A high magnesium concentration in citrate dialysate prevents oxidative stress and damage in human monocytes in vitro

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ABSTRACT

Background. The use of dialysis fluids (DFs) during haemodialysis has been associated with increased oxidative stress and reduced serum magnesium (Mg) levels, contributing to chronic inflammation. Since the role of Mg in modulating immune function and reducing oxidative stress has been demonstrated, the aim of this study was to characterize in vitro whether increasing the Mg concentration in DFs could protect immune cells from oxidative stress and damage.

Methods. The effect of citrate [citrate dialysis fluid (CDF), 1 mM] or acetate [acetate dialysis fluid (ADF), 3 mM] dialysates with low (0.5 mM; routinely used) or high (1 mM, 1.25 mM and 2 mM) Mg concentrations was assessed in THP-1 human monocytes. The levels of reactive oxygen species (ROS), malondialdehyde (MDA) and oxidized/reduced (GSSG/GSH) glutathione were quantified under basal and inflammatory conditions (stimulation with lipopolysaccharide, LPS).

Results. The increase of Mg in CDF resulted in a significant reduction of ROS production under basal and inflammatory conditions (extremely marked in 2 mM Mg; P < 0.001). These effects were not observed in ADF. Interestingly, in a dose-dependent manner, high Mg doses in CDF reduced oxidative stress in monocytes under both basal and inflammatory conditions. In fact, 2 mM Mg significantly decreased the levels of GSH, GSSG and MDA and the GSSG/GSH ratio in relation to 0.5 mM Mg.

Conclusions. CDF produces lower oxidative stress than ADF. The increase of Mg content in DFs, especially in CDF, could have a positive and protective effect in reducing oxidative stress and damage in immune cells, especially under inflammatory conditions.
Keywords: acetate, chronic kidney disease, citrate, dialysate, haemodialysis, inflammation, magnesium, monocytes, oxidative stress

INTRODUCTION

Cardiovascular diseases (CVDs) remain the main cause of morbidity and mortality among chronic kidney disease (CKD) patients, especially in those undergoing haemodialysis (HD) [1–3]. Several risk factors inherent to the dialysis procedure itself could contribute to this high incidence/prevalence of CVDs [4, 5]. Several sources of evidence support the theory that the pathological mechanism by which these risk factors induce CVDs is closely related to their ability to generate systemic inflammation, which is characterized by excessive production and release of pro-inflammatory compounds [4–7]. These mediators also lead to the activation of signalling cascades that trigger the overproduction of oxidant compounds, such as reactive oxygen species (ROS), contributing to oxidative stress and amplifying cellular damage [7]. Since a relationship between oxidative stress, vascular cell damage and the systemic inflammation has been extensively reported in HD patients with CVDs, preventing oxidative stress in these subjects has become a priority therapeutic objective [6, 8].

Immune cells continuously generate oxidants and inflammatory compounds to perform their protective functions. However, when the overproduction of oxidant compounds is not well controlled by the antioxidant defences, an alteration occurs in the redox balance, leading to oxidative stress; thus, certain biomolecules are oxidized, promoting remarkable detrimental consequences on cellular functioning [9]. Several sources of evidence indicate the strong involvement of oxidative–inflammatory stress in the progression of renal injury and atherosclerosis in CKD patients [6, 8, 10–12]. Moreover, uremic- and dialysis-associated factors contribute to oxidative-inflammatory stress in HD patients, including the use of biocompatible dialyser membranes and dialysates [12].

