The Effect of Magnesium on Halothane-Induced Intracellular Calcium Concentration Changes in Cardiomyocytes

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Background: Halothane, a potent inhalational anesthetic, is known to cause arrhythmia, probably due to Ryanodine Receptor (RyR) activation, triggering Ca2+ release from sarcoplasmic reticulum (SR) to the cytosol. Mg2+ hypothetically prevents Ca2+ release by inhibiting RyR and increasing Ca2+ reuptake to SR. Methods: An in vitro experiment was done on cultured cell of rat cardiomyocytes. Cells were divided into six groups. Five groups were exposed to 2 mM halothane (1–3 MAC) for 5 minutes and one was not. Of the 5 halothane-exposed groups, group 1 received no additional treatment and was observed at minute-0, 5, 10, 15 and 20 after discontinuation. Group 2 and 3 received 11 mM and 22 mM MgSO4 after halothane exposure, respectively. Group 4 and 5 received corresponding MgSO4 treatment before exposure. Cytosolic Ca2+ change was observed by a confocal microscope and emission was measured by pixel analysis. Results: Halothane increased cytosolic Ca2+ concentration in rat cardiomyocytes, in which was not substantially altered by MgSO4 given before or after the exposure. Higher dose of Mg2+ resulted in decreasing Ca2+ concentration. 22 mM MgSO4 decreased cytosolic Ca2+ concentration to the same extent as halothane discontinuation for 10 minutes. Fifteen minutes halothane discontinuation significantly decreased cytosolic Ca2+ concentration and 20 minutes discontinuation returned the Ca2+ concentration to the basal level. Conclusion: Halothane increases cytosolic Ca2+ concentration in rat cardiac myocytes. Neither pre-nor post-halothane exposure administration of MgSO4 substantially alters this phenomenon. Discontinuation of halothane for 15 minutes significantly reduces cytosolic Ca2+ concentration.

Keywords: Halothane, Cytosolic Ca2+, Cardiac Myocyte, Mg2+.

1. INTRODUCTION

Halothane is a potent inhalational anesthetic, but its use has been limited due to its widely-known arrhythmicogenic effect.1 Previous studies on halothane side effect were mostly done on skeletal muscle in association with malignant hyperthermia (MH), revealing that hypercontracture of muscle cells is caused by cytosolic Ca2+ accumulation due to the activation of ryanodine (RyR) receptor by halothane.2 A similar mechanism probably occurs in cardiomyocytes, leading to cardiac cytosolic Ca2+ accumulation and arrhythmia. In normal cells, this cytosolic Ca2+ accumulation does not occur as clearance mechanisms will return cytosolic Ca2+ concentration to the baseline value.

Sarcoplasmic-endoplasmic reticulum Ca-ATPase (SERCA) probably plays the major role in clearance by returning 70% of cytosolic Ca2+ to its storage in the sarcoplasmic reticulum (SR).3 SERCA is an active pump, requiring energy from ATP hydrolysis and cytosolic magnesium (Mg2+).4,5 In a previous in vivo study on MH skeletal muscle, magnesium attenuated the severity of clinical manifestations of MH.5 Theoretically, Mg2+ inhibits RyR to prevent Ca2+ depletion from SR and plays a role in optimizing SERCA activity to reuptake Ca2+ which has been released from SR to the cytosol.

There have been few studies on the role of Mg2+ in modifying the effects of halothane exposure on cardiac myocytes. Although magnesium has long time been recognized as an antiarrhythmic agent, its clinical use has been limited to pre-eclampsia/eclampsia as well as perioperative open heart surgery
management. We aimed to evaluate the effects of magnesium administration on cytosolic Ca^{2+} concentration in cultured cells being exposed to halothane. We secondarily also looked at the effects of halothane exposure cessation on cytosolic Ca^{2+} concentration.

2. METHOD
This was an in vitro experimental study on cardiomyocyte cultured cells. After being thawed, cultured cells from rat neonatal heart ventricles (RCM-561, Lonza Clonetics) were divided to 10 aliquots, each contains 1 mL. Cells were embedded with cell-loading medium (RPMI, FCS 2% and HEPES at pH 7.4). Prior to dye-loading, Indo-1 working solution, containing 5 μM Indo-1 (Indo-1 AM, I-1223, Molecular Probes™) in dye-loading medium (HEPES at pH 7.4, 119 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl_2, 0.8 mM MgCl_2 and 13.8 mM glucose), was prepared. Pluronic F-127 was added to facilitate Indo-1 diffusion through the thick cell membrane. Cell medium then was changed with Indo-1 working solution (in DLM). Cells were protected from light and then incubated for 20 minutes at 37 °C in a 5% CO_2 incubator.

Cells were washed twice with DMEM and 2% FCS, then were resuspended in a cell-loading medium and were stored in a dark room for 1 hour. Cell washing needs centrifugation, which is actually not recommended for RCM-561, so we decided to centrifuge the cells in a very low setting (300 rpm). Supernatant was then removed and 2% FBS was added. Before the experiment, cells were incubated for 30–60 minutes in room temperature. Cells then divided into 6 groups. The control group was not being exposed to halothane. The image of a control cell is shown in Figure 1.

