International Journal of Pharmacology

ISSN 1811-7775

science alert

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Asian Network for Scientific Information

International Journal of Pharmacology 5 (1): 13-21, 2009
ISSN 1811-7775

Anticancer and Biochemical Effects of Calcium Chloride on Ehrlich Carcinoma Cell-Bearing Swiss Albino Mice

Yousif A. Asiri
Department of Clinical Pharmacy, College of Pharmacy, King Saud University,
P.O. Box 2457, Riyadh-11451, Saudi Arabia

Abstract: The anticancer activity of calcium chloride was evaluated from the total count and viability of Ehrlich Ascites Carcinoma (EAC) cells and their proteins, nucleic acid, malondialdehyde (MDA) and Nonprotein sulfhydryl (NP-SH) groups in addition to observations on survival and the body weight changes. The tumors at site of injection were investigated for histopathological changes. The treatment with calcium chloride (50, 100 and 200 mg/kg/day) caused cytotoxic activity. These data are substantiated by biochemical and histopathological changes and are attributed to calcium ions, which are known to disrupt calcium homeostasis, produce ROS, damage mitochondria and cause DNA breaks. Nevertheless, these data were not in agreement with the results on survival and body weight of the same animals. Treatment at the higher doses of calcium chloride increased the body weight and restricted the life span much earlier than the lower dose of calcium chloride and ADM. The discrepancy between these results might be due to the difference in the time of observation. While the experiments on cytotoxic activity, biochemical investigation and histopathology were conducted 10 and 5 days after the implantation of EAC cells and treatment, respectively, the observations on body weight and mortality were continued until death of all the animals or up to a maximum of 50 days. Data obtained in the present study demonstrate that treatment of calcium chloride at the higher doses had no influence on body weight and cause mortality as a long term effect, which might be due to co-morbidity of several diseases, caused by hypercalcemia and impairment of mitochondria. Further experiments are warranted on the use of a sufficient number of lower doses of calcium chloride to determine a pharmacologically effective and non-toxic dose.

Key words: Calcium chloride, Ehrlich ascites carcinoma cells, cytotoxicity, nucleic acids, malondialdehyde, nonprotein sulfhydryl groups, histopathology

INTRODUCTION

Calcium chloride is a chloride salt of calcium used in a variety of industrial and consumer products. In addition to its medicinal, industrial and laboratory use, calcium chloride has been evaluated to be a food substance and/or dietary supplement of a very low toxicity and is generally recognized as safe (GRAS) by the US Food and Drug Administration (UNEP, 2002a). Calcium chloride dissociates easily in water to form calcium and chloride ions. Calcium is useful in the treatment of hypocalcemic tetany, laryngospasm and osteoporosis (Marcus, 1996), in addition to its general use in formation of skeletons, neural transmission, muscle contraction and coagulation of the blood. Chloride ions are required for normal cellular functions in animals and humans and serve as a micronutrient that play important role in photosynthesis and osmoregulation of plants (UNEP, 2002b).

A large number of reports in the literature suggest that cytotoxicity, in general, is mediated by mechanisms which are dependent on calcium ions. Khanna *et al.* (1991) showed that the presence of calcium ions is compulsory for the cytotoxic activity in the susceptible target cells, namely T lymphocytes and macrophages. A sustained increase in cytosolic calcium has been associated with the onset of cytotoxicity characterized by disruption of cytoskeleton, DNA fragmentation and extensive damage to other sub cellular components leading to cell death (Orrenius *et al.*, 1989). Endogenous increase in cytosolic calcium level has been associated with cytotoxicity in response to a variety of agents in different cell types (Rahimtula and Chong, 1991). Calcium is reported to play a major role in ATP-induced cytotoxicity and cell death of hepatocytes (Zoetewij *et al.*, 1993). An overload of calcium is shown to activate cell death via apoptosis (Pu *et al.*, 2002) and ROS (Anane *et al.*, 1996; Obata, 2003) or may lead to disintegration of cells (necrosis) through the activity of calcium-sensitive protein digesting enzymes (Berridge *et al.*, 1998).

