Effects of nebulised magnesium sulphate on inflammation and function of the guinea-pig airway

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ABSTRACT

Magnesium sulphate is a potential treatment for acute severe asthma. However, the mechanisms and dose-response relationships are poorly understood. The first objective of this study was to examine whether inhaled magnesium sulphate exerts bronchodilator activity measured as bronchoprotection against histamine-induced bronchoconstriction in conscious guinea-pigs alone and combined with salbutamol. Secondly, we examined whether inhaled magnesium sulphate inhibits airways inflammation and function in models of neutrophilic and eosinophilic lung inflammation induced, respectively, by inhaled lipopolysaccharide or the inhaled antigen, ovalbumin (OVA). Airway function was measured in conscious guinea-pigs as specific airway conductance (sG_{aw}) by whole-body plethysmography. Anti-inflammatory activity was measured against lung inflammatory cell influx induced by OVA inhalation in OVA-sensitised animals or by lipopolysaccharide (LPS) exposure of non-sensitised animals. Airway function (sG_{aw}) was measured over 24 h after OVA exposure. Airway hyperresponsiveness to inhaled histamine and inflammatory cells in bronchoalveolar lavage fluid were recorded 24 h after OVA or LPS challenge. Histamine-induced bronchoconstriction was inhibited by inhaled magnesium sulphate or salbutamol alone and in combination, they produced synergistic bronchoprotection. LPS-induced neutrophil influx was inhibited by 6 days pretreatment with magnesium sulphate. Early and late asthmatic responses in OVA sensitized and challenged animals were attenuated by magnesium

sulphate. Lung inflammatory cells were increased by OVA, macrophages being significantly reduced by magnesium sulphate. Nebulised magnesium sulphate protects against histamine-induced bronchoconstriction in conscious guinea-pigs and exerts anti-inflammatory activity against pulmonary inflammation induced by allergen (OVA) or LPS. These properties of magnesium sulphate explain its beneficial actions in acute asthma. (246 words)

Key words

Magnesium sulphate; asthma; bronchoprotection; guinea-pigs; airways inflammation; lipopolysaccharide

1. Introduction

The use of magnesium sulphate ($MgSO_4$) for acute asthma was first described in 1936, and since then there has been inconsistent evidence to support its use in adults and children with asthma (Mohammed and Goodacre, 2007). Recent systematic reviews have suggested that both inhaled and intravenous $MgSO_4$ has a potential role in patients with a more severe exacerbation of acute asthma (Mohammed and Goodacre, 2007; Powell et al., 2012). However, the exact role of $MgSO_4$ is not fully delineated (Rowe, 2013). A large randomized controlled trial of nebulised $MgSO_4$ with 508 children with severe acute asthma has shown a minimal effect on asthma severity scores but in those children with a more severe exacerbation and those with shorter duration of symptoms the effect appeared to be more clinically relevant (Powell et al., 2013). A randomised controlled trial with 1109 adult patients, has shown no significant clinical benefit from the addition of nebulised $MgSO_4$ to standard treatment in acute asthma, although a minimal benefit was shown in severe asthma when given intravenously (Goodacre et al., 2013).

The exact mechanism of action of $MgSO_4$ in asthma is not understood. It appears to have some bronchodilator or bronchoprotective effects for which there are a number of proposed mechanisms. *In vitro* studies demonstrate an inhibitory effect of $MgSO_4$ on contraction of bronchial smooth muscle, the release of acetylcholine from cholinergic nerve terminals, and of histamine from mast cells

(Blitz et al., 2005). The main effect of $MgSO_4$ is to block the influx of calcium ions into smooth muscle cells of the airways (Georgoulianis et al., 2001) which results in bronchodilatation. One previous study demonstrates bronchoprotective effects of magnesium fluoride and $MgSO_4$ against inhaled methacholine in rats (Gandia et al., 2010), although Lindeman et al. (1985) failed to show any effect of intravenous $MgSO_4$ against acetylcholine-induced bronchoconstriction in anaesthetized dogs. There is evidence that $MgSO_4$ may also act as an anti-inflammatory agent by inhibiting the neutrophil respiratory burst in adults with asthma (Cairns and Kraft, 1996).

This study was undertaken to explore the actions of nebulised $MgSO_4$ in the airways of conscious guinea-pigs. There were two main objectives: Firstly, we examined whether inhaled magnesium sulphate exerts bronchodilator activity by measuring whether it has bronchoprotective properties against histamine-induced bronchoconstriction. We examined the speed of onset and dose-response relationships. Magnesium sulphate was examined on the airways function responses to inhaled histamine either alone or in combination with the β_2-adrenoceptor agonist, salbutamol. Secondly, we examined the hypothesis that inhaled magnesium sulphate inhibits airways inflammation and airways function in models of neutrophilic and eosinophilic lung inflammation. Neutrophilic inflammation was induced by inhaled lipopolysaccharide. Eosinophilic inflammation and the accompanying early and late asthmatic responses and airways hyperresponsiveness were induced by the inhaled antigen, ovalbumin, in

ovalbumin-sensitised animals.

2. Materials and methods

2.1. Guinea-pig airways function and inflammation models

Naïve conscious guinea-pigs challenged with nebulised histamine exhibit an immediate bronchoconstrictor response that recovers to baseline within 10 min. Immediate bronchodilator effects of agents such as magnesium sulphate can be measured as a reduced bronchoconstriction by inhaled histamine when administered immediately before the histamine; this is known as bronchoprotection (Turner et al., 2012). To examine the effects of a drug such as magnesium sulphate against models of asthma and chronic obstructive pulmonary disease (COPD) the main features of these lung diseases need to be induced. The four main features of asthma, namely airways hyperreactivity or hyperesponsiveness (AHR), inflammatory cell influx into the airways (predominantly eosinophils) and early (EAR) and late asthmatic responses (LAR), can be reproduced in conscious guinea-pigs sensitised and challenged with ovalbumin (Toward and Broadley, 2004). Guinea-pigs challenged with inhaled lipopolysaccharide (LPS) exhibit neutrophil driven pulmonary inflammatory responses similar to COPD (Toward and Broadley, 2000). Magnesium sulphate was administered as a single dose prior to OVA challenge or as several doses over a few days before LPS challenge.

