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ORIGINAL ARTICLE

# Functional and histological effects of inhaled magnesium alone or associated to fluoride: An experimental study in rats

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**Abstract** The study was carried out to investigate the effects of inhaled Mg alone and associated with F in the treatment of bronchial hyperresponsiveness. 43 male Wistar rats were randomly divided into four groups and exposed to inhaled NaCl 0.9%, MeCh, $MgSO_4$ and $MgF_2$. Pulmonary changes were assessed by means of functional tests and quantitative histological examination of lungs and trachea. Results revealed that delivery of inhaled Mg associated with F led to a significant decrease of total lung resistance better than inhaled Mg alone ($p < 0.05$). Histological examinations illustrated that inhaled Mg associated with F markedly suppressed muscular hypertrophy ($p = 0.034$) and bronchoconstriction ($p = 0.006$) in MeCh treated rats better than inhaled Mg alone. No histological changes were found in the trachea. This study showed that inhaled Mg associated with F attenuated the main principle of the central components of changes in MeCh provoked experimental asthma better than inhaled Mg alone, potentially providing a new therapeutic approach against asthma.



## Introduction

Asthma is a serious global health problem, throughout the world affecting over 300 million people of all ages. Asthma is a chronic disease characterized by a variety of features, including increased airway responsiveness, airway inflammation and reversible airway obstruction. According to the Global Initiative for Asthma (GINA), the definition of asthma is based on an operational description. In these terms:

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"*Asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. The chronic inflammation causes an associated increase in airway hyperresponsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment*" [1].

Several studies [2–4], had confirmed the bronchodilating effects of intravenous Mg, but its effects through inhalation are controversial [5–8]. Mg has been reported in many researches to inhibit the $Ca^{2+}$ influx by blocking the voltage-dependent calcium channels, modulate the vaso-activity by affecting the influx of extracellular $Ca^{2+}$ through dihydropyridine-sensitive, voltage-dependent channels, which accounts for much of its relaxing action on airway [9,10]. In vitro studies showed that the magnesium ion ($Mg^{2+}$) modulates smooth muscle contractility and mediates release by antagonism of the action of calcium [11,12].

The bronchodilator effect of fluoride is poorly documented. Cushing et al., found that NaF relaxed arteries by releasing an endothelium derived relaxing factor and one or more prostanoids [13]. Zaho et al. proved that NaF induced bronchial relaxation on precontracted bovine bronchi in vitro and rats in vivo. In fact, fluoride is an inhibitor of enolase, an enzyme of the glycolysis pathway leading to phosphoenolpyruvate [14,15].

As compared with physiological studies, there are a few detailed histological studies on the respiratory system, especially in the lung, of humans or rats exposed by inhalation to Mg alone or when associated with fluoride.

The purposes of this study were to investigate the changes in the pulmonary function as well as in the histology in the lung of Wistar rats which were challenged with MeCh and then exposed by inhalation to Mg alone and when associated with fluoride.

## Materials and methods

The study protocol used in the present study was approved by the Animal Ethics Committee of the Faculty of Medicine of Sousse, Tunisia, where the experiments were carried out.

### *Animals*

Forty-three male Wistar adult rats (180 g ± 30 g) were included in the study. Rats were randomly assigned to four groups: Controls ($N = 14$), MeCh alone ($N = 10$), $MgSO_4$ ($N = 9$) and $MgF_2$ ($N = 10$).