Dialysis fluid (DF) composition is critical to optimize uraemic toxin removal while preserving fluid, electrolytes and acid-base balance [13]. DFs are produced by treated purified water, bicarbonate concentrate and an acid concentrate, which is needed to prevent calcium/magnesium carbonate precipitation occurring by the addition of bicarbonate. The most commonly used acid is acetic acid (3–10 mM) [14]. However, acetate dialysis fluid (ADF) is associated with several side effects and induces leukocyte activation, resulting in increased ROS production and the release of inflammatory compounds [15–17]. Thus, citrate dialysis fluid (CDF) was developed to improve HD biocompatibility by replacing the small amount of acetic acid with more physiological citric acid [18]. At the concentrations routinely used in clinical practice, ADF increases oxidative stress and activates other pro-inflammatory stimuli typical of HD patients, whereas CDF does not induce this activation, which could make it a suitable alternative in clinical practice [17].

Magnesium (Mg) plays a key role in numerous biological processes [19, 20]. Low Mg levels are associated with CVDs and all-cause mortality in HD patients [20, 21]. Moreover, hypomagnesaemia is associated with increased oxidative–inflammatory stress in CKD, suggesting that an increase in Mg levels is a useful therapy [20, 21]. Routine HD with the standard 0.5 mM Mg dialysate reduces Mg in most patients [22, 23], who often need an increase in dialysate Mg concentration [19]. The maintenance of serum Mg levels in HD patients depends on intestinal absorption, uptake and release by bone, and residual renal function and the composition of the DFs in relation to both the Mg concentration and the type of acid [19]. A recent multicentre, randomized, prospective study has described Mg reduction in relation to the use of CDF [24]. Several investigators suggested a CDF formulation with an Mg concentration higher than usual (0.5 mM), because higher Mg levels might be beneficial or indeed needed when using 1 mM citrate concentrates [18, 25]. Furthermore, the increase of Mg concentration in DFs such as in ADF is reportedly associated with a decrease of intra- and post-dialysis arrhythmias [26], the serum’s propensity to inhibit calcification [27] and cardiovascular mortality in HD patients [28, 29]. Therefore, higher Mg concentrations in DFs could be a potential mechanism to modify oxidative–inflammatory stress in HD patients [25]. However, more studies are necessary to reinforce clinical translation in this field.

This study aimed to evaluate, in vitro, whether the increase of Mg concentrations in different DFs could have a beneficial/protective effect on oxidative stress in immune cells. Several oxidative stress and damage parameters were assessed in human monocytes culture in two DFs generally used in clinical practice: citrate (CDF, 1 mM) or acetate (ADF, 3 mM) dialysates with standard (0.5 mM) or high (1 mM, 1.25 mM and 2 mM) Mg concentrations.

MATERIALS AND METHODS

HD fluids

Two types of DFs were used:

- ADF: containing acetate (3 mM)
- CDF: containing citrate (1 mM)

The composition of the DFs is shown in Table 1. Samples were taken from the Hansen connector to the dialysate input, once the machine had been stabilized (conductivity and temperature). Hank’s medium with similar standard Mg concentration (0.5 mM) to DFs was used as control solution. A vial of magnesium sulphate (150 mg/mL solution of MgSO4 heptahydrate; Genfarma®, Altan Pharma, Madrid, Spain) was used for supplementing ADF, CDF and control solution with 1, 1.25 and 2 mM Mg concentrations.

Human monocytes cultures

Human monocytes (THP-1 cell line, Cat#88081201; Sigma-Aldrich, St Louis, MO, USA) were cultured in RPMI-1640 medium with 1% penicillin–streptomycin (Basel, Switzerland) and 10% fetal bovine serum (FBS, Sigma-Aldrich).

All different assays were performed under basal and stimulating pro-inflammatory conditions, such as those induced by bacterial lipopolysaccharide (LPS), to assess whether the antioxidant capacity of Mg could protect patients from potential high-risk situations.

