Chemopreventive Effects of Magnesium Chloride Supplementation on Hormone Independent Prostate Cancer Cells

Saheed Oluwasina Oseni, *Elsa Quiroz, and James Kumi-Diaka

Department of Biological Sciences, Florida Atlantic University, Davie, FL 33314, USA

*Corresponding Author: Elsa Quiroz, Department of Biological Sciences, Florida Atlantic University, Davie, FL 33314, USA

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ABSTRACT

Background: Lifestyle significantly impacts the risk factors associated with prostate cancer, out of which diet appears to be the most influential. An emerging chemopreventive approach, which involves the adequate intake of dietary constituents, has shown great potential in preventing the occurrence or progression of cancer. Magnesium is known to be an essential cofactor for more than 300 enzymatic processes, and is responsible for the regulation of various cellular reactions in the body. A plethora of studies have shown evidence that changes in the intracellular levels of magnesium could contribute to cell proliferation and apoptosis in some normal and malignant cells. The aim of the study was to investigate the effects of magnesium chloride (MgCl$_2$) in DU-145 prostate cancer cells.

Methodology: Cultured DU-145 cells were subjected to graded concentrations or doses (50-500 µM) of MgCl$_2$ for 48 hours. The cell viability was assessed using MTT and Resazurin reduction assays. NBT assay was also used to assess the treatment-induced intracellular ROS levels. Acridine Orange/Ethidium Bromide (AcrO/EtBr) and Rh123/EtBr fluorescent stains were used to assess the cell death type and mitochondrial membrane potential (Δψm) respectively.

Results: The results revealed a dose-dependent decrease ($P < 0.05$) in cell viability in treated DU-145 cells after 48 hours. The NBT assay also revealed a dose dependent biphasic response ($P < 0.05$) in intracellular levels of ROS. There was a drop ($P < 0.05$) in ROS levels in all groups except at 100 µM, where ROS level was higher than the control. Apoptosis was the primary mode of cell death as observed in the fluorescence analysis.

Conclusion: Our finding suggests that MgCl$_2$ may be potentially chemopreventive for prostate cancer. This justifies further studies into its mechanism of action in DU-145 and other prostate cancer cell types.
**INTRODUCTION**

Prostate cancer (PCa) is the 2nd leading cause of cancer in U.S. men, and it represents 13.3 % of all cancer cases in the U.S. [1]. In 2015 alone, an estimated 220,800 new cases and 27,540 deaths are expected to occur due to prostate cancer, adding to the burden of the over 2.6 million men currently living with the disease in the country [2]. Lifestyle coupled with aging has been shown to be one of the predisposing factors that may account for about 75 percent of the prostate cancer cases, and of all the environmental variables, diet appears to be the most influential [3]. An emerging chemopreventive approach involving the adequate intake of dietary constituents has shown great potential in preventing the occurrence or progression of cancer [4].

Magnesium is known to be an essential cofactor for more than 300 enzymatic processes responsible for the regulation of various cellular reactions in the body [5]. Magnesium has been shown to be an indispensable element in all of the body's natural self-cleansing and detoxification responses due to its ability to chelate with intracellular ATP and competitively bind with calcium for binding sites on proteins and cell membranes [6]. Without sufficient magnesium, toxic waste and acid residues accumulate in cells and tissues, setting the stage for chronic degenerative conditions, cancer, and rapid aging symptoms [7]. Plethora of studies have shown evidences that change in the intracellular levels of minerals and micronutrients, such as magnesium, could contribute to the modulation or regulation of cell proliferation and apoptosis in some normal and malignant cells [8].

Magnesium occurs naturally in many forms, but is most readily assimilated and utilized for metabolic purposes. The distribution of magnesium in the body is as such; 60 % in bone, 20 % in skeletal muscle, 19 % other soft tissues, and less than 1 % in blood serum [9]. It has been shown that the average diet in the U.S. is deficient in magnesium [10]. This was confirmed through a survey that was performed to determine the daily intake of magnesium in U.S. population, and was found to be 32 % and 20 % less than the daily recommended dietary allowance (RDA) in women and men respectively [11]. This was also found to be similar in most other countries.