### *Total lung resistances measurement*

Rats were anesthetized intraperitoneally with ketamine (150 mg/kg). After dissecting the neck, a tracheal cannula was inserted into a mid-line incision of the trachea. A catheter was inserted into the esophagus and connected to a pressure transducer to measure the intra-esophageal pressure. A small pneumotachograph (PTG, 8431B, Hans Rudolph, Kansas, USA) was connected to tracheal cannula. The period of measurement of the flow rate with the PTG was set at 10 s to avoid a change in ventilation due to the PTG dead volume. The PTG was connected to a differential pressure transducer. Both pressure and flow transducers were assembled together with connecting valves to ease the calibration. Calibration in volume was done daily with a 10 ml syringe. Total lung resistance ($R$) was calculated by using a first order mechanical model of the lung. Aerosols were made through a DeVilbiss nebulizer (Ref 123016 Marquette Medical products, Englewood co. USA) connected to a compressor (flow rate 100 ml/s). Aerosols were delivered at a flow rate of 0.1 ml/s in a rigid plastic chamber placed over the rat body. Bronchoconstriction was induced by gradually increasing concentrations of MeCh: 0.5 mg/L, 1 mg/L, 2.12 mg/L, 4.25 mg/L, 8.5 mg/L, 17 mg/L, 34 mg/L and 68 mg/L. MeCh solutions were aerosolized within the chamber for 1 min with 3 min intervals between doses.

$MgSO_4$ and $MgF_2$ inhaled aerosols were delivered for one minute after each dose of MeCh from the fourth dose of MeCh. The total lung resistances ($R$) were measured before the challenge, after an aerosol of isotonic saline and 2 min after each dose of MeCh.

### *Histology*

At the end of the protocols, the rats were sacrificed; the lungs and trachea were removed. Longitudinal sections were taken from the left and right lung and trachea sections were cut transversely. The lungs and trachea sections were processed by routine histological procedures for paraffin embedding. Five-micrometer thick histological sections were stained with hematoxylin and eosin, and examined by light microscopy. Histological modifications of the lungs and trachea were assessed by means of a quantitative histological score. The degrees of inflammation, mucus, muscular hypertrophy, bronchial dilatation and emphysema were scored from 0 (absent) to 3 (intense) by two pathologists who examined the slides at the same time under a double-observation microscope. The histological slides were coded and the two investigators were unaware of the origin of the material during scoring.

### *Chemicals*

$MgSO_4$, acetic acid and ketamine were purchased from Sigma (St. Louis, MI, USA) and MeCh from Allerbio (Lavarenne, France). $MgF_2$ was dissolved in acetic acid to improve the solubility.

Magnesium fluoride, random crystals, 99.99 + %, optical grade: purchased from (Sigma, Aldrich).

### *Solutions synthesis*

$MgF_2$ was dissolved in acetic acid to improve the solubility. $MgF_2$ solution was prepared and stored in polyethylene or polypropylene bottles in order to prevent attack on glass surfaces.

### *Data analysis*

All data are reported as mean ± SEM. Mean values of $R$ between control and other groups were compared using the Mann–Whitney's $U$ test. Comparison of rat's resistance ($R$) values among the same group of rats at different concentrations of MeCh was made using the paired Student's t-test.

Changes in *R* during the MeCh challenge in different groups were analyzed with a two-way ANOVA.

The histological scores were analyzed by the Kruskal–Wallis test and multiple comparison procedures. When comparing controls with the two groups ($MgSO_4$ and $MgF_2$), Student's *t*-test and the Mann–Whitney's test were employed. A *p* value $<0.05$ was considered significant.

## Results

### *Effects of inhaled Mg alone and Mg associated with F on* ***R*** *values*

Basal resistances were not different for all groups. *R* value in the control group receiving MeCh increased significantly with the cumulative doses of MeCh. Compared to the base value of *R*, at the start of the challenge, the increase was significant at the fourth dose = 4.25 mg/L ($p < 0.05$).

### *Histological findings*

Table 1 shows the results of the histological scoring of the lung (inflammation, mucus, muscular hypertrophy, bronchial dilatation and emphysema). Histological examination of the transversal sections of the trachea of the different groups does not show significant histological abnormalities or changes.