Measurement of ROS

Intracellular ROS production was measured in THP-1 monocytes using a method [30] based on the fluorescence emitted by 2’,7’-dichlorodihydrofluorescein (DCF-DA, Sigma-Aldrich) which is
oxidized in the cytoplasm by ROS to 2,7'-dichlorofluorescein, with several modifications [25]. Briefly, THP-1 cell suspension adjusted to 0.2 × 10⁶ cells/well in the different DFs or control solution were loaded into 96-well plates (NuncIon Delta, Thermo Fisher Scientific, Waltham, MA, USA) and incubated for 30 min (37°C) with or without LPS (1 µg/mL), Escherichia coli serotype 0127: B8 endotoxin, Sigma-Aldrich). Previously, monocytes were pre-treated 30 min with different Mg concentrations (1, 1.25 and 2 mM). After LPS incubation, monocytes were incubated with DCF-DA (1 mM; 30 min; 37°C). Fluorescence of samples was measured with excitation/emission = 485–535 nm. Results were expressed as units ‘relative of fluorescence’ (URF). The percentage (%) of ROS production in response to LPS stimulation (related to the basal value without LPS) was also calculated.

To simulate the chronic inflammatory state that CKD patients suffer in HD, we chose LPS (1 µg/mL) to induce cell activation, because bacterial cell wall components mimic the infectious state and promote inflammatory conditions [31, 32].

**Glutathione content assay**

Both reduced (GSH) and oxidized (GSSG) glutathione levels were quantified using a fluorimetric method [33, 34], with several modifications [25]. This assay was carried out on frozen THP-1 monocytes (3 × 10⁶ cells) aliquots, which were previously cultivated in CDF or control solution with different Mg concentrations (0.5, 1, 1.25 and 2 mM) and then stimulated or not with LPS (1 µg/mL). Monocytes were lysed (lysis buffer with butylated hydroxytoluene 0.1 mM), sonicated and centrifuged (16 000 g; 10 min). The protein contents of different samples were assessed using the ‘Lipid peroxidation MDA Assay’ kit (Biovision, Milpitas, CA, USA), which quantified the adduct formation of MDA with thiobarbituric acid (TBA; Sigma-Aldrich). The assay was carried out on frozen THP-1 monocytes (3 × 10⁶ cells) aliquots, which were previously cultivated in CDF or control solution with several Mg concentrations (0.5, 1, 1.25 and 2 mM) and incubated (4 h; 37°C; 5% CO₂) with or without LPS (1 µg/mL). Monocytes were lysed (lysis buffer with butylated hydroxytoluene 0.1 mM), sonicated and centrifuged (13 000 g; 10 min). Later, supernatants were mixed with TBA, incubated in a water bath (1 h; 95°C) and iced (10 min) to stop the reaction. Finally, samples were mixed with n-butanol (Sigma-Aldrich) and centrifuged (13 000 g; 10 min) to collect the upper organic phase for spectrophotometric measurement at 532 nm. The protein levels of the samples were quantified as mentioned above. Results were expressed as nmol MDA/mg protein.

**Lipid peroxidation assay**

Malondialdehyde (MDA) content was assessed using the ‘Lipid peroxidation MDA Assay’ kit (Biovision, Milpitas, CA, USA), which quantified the adduct formation of MDA with thiobarbituric acid (TBA; Sigma-Aldrich). The assay was carried out on frozen THP-1 monocytes (3 × 10⁶ cells) aliquots, which were previously cultivated in CDF or control solution with different Mg concentrations (0.5, 1, 1.25 and 2 mM) and then stimulated or not with LPS (1 µg/mL). Monocytes were lysed (lysis buffer with butylated hydroxytoluene 0.1 mM), sonicated and centrifuged (13 000 g; 10 min). Later, supernatants were mixed with TBA, incubated in a water bath (1 h; 95°C) and iced (10 min) to stop the reaction. Finally, samples were mixed with n-butanol (Sigma-Aldrich) and centrifuged (13 000 g; 10 min) to collect the upper organic phase for spectrophotometric measurement at 532 nm. The protein levels of the samples were quantified as mentioned above. Results were expressed as nmol MDA/mg protein.