2.2. Animal husbandry

Groups of six male Dunkin-Hartley guinea-pigs (200-250 g) were obtained from Charles River, Sulzfeld, Germany and housed in solid bottomed cages. A total of 96 animals were used. Light was maintained on a 12 h cycle and food and water were available *ad libitum*. All experiments complied with the Animals (Scientific Procedures) Act, 1986 and protocols were reviewed by Cardiff University Ethical Review Panel. The ARRIVE Guidelines on Animal Research: Reporting *In Vivo* Experiments have been adhered to in the design and execution of the study (Kilkenny et al., 2012; McGrath et al., 2010).

2.3. Measurement of lung function

Airways function was measured by whole-body plethysmography as specific airway conductance (sG_{aw}) in conscious guinea-pigs using a non-invasive double-chamber plethysmograph (Buxco Systems, Wilmington, North Carolina, USA). Guinea-pigs were placed in the double chamber plethysmograph and restrained by use of a neck restraint which also separated the nasal and thoracic compartments of the chamber making them both airtight. Air temperature and gas percentages were kept constant by use of a bias flow supply unit. Box pressure changes in both compartments were measured by pressure transducers. The pre-amplified output was converted to inspiratory and expiratory waveforms by Finepoint software (Buxco system Ltd), which also derives sG_{aw}. Readings were taken every 2 s and at least 20 breaths were recorded during any time point measurement. For calculation of sG_{aw} at a particular time point, the

average of 15 values taken at random was calculated.

Prior to the commencement of lung function measurements, all guinea-pigs were acclimatised to being restrained in the plethysmograph for at least 20 min on two separate occasions. This reduces movement-related signal 'noise' during measurements and reduces animal stress minimising interference from stress-related hormones such as cortisol. A decrease in sG_{aw} represents bronchoconstriction while an increase is a bronchodilator response.

2.4. Bronchoprotection by nebulised $MgSO_4$ and salbutamol

Baseline sG_{aw} readings were determined before challenge of naïve unsensitised guinea-pigs. With them still in the plethysmograph, they were challenged with nebulised histamine (0.5 mM solution for 2 min) delivered by use of a Buxco nebuliser at 0.5 l/min and 20% duty (% every 6 s of nebulising) per chamber. sG_{aw} was measured immediately after completing the challenge and at 5 and 10 min. After forty-eight hours, the same animals were treated with inhaled $MgSO_4$, salbutamol or vehicle (0.9% saline) and the histamine challenge repeated 15 min after completion of the drug or vehicle exposure. Lung function was then re-assessed as above. Each animal received one challenge with $MgSO_4$, salbutamol or vehicle in any one week.

Administration of nebulised $MgSO_4$ (62.5-250 mM), salbutamol (0.035-0.1 mM), vehicle (0.9% saline) or a combination of $MgSO_4$ and salbutamol was achieved with a DeVilbiss nebulizer (Somerset, Pennsylvania, USA) by placing the guinea-

pigs in an in-house Perspex chamber (20x30x15 cm) for 15min.

2.5. Effect of nebulised $MgSO_4$ on an ovalbumin model of asthma

Guinea-pigs were sensitised to ovalbumin on days 1, 4 and 7 by bi-lateral intra-peritoneal injection of ovalbumin (100 µg) and aluminium hydroxide (150 mg) suspended in saline (1 ml). On day 19, baseline sG_{aw} values were determined before placing the guinea-pigs into the Perspex exposure chamber for challenge with nebulised ovalbumin (0.03%) for 1 h using a DeVilbiss nebuliser. Guinea-pigs were removed from the chamber and sG_{aw} was measured every 15 min for the first hour following ovalbumin challenge and every hour thereafter for 12 h. A further reading was taken 24 h after the ovalbumin challenge. sG_{aw} was expressed as the percentage change from baseline. Animals were treated with nebulised $MgSO_4$ (250 mM) or vehicle for 15 min before ovalbumin challenge.

2.6. Effect of nebulised $MgSO_4$ on LPS-induced inflammation

Naïve unsensitised guinea-pigs were exposed daily to nebulised saline or $MgSO_4$ (250 mM) for 15 min in the Perspex exposure chamber (30x40x30 cm) on days 1 to 6. On day 4, 30 min after treatment with saline or $MgSO_4$, animals were exposed to a priming challenge with nebulised LPS (30 µg/ml) for 1 h. On day 6 they received a second identical challenge with LPS 30 min after saline or $MgSO_4$ and 24 h later, bronchoalveolar lavage was performed to determine inflammatory cell influx into the airways.

2.7. Airway responsiveness to inhaled histamine

Airway reactivity to a dose of inhaled histamine (0.3 mM) that produced non-significant threshold bronchoconstriction was evaluated 24 h before and 24 h after ovalbumin challenge. Baseline sG_{aw} values were determined before 0.3 mM histamine was delivered via inhalation (2 min at 20% duty) and sG_{aw} was measured immediately and at 5 and 10 min.

2.8. Bronchoalveolar lavage and inflammatory cell counts

After the final histamine challenge of the ovalbumin experiments or 24 h after the second LPS exposure, guinea-pigs were euthetized with an overdose of sodium pentobarbitone (Euthetal, 400 mg/kg i.p.). The trachea was cannulated and the lungs removed before instilling the left lung with saline (1 ml per 100 g body weight) for 3 min before withdrawal. This was repeated and the samples pooled for counting the total number of cells/ml with a Neubauer haemocytometer (Sigma-Aldrich, Gillingham, Dorset, UK). Differential counts of eosinophils, macrophages, lymphocytes and neutrophils were performed after centrifuging 100 µl of lavage fluid onto a glass microscope slide using a Shandon Cytospin centrifuge (ThermoFisher Scientific, Hemel Hempstead, UK) at 110 x *g* for 7 min. Slides were then stained with 1.5% Leishman's solution in 100% methanol for 6 min. A minimum of 200 cells were counted.