The comparison of the lungs histological scoring of the four groups by the Kruskal–Wallis test, mucus, muscular hypertrophy and bronchial dilatation exhibited significant differences among groups ($p = 0.0259$, $p = 0.0338$ and $p = 0.001$,

**Table 1** Effect of inhaled magnesium on lung histology score.

| Groups | N° rat | Inflammation | Mucus | Muscular hypertrophy | Bronchial lumen dilation | Emphysema |
|---|---|---|---|---|---|---|
| Control | 1 | 3 | 0 | 0 | 1 | 0 |
| | 2 | 0 | 0 | 1 | 1 | 1 |
| | 3 | 1 | 1 | 0 | 1 | 1 |
| | 4 | 0 | 0 | 0 | 1 | 0 |
| | 5 | 0 | 1 | 1 | 1 | 0 |
| | 6 | 1 | 0 | 1 | 1 | 0 |
| | 7 | 0 | 0 | 0 | 1 | 0 |
| | 8 | 0 | 0 | 1 | 2 | 0 |
| | 9 | 2 | 0 | 0 | 1 | 0 |
| | 10 | 0 | 0 | 0 | 2 | 0 |
| | 11 | 0 | 0 | 2 | 1 | 0 |
| | 12 | 1 | 0 | 0 | 2 | 0 |
| | 13 | 0 | 1 | 0 | 0 | 0 |
| | 14 | 1 | 0 | 1 | 1 | 0 |
| MeCh | 1 | 0 | 1 | 0 | 0 | 0 |
| | 2 | 1 | 1 | 1 | 0 | 0 |
| | 3 | 1 | 1 | 0 | 0 | 0 |
| | 4 | 2 | 0 | 1 | 0 | 0 |
| | 5 | 2 | 1 | 1 | 0 | 0 |
| | 6 | 2 | 0 | 2 | 0 | 0 |
| | 7 | 1 | 1 | 2 | 1 | 0 |
| | 8 | 1 | 0 | 2 | 0 | 0 |
| | 9 | 2 | 1 | 1 | 0 | 0 |
| | 10 | 2 | 1 | 1 | 0 | 0 |
| $MgF_2$ | 1 | 1 | 0 | 0 | 3 | 0 |
| | 2 | 0 | 0 | 0 | 0 | 0 |
| | 3 | 2 | 0 | 1 | 3 | 0 |
| | 4 | 1 | 1 | 0 | 2 | 0 |
| | 5 | 1 | 0 | 0 | 2 | 0 |
| | 6 | 0 | 0 | 1 | 1 | 0 |
| | 7 | 2 | 0 | 1 | 2 | 0 |
| | 8 | 0 | 0 | 0 | 2 | 0 |
| | 9 | 1 | 0 | 0 | 2 | 1 |
| | 10 | 0 | 0 | 0 | 3 | 0 |
| $MgSO_4$ | 1 | 1 | 1 | 1 | 1 | 0 |
| | 2 | 0 | 0 | 1 | 2 | 0 |
| | 3 | 2 | 0 | 1 | 2 | 0 |
| | 4 | 1 | 1 | 0 | 0 | 0 |
| | 5 | 3 | 0 | 1 | 1 | 0 |
| | 6 | 1 | 0 | 0 | 1 | 0 |
| | 7 | 2 | 0 | 2 | 0 | 0 |
| | 8 | 0 | 1 | 1 | 0 | 0 |
| | 9 | 2 | 0 | 1 | 2 | 0 |
| Kruskal–Wallis (valeur de *p*) | | 0.07 | 0.025 | 0.033 | 0.0001 | 0.447 |

*P* value for comparison of the four groups (Kruskal–Wallis test).

respectively). In a comparison of the $MgSO_4$ group to the $MgF_2$ group by the Mann–Whitney's test, the $MgF_2$ group presented a significant difference in bronchial dilatation ($p = 0.006$) and muscular hypertrophy ($p = 0.034$) (Table 1).