Prostate cancer is a slow growing tumor, and has been documented to be strongly correlated with aging, with the highest prevalence indicated in men above 60 years of age, but not below 40 [1]. A link between magnesium and aging has been a topic of discussion in literatures, with insufficient intake of magnesium in diet or variance in magnesium metabolism associated with aging process and susceptibility to age-related diseases [12]. For instance, magnesium imbalance in elderly people was found to be associated with inflammation, cardiovascular diseases, diabetes, and stress vulnerability [7]. However, metabolism in normal and abnormal cells is markedly different, and the links between cancer and magnesium status perhaps may rely on cellular and subcellular backgrounds. The aim of the study was to investigate the chemopreventive effects of magnesium chloride (MgCl₂) supplementation on DU-145 hormone refractory prostate cancer cells.
MATERIALS AND METHODS:

Cell Line and Cell Culture: DU-145 cell line was purchased from ATCC (Manassas, Virginia, USA). It is a hormone independent prostate cancer cell line. The DU-145 cells were cultured and maintained in complete RPMI 1640 (Sigma Aldrich Chemical Co., St Louis, Mo, USA) media with 10 % Fetal Bovine Serum and 1 % penicillin/streptomycin.

Micronutrient: Magnesium Chloride (MgCl₂) (100 % pure) was purchased from Seychelles Organics, Inc. (Life-Flo, Bowling Green, Florida, USA) and dissolved into a stock solution (1 mM) with deionized water. Stock solution of MgCl₂ was further diluted with RPMI-media to produce aliquots ranging in concentration from 50 - 500 μM.

Treatment: The DU-145 cells were cultured in 25 cm³ flask at 37°C, 5 % CO₂ and 89 % - 90 % humidity to achieve 80 % - 90 % confluence. The cells were harvested, centrifuged and reconstituted into suspension with fresh RPMI 1640 media. 1 × 10⁴ cells in 100 μL of media were dispensed into each well of 96 well microtiter plates and cultured for 48 hours to allow adherence and to obtain greater than 80 % confluence. The media (supernatants) for each well were aspirated and the adhered cells were treated with graded concentrations of MgCl₂ (50, 60, 70, 80, 90, 100, 200, 300, 400, 500 µM). Experiments had a control group consisting of DU-145 cells cultured in RPMI 1640 media (with 10 % FBS, 1 % penicillin/streptomycin and L-glutamine) without treatment (0 µM). All treatments were done in quadruplicates, and cultured for 24 - 48 hours in a humidified incubator at 37°C and 5 % CO₂. At 24 and 48 hours post-treatment/culture, cells were subjected to bioassay analyses as follows:

Trypan Blue Exclusion Assay: Trypan blue exclusion assay was used to assess the cell viability and initial concentration of the DU-145 cells added to the microplate wells prior to treatment, according to standard procedures using a Neubauer counting chamber. The trypan blue exclusion assay allows for a direct identification and enumeration of live (unstained) and dead (blue) cells in a given population. Briefly, the trypan blue stock (0.4 %) was diluted with PBS to 0.2 % and filtered the trypan blue with 0.2 micron filter. The cell suspension was then mixed with the 0.2 % trypan blue at 1:1 ratio. Counting chamber slide was loaded and analyzed.

MTT Assay: MTT [3-(4, 5-dimethyl thiazolyl-2)-2, 5-diphenyltetrazolium bromide], a tetrazolium dye, is used to determine metabolic status and cell viability. Since the resulting color intensity correlates directly with the amount of metabolically active cells in each well, cell viability can be quantitatively determined by measuring the optical density (OD) in individual wells. After briefly treating the cells as described previously, a volume of 20 μL of MTT reagent was added to each well at 48 hours post-treatment. The plates were further incubated at 37°C and 5% CO₂ for 4 hours in the dark, after which a volume of 100 μL of DMSO was added to the cells to solubilize the formazan. Absorbance (OD) of the resultant solution was read at 490 nm using a BioTek microplate reader (Winooski, Vermont, USA). The relative population of live cells could therefore be determined based on the optical absorbance (optical density, OD) of the sample. The OD obtained was graphed against the concentrations of the MgCl₂. The values of the blank wells
were subtracted from each well of treated and control cells; and the mean percentage of post-
treatment viable cells relative to the controls was calculated as shown:
Cell viability (%) = \( \frac{A_T - A_B}{A_C - A_B} \)

Where \( A_C \) is the absorbance of the control (mean value), \( A_T \) is the absorbance of the treated
cells (mean value), and \( A_B \) is the absorbance of the blank (mean value). The IC\(_{50}\) was
extrapolated from the graphs as a measure to determine the concentration required to obtain
expected results (potency).