2.9. Calculation of responses

sG_{aw} values were determined at baseline before histamine or ovalbumin inhalation and at each time point after exposure. The percentage change from baseline was calculated. The peak fall in sG_{aw} in response to histamine was then determined for each animal. The percentage changes in sG_{aw} values for histamine after inhalation of $MgSO_4$, salbutamol or their combination were then expressed as a percentage of the pre-treatment peak fall in sG_{aw}. The mean percentage inhibitions of the histamine responses were then plotted against molar concentrations and the potency-ratio calculated at the IC_{50}. All data is presented as the mean ± standard error of the mean (S.E.M.). Because the time of peak fall in sG_{aw} occurring during the late asthmatic response following ovalbumin inhalation varied between animals, only the peak fall in sGaw between 9 and 11 h was plotted.

2.10. Statistical analysis

Student's paired *t*-test was used to determine significance of inhibitory effects on the histamine-induced bronchoconstriction, from the pre- and post-drug responses to histamine. One-way analysis of variance (ANOVA), followed by *post-hoc* Tukey's or Dunnett's test was used to determine significant differences between different treatment groups. Significance was determined as $P<0.05$.

2.11. Drugs

Magnesium sulphate was provided as isotonic $MgSO_4$ (250 mmol/l, tonicity 289 milliosmole; 151 mg per dose manufactured by St Mary's Pharmaceutical Unit,

Cardiff, UK). The dose range of $MgSO_4$ was selected to equate with clinical experience in which 250 mM has been used in children with acute asthma (Powell et al., 2013). Aluminium hydroxide, histamine diphosphate, ovalbumin and salbutamol hemisulphate were obtained from Sigma-Aldrich, Gillingham, Dorset, UK. Sodium pentobarbitone (Euthetal) was purchased from Merial, Harlow, UK. Drugs and materials were dissolved in saline purchased from Baxter Healthcare, Newbury, UK.

3. Results

3.1. Bronchoprotection by nebulised $MgSO_4$ and salbutamol

Bronchoprotection was measured as a reduction of histamine-induced bronchoconstriction. Histamine (0.5 mM) induced a significant fall in sG_{aw} in conscious guinea-pigs, indicating a substantial bronchoconstriction that recovered to baseline 10 min post-challenge. Guinea-pigs treated 48 h later with the saline vehicle 15 min prior to the histamine challenge also demonstrated a significant bronchoconstriction to histamine, which was not significantly different from the response before saline (7.9±10.7% inhibition) (Fig. 1A). Nebulised $MgSO_4$ dose-dependently inhibited the histamine response over the dose range of 62.5-250 mM, inhibiting by 23.4±6.5% at 62.5 mM, and 46.8±5.6% and 85.4±18.1% at 125 and 250 mM, respectively (Fig. 1A). Inhibition of the histamine response was significant at 250 mM (P<0.01).

Nebulised salbutamol caused dose-dependent inhibition of histamine-induced bronchoconstriction over the dose range of 0.035 - 0.1 mM, inhibiting the

histamine response at 0.035 mM by 21.8±13.2% and increasing to 90.6±11.2% at 0.1 mM (Fig. 1B). The dose-response curves (Fig. 1C) showed that salbutamol was more potent than $MgSO_4$ with a potency-ratio of 2049 at the IC_{50}.

Co-administration of salbutamol (0.035 mM) and $MgSO_4$ (62.5 mM), which alone had no significant inhibitory effects, significantly inhibited histamine-induced bronchoconstriction by 104.7±16.6% (Fig. 1D). It is possible to achieve more than 100% inhibition because sG_{aw} may fall below baseline after the second exposure to histamine. To determine whether this was a synergistic effect, where the effect of the two bronchodilators together is greater than the sum of their individual effects, we applied the principles described by Tallarida (Tallarida et al., 1999; Tallarida, 2001). From the potency-ratio (R) between salbutamol and $MgSO_4$ (2049), the dose (A_{eq}) of $MgSO_4$ to yield the same response as the combination can be calculated from the equation $A_{eq} = a + bR$, where a = dose of $MgSO_4$ and b = dose of salbutamol in the combination. This yielded a value of A_{eq} of 134.2 mM. The dose-response curve for $MgSO_4$ alone shows that the actual response to the combination (104.7±16.6%) would be generated by a corresponding dose (A_{corr}) of 400mM of $MgSO_4$. The interaction index α is the ratio $A_{eq}/A_{corr} = 0.33$. This value is less than unity and therefore indicates that a synergistic interaction has occurred between $MgSO_4$ and salbutamol.

3.2. Effects of nebulised $MgSO_4$ on ovalbumin-induced changes in lung function, airway reactivity and inflammatory cell influx

3.2.1. Lung function – early and late asthmatic responses Exposure of sensitised

guinea-pigs to inhaled ovalbumin elicited an immediate fall in sG_{aw} of -65.8±3.8% (n=6). This bronchoconstriction was the early asthmatic response (EAR), which returned to baseline after 5 h. A second fall in sGaw of -34.7+14.1% was observed between 7-12 h. This bronchoconstriction was the late asthmatic response (LAR) which returned to baseline 12 h after the ovalbumin challenge.

Guinea-pigs sensitised and challenged with ovalbumin but pre-treated with vehicle (0.9% saline) exhibited an EAR, the peak nadir change in sG_{aw} being -64.1$\pm$4.3%. A second fall in sG_{aw} occurred between 9 and 11 h, the mean peak being -20.5$\pm$5.7% (Fig. 2). This was not significantly different from the ovalbumin alone group. Animals treated with a single dose of nebulised $MgSO_4$ 30 min before ovalbumin challenge had a significantly reduced EAR compared to the vehicle group with a peak fall in sG_{aw} of -32.3$\pm$9.3%, which was significantly less than the vehicle group (Fig. 2). The mean peak LAR between 9 and 11 h (-3.7$\pm$2.0%) was also significantly attenuated in $MgSO_4$-treated guinea-pigs (Fig. 2).