Cells in group 1 were exposed to halothane (halothane group) for five minutes, then were discontinued from halothane exposure. The change of cytosolic Ca^{2+} concentration was evaluated immediately. Evaluation then was continued for 5, 10, 15 and 20 minutes after halothane discontinuation. Cells in group 2 and 3 were given MgSO_4 11 mM and 22 mM after the exposure of halothane, respectively, and cells in group 4 and 5 were given MgSO_4 11 mM and 22 mM respectively, prior to the exposure of halothane. Halothane exposure was set at a concentration of 2 mM (equal to 1–3 MAC) for five minutes duration. The change in cytosolic Ca^{2+} was observed by laser scanning confocal microscope (Olympus FluoView 1000) and measured by pixel analysis for the emission.

Findings are presented as mean difference of cytosolic Ca^{2+} concentration compared to the control group or to the halothane group and evaluated by independent T-test or Mann Whitney test in SPSS. A p value of less than 0.05 was considered statistically significant.

3. RESULT
Halothane significantly increased cytosolic Ca^{2+} concentration in group 1 (Fig. 2). The administration of MgSO_4 before halothane exposure did not prevent this phenomenon in which the cytosolic Ca^{2+} concentrations were similar to that of the reference group (Table I).

Discontinuation of halothane decreased the cytosolic Ca^{2+} concentration in a time linear manner (Fig. 3). Five minutes discontinuation decreased the Ca^{2+} concentration by 14.6% of the initial concentration after halothane exposure, but this was not statistically significant.

Ten and 15 minutes discontinuation further decreased Ca^{2+} concentration by 30% and 57% of the level after initial concentration.
Table I. The effect of MgSO$_4$ before halothane exposure on cytosolic Ca$^{2+}$ concentration compared to control group.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean (±SD)</th>
<th>$p^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgSO$_4$ 0.5 $\mu$L</td>
<td>116.7 (±43.7)</td>
<td>0.8327</td>
</tr>
<tr>
<td>MgSO$_4$ 1 $\mu$L</td>
<td>122.7 (±32.7)</td>
<td>0.8217</td>
</tr>
</tbody>
</table>

Notes: *Statistical test used was T-test, $p < 0.05$ was significant.

Table II. Change of cytosolic Ca$^{2+}$ concentration as time of discontinuation of halothane.

<table>
<thead>
<tr>
<th>Discontinuation of halothane</th>
<th>Mean/median (±SD/min–max)</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 minutes</td>
<td>97.79 (±46.41)</td>
<td>0.6795</td>
</tr>
<tr>
<td>10 minutes</td>
<td>79.68 (±8.193)</td>
<td>0.3638</td>
</tr>
<tr>
<td>15 minutes</td>
<td>48.75 (32.98–79.89)</td>
<td>0.0286</td>
</tr>
<tr>
<td>20 minutes</td>
<td>1.00 (1.00 – 1.00)</td>
<td>0.0286</td>
</tr>
</tbody>
</table>

Notes: *Statistical test used was T-test, $p < 0.05$ was significant.
†Statistical test used was Mann-Whitney-U test, $p < 0.05$ was significant.

exposure, respectively, in which the later was statistically significant (Table II). Twenty minutes discontinuation returned the Ca$^{2+}$ concentration to the basal level before halothane exposure.

MgSO$_4$ administration after halothane exposure also did not significantly decrease cytosolic Ca$^{2+}$ concentration, but there was a trend of decreasing Ca$^{2+}$ concentration with higher dose of Mg$^{2+}$ (Figs. 3–5). The administration of 11 mM of MgSO$_4$ (0.5 $\mu$L) resulted in a decrease in cytosolic Ca$^{2+}$ concentration by more than 20%, while 22 mM (1 $\mu$L) of MgSO$_4$ decreased the cytosolic Ca$^{2+}$ concentration by more than 30%, which was similar to the effect of 10 minutes discontinuation of halothane exposure.

4. DISCUSSION

In this study, we found that the administration of MgSO$_4$, either before or after halothane exposure, did not alter the increase in cytosolic calcium concentration induced by halothane exposure. However, discontinuation of halothane exposure returned the calcium concentration to basal level after 20 minutes.

The release of Ca$^{2+}$ from SR to cytosol is the basic mechanism that initiates contraction in skeletal and cardiac muscles. In physiologic condition, the activation of RyR by action potential (in skeletal muscles) or by Ca$^{2+}$ influx through dihydropiridine...
(DHPR) is the key step in inducing SR to release its Ca\(^{2+}\) content. Halothane has been shown to activate RyR in skeletal muscle. In MH-susceptible patients, this will cause a large Ca\(^{2+}\) release, which further increases cytosolic Ca\(^{2+}\) concentration and induces muscle hypercontracture.\(^5\) In this study, halothane also increased cytosolic Ca\(^{2+}\) concentration. The accumulation of Ca\(^{2+}\) in the cytosol is the probable mechanism underlying cardiac arrhythmia caused by halothane exposure. In normal condition, this accumulation does not occur because of the availability of Ca\(^{2+}\) clearance mechanisms, including the SERCA activity.