**Cell viability assay**

In all experiments, the viability of THP-1 monocytes was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit (Sigma-Aldrich) [25]. THP-1 cells (0.2 × 10⁶ cells/well) in 96-well, flat-bottomed plates were incubated in ADF, CDF or control solution with different Mg concentrations (0.5, 1, 1.25 and 2 mM) and then stimulated or not with LPS (1 µg/mL) for 4 h. Later, the solutions were replaced with MTT solution (0.5 mg/mL) for 4 h (37°C; 5% CO₂). Then, the MTT solution was removed and the formazan crystals were solubilized (1 h; 37°C) using a lysis buffer (DMSO, Sigma-Aldrich).

![FIGURE 1: Intracellular ROS production in human monocyte (THP-1) activated with LPS.](image-url)
Absorbance was measured at 570 nm. Results are expressed as percentages (%) relative to results obtained with control cells.

Statistical analysis
Statistical analyses were performed with the SPSS 21.0 statistical package (SPSS Inc., Chicago, IL, USA). Data are expressed as mean ± standard deviation (SD). All tests were two-tailed, with a significance level of 0.05. Both normality and homogeneity of the variances were checked by the Kolmogorov–Smirnov and Levene’s tests, respectively. Differences between groups were studied by one-way analysis of variance followed by post hoc test analysis. The Tukey test was used for post hoc comparisons when variances were homogeneous, whereas its counterpart Games-Howell was used when variances were not homogeneous.

RESULTS
Effect of the addition of high Mg concentrations to the DFs on ROS production under basal and inflammatory conditions
First, THP-1 monocytes were cultured in ADF, CDF or control solution, all with similar standard Mg concentration (0.5 mM). ROS generation was quantified under basal and LPS-stimulated conditions (Figure 1). Monocytes cultured in both ADF and CDF (P < 0.001 and P < 0.01, respectively) produced a significantly higher basal ROS production than the control solution.
Magnesium and oxidative stress in human monocytes

DISCUSSION

The use of DFs during HD has been associated with increased oxidative stress and reduced serum Mg levels, contributing to oxidative damage and chronic inflammation in HD patients [10, 17]. Herein, we analysed in vitro the effect of increasing Mg concentration (1, 1.25 and 2 mM) in different DFs on oxidative stress and damage in human monocytes, especially under inflammatory conditions. In culture, THP-1 cells can be activated by LPS, serving as an effective model to investigate the mechanisms.
involved in vascular damage associated with inflammatory activity [17, 35]. Given that previous studies had shown that dialysates themselves differentially induce ROS production [17, 25], we included the most common DFs used in clinical practice, ADF (3 mM) and CDF (1 mM). Our study clearly showed that the increase in Mg concentrations in ADF or CDF significantly modulates changes in oxidative stress in monocytes. CDF produces lower ROS production than ADF. Interestingly, in a dose-dependent manner, high Mg concentrations in CDF resulted in a marked reduction of oxidative stress (decreased ROS, GSSG and GSSG/GSH) and lipid damage (decreased MDA) in monocytes, especially under LPS stimulation.

HD promotes excessive oxidative stress, due to the loss of antioxidants and the accumulation of oxidative products during HD procedures [10, 11]. Our results confirmed that ADF and CDF induced an increased ROS basal production in monocytes. Since oxidation and inflammation processes are interlinked processes [7], we hypothesized that ROS production in monocytes could also be triggered by an increase in basal inflammation. Thus, under LPS stimulation, a marked ROS production was observed in the monocytes cultured with ADF and CDF. However, substantial differences between ADF and CDF were observed despite their identical Mg concentration (0.5 mM). Interestingly, CDF induced significantly lower ROS production than ADF.