3.2.2. Lung function - airway hyperreactivity to histamine Guinea-pigs did not demonstrate a bronchoconstriction to the low dose (0.3 mM) of histamine prior to the ovalbumin challenge (Fig. 3). However, 24 h after the ovalbumin challenge, in animals receiving no treatment or those receiving inhaled vehicle, there were immediate, significant falls in sG_{aw} of -27.4+1.7% and -33.8$\pm$6.5%, respectively, after histamine exposure. This response was diminished in guinea-pigs treated

with $MgSO_4$, with a non-significant change in sG_{aw} of -11.5±6.4% at zero time (Fig. 3). $MgSO_4$ treatment appeared to alter the shape of the histamine response which was now delayed with a small significant fall in sG_{aw} at 10 min.

3.2.3. Airway inflammation Ovalbumin-sensitised and challenged animals had a significant influx of total inflammatory cells into the airway (10.7±0.5x10^6/ml), compared with naïve animals (Fig. 4). This increase in inflammatory cells was predominantly associated with an influx of eosinophils (4.4±0.5x10^6/ml) and macrophages (4.3±0.3x10^6/ml), although lymphocytes (0.4±0.06x10^6/ml) and neutrophils (0.9±0.2x10^6/ml) were also evident (Fig. 4).

Vehicle-treated OVA challenged animals also demonstrated an increased number of total cells in the airways (9.2±1.3x10^6/ml) and these together with individual cell types were not significantly different from OVA challenged animals without vehicle. $MgSO_4$ treatment did not significantly reduce total cells (8.4±0.9x10^6/ml), eosinophils (5.32±0.8x10^6/ml), lymphocytes (0.51±0.08x10^6/ml) or neutrophils (0.66±0.17x10^6/ml). However, macrophages were significantly lower in the $MgSO_4$-treatment group (1.9±0.2x10^6/ml) than the vehicle group (2.90±0.53x10^6/ml) (Fig. 4).

3.3. Effect of nebulised $MgSO_4$ on LPS-induced inflammation

Guinea-pigs exposed to LPS had significantly more total cells in their airways than naïve animals (24.4±1.3x10^6/ml and 1.5±0.2x10^6/ml, respectively) (Fig. 5).

This increase was predominantly a result of neutrophil influx (15.2±0.7x10^6/ml, compared to 0.035±0.005x10^6/ml^{-1} in naïve animals). Eosinophils and lymphocytes were also increased in LPS-treated animals compared to naïve animals.

Animals treated with saline vehicle had a significant increase in total cell counts (23.5±2.0x10^6/ml) (Fig. 5), which were not significantly different from untreated LPS-challenged animals. $MgSO_4$ significantly reduced the total cells to 16.8±1.4x10^6/ml (Fig. 5). Macrophages, lymphocytes and eosinophils were not different between vehicle- and $MgSO_4$-treated animals. However, a significant reduction in neutrophils was observed following treatment with $MgSO_4$ (8.1±0.6x10^6/ml) compared to vehicle (16.3±1.5x10^6/ml) (Fig. 5).

4. Discussion

These studies have demonstrated for the first time the actions of nebulised magnesium sulphate on airways function and inflammation in conscious guinea-pigs and indeed in any laboratory species. Our results clarify the clinical application of inhaled $MgSO_4$ in the treatment of acute asthma in children.

Firstly, we have shown that inhaled $MgSO_4$ causes a dose-dependent bronchoprotection against histamine-induced bronchoconstriction (Fig. 1), which can be attributed to a direct bronchodilator action on the airways smooth muscle.

This confirms observations that $MgSO_4$ relaxes rabbit isolated tracheal smooth muscle (Georgoulianis et al., 2001) and bronchoprotection against methacholine-induced bronchoconstriction in rats (Gandia et al., 2010). However, Yoshioka et al. (2001) and Lindeman et al. (1989) failed to show any inhibition of bronchoconstriction to intravenous 5-hydroxytryptamine or inhaled acetylcholine, respectively, in anaesthetized dogs. In clinical studies, a number of different doses of nebulised $MgSO_4$ have been used (Powell et al., 2012) but we still do not know the appropriate dose nor indeed the frequency of nebulised $MgSO_4$ to achieve a maximal response in either adults or children. From our results, it would appear that the 250 mM dose produces optimum effect and this relates directly with the inhaled dose used in the MAGNETIC study (Powell *et al*., 2013). We also showed that the short-acting β-adrenoceptor agonist, salbutamol, caused dose-related inhibition of the histamine bronchoconstriction. This confirms our previous studies (Turner et al., 2010) and clearly demonstrates the ability of this model to display the effectiveness of inhaled bronchodilators such as β-adrenoceptor agonists. The combination of ineffective doses of $MgSO_4$ and salbutamol exerted a significant bronchoprotective effect which our calculations showed to be greater than the sum of their individual effects, namely synergism. The exact nature of this synergistic interaction is not understood but may represent an interaction between magnesium and salbutamol at the β-adrenoceptor. The β-adrenoceptor binding sites for agonist binding (i.e. salbutamol) are converted to high affinity binding by Mg^{2+} (Broadley, 1996) and in the presence of Mg^{2+}, the affinity of agonist binding to the β-adrenoceptor is

increased (Cech and Maguire, 1982). An increased agonist binding affinity to the β-adrenoceptor in the presence of Mg^{2+} would thus explain the enhanced response to salbutamol. It would be interesting to confirm that this is a class effect and applies to all β-adrenoceptor agonists. This finding has important clinical implication in that co-administration of a β-agonist with $MgSO_4$ will allow the dose of the agonist to be reduced thereby minimizing any undesirable side effects.

Secondly, we examined the effects of $MgSO_4$ in an ovalbumin model of asthma which displays three important functional features of the asthmatic airways: the early (EAR) and late asthmatic bronchoconstrictor responses (LAR) and airways hyperreactivity (AHR). Both the EAR and LAR were significantly reduced after a single inhaled dose of $MgSO_4$ (Fig. 2). Airways hyperreactivity to histamine was reduced in those animals pre-exposed to $MgSO_4$ (Fig. 3). The MAGNETIC study has suggested that those children with a short duration of symptoms, which would equate with the EAR, seem to respond better to $MgSO_4$ (Powell et al., 2013). Although this needs to be explored further in the clinical situation, the marked effect on the early asthmatic response seen in this guinea-pig model would support that hypothesis. The inhibition of the LAR and AHR by $MgSO_4$ administered immediately before the ovalbumin exposure is unlikely to be due to its bronchodilator action since this action would have diminished by the time of the LAR (9-11 h) and measurement of AHR, 24 h after ovalbumin administration. A similar result to the one presented here was protection of guinea-pig hearts

against allergen-induced anaphylactic shock by Intraperitoneal magnesium chloride (Kusniec et al., 1994).