## Discussion

In this study we investigated the effects of inhaled magnesium alone and when associated with fluoride on rats pre-contracted by cumulative doses of MeCh. We found that inhaled magnesium (0.5 M) reversed bronchospasma but the association of magnesium and fluoride ($MgF_2$) ([Mg] = 0.08 mM, [F] = 0.084 mM) had a better bronchodilator effect (decrease of total lung resistances, $p < 0.05$, bronchial lumen dilatation, $p = 0.006$ and muscular hypertrophy, $p = 0.034$) (Fig. 1) (Table 1).

MeCh was used to challenge the rat before any administration. In fact, it was reported as a synthetic muscarinic agonist more stable than acetylcholine and better tolerated than histamine or carbachol [16]. In addition, MeCh rarely induced cough. Our experimental results demonstrated that MeCh induced severe bronchoconstriction in experimental rats. In fact, the comparison of the control and MeCh groups using the Mann–Whitney's *U*-test revealed significant differences in the total lung resistances (*R*) ($p < 0.05$) that start at the fourth dose of MeCh (4.25 mg/L). Moreover, the comparison of the histological score of the same groups by the Mann–Whitney's test, revealed a significant difference in the mucus presence ($p = 0.019$), muscular hypertrophy ($p = 0.049$) and in the bronchial lumen dilatation ($p = 0.000132$) (Table 1). Histological observation demonstrated a narrowed and scalloped lumen bronchiole surrounded by a thick layer of muscle (bronchoconstriction) with presence of mucus in the bronchial lumen (Fig. 3).

While the bronchodilating effect of magnesium administered intravenously has been confirmed by several studies [2–4], its effect through inhalation is controversial and poorly documented. Recent and previous studies concluded that treatment with nebulised $MgSO_4$ alone was difficult to draw due to lack of studies in this area [17–19]. Rolla following a study of 9 cases showed that asthma administered inhaled $MgSO_4$ led to a reduction in bronchial reactivity to histamine [20]. Indeed, Bessmertny et al. showed that $MgSO_4$ can directly inhibit histamine release from mast cells and stimulate the production of NO and prostacyclin synthesis [21]. However, Hill has shown that inhaled $MgSO_4$ may increase bronchial responsiveness in these patients [22]. In another trial, carried out by Tiffany et al., a beneficial effect of magnesium was not, however, found [8]. We have therefore undertaken this study to try to clarify this controversy. Magnesium was administered as $MgSO_4$ at 0.5 M. At high concentrations, this cation produces significant toxicity. Infact, hypermagnesemia induce paralysis of skeletal muscles, reduce lung capacity and sometimes even causes coma and death. The results of this study were in agreement with previous studies of Rolla and colleagues [20] that confirmed the bronchorelaxant effect of inhaled $MgSO_4$. Inhaled $MgSO_4$ by hyperactive asthmatic patients during (MCT), had bronchodilating effects. In our study, inhaled $MgSO_4$ decrease *R* values ($p < 0.05$), inflammation ($p = 0.01$) and bronchial dilatation ($p = 0008$). Histological observation illustrated a bronchiole with a little scalloped lumen (moderate bronchodilation), low abundance of mucus and a thin muscle layer (Figs. 1 and 3). Magnesium's pharmacological action is based upon its ability to inhibit the release of calcium from vesicles in the sarcoplasmic reticulum, resulting in bronchial smooth muscle relaxation [9,10].

The histological results were consistent with the physiological data; the rats treated with inhaled Mg associated with Fluoride ($MgF_2$) had better improvement in *R* values, bronchoconstriction and muscular hypertrophy than rats treated with inhaled Mg alone. Our histological results revealed that there was a bronchodilation with thinning of the muscles and no mucus. However, there was persistent peribronchial inflammation (lymphoplasmocytis inflammation) in MeCh, $MgSO_4$ and $MgF_2$ groups ($p > 0.05$) (Fig. 3). Further, transversal sections of trachea of the different groups did not show significant histological changes (regular diameter, no mucus, and absence of thickening of tracheal muscle); this result can be explained by the low abundance of smooth muscle in the trachea (Fig. 2).