**Resazurin (Alamar Blue) Reduction Assay:** Resazurin (Alamar blue) is a cell permeable redox
indicator that can be used to monitor viable cell number with protocols similar to those utilizing
the tetrazolium compounds, but slightly more sensitive than tetrazolium reduction assays. Viable
DU-145 cells with active metabolism can reduce resazurin into the resorufin product, which is
pink and fluorescent. This resorufin can then be quantified by measuring a change in absorbance.
Briefly, high purity resazurin was dissolved in DPBS (pH 7.4) to 0.15 mg/ml to make a stock
solution. This was then Filter-sterilized through a 0.2 μm filter into a sterile, light protected
container and stored from light at 4°C for frequent use. 20 μL resazurin solution to each well
containing MgCl\(_2\) treated DU-145 cells (in 96-wells microplate at a final volume of 100 μL/well
after 48 hours of incubation at 37°C and 5 % CO\(_2\)). This was thereafter incubated for 4 hours at
37°C, and the absorbances (OD) of the resultant solution were read at 490 nm using a BioTek
microplate reader (Winooski, Vermont, USA).

**Nitro-Blue Tetrazolium (NBT) Assay:** On the other hand, Nitroblue tetrazolium (NBT) is a
yellow water-soluble nitro-substituted aromatic tetrazolium compound that reacts with cellular
superoxide ions to form formazan derivative that can be monitored spectrophotometrically. The
cytoplasmic NADPH, which is produced by oxidation of glucose through the hexose
monophosphate shunt, serves as an electron donor, therefore the oxidase system available in the
cytoplasm helps transfer electrons from NADPH to NBT and reduce NBT into formazan. Thus,
the NBT reaction indirectly reflects the ROS-generating activity in the cytoplasm of DU-145
cells. Briefly, DU-145 cells were seeded at 1× 10\(^4\) cells per well in 96- well microplates and
allowed to attach for 24 hours at 37°C and 5 % CO2. Relevant plates were treated as discussed
previously. NBT (1 mg/mL) in HBSS medium was added to the wells 48 hours after treatment,
and incubated for 4 hours at 37°C in the dark. NBT formazan formed in each wells were then
solubilized with 100 μl of DMSO for 30 minutes. ROS production levels were quantified using
an absorbance microplate reader at 490 nm. The results were expressed as relative percentage of
ROS, and then normalized in which the control was zeroed.

**Acridine Orange/Ethidium Bromide Fluorescence Assay:** The acridine orange
(AcrO)/ethidium bromide (EtBr) fluorescence assay was used to differentiate between viable,
apoptotic, and necrotic cells based on fluorescence emission characteristics of acridine orange
and ethidium bromide. Acridine orange permeates both viable and nonviable cells, causing the
nuclei to emit green fluorescence. Since absorption of ethidium bromide is based on the
disruption of cell membrane integrity, ethidium bromide selectively stains the nuclei of dead
(non-viable) cells to produce orange or red fluorescence. Cells that emit orange/brown colored
fluorescence are indicative of apoptosis, while necrotic cells emit red fluorescence. Briefly, ethidium bromide (25 μL) and acridine orange (75 μL) were mixed to make a cocktail in which 3 μL of it was added to 25 μL of the cell suspensions. Wet-mounts were prepared using 10 μL of each cell suspension and analyzed under a fluorescent microscope with a bandpass filter. Apoptotic cell death was quantified by counting a total of 100 cells per 2 to 3 fields of view. For each relevant treatment regimen, fluorescence microphotographed pictures were taken directly from under the fluorescence microscope using a digital camera (Nikon: Coolpix VR & ISO 2000, Japan).