The LAR and AHR seen in our asthma model after OVA challenge have been shown to be closely associated in human asthmatics (Hargreave et al., 1986) and in guinea-pig models of asthma (Smith and Broadley, 2007). Both responses to OVA are linked to the lung inflammation, in particular the observed increase in eosinophils (Bousquet et al., 1990; Laitinen et al., 1996). Pretreatment with $MgSO_4$ did not affect eosinophil numbers but significantly reduced numbers of macrophages (Fig. 4). Macrophages can be pro-inflammatory or anti-inflammatory as there are three different macrophage phenotypes with varying functions (Draijer et al., 2013; Mosser and Edwards, 2008; Stout and Suttles, 2004). Recent data suggests that macrophages play an important role in the development of severe asthma especially steroid-resistant asthma (Yang et al., 2012). One subset of macrophages can be a major source of IL-13 production which would drive increased mucus production and AHR (Byers and Holtzman, 2011). Thus, the reduction in macrophage numbers could explain the concomitant inhibition of AHR observed after $MgSO_4$ inhalation. It is possible that while the dose of $MgSO_4$ used here only affected macrophages, higher doses or repeated dosing, for example at 6 h after ovalbumin challenge, may have inhibited the other inflammatory cell types. Indeed, in the neutrophilic LPS model, which received multiple dosing with $MgSO_4$, there was significant attenuation of neutrophil numbers (see below). The LAR is also associated with lung

inflammation and it is possible that its inhibition by $MgSO_4$ was due to its anti-inflammatory activity. In rodents, the LAR may also in part be due to parasympathetic reflex activity (Raemdonck et al., 2012) and its inhibition may therefore be due to inhibition of this reflex. However, this is unlikely to occur at the muscarinic receptor site as others have found that acetylcholine responses are not inhibited by $MgSO_4$ (Lindeman et al., 1989).

In contrast to ovalbumin, LPS inhalation caused neutrophil-driven rather than eosinophil-driven airways inflammation and is therefore analogous to the inflammation associated with chronic obstructive pulmonary disease (COPD). This confirms previous observations in guinea-pigs from this (Nevin and Broadley, 2004; Toward and Broadley, 2000) and other laboratories Pera et al., 2011). $MgSO_4$ significantly reduced the neutrophil count in the airways (Fig. 5). There is evidence that Mg^{2+} can exert anti-inflammatory effects against neutrophils (Cairns et al., 1996), since the respiratory burst induced by N-formyl-methionyl-leuyl-phenylalanine (fMLP) in neutrophils isolated from asthmatics was reduced by Mg^{2+}, an effect attributed to Mg^{2+} interfering with the influx of extracellular Ca^{2+}. The increase in cytosolic Ca^{2+} of rat basophilic leukaemia cells due to antigen challenge was shown to be reduced by an elevation of Mg^{2+} in the medium (Kusniec et al., 1994). Other inflammatory cells, including T-lymphocytes, upregulate dihydropyridine-sensitive Ca^{2+} channels in asthma and these control calcium signaling and cytokine production. Dihydropyridine calcium antagonists prevent airway inflammation and hyperresponsiveness in

experimental models of asthma (Pelletier and Guéry, 2008). Interference with Ca^{2+} flux may be a common mechanism for the inhibitory actions of $MgSO_4$ seen throughout the present study. Ca^{2+} is involved in the contraction of smooth muscle and in the mobilization of inflammatory cells in both the ovalbumin and LPS models. Ca^{2+} has a central role in airway epithelial cells to induce proinflammatory gene transcription and the migration of leukocytes (Chun and Prince, 2009). Mast cells may be a further site of $MgSO_4$ and Ca^{2+} interaction, since influx of Ca^{2+} is required for degranulation and release of cytokines (Ma and Beaven, 2011). Thus, $MgSO_4$ probably exerts both airways smooth muscle relaxation (bronchoprotection) and anti-inflammatory effects through a mechanism similar to the calcium antagonists such as nifedipine. Indeed, nifedipine and $MgSO_4$ have been shown to blunt hypocapnia- but not acetylcholine-induced bronchoconstriction (Lindeman et al., 1989). These authors concluded that they preferentially inhibited voltage sensitive calcium channels rather than receptor-operated calcium channels. As well as through ion channels, Ca^{2+} modulates cell function via the calcium-sensing receptor (CaSR) which is involved in calcium homeostasis. Ca^{2+} and other divalent cations bind to and activate this receptor which is expressed on vascular endothelial cells and immune cells, including macrophages (Molostvov et al., 2009). Whether Mg^{2+} activates or inhibits the CaSR is not known, but it remains a likely site of the anti-inflammatory action of inhaled $MgSO_4$.

Conclusions

This study has shown that inhaled $MgSO_4$ exerts a direct immediate bronchodilator action in conscious guinea-pig airways, measured as bronchoprotection against histamine-induced bronchoconstriction. There was a synergistic bronchoprotection between $MgSO_4$ and the β_2-adrenoceptor agonist, salbutamol. It also exerts a similar bronchoprotection against the immediate bronchoconstriction following inhalation of ovalbumin (OVA) in sensitized animals, the EAR. A single dose of $MgSO_4$ administered before OVA also inhibited the LAR, AHR and inflammatory cell influx. Multiple doses were employed against the neutrophilic lung inflammation following LPS challenge and were shown to exert anti-inflammatory action against the neutrophil influx.