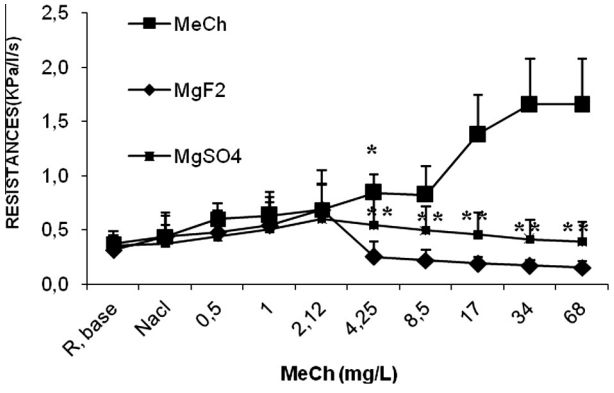


**Figure 1** Effect of inhaled MeCh, $MgSO_4$ and $MgF_2$ on *R* values. $^{*}p < 0.05$, significant increase in bronchial resistance that starts at the fourth dose of MeCh (4.25 mg/L). $^{**}p < 0.05$, Mann–Whitney *U* test. The comparison between $MgF_2$ and $MgSO_4$ groups using the Mann–Whitney's *U*-test revealed significant differences in airway resistance ($P < 0.05$) that starts at the fourth dose of MeCh (4.25 mg/L).

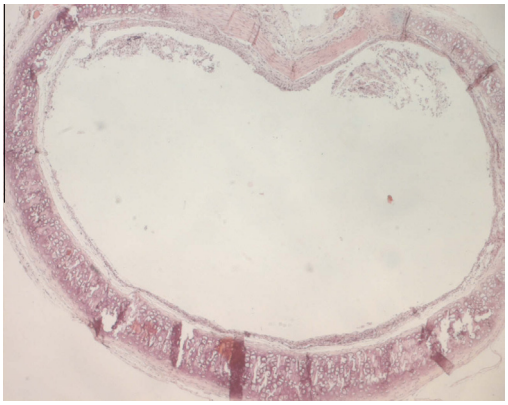

**Figure 2** Section of trachea rat after inhaled magnesium exposure. Transversal sections of the trachea of the different groups (control, MeCh, $MgSO_4$ and $MgF_2$) did not show significant histological changes (regular diameter, no mucus and absence of thickening of tracheal muscle). Hematoxylin–eosin staining. Original magnification was 40×.