**Rhodamine 123/Ethidium Bromide Fluorescence Assay:** The rhodamine 123 (Rh123)/ethidium bromide (EtBr) fluorescence assay was used to assess the mitochondrial membrane potential (ΔΨm) in treated cells and also used to differentiate between viable, apoptotic, and necrotic cells, as well as to determine the possible mechanism of apoptosis, based on mitochondrial transmembrane potential. Rh123 is a cationic fluorochrome, which utilizes the transmembrane potential of active mitochondria to diffuse into cells. The intact mitochondrial membranes of viable DU-145 cells allow for the absorption of the dye, resulting in the emission of bright green fluorescence. DU-145 cells, in which the integrity of the mitochondrial membrane has been impaired (non-viable cells), stain lightly with Rh123. Meanwhile, ethidium bromide selectively enters the disrupted membranes of dead cells and stains the nuclei to produce orange and red fluorescence. Cell samples were briefly washed three times in PBS and re-suspended in a final volume of 20 μL. 2 μL of the Rh123 stock solution was added to each cell suspension and was incubated at 37°C for 5 minutes. 20 μL of EtBr stock solution was then added to each cell suspension and this was incubated at room temperature for 5 minutes. Finally, 10 μL of each cell mixture was transferred onto a microscope slide covered with a coverslip and examined/analyzed under a fluorescent microscope with a band-pass filter. Green fluorescence is indicative of viable cells; orange/brown cells are apoptotic while necrotic cells emit red fluorescence. The percentage of apoptotic cell death was quantified from an average of 100 cells spread across two to three regions/views on each slide.

**Statistical analysis:** Experiments were performed in quadruplicate and repeated twice to confirm results. Significance of the differences in mean values was determined using Microsoft excel (v. 2013) and the Student’s t-test. Statistical significance was defined as $P < 0.05$.

**RESULTS:**

**MgCl$_2$ supplementation inhibited growth and proliferation of DU-145 prostate cancer cells:** DU-145 human prostate cancer cell line was used to determine the chemosensitivity of hormone independent prostate cancer to MgCl$_2$ supplementation in vitro using MTT and Resazurin (Alamar blue) reduction bioassays. MgCl$_2$ was found to significantly inhibit cell growth and also decrease cell survival or viability [Figure 2 & 3]. The data indicated that DU-145 sensitivity to MgCl$_2$ supplementation is dose-dependent. At lower concentrations, the DU-145 was significantly more sensitive ($P < 0.05$) at 90 and 100 μM concentration with cell growth inhibition of 41% and 47% respectively from the MTT analysis, and 49 % and 42 % from the
Alamar blue analysis. At higher concentrations, the greatest effect in this study was significantly greatest \((P < 0.05)\) at 500 \(\mu M\) concentration. From the MTT and Alamar blue cytotoxicity results, the IC\(_{50}\) was determined to be 460 \(\mu M\) and 440 \(\mu M\) respectively or within 440 - 460 \(\mu M\) range when put together [Figure 2 & 3]. Figure 1A & B are showing the micrographs of the control and MgCl\(_2\) induced cytotoxic effect at 500 \(\mu M\) respectively; characterized by massive population of apoptotic cells compared to the control.

**Figure 1 A & B:** Phase contrast micrographs (200X) of untreated and treated DU-145 cells. Figure 1A: Showing the micrographs of DU-145 cells on first day of culture in RPMI 1460 media. Figure 1B is showing the micrographs of untreated viable DU-145 cells at 85 % confluence. Figure 1B is showing micrograph of DU-145 cells following treatment with MgCl\(_2\) at 500 \(\mu M\). The red arrow points to a cell that is sensitive (apoptotic) to the treatment. Other cells showing different hallmarks of apoptosis were observed. This micrograph was taken 48 hours post-treatment.

**Figure 2:** Graph showing the dose-effect curve of MgCl\(_2\)-induced cytotoxicity in DU-145 PCa cells via MTT assay. There was a dose-dependent decrease \((P < 0.05)\) in cell viability in treated DU-145 cells with graded concentrations from 50 to 500 \(\mu M\) of MgCl\(_2\) after 48 hrs. Data points represent the mean values ± SEM of two independent experiments performed in quadruplicate \((P < 0.05)\).
**Figure 3:** Graph showing the dose-effect curve of MgCl\(_2\)-induced cytotoxicity in DU-145 PCa cells via Resazurin (Alamar Blue) Reduction Assay. There was a dose-dependent decrease (\(P < 0.05\)) in cell survival in treated DU-145 cells with graded concentrations from 50 to 500 μM of MgCl\(_2\) after 48 hrs. Data points represent the mean values ± SEM of two independent experiments performed in quadruplicate (\(P < 0.05\)).