The dose-response relationship for $MgSO_4$, the frequency of administration and the synergy between $MgSO_4$ and a β-adrenoceptor agonist need to be examined in humans to identify the ideal dose and timing of administration during an exacerbation of acute asthma and further explore whether the two drugs together rather than individually has improved outcomes. It will also be necessary to examine whether the combination therapy has a synergistic effect upon the inflammatory response and associated airways function changes, such as the LAR and AHR. Interaction between cellular Ca^{2+} and Mg^{2+} may provide a mechanism for beneficial actions of inhaled $MgSO_4$ against both the inflammation and bronchoconstriction in asthma (Tam et al., 2003). The anti-inflammatory actions of $MgSO_4$ observed here suggest that its use in the treatment of asthma should be extended to more chronic administration than is currently

recommended. Although L-type calcium channel blockers such as nifedipine showed promise in early studies, this has not translated into therapeutic effectiveness in asthma (Fanta, 1985). If the same synergism exists between nifedipine and β-adrenoceptor agonists as seen here with $MgSO_4$, then these studies open the possibility for effective combination therapy in asthma.

Author contributions

An equal contribution to the original idea, study design, analysis and manuscript preparation by D.L.T., E.J.K., W.R.F., K.J.B. and C.P. The experimental contribution was made by D.L.T.

Conflict of interest

The authors declare no conflicts of interest.

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Figure Legends

Fig. 1. Effects of $MgSO_4$ and salbutamol on histamine-induced bronchoconstriction in conscious guinea-pigs. **A.** Inhibition of histamine-induced

bronchoconstriction following treatment with nebulised $MgSO_4$ (62.5-250 mM) or vehicle (O). $MgSO_4$ significantly inhibited the histamine response at 250mmol; ***P*<0.01, one-way ANOVA and Dunnett's post hoc test. **B.** Inhibition of histamine-induced bronchoconstriction following treatment with nebulised salbutamol (0.035-0.1 mM) or vehicle (O). Histamine-induced bronchoconstriction was significantly inhibited by salbutamol at 0.07 and 0.1 mM; **P*<0.05, ***P*<0.01, one-way ANOVA followed by post hoc Dunnett's. **C.** Dose-response curves for inhibition of histamine-induced bronchoconstriction by salbutamol (●) and $MgSO_4$ (■) showing the IC_{50} values (mM) and the dose-ratio (R) at the IC_{50}. **D.** The individual and combined bronchoprotection by nebulised $MgSO_4$ (62.5 mM) and salbutamol (0.035 mM) against histamine-induced bronchoconstriction. The combination of $MgSO_4$ and salbutamol inhibited the histamine response to a greater extent than either drug alone; **P*<0.05, one-way ANOVA followed by post hoc Tukeys. Responses are the mean n=6 % inhibition of the peak histamine response ± S.E.M.

Fig. 2. Changes in airways function of conscious ovalbumin-sensitized guinea-pigs following inhalation of ovalbumin (0.03% for 1 h). Guinea-pigs were treated with either saline control (●) or $MgSO_4$ (■, 250 mM) for 15 min before the ovalbumin challenge. Changes in sG_{aw} are expressed as a percentage of the baseline (BL) immediately before ovalbumin exposure. Mean values (n=6) ± S.E.M. are shown including the mean peak fall in sG_{aw} occurring between 9 and 11 h. * Significant difference between the $MgSO_4$- and saline-treated groups by Student's unpaired t-test *P*<0.05.

Fig. 3. Time courses for the changes in sG_{aw} following exposure of conscious ovalbumin-sensitized guinea-pigs to nebulised histamine (0.3 mM, 2 min). Responses to histamine were obtained before (Pre-OVA, ●) ovalbumin exposure (0.03% for 1 h) and at 24 h after OVA exposure (Post-OVA, ■). **A.** Guinea-pigs received no treatment (control). **B.** Guinea-pigs received nebulised vehicle (saline) for 15 min before ovalbumin exposure. **C.** Guinea-pigs received nebulised $MgSO_4$ (250 mM) for 15 min before ovalbumin exposure. Responses are the mean changes (n=6) in sG_{aw} expressed as a % of the baseline value (BL) obtained before histamine exposure ± S.E.M. * Significantly different from the Pre-OVA value by Student's paired *t*-test *P*<0.05.

Fig. 4. Leukocyte numbers in bronchoalveolar lavage fluid removed from ovalbumin-sensitized guinea-pigs 24 h after ovalbumin (OVA, 0.03% for 1 h) challenge. Total cells, macrophages, eosinophils, lymphocytes and neutrophils are expressed as the mean ± S.E.M cell number ($x10^6$/ml n=6). Cells were obtained from guinea-pigs that were untreated (naïve) and from ovalbumin sensitized and challenged guinea-pigs that received no treatment (OVA), received saline (OVA + vehicle) or received $MgSO_4$ (250 mM, for 15 min before OVA challenge) (OVA + $MgSO_4$). * Significant difference between vehicle-treated and $MgSO_4$-treated animals by Student's unpaired *t*-test *P*<0.05.

Fig. 5. Leukocyte numbers in bronchoalveolar lavage fluid removed from non-sensitised guinea-pigs 24 h after the second of two inhalation exposures to lipopolysaccharide 48 h apart (LPS, 30 μg/ml for 1 h). Total cells, macrophages, eosinophils, lymphocytes and neutrophils are expressed as the mean ± S.E.M. cell number ($x10^6$/ml n=6). Cells were obtained from guinea-pigs that were untreated (naïve) and from LPS challenged guinea-pigs that received no treatment (LPS), received saline (LPS + saline) or received $MgSO_4$ (250 mM, 15 min) daily for 6 days 15 min before LPS challenge (LPS + $MgSO_4$). *** Significantly different from saline-treated animals by Student's unpaired *t*-test $P<0.001$.