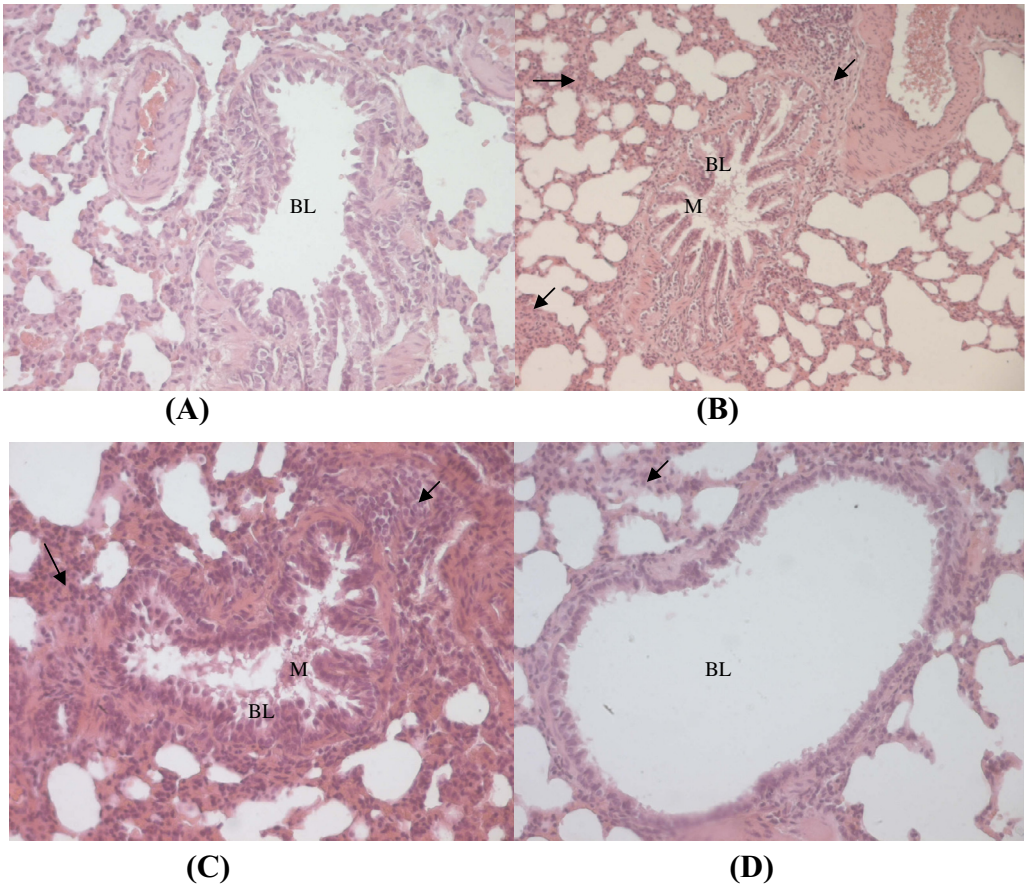


**Figure 3** Histological changes in rat lung following inhaled magnesium exposure. Sections of lung rats (control, A), (MeCh, B), ($MgSO_4$, C) and ($MgF_2$, D). BL, bronchial lumen, M, mucus. Arrowheads indicate peribronchial inflammation (B–D). Hematoxylin–eosin staining. (Original magnification: A, C and D = 200×; B = 100×).

The bronchodilator effect of fluoride is poorly documented. Fluoride had been reported to stimulate adenylate cyclase activity on smooth muscles and induced NO synthesis which would relax bronchi [11,23]. The better known bronchodilator mechanism of fluoride is induced by inhibition of the glycolytic enzyme, enolase, which converts 2-phospho-glycerate to phosphoenolpyruvate according to [15]. The inhibition of glycolysis induced by fluoride is illustrated by the sharp decrease in lactate production in its presence. Inhibition of this enzyme would be expected to reduce glycolytic ATP production and impair smooth muscle contraction [14].

Then, the association of magnesium and fluoride makes a powerful bronchodilator that acts in micromolar dose ranges compared to $MgSO_4$ (0.5 M) alone. In the present study, inhaled doses of $MgSO_4$, NaF and $MgF_2$ were very low and far from the toxic doses of magnesium and fluoride [24,25]. It is difficult to assess the dose of $MgF_2$ needed for the treatment of asthma as this study seems to be the first to demonstrate its bronchodilating effect in vivo. The duration of the bronchorelaxant effect of $MgSO_4$ and $MgF_2$ is not known and needs further study.

## Conclusion

Magnesium associated with fluoride and administered as inhaled $MgF_2$ is a double combination able to induce a significant and constant bronchodilating effect through two different pathways. Their effects were additive.

In addition, the association of Mg with F provides immediate bronchodilation when inhaled in a state of bronchoconstriction. This supports the use of this association for both relief and prevention of asthma symptoms. However, the optimum dose–response relationship needs to be addressed in future studies. In addition, further research in this area should be encouraged to determine the potential mechanism in which inhaled $MgF_2$ enhanced bronchospasma.

## Conflict of interest

The authors declare that there is no conflict of interest.

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