad model 550 (Hercules, CA, USA).

Statistical analysis. The assays were done at least in triplicate. Data were expressed as means ± S.D. and analysed by Student's t-test. $P < 0.05$ was accepted as the level of statistical significance.

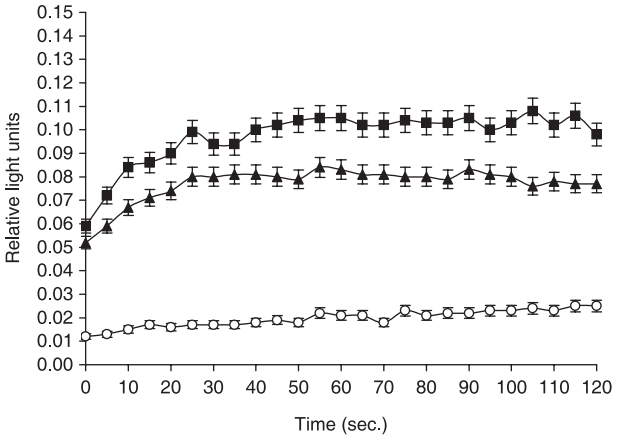

Fig. 1. Stimuli of reactive oxygen species in human neutrophils at different times of incubation with chloramphenicol were measured by chemiluminescence assay. The graph shows that the treatment with 4 μg/ml generated a greater increase of relative light unities at 3 min. (■) than at 5 min. (▲) of incubation. $P < 0.05$, compared to the untreated sample (○).

Results

Determinations of ROS and nitrite.

Reactive oxygen species production measured by chemiluminescence was significantly greater in neutrophils treated with chloramphenicol in comparison with the untreated ones (fig. 1). The oxidative stress was found to be dose-dependent, and in quantitative terms, neutrophils incubated with chloramphenicol at concentrations of 2, 4 and 8 μg/ml resulted in a significant increase in ROS production in comparison with the untreated sample. However, when neutrophils were incubated with 32 μg/ml of antibiotic, they exhibited lower levels of ROS than those with 8 μg/ml, as a consequence of excessive stress (fig. 2).

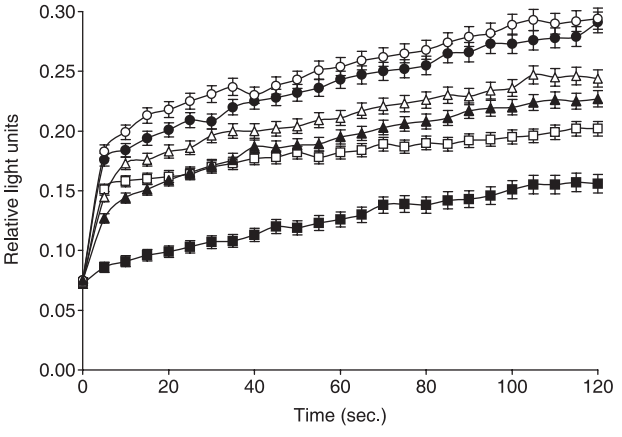

Fig. 2. Chloramphenicol increased reactive oxygen species in human neutrophils by chemiluminescence assay. The graph shows the treatment at different concentrations for 120 sec. with chloramphenicol; 2 μg/ml (□), 4 μg/ml (Δ), 8 μg/ml (●), 16 μg/ml (○) and 32 μg/ml (▲). $P < 0.05$, compared to the untreated sample (■).

Table 1.

Intracellular and extracellular generation of reactive oxygen species (ROS) in neutrophils treated with chloramphenicol by nitroblue tetrazolium assay.

	ROS (OD_{575})	
Chloramphenicol (μg/ml)	Intracellular (mean ± S.D.)	Extracellular (mean ± S.D.)
0	0.015 ± 0.002	0.040 ± 0.009
2	0.025 ± 0.005	0.030 ± 0.002
4	0.062 ± 0.002[1]	0.038 ± 0.006
8	0.044 ± 0.005[1]	0.035 ± 0.006
32	0.030 ± 0.002[1]	0.079 ± 0.003[1]

Results are given as mean ± S.D. [1]$P < 0.05$, with respect to the untreated sample. OD, optical density.

The NBT method was useful to investigate intracellular and extracellular changes of ROS. This assay demonstrated an oxidative alteration in neutrophils treated with chloramphenicol, manifested by an intracellular increase in ROS, which extended to the extracellular media. It was observed that the stimuli of intracellular ROS were optimum at 4 μg/ml of antibiotic, but above this concentration the oxidative stress was too toxic for neutrophils, leading to a decrease in ROS. For values over 8 μg/ml of chloramphenicol, the extracellular ROS rose in the neutrophils (table 1). The investigation by NBT assay showed that ROS increased by 313% in neutrophils treated with 4 μg/ml of chloramphenicol. On the other hand, there were no significant increments in the nitrite levels in neutrophils (table 2).

Determination of enzymatic activities and non-enzymatic activities in neutrophils.