Figure 1

Figure 1

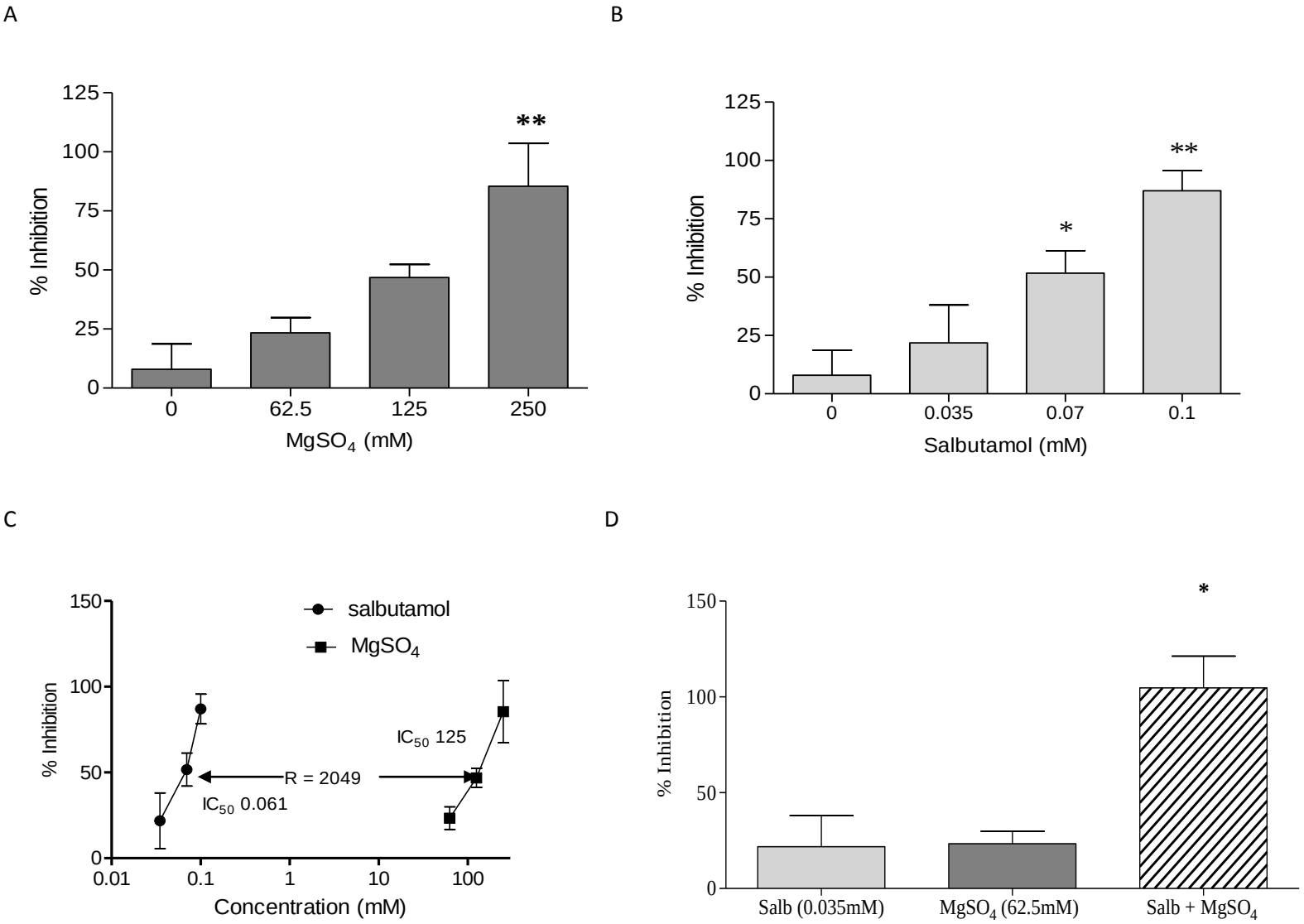


Figure 2

Figure 2

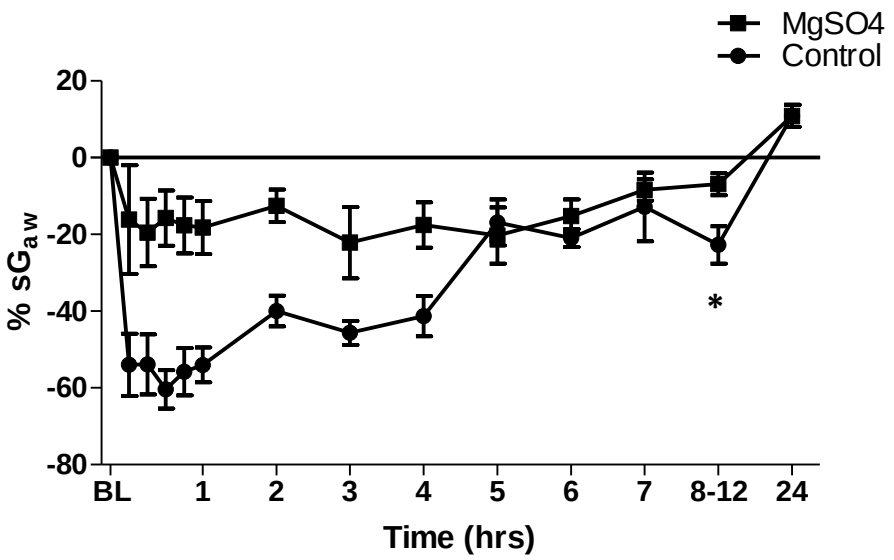
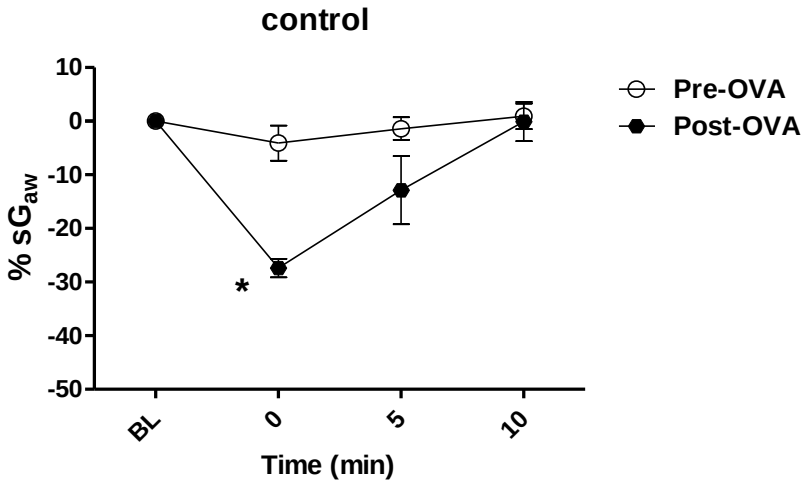