**MgCl\(_2\) supplementation induced a compromise in the mitochondrial membrane potential in DU-145 human prostate cancer cells:** In order to determine the effect of different doses (0, 50, 100, 500 μM) of MgCl\(_2\) supplementation on the mitochondrial membrane integrity, Rhodamine 123/ Ethidium bromide fluorescent stain was used. This also gives us an assessment of the type of cell death and the mechanism by which apoptosis was programmed. The Rh123/EtBr fluorescent revealed that about 97 % of the cells in the control (0 μM) stained green, which demonstrate an intact mitochondrial 3 % undergoing stress induced apoptosis. Following 48 hours incubation of DU-145 treated with MgCl\(_2\) at doses of 50, 100 and 500 μM, we observed a significant dose-dependent increase (\(P < 0.05\)) in apoptotic cells (27 %, 48 % and 54 % respectively) with compromised mitochondria and reduced transmembrane potential [Figure 4]. They visibly stained orange-brown. This probably suggests that MgCl\(_2\) supplementation utilize the intrinsic-mitochondrial apoptotic pathway and the activation of caspase proteases for its chemopreventive effects in hormone independent prostate cancer.
Figure 4: Bar-charts showing MgCl₂-induced effects on the mitochondrial membrane potential (Δψm) of DU-145 PCa cells via Rh123/ EtBr Fluorescence Assay. There was a significant dose-dependent increase (P < 0.05) in compromise of the Δψm. Apoptosis appears to be the major mode of cell death. At the highest concentration (500 μM) more apoptotic cells were observed with reduced mitochondrial transmembrane potential. Data points represent the mean values ± SEM of two independent experiments performed in quadruplicate (P < 0.05).

MgCl₂ supplementation induced apoptosis in DU-145 human prostate cancer cells

Extensive cell deaths if the treatment induced cell death occurred through cytotoxic necrosis and/or apoptosis, cells were fluorescently stained with AcrO/EtBr after 48 hrs. of incubation, in which live cells pick up the acridine orange dye and stain green, while the damaged cells pick up the ethidium bromide dye and stain orange-brown, depending on the stage of apoptosis. This can also give us a qualitative state of apoptotic cell death in different cells exposed to different levels of treatment, especially at 0, 50, 100, and 500 μM concentrations of MgCl₂. The micrographs qualitatively show that cell death was mostly due to apoptosis in a dose-dependent manner [Figure 7 A - F]. More apoptotic cell death (orange-brown stained cells) was observed in micrographs containing DU-145 exposed to MgCl₂ at 500 μM compared to at 50 μM.
Figure 7 A to F: Micrographs (200X) of Acridine Orange / Ethidium Bromide fluorescently stained DU-145 cells following treatment with MgCl₂ at 50 μM, 100 μM, and 500 μM. Showing qualitatively apoptosis as the main form of induced cell death in a dose-dependent mode. Figures 7A, 7C and 7E are showing the micrographs of AcrO fluorescently stained DU-145 cells at 50 μM, 100 μM, and 500 μM respectively. Figures 7B, 7D, and 7F are showing the micrographs of EtBr fluorescently stained DU-145 cells at 50 μM, 100 μM, and 500 μM correspondingly. Acridine orange stained live cells green and Ethidium bromide stained apoptotic cells orange-red depending on the stage of apoptosis. This micrograph was taken 48 hours post-treatment.
MgCl₂ supplementation increased ROS generation in DU-145 human prostate cancer cells
Since we already elucidated the effects of MgCl₂ supplementation in DU-145, our next goal was to assess the role of MgCl₂ on intracellular ROS generation. The NBT assay was used to assess the change in intracellular ROS levels following exposure of DU-145 cells to different concentrations of MgCl₂ at 50, 60, 70, 80, 90, 100, 200, 300, 400, 500 µM compared to the control (0 µM). The data was zeroed to show the increase or decrease in ROS levels relative to the control or untreated group. NBT assay ROS data also revealed a dose dependent biphasic response ($P < 0.05$) in intracellular levels of ROS in DU-145 cells. There was a drop ($P < 0.05$) in ROS levels in all treated groups except at 100 µM, where ROS levels were found to be higher than the control [Figure 5]. This suggests that at 100 µM supplementation, there was a shift from its antioxidant activity to pro-oxidant activity, but further increase in the dose of MgCl₂ returns the ROS levels to below the control again. Put together, there is the possibility that MgCl₂ inhibit DU-145 cell growth via free radical scavenging in which the ROS levels are dropped below the normal required for the normal proliferation of the cancer cell. At 100 µM, a significant rise ($P < 0.05$) in ROS generation was observed. Either way, the cancer cell viability rate was still drastically reduced [Figure 6].

