# The Supplement of Magnesium Element to Inhibit Colorectal Tumor Cells

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# Abstract

Magnesium ions are essential elements to the human body, which need to intake about 350 mg per day for an adult. Recently, a meta-analysis reported that magnesium ions intake is related to a reduced risk of colorectal tumors. In addition, implantation of biodegradable magnesium pins after colorectal tumor resection has potential to inhibit the residual tumor cells. These impressive results implied that magnusium ions possess the posibility to against colorectal carcinoma. However, this hypothesis awaits confirmation by experimental results. In this work, we modulated different concentrations of magnesium ions and investigated the inhibitory effect on the cell viability through the cell cycle arresting, which subsequently induces apoptosis by activating the caspase-3 pathway. In animal experiments, the injection of magnesium restricted the size of tumor tissues for three weeks compared to the control group. The remarkable effect is attributed to the promoting apoptotic rate of tumor cells according to the results of immunohistochemistry and transmission electron microscopy. The promising evidence provides feasibility for the clinical use of magnesium implants to prevent recurrence after colorectal tumor surgery. The promising evidence provides feasibility for the clinical use of magnesium ions supplement to prevent recurrence after colorectal tumor surgery.


# 1. Introduction

Colorectal carcinoma (CRC) is the most widespread cancer in the intestinal tract system, which is a major public health issue. In 2020, CSC with a 10.0% incidence and 9.4% mortality rate are in the top three of all oncological diseases and being a trend towards younger age [1–3]. Combination therapy of surgical removal with chemotherapy is a typical treatment for CRC. However, postoperative recurrence and metastases are an ongoing problem for surgeons and patients, with the risk of secondary surgery or even life-threatening consequences. Recently, epidemiologic studies indicated that magnesium (Mg) intake may decrease the risk of CRC, although this observation is not universal [4, 5]. Zan et al [6] reported that the high-purity magnesium staples inhibit the viability and migration of CRC cells mainly due to the released hydrogen inducing tumor cells apoptosis through a p53-lysosome-mitochondria pathway[7]. However, the effect of magnesium ions ($Mg^{2+}$), the main degradation product of magnesium implants, on the CRC need to be uncovered.

$Mg^{2+}$, the second most plentiful divalent cation in the human body, is involved in hundreds of physiologic processes associated with energy metabolism and nucleic acid synthesis [8]. An adult requires 220–350 mg of $Mg^{2+}$ per day to maintain health recommended from the “Dietary Reference Intakes (DRIs) [9]. As a nutritional element in the body, $Mg^{2+}$ supplementation is advantageous for several medical conditions, such as inflammation [10], bone regeneration [11, 12], arrhythmia[13], and depression[14]. The homeostasis of $Mg^{2+}$ is regulated through the circulatory system and excessive $Mg^{2+}$ can be exerted by the feces and urine [15], without adverse effects due to $Mg^{2+}$ accumulation. A pioneering studies reported that serum $Mg^{2+}$ levels are associated with the immune system, which guarantees CD 8 + T cells to eliminate abnormal or infected cells in an $Mg^{2+}$-rich environment[16]. Besides, $Mg^{2+}$ inhibits the

proliferation and migration of bone tumor cells thtough a parallel pathway snail1-microRNA181c-NLK and snail1-microRNA181d-TIMP3[17]. Moreover, $Mg^{2+}$ has the potential to alleviate the side effects caused by platinum-based chemotherapeutic agents via the transient receptor potential melastatin 7 channel [18].

Here, we studied the efficacy of $Mg^{2+}$ on the viability, apoptosis, and cell cycle of colorectal tumor cells *in vitro*. Additionally, we investigated the effect of $Mg^{2+}$ on tumor tissue in animal experiments, which was constructed through a cell-derived xenograft model in nude mice. It is worthy to believe that magnesium acts an invaluable role in the future after the surgery of CRC.

## 2. Experimental Section

## 2.1 Cell culture

The colorectal adenocarcinoma cell line DLD-1and RKO was purchased from the Cell Bank of the Chinese Academy of Sciences. The medium of RPMI-1640 supplemented with 10% fetal bovine serum (FBS, Gibco, USA) was used to culture cells according to the cell culture guidelines. The cells were passaged twice a week using trypsin (Gibco, USA).

## 2.2 Cell viability

The number of 3000 DLD-1 or RKO cells were seeded in each well of 96-well plates. After 24h attachment, the culture medium was exchanged to culture medium supplemented with different concentrations (10 mM and 30 mM) of $MgCl_2$ powder. Cell viability was tested at the 24, 48, and 72 h time points by CCK8 assay (DOJINDO, Japan). After adding 100 μL of medium-CCK8 mix solution for 4 h, the results were measured by a microplate reader (Spark, Tescan, Czech Republic).

## 2.3 Live/dead staining

After $MgCl_2$ addition for 72 h, the DLD-1 cells were conducted Live/Dead staining. The cells were washed with PBS and stained by the LIVE/DEAD staining kit (Biotium, USA). Then, the plates were placed into a cell incubator for 20 min. Finally, the cells were washed once and photographed.