Although, calcium is suggested to cause cytotoxicity by induction of ROS, DNA fragmentation and apoptosis, there is a paucity of literature on the anticancer activity of calcium chloride. However, calcium chloride is well known for its protective activity against some toxicants. Furhata *et al*. (1989) reported calcium chloride to inhibit stimulation of replicative DNA synthesis induced by sodium chloride in pyloric mucosa of male Fischer 344 rats. The mutagenic activity and cytotoxicity of dimethylnitrosamine (DMN) in CHO/HGPRT system (Chinese hamster ovary cell/Hypoxanthine-guamine phosphoribosyl transferase system) were greatly increased, upon addition of calcium chloride to rat liver metabolic activation system (S9) buffered with sodium phosphate (Tan and Hsie, 1981). Aboul-Ela (2002) found it to protect against the chromosomal aberrations and spermatozoa abnormalities caused by lead acetate.

The present study on the anti-carcinogenic and biochemical potentials of calcium chloride was undertaken in view of the literature reports on the cytotoxic activity of calcium, the extensive use of calcium chloride as a food supplement, drug and a protective agent against toxicants and a paucity of reports on the cytotoxic and anticancer activity of calcium chloride, as a whole compound.

MATERIALS AND METHODS

The present study on the anticancer and biochemical effects of calcium chloride on Ehrlich carcinoma cell-bearing Swiss albino mice was conducted in the Department of Clinical Pharmacy, King Saud University. The experimental part was undertaken during the period February 07 to August 08.

Test compound, chemicals and reagents: Calcium chloride (CAS No. 10043-52-4) obtained from Sigma Chemical Co., St. Louis, MO, USA, was used as the test compound. All the other chemicals and reagents were of analytical grade purchased from commercial sources. Adriamycin was purchased from Endoxan-Asta, Asta Medica AG, Frankfurt, Germany, was used as a standard drug.

Animal stocks: Female Swiss albino mice (SWR) aged 5-6 weeks and weighing 24-26 g were obtained from the Experimental Animal Care Center, King Saud University, Riyadh, Saudi Arabia. The animals were fed on Purina chow diet and water *ad libitum* and were maintained under standard conditions of humidity, temperature and light (12 h, light/12 dark cycle).

Dose and mode of administration: The doses of calcium chloride selected were 50, 100 and 200 mg/kg/day determined on the (i) experiments conducted on maximum tolerated dose and a trial experiment, which showed it to be pharmacologically effective at a dose of 100 mg kg^{-1} (ii) reports in the literature (Aboul-Ela, 2002; Wong *et al*., 1991) and (iii) Human Therapeutic Dose (HTD) with reference to the surface area rule. The average intake of calcium chloride has been estimated to be 160-345 mg/kg/day for humans (1). According to the rule of surface area ratio of mice (20 g) and human (60 kg), the calculated ratio is 0.0026 and hence the dose of calcium chloride (per 50 mice weighing 1 kg) for the upper limit of HTD would be (0.0026×345.0×50 = 44.85 mg/kg/day). However, the dose used experimentally is generally six times more than the calculated value (44.85×6 = 269.0 mg/kg/day). This is because the metabolic rate is more in mouse as compared to human being (Wallace Hayes, 1989). The different doses (50-200 mg/kg/day) used in the present study were less than the maximum calculated dose. Aqueous solution of calcium chloride was administered (i.p.) daily for 5 days. The intra-tumoral route of administration was adopted in view of its reported efficacy in the acquisition and retention of the drug by the Ehrlich ascites carcinoma cells (Unnikrishnan and Kuttan, 1990; Hall *et al*., 1992; Devi *et al*., 1994). ADM (1 mg/kg/day for 5 days) was used as a reference drug.