Figure 3

A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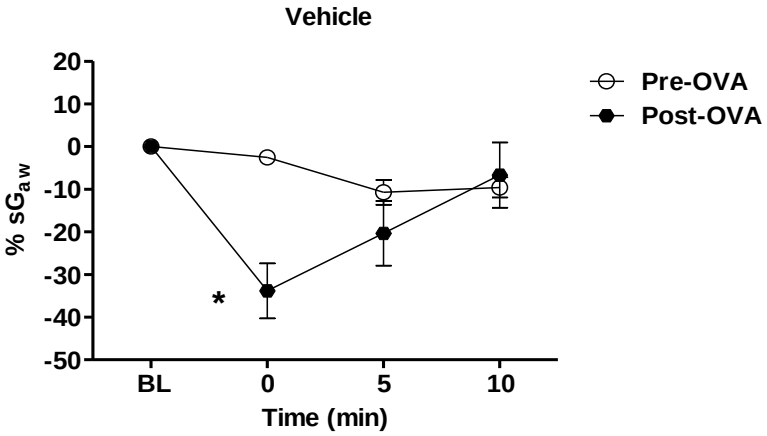
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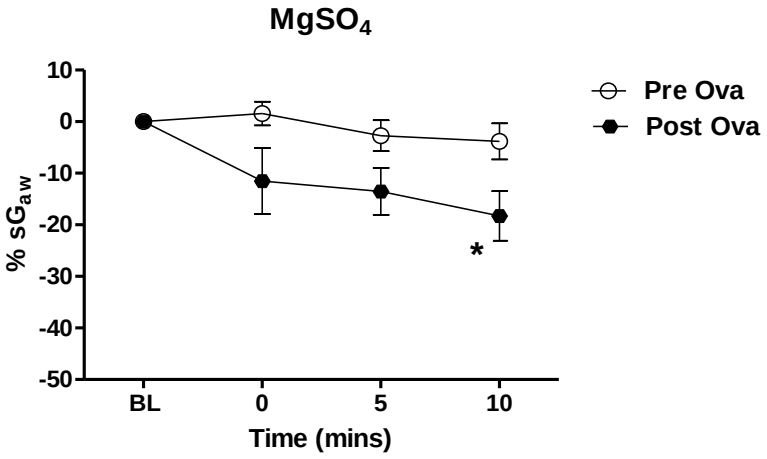
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Figure 4

Figure 4

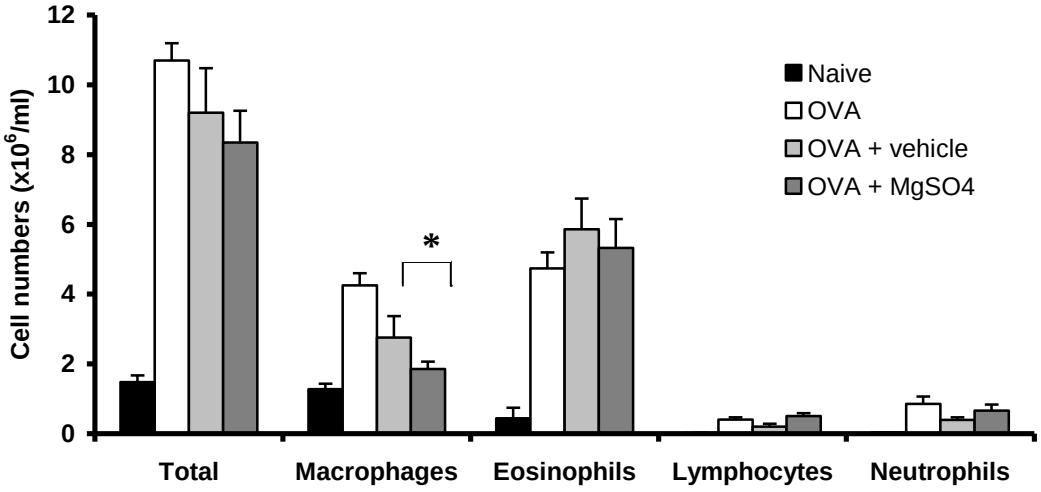
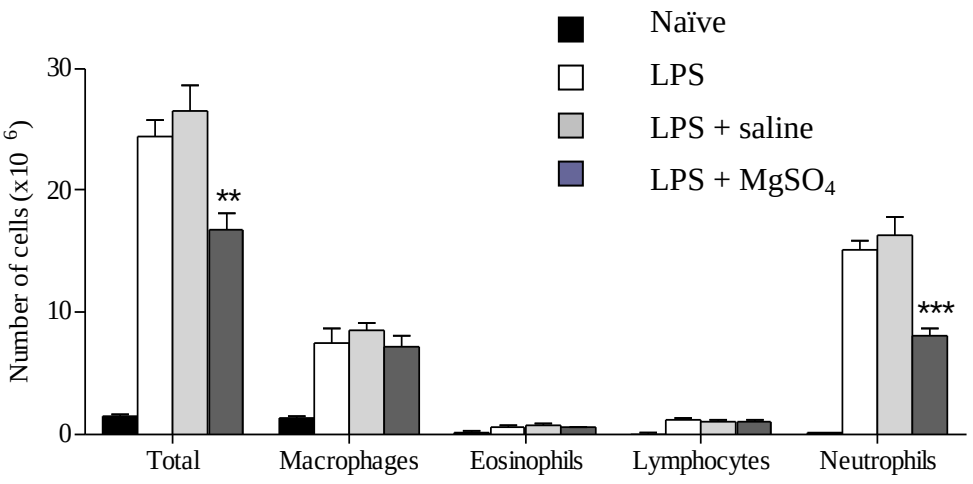


Figure 5

Figure 5



***Response to Reviewers**

European Journal of Pharmacology EJP-44410R1

Effects of nebulised magnesium sulphate on inflammation and function of the guinea-pig airway by

Dawn L Turner, William R Ford, Emma J Kidd, Kenneth J Broadley, Colin Powell.

Dear Editor

Thank you for your letter of acceptance of our manuscript.

I have retyped the manuscript with the corrections that were requested which I hope is now satisfactory.

With thanks for your help in getting this work published

Yours faithfully

Ken Broadley

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