Theoretically, free cytosolic Mg\(^{2+}\) may augment SERCA activity,\(^7\) decreasing cytosolic Ca\(^{2+}\) concentration. In this study, Mg\(^{2+}\) in the form of MgSO\(_4\) did lower cytosolic Ca\(^{2+}\) concentration as seen by decreased laser emission of the cells, and the effect was more prominent in higher magnesium dose, however it was not statistically significant. There are several possible explanations for this. First, the doses of administered Mg\(^{2+}\) might not be appropriate for cellular setting. We used doses similar to that are used in clinical practice (around 2 g or 50 mg/kg, maximum dose 4 g, equals 5 to 10 mL of 40\% MgSO\(_4\)). According to this, when applied to culture cells, the doses were very small, which were 0.2 mg and 0.4 mg or 11 mM and 22 mM. The administration of MgSO\(_4\) was done in the cell suspension. Increasing the dose results in cell dilution, making microscope visualization difficult. Another possible reason is that the duration of MgSO\(_4\) administration was probably too short and the amount of cells analyzed was insufficient.

Although statistically not significant, the trend of decreased cytosolic Ca\(^{2+}\) concentration with higher dose of MgSO\(_4\) may indicate some clinical benefits. For anesthesia practitioners in rural areas or small hospitals who still often use halothane, the administration of MgSO\(_4\) could be an option to manage intra-anesthesia arrhythmia.

The effect of Mg\(^{2+}\) in reducing cytosolic Ca\(^{2+}\) concentration after halothane exposure in this study was probably mediated by the augmentation of SERCA activity since the major mechanism of Ca\(^{2+}\) clearance from the cytosol is the reuptake by SERCA. Calcium efflux through Ca\(^{2+}\)-ATPase only occurs for Ca\(^{2+}\) in the T-tubule and not for cytosolic Ca\(^{2+}\). Sarcolemmal Ca\(^{2+}\) efflux via NCX eliminates both Ca\(^{2+}\) in the T-tubule and cytosol, but only accounts for 20\% of the total cytosolic Ca\(^{2+}\) concentration,\(^3\) while up to 70\% of cytosolic Ca\(^{2+}\) are restored in the SR by SERCA pump.

We found that the administration of MgSO\(_4\) prior to halothane exposure did not prevent the increase in cytosolic Ca\(^{2+}\). This is not in line with previous finding,\(^6\) showing that a cytosolic free Mg\(^{2+}\) concentration of 1 mM would inhibit RyR and diminish Ca\(^{2+}\) release from SR. Physiologic concentration of intracellular free Mg\(^{2+}\) is 0.8–1.2 mM.\(^11\) In high cytosolic concentration, Mg\(^{2+}\) has the antagonistic effect on A (activation)-site of RyR and acting as agonist on the I (inhibitory)-site as shown in MH skeletal muscle.\(^6\) Balog et al. administered an exact dose of 50 \(\mu\)M of Mg\(^{2+}\) directly to the cytosol by micro pipette. However, in this current study, the MgSO\(_4\) was added as an extracellular Mg\(^{2+}\). Although Mg\(^{2+}\) can easily diffuse into the cell, we did not measure the actual Mg\(^{2+}\) concentration that diffused into the cytosol as free ions.

Time is another consideration. The active form of Mg\(^{2+}\) is in the dehydrated form. To be able to enter sites or small canals and to compete for Ca\(^{2+}\), Mg needs to detach two water layers, while Ca\(^{2+}\) only detaches one layer.\(^1\) This probably explains why Mg\(^{2+}\) given prior to halothane exposure failed to inhibits RyR in this study. More time is probably needed to allow Mg\(^{2+}\) diffusion into the cytosol. Further studies evaluating the effects of Mg\(^{2+}\) should take this diffusion time into account.

We found that cessation of halothane exposure decreased cytosolic Ca\(^{2+}\) concentration in time linear manner. Specifically, cessation for 15 minutes resulted in an approximately 60\% decrease of the emission after halothane exposure, and after 20 minutes the emission totally disappeared. This was in line with previous findings (Drici et al.), showing reversal of halothane-induced arrhythmia after drug wash out.\(^14\) From clinical practice point of view, this finding suggests that in surgeries involving epinephrine injection, halothane exposure should be stopped for 15 minutes before injection, instead of the traditional practice of 5 minutes.

As a conclusion, our findings suggest that halothane increases cytosolic Ca\(^{2+}\) concentration in cardiac myocytes. The administration of Mg\(^{2+}\) before halothane exposure does not prevent this halothane-induced increase of cytosolic Ca\(^{2+}\) concentration, whereas high dose administration after halothane exposure, although not statistically significant, may decrease cytosolic Ca\(^{2+}\) concentration. Cytosolic Ca\(^{2+}\) concentration decreases significantly after discontinuation of halothane exposure for 15 minutes and returns to basal level after 20 minutes.

**Conflict of Interest**

There is no conflict of interest, including financial and scientific interest.

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**References and Notes**


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