**FIGURE 4:** Effect of citrate (CDF, 1 mM) dialysate with standard 0.5 mM Mg (control) or with high Mg concentrations (1, 1.25 and 2 mM) on the intracellular levels of (A) reduced (GSH) and (B) oxidized (GSSG) glutathione (nmol/mg protein), as well as on (C) the redox balance GSSG/GSH ratio assessed in human monocyte cultures (THP-1) cultivated under basal or inflammatory conditions (stimulation with LPS, 1 μg/mL). Each column represents the mean ± SD of 5–10 values corresponding to that number of experiments, and each value being the mean of duplicate assays. *P < 0.05, **P < 0.01 and ***P < 0.01 versus Mg 0.5 mM (control). #P < 0.05 and ##P < 0.01 versus Mg 1 mM. $P < 0.05 versus Mg 1.25 mM. †P < 0.05 versus basal conditions.

[Image of bar graphs showing the effect of citrate dialysate on intracellular levels of GSH, GSSG, and GSSG/GSH ratio in human monocyte cultures.]
These results confirm previous studies, which revealed that CDF does not induce oxidative stress or inflammation both in vitro and in vivo, as compared with ADF [18, 25, 36, 37]. In fact, fluids with acetate induced greater ROS activity in immunocompetent cells of both healthy subjects and HD patients than citrate-containing fluids [18]. ADF reportedly induces leucocyte activation and cytokine production [38], whereas both ROS production and inflammatory cytokine release were minimal during acetate-free biofiltration [15, 39]. Until recently, acetate has been used as a buffer in conventional dialysates. However, ADF leads to immune cell activation, promoting inflammation and arterial hypotension [15]. Because CDF does not induce cell activation [18] and has a positive impact on haemodynamic tolerance [24], CDF could be used as an alternative in clinical practice.

Dialysis patients are particularly vulnerable to the effect of Mg deficiency, which may explain the increased risk of CVs in these subjects [40]. The use of low Mg dialysates (0.25–0.5 mM; routinely prescribed) is a risk factor for hypomagnesaemia in HD patients [40]. However, there is still no consensus regarding the optimal Mg concentration for dialysis. Although the use of a low Mg dialysate may have harmful haemodynamic effects [40, 41], the impact of Mg in HD patients still remains unclear in clinical routine practice. Chronic hypomagnesaemia could result in excessive ROS production, supporting a role for Mg in altering the threshold antioxidant capacity [40]. Thus, increased ROS production is associated with low-intracellular Mg levels [42]. Our study found that the increase in Mg concentration (1.25 and 2 mM) in dialysates had a dual effect on ROS production by monocytes, depending on the type of DFs used. Thus, high Mg concentrations decreased ROS production in monocytes cultured with CDF but not with ADF. Interestingly, this effect was more exacerbated under inflammatory conditions. In fact, the highest Mg concentration (2 mM) in CDF produced a marked reduction of ROS generated in response to LPS stimulation. By contrast, this highest Mg concentration in ADF promoted an increase in ROS production. Our results are in consonance with a previous in vitro study that supports a different modulation of oxidative stress in monocytes depending on the Mg concentration in several DFs [25]. Moreover, low serum Mg and immune system activation seem to co-operate to promote oxidative stress associated with endothelial dysfunction [43]. Increased ROS production and DNA damage were found in Mg-deprived endothelial cultured cells [43]. Therefore, increasing Mg concentrations in DFs, especially in CDF, could have a positive effect in decreasing ROS associated with inflammation, thereby improving the redox balance in immune cells.

Low serum Mg levels are common in many patients on HD and 5% are overtly hypomagnesaemic in those dialysed against a concentration of 0.5 mM [44]. This low dialysate Mg concentration inevitably induces a post-dialysis hypomagnesaemia [45], promoting acute haemodynamic effects [40, 41]. In relation to our data, it is important to note that the use of CDF might affect Mg concentration, because citrate may form a complex with Mg, which is dialysable, and increase the intradialytic removal of Mg. Therefore, some authors propose the use of CDF with higher Mg levels than usual (0.5 mM) to solve the problem of the reduction in Mg described in relation with the use of CDF [24], which could also have a beneficial effect on cardiovascular mortality in CKD patients [29].