## 2.4 The analysis of cell apoptosis and cell cycle

Two groups were set up (Mg added group and normal medium group) to study the influence of Mg on tumor apoptosis. After 48 hours of culture, DLD-1 or RKO cells were stained by the apoptosis detection kit (Sony Biotechnology, Japan) and analyzed using flow cytometry.

Mg added medium and normal medium was used to culture DLD-1 or RKO cells for 48 hours. Cells were washed twice with PBS and fixed in 75% ethanol overnight in a -20 ℃ fridge. After being stained by a cell cycle kit (BD biosciences, USA), the cell cycle distribution was tested.

## 2.5 The immunofluorescence image of cleaved Caspase 3

After $Mg^{2+}$ treatment for 72 h, tumor cells were fixed and washed twice. Then, the cells were permeabilized and blocked for 1 h. Subsequently, the cells were stained by the antibody of cleaved caspase-3 (Abcam, UK) for overnight and counterstained with a secondary antibody. Confocal laser scanning microscopy was used to capture immunofluorescence images.

## 2.6 The effect of Mg on tumor model *in vivo*

The animal study was approved by the Approval of the Medical Research Ethics Review Committee of General Hospital of Ningxia Medical University. We used the cell line-derived xenograft (CDX) model to construct tumors in BALB/c nude mice (eight weeks, male). In brief, the density of $2\times10^6$ DLD-1 cells was subcutaneously injected into the back of mice. When the size of the tumor was over 125 $mm^3$, 10 mice were randomly assigned into the Mg injection group (namely Mg group) and control group. The tumor-bearing mice were injected with 10 μL saline as a control group. The Mg group got a 500 mM $MgCl_2$ injection every three days. On the last day, all mice are sacrificed and the tumor tissue, normal organs are collected to stain by H&E. The tumor weight was weighted and volume (V) of tumor tissue was calculated. The tumor tissues from each group were harvested for H&E staining, Ki67 immunohistochemical analysis and transmission electron microscope observation.

## 2.7 Statistical analysis

All data were presented as mean ± SD, and statistical analysis between multiple groups

was performed by the one-way analysis of variance using GraphPad Prism 8 software (GraphPad, USA). Statistical significance is represented by *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$..

# 3 Results And Discussion

## 3.1 The cell viability

To investigate the effect of $Mg^{2+}$ on the viability of colorectal adenocarcinoma cells, we adjusted the culture medium with 15 mM or 30 mM of $MgCl_2$ and cultured the DLD-1 cells and RKO cells. Meanwhile, the culture medium without $Mg^{2+}$ addition was set as the control group. The $Mg^{2+}$ concentration was chosen to be within the optimal osmolality range of the cells which does not directly disrupt cell growth due to the increase in osmotic pressure [17, 19]. As shown in Fig. 1a, the cell viability of the DLD-1 cells was significantly alleviated after 48 h treatment of the $Mg^{2+}$ and gradually decreased with the increase of $Mg^{2+}$ concentration. After 72 h treatment, the cell viability decreased 71.7% in the 30 mM $Mg^{2+}$ group and 11.7% in the 15 mM $Mg^{2+}$ group compare to the control group. The RKO cells showed less sensitivity to $Mg^{2+}$ when its concentration is 15 mM. After 72 h treatment, the cell viability decreased 85.8% in the 30 mM $Mg^{2+}$ group and 5.1% in the 15 mM $Mg^{2+}$ group compare to the control group.

The live/dead staining also showed that $Mg^{2+}$ suppressed the viability of DLD-1 cells in a dosed manner. As shown in Fig. 2, many dead cells (red) with few live cells are found in the group supplement with 30

mM $Mg^{2+}$, while more live cells and fewer dead cells were found in the control group.

## 3.2 The apoptosis

Apoptosis is a programmed cell death process, which has been an essential factor in clinical oncological therapy to eliminate the tumor cells [20, 21]. In recent studies, compelling evidence indicates that the initiation of apoptosis effectively inhibits tumor cell recurrence, subsequently influences proliferation and differentiation [22]. Therefore, we investigated the interaction between $Mg^{2+}$ and the incidence of apoptosis in DLD-1 cells and RKO cells. After 72 h treatment, the apoptosis rate of DLD-1 cells was increased with the enhancement of the dose of $Mg^{2+}$, and the apoptosis rates in the 15 mM and 30 mM groups were 13.2 ± 0.6% and 33.0 ± 3.0%, respectively, which shows significant difference from the control group (10.7 ± 2.8%) (Fig. 3a). Meanwhile, the apoptosis rates of RKO cells in the 15 mM was 8.4 ± 0.9%, and 30 mM groups was and 98.2 ± 0.5%, respectively. $Mg^{2+}$ exhibit encouraging apoptotic efficacy on RKO cells.

The family of caspase, especially caspase-3, holds a critical function in programmed cell death and activated protease in apoptosis [23]. We used FITC-conjugated cleaved caspase-3 to evaluate the expression in DLD-1 cells after $Mg^{2+}$ therapy. Among all groups, the expression of cleaved caspase-3 (green) is the highest in the 30 mM $Mg^{2+}$ group (Fig. 4), indicating an increased pro-apoptotic protein to amplify the apoptosis of tumor cells [24]. The results of the corresponding apoptosis assay and caspase-3 expression jointly indicate that $Mg^{2+}$ can induce apoptosis in colorectal adenocarcinoma DLD-1 cells *in vitro*.