![Figure 5: Bar-chart showing the MgCl₂-induced ROS level in DU-145 PCa cell lines via Nitro-blue tetrazolium (NBT) assay. There was a biphasic dose-dependent response ($P < 0.05$) in intracellular levels of ROS in DU-145 PCa cells with graded concentrations from 50 to 500 µM. Significant decrease in ROS levels below the control (0 µM) were observed except at 100 µM concentration in treated DU-145 cells after 48 hrs, where the ROS level was significantly higher ($P < 0.05$) than the control (0 µM). Data points represent the mean values ± SEM of two independent experiments performed in quadruplicate ($P < 0.05$).]
**DISCUSSION:**

The role of magnesium in cancer prevention and progression is still emerging and at present tainted with ambiguity from reports in the literature. This is due to multiple role of magnesium in the body, and it’s overly distribution in different tissues in the body [13]. The status of magnesium has been shown to be biphasic, in which changes in cellular magnesium can either stimulate or inhibit cancer growth and progression. In other words, this means that it has both anticancer effect and also carcinogenic effect. However, we believe that this variation in activity may be related to activation of cellular transduction pathways as a result of disparity in the concentration of magnesium in extracellular and intracellular compartments. Many studies have focused on the extracellular serum levels of magnesium as prognostic and diagnostic tools for hypomagnesemia or hypomagnesemia [5]; hence neglecting the intracellular regulation of magnesium in target tissues.

This inequality in intracellular and extracellular levels of magnesium becomes challenging when diagnosing magnesium deficiency-related disorders, and further supports the fact that serum magnesium levels may not precisely reflect the overall magnesium status of a patient. Moreover, tumor cells have been demonstrated to function independently of the extracellular status of magnesium [14].

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**Figure 6:** Graphs showing the correlation between the MgCl₂-induced Cell viability vs ROS levels. Both cell viability and ROS generation showed a dose-dependent response in DU-145 prostate cancer cells treated with different concentrations of MgCl₂. Even though at 100 μM, a significant increase \((P < 0.05)\) in ROS generation was perceived, cell viability was positively correlated. Data points represent the mean values ± SEM of two independent experiments performed in quadruplicate \((P < 0.05)\).
Drugs that have the ability to scavenge for magnesium, such as Cisplatin have been shown to lower the intracellular levels of magnesium in the body, hence causing hypomagnesemia [15 & 16]. Approximately 50% of the testicular cancer patients treated with Cisplatin were reported to have low levels of magnesium due to defect that may be related to magnesium absorption by the cells. This suggests that disproportionality in the intracellular levels of magnesium in cancer cells compared to normal cells in the body, as well as the level that can be found in the blood are factors that should be considered, when making a prognostic and diagnostic inferences, especially considering the role magnesium may be playing in cancer progression and inhibition.

In addition, hypomagnesaemia has also been associated with the incidence and decreased survival rate in chronic pathologies such as diabetes, cancer, coronary heart disease and hypertension [9]. In a histopathological study of a carcinomatous prostate gland, the prostate cancer cells failed to show staining reactions for magnesium despite appearing morphologically differentiated. Unlike in the cancer cells, the prostate epithelial cells showed a strong positive staining of the prostate acini [17].

The scope of this study was to investigate the role of magnesium supplementation in the prostate cancer development, and also assess the dose-dependent effects of magnesium chloride in DU-145 - a hormone independent prostate cancer cell line. The cancer cells were subjected to varied concentrations of magnesium chloride and the level of dose-response was assessed using batteries of cell proliferation and inhibition assays. In this study, DU-145 PCa cells were found to be responsive to MgCl₂ levels dose-dependently. A significant increase (P < 0.05) in cell growth inhibition was observed with an increase in concentration of MgCl₂. This is in congruity with prior studies, in which magnesium supplementation was found to show anti-carcinogenic effect on 3-MC-induced fibrosarcoma [13].