Superoxide dismutase activity increased 3-fold in neutrophils incubated with 4 μg/ml of chloramphenicol, compared to the untreated sample. However, a significant decrease in SOD activity was observed with 32 μg/ml of chloramphenicol with respect to the value obtained with 4 μg/ml of antibiotic (table 3). In addition, as a consequence of oxidative stress, diaphorase and CAT increased in neutrophils treated with 4 μg/ml of chloramphenicol, while an excess of antibiotic (32 μg/ml) reduced the diaphorase activity.

Determination of GSH.

Intracellular GSH levels increased in neutrophils incubated with 4 μg/ml of chloramphenicol, while they decreased in the presence of 32 μg/ml (table 4).

Table 2.

Nitrite production by leucocytes with chloramphenicol.

Chloramphenicol (μg/ml)	Nitrite (μM/10^6 cell)
0	6.07 ± 0.40
4	6.51 ± 0.24
32	6.26 ± 0.10

Results are given as mean ± S.D.

Table 3.

Enzymatic activities of superoxide dismutase, catalase and diaphorase in neutrophils treated with chloramphenicol.

Chloramphenicol (μg/ml)	Superoxide dismutase ($U/10^6$ cells) (mean ± S.D.)	Diaphorase (ΔOD per minute/10^6 cells) (mean ± S.D.)	Catalase ($U/10^6$ cells) (mean ± S.D.)
0	0.452 ± 0.030	0.0016 ± 0.0003	0.014 ± 0.005
4	1.478 ± 0.045[1]	0.0027 ± 0.0005[1]	0.287 ± 0.026[1]
32	1.133 ± 0.011[1]	0.0018 ± 0.0002	0.394 ± 0.032[1]

Results are given as mean ± S.D. [1]$P < 0.05$, with respect to the untreated sample. OD, optical density.

Discussion

The results obtained show that chloramphenicol stimulated oxidative stress with a corresponding increase in ROS at 4 μg/ml. However, for higher concentrations, there was a decrease in this stress promoter.

In addition, the antibiotic altered the behaviour of the enzymes related to oxidative stress in neutrophils, thereby affecting the SOD, CAT and diaphorase activities. In response to the increased amounts of ROS generated by low doses of chloramphenicol, there was a rise in the activity of the three enzymes investigated. Because SOD dismutes the superoxide anion to hydrogen peroxide (H_2O_2), the intracellular increase in the SOD level led to an increasing H_2O_2 concentration, and consequently, to activation of CAT. However, when increasing the concentration of chloramphenicol up to 32 μg/ml, there was a subsequent decrease in the SOD and diaphorase activities, thus affecting the first defence mechanism of the cell against ROS.

At present, determining the capacity of chloramphenicol and its derivatives to generate aplastic anaemia requires evaluation of the metabolic alterations that precede this severe affection [33,34]. Recent advances in experimental studies have indicated that chloramphenicol generates a reversible and dose-related anaemia during treatment, but this antibiotic can also cause a non-dose-related aplastic anaemia. Chronic bone marrow aplasia is one of the major haematoxic effects induced by this antibiotic, and the mechanisms that are derived in this irreversible damage need further investigation. At present, the elucidation of the alterations involved in this toxic effect have failed as a consequence of the lack of an animal model [35]. Thus, the development of new toxicity assays could be useful in ameliorating this severe risk.

Table 4.

Comparison of reactive oxygen species (ROS) and reduced glutathione (GSH) in neutrophils treated with chloramphenicol.

Chloramphenicol (μg/ml)	ROS (RLU)	GSH (μM GSH/mg protein)
0	0.015 ± 0.002	1.618 ± 0.054
4	0.211 ± 0.007[1]	1.785 ± 0.210
32	0.195 ± 0.004[1]	1.425 ± 0.430[1]

Results are given as mean ± S.D. [1]$P < 0.05$, with respect to the untreated sample. RLU, relative light unities.

Previous results obtained in our group with ciprofloxacin [25,36] have led us to realize that this antibiotic does not stimulate the production of ROS in neutrophils. In addition, chloramphenicol generates the stimuli of ROS and nitrites in neutrophils, as has been demonstrated in the present investigation. The results obtained for both antibiotics showed a higher oxidative capacity in the case of chloramphenicol compared to ciprofloxacin, so the leukotoxicity of the former antibiotic could be related to elevated oxidative damage of the neutrophils. Furthermore, the release of ROS to the media has not been previously observed for ciprofloxacin. It must be pointed out that the oxidative stress caused by this fluoroquinolone in neutrophils did not provoke an extracellular increase in ROS, as was seen for chloramphenicol. Consequently, ROS and nitrite production, together with the alteration of antioxidant enzymes, could explain the leukotoxic action of chloramphenicol in neutrophils.

Cells contain several antioxidant systems to protect themselves from the injury induced by increased intracellular ROS. Many studies have reported that the levels of GSH are modified by anaesthetics and other drugs [37–39], while the effect of chloramphenicol was not investigated for this aspect.

It was evident that chloramphenicol altered more than one antioxidant system and that the intensity of these effects was concentration-dependent. The present study is a contribution to the current knowledge about oxidative stress caused by chloramphenicol in human leucocytes, suggesting that an investigation of ROS, nitric oxide and antioxidant enzymes should be performed in order to detect patients with different responses to chloramphenicol, taking into consideration that haematological alterations after therapy with this antibiotic may be a consequence of oxidative damage.

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