## 3.3 The cell cycle

The link between proliferation and apoptosis is regulated by the cell cycle proteins, such as p21, cdk2 [25–27]. It has been reported that antitumor agents target the predisposition of tumor cells to rapidly duplicate their DNA, and arrest tumor cells in the G0/G1 phase to induce apoptosis [28]. Here, we found that the addition of $Mg^{2+}$ is a feasibility to induce DLD-1 cells arrest in G0/G1 phase. The percentages of G0/G1 phase in the control group, 15 mM $Mg^{2+}$ added group, and 30 mM $Mg^{2+}$ added group are 45.7 ± 1.7%, 51.6 ± 1.8% and 60.6 ± 1.4%, respectively (Fig. 5). In comparison to the control group, both the $Mg^{2+}$ added groups markedly arrested in the G0/G1 phase ($P$ 0.001). This finding reveals that $Mg^{2+}$ inhibits proliferation and promotes apoptosis of colorectal adenocarcinoma cells by increasing the percentage of G0/G1 phase [29].

Based on the above results, the supplement $Mg^{2+}$ can promote apoptosis in a dosed manner through the regulation of the cell cycle of the DLD-1 tumor cells, subsequently inhibiting the proliferation *in vitro*.

## 3.4 The effect of Mg on the tumor model in mice

To evaluate the therapeutic effect of $Mg^{2+}$ *in vivo*, we establish a subcutaneous tumor model in BALB/c nude mice. Furthermore, administration of the $Mg^{2+}$ injection group with the control group is assessed the

therapeutic performances, respectively, which received an injection of saline (control group) or $MgCl_2$ solution (Fig. 6a). Furthermore, the dose of $Mg^{2+}$ injection was referred according to the ref. [30]. After treatment for 21 days, all tumor tissues of mice were harvested for volume measurement and weighting. As demonstrated in Fig. 6, the volume and weight of tumor tissue are significantly decreased in the Mg group in contrast to the control group. Hence, it was found that sufficient $Mg^{2+}$ addition could efficiently decrease the growth of the tumor tissue *in vivo*.

As shown in the H&E staining images, the purple staining cells indicate that the majority of the tumor cells were alive in the control group. On the contrary, the Mg group induced more tumor cells apoptosis, performing some necrotic areas occurred without abundant inflammatory cell infiltration. In addition, the analysis of Ki67 staining was consistent with the H&E staining, showing fewer proliferate cells after Mg treatment. We also found many nuclei of tumor cells in the Mg group have undergone karyorrhexis and karyolysis by transmission electron microscope (TEM), which is typical apoptotic performance (Fig. 7). These results indicated that Mg effectively inhibits the growth and induces apoptosis of colorectal adenocarcinoma DLD-1 cells *in vivo*, in agreement with the results found in cell experiments.

# 4. Conclusion

In summary, we demonstrated the anti-tumor property of Mg ions in colorectal adenocarcinoma. The Mg ions induce apoptosis of tumor cells in a dosed manner through the cell cycle G0/G1 arresting, then inhibit the proliferation. The intra-articular injection of Mg ions alleviates the growth of tumor tissue in the nude mice. The supplementary Mg ions induce apoptosis of the tumor in the tumor-bearing mice. Combining all results, we provide essential evidence for Mg and Mg implants for clinical use in future surgery.

# Declarations

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Author Contribution: Heng Li and Xiaonan Feng designed this study. Hai Li conducted the cell experiments. Shuo Ma did the animal experiments. Wei Song wrote the first version of the manuscript. Bao Yang did the data analysis. Jiang Tao and Chun Yang reviewed the manuscript.

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Data availability: The data are available from the corresponding author.

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# Figures

## Figure 1

The effect of $Mg^{2+}$ on the viability of DLD-1 (a) and RKO (b) cells for 24, 48 and 72 h, respectively. n=12, *p<0.05, **p<0.01, ***p<0.001.

## Figure 2

The live/dead staining of DLD-1 cells with different concentrations of $Mg^{2+}$ treatments for 72 h.

## Figure 3

The apoptosis rate and corresponding statistics of DLD-1 (a) and RKO (b) cells with different treatments in 72 h. n=4

## Figure 4

The expression of cleaved caspase 3 of DLD-1 cells for 72 h treatment.

## Figure 5

The cell cycle of DLD-1 cells in different groups after 0, 15 and 30 mM $Mg^{2+}$ treatment for 72 h. n=4

## Figure 6

(a) Schematic diagram of the Mg ions treatment protocol for tumor therapy *in vivo*. (b) The photograph of the isolated tumor tissue after 21 days of treatment. Scale bar: 1 mm. (c, d) The volume (c) and weight (d) of tumors in different groups after 21 days of treatment. n=6

## Figure 7

H&E, Ki67 and TEM analysis of the tumor tissue of the mice on day 21st.