The major mode of cell death was found to be apoptosis, and also found to increase with an increase in the concentration of MgCl₂ supplementation. Apoptosis has been used for many years by researchers as a dosimeter for programmed cell death, which is controlled by the disparity in the expression of pro-death and anti-death molecules in the body [18]. This means that the presence of damaged or mutated cell in a tissue create a pathway geared towards the elimination of such abnormal cell. Magnesium has also been demonstrated to be involved in membrane transport and cellular repair [19].

In this study, DU-145 cells treated with MgCl₂ were found to show more sensitivity to magnesium levels, especially at a higher concentration of MgCl₂ (100 - 500 μM). We also observed that high concentrations of MgCl₂ caused a relative decrease in the mitochondrial membrane potential visualized from the fluorescence imaging and staining with Rh123/EtBr. This is in concert with prior studies, in which magnesium was shown to act as a second intracellular messenger involved in the regulation of apoptosis downstream. In addition, an increase in magnesium levels was correlated with a rise in cytochrome C release from the mitochondria, which is known as a driver for the post-mitochondrial, caspase-mediated apoptotic events in cells [20]. Another study conducted on drinking water containing magnesium in Taiwan show that a protective effect from the risk of prostate cancer was apparent in groups with the highest levels of magnesium in their body [21].

Moreover, the normal range for serum magnesium concentration in humans is between 750 and 950 μM/L [22]. In this study, our IC₅₀ was within 440 - 460 μM range, suggesting that the
presence of magnesium at high levels might be linked with decrease in tumor proliferation or growth; and hence may be of clinical significance in increasing the survival rate of prostate cancer patients.

Majority of the cell death recorded in this study were due to apoptosis, reflected by cells showing different hallmarks of apoptosis. Some of the observed hallmarks include; cell shrinkage, nuclear condensation, membrane blebbing, and formation of apoptotic bodies similar to what was previously reported [23]. In a prior study, an increase in the level of magnesium compounds to 1 mM concentration, was found to increase the apoptotic fragmentation of DNA in cells after 15 hours of culture and reported to be more pronounced at 30 hours post-treatment compared to when the cells were not exposed to magnesium compounds at all [24]. Another study reported Fas-initiated apoptosis as a result of Mg mobilization in B cells [25]. They also noticed that the increase in Magnesium mobilization correspond with an increase in DNA fragmentation and externalization of phosphatidylinerine, in which the cytosolic increase in levels of magnesium was found to be higher in cells undergoing apoptosis [12]. Thus, emphasizing the role of magnesium as a phase II intracellular messenger involved in downstream events in apoptosis.

Meanwhile, in another study, co-administration of magnesium with antioxidants such as vitamin C and vitamin E inhibited the apoptotic pathway in more than 50 % cases, indicating that magnesium may be involved in the cellular redox reactions and extracellular signaling for apoptosis [26]. This further buttress our finding in which dose-dependent biphasic ROS generation responses to magnesium chloride supplementation-induced cytotoxicity was observed. The results presented herein indicate that magnesium chloride supplementation has dose-dependent modulatory effects - anti-proliferative and apoptotic - on DU-145 prostate cancer cells, thus emphasizing its potential as a chemopreventive mineral for prostate cancer.

CONCLUSION:
Our finding suggests that magnesium chloride supplementation or a diet rich in magnesium may be potentially chemopreventive for prostate cancer. This justifies further studies into its mechanism of action in DU-145 and in other prostate cancer cell types.

Abbreviations: PCa: Prostate cancer; MgCl₂: Magnesium chloride; AcrO/EtBr: Acridine orange/Ethidium bromide; ROS: Reactive oxygen species; Rh123/EtBr: Rhodamine123/Ethidium bromide; Δψm: Mitochondrial membrane potential; NBT: Nitroblue tetrazolium assay; MTT: 3-(4, 5-dimethyl thiazolyl-2)-2, 5-diphenyltetrazolium bromide.

Competing Interests: All authors have declared that there is no conflicting interest.

Authors’ Contributions: All authors contributed equally to this study.

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