An increase in oxidative lipid damage and reduced plasma concentrations of antioxidants are closely associated with the HD procedure [10–12, 45, 46]. Our study also showed that LPS stimulation remarkably decreased the GSH content and increased both GSSG and MDA levels and the GSSG/GSH ratio in monocytes cultured in CDF with 0.5 mM Mg. Interestingly, in a dose-dependent manner, high Mg concentrations in CDF decreased GSH, GSSG and MDA content in monocytes, especially with 2 mM Mg under LPS stimulation. A disturbance of the glutathione antioxidant system, including overall compromised blood levels of GSH and increased GSSG, has been reported in CKD and HD patients [46]. Moreover, the GSSG/GSH ratio has been proposed as a good maker of redox balance in dialysed and not yet dialysed patients [47]. Although reduced GSH could have detrimental effects on promoting oxidative damage, the observed and marked GSSG/GHS reduction indicates a good maintenance of redox balance. Although we have not yet elucidated the molecular mechanisms underlying the potential action of high Mg levels in CDF in decreasing GSH/GSSG, several studies demonstrate a direct relationship between cellular Mg status and circulating GSSG/GSH [42, 47]. Thus, a lower GSSG/GSH ratio was associated with higher intracellular levels of Mg in red blood cells of hypertensive patients [42]. Regarding lipid peroxidation, several studies revealed that CDF decreases lipid oxidation and lipid peroxidation products compared with conventional ADF [45], as well as decreasing the degradation of polymorphonuclear leukocytes and platelets, and contributing to reduce oxidative damage during HD [47]. Mg deficiency is also associated with increased lipid oxidative tissue damage [42]. Mg deficiency is reportedly related to a marked increase in plasma and kidney MDA levels [48], whereas increased Mg intake reduced lipid peroxidation and improved hyperlipidaemia in rats [49]. Therefore, our results support the notion that the use of CDF containing high Mg concentrations has possible beneficial effects through reducing lipid damage during HD. This could be because the citrate would exert direct or indirect antioxidant effects due to its iron chelation activity and interaction with redox signalling pathways (regulation of the cellular antioxidant response) [50].

Our study provides useful information about the beneficial effects of the increase of Mg concentration in CDF on reducing oxidative stress and damage in monocytes. However, there are...
two major limitations in this study that could be addressed in future research. First, our results are based on in vitro experiments using a human monocyte cell line. Thus, experimental results must be viewed with caution and conclusions must not be inappropriately extrapolated. Secondly, our results might have been affected by other electrolytes present in the DFs. Therefore, further pre-clinical and clinical studies, especially randomized trials, will be required to validate our findings and to confirm the positive effects of the increase of Mg in DFs on improving clinical outcomes in HD patients.

**CONCLUSION**

This is the first study to reveal that the increase of Mg content in DFs has different effects on oxidative stress in human monocytes. Interestingly, high Mg concentrations (1.25 mM and 2 mM) in CDF have a positive effect, reducing oxidative stress and lipid damage, especially under inflammatory conditions. By contrast, high Mg in ADF enhances ROS production. Although further studies in clinical practice are needed, the increase of Mg concentration in CDF may provide a potential therapeutic tool to prevent excessive oxidative stress and damage and subsequent CVDs in HD patients.

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**AUTHORS’ CONTRIBUTIONS**

C.V., M.A., P.d.S., J.C. and R.R. were involved in the concept and design. C.V. was involved in the experiment and the acquisition of data. C.V., M.A., P.d.S., G.B., J.C., R.P. and R.R. were involved in the analysis and/or interpretation of data and contributed to manuscript drafting and critical revision. Final approval of the version to be published was provided by all authors.

**CONFLICT OF INTEREST STATEMENT**

P.d.S. holds a grant from Baxter and received honoraria for participation as a speaker at meetings of Baxter and sponsorships of scientific congresses by Nipro and Baxter. The authors declare no other conflicts of interest.

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