Implantation of Ehrlich ascites carcinoma cells in the peritoneal cavity of mice: EAC cells supplied by Dr. C. Benckuijsen (Amsterdam, Holland) were maintained by serial transplantations in female Swiss albino mice every 8 days. A total of 150 female mice were randomly allotted to different control and treatment groups (25 mice in each group divided as 10 and 15 animals in two separate sets). Ten mice in each group (set 1) were used for the evaluation of parameters on cytotoxicity, biochemistry and histopathology and the remaining 15 mice in each group (set 2) were used for the studies on body weight and survival. The EAC cells (2.5×10^6 cells mouse) were implanted (i.p.) into all the experimental mice. The treatment was initiated 5 days after tumor implantation and continued for 5 consecutive days.

Experimental groups: The experimental groups of mice consisted of the following: (1) positive control (EAC-cell bearing), tap water; (2) ADM (1 mg/kg/day); (3) calcium chloride (50 mg/kg/day); (4) calcium chloride (100 mg/kg/day); (5) calcium chloride (200 mg/kg/day). In each case, five animals were killed 24 h after the last treatment. The peritoneal fluid samples from each mouse were collected in two different vials. One of these samples was immediately processed for parameters on cytotoxicity and viability, while the second vial was stored at -70°C until used for the determination of MDA, NP-SH, nucleic

acids and proteins. Tumor tissues obtained from the site of inoculation, were preserved in buffered formalin and processed by routine procedures for histopathological investigations.

Evaluation of body weight and survival: The sets of animal groups allotted for observations on body weight changes and survival were maintained separately. These animals were observed for their weight and mortality daily until their death or unto maximum of 50 days.

Cytotoxicity and viability: The aliquots of peritoneal fluid collected were immediately processed for the observation of viability and cytotoxicity of EAC cells with a hemocytometer using a dye-exclusion technique (Qureshi *et al*., 2001).

Biochemical studies: The frozen peritoneal fluid samples were used for estimation of nucleic acids, protein, MDA and NP-SH.

Estimation of proteins and nucleic acids: Total protein was determined by the modified Lowry method of Schacterle and Pollack (1973). The method described by Bregman (1983) was used to determine the levels of nucleic acids. Peritoneal fluid containing EAC cells was homogenized and suspended in ice-cold trichloroacetic acid (TCA). After centrifugation, the pellet was extracted with hot TCA. The levels of DNA were determined by treating the nucleic acid extract with diphenylamine reagent and reading the intensity of blue color at 600 nm. For quantification of RNA, the nucleic acid extract was reacted with orcinol reagent and the absorbance of green color was read at 660 nm. Standard curves were used to determine the amounts of nucleic acids present.

Determination of MDA concentrations: The method described by Ohkawa *et al*. (1979) was followed. Peritoneal fluid containing EAC cells was homogenized in KCl solution and incubated with thiobarbituric acid reagent. After centrifugation, the optical density of the clear pink supernatant was read at 532 nm. Malondialdehyde bis (dimethyl acetal) tetra ammonium was used as an external standard.

Quantification of NP-SH levels: The levels of NP-SH were determined according to the method described by Sedlak and Lindsay (1968). Peritoneal fluid containing EAC cells was homogenized in ethylenediaminetetraacetic acid disodium (EDTA). Aliquots of homogenate were treated with 50% w/v TCA and centrifuged at 3000 g. The supernatant fractions were mixed with Tris buffer and 5, 5-dithiobis-(2 nitrobenzoic acid) (DTNB) and the absorbance was read at 412 nm against a reagent blank with no homogenate.

Histopathological procedure: The tumors that developed at the site of injection of EAC cells were excised and fixed in 10% formaldehyde. The preserved tumor tissue was dehydrated, cleared and processed for routine paraffin-block preparation using an American optical rotary microtome section of about 5 μm thickness were cut, stained with hematoxylin and counter-stained with eosin (Al-Harbi *et al*., 1995). The slides were examined for histopathological changes such as inflammatory reaction, necrosis, number of hair follicles, mitotic figures and size of tumor by an observer who was blind with respect to the treatment groups.

Statistical analysis: Data are presented as Mean±SE. Statistical comparison were made by using analysis of variance (ANOVA) with significance defined as $p<0.05$. Post-hoc analyses were done by using Student's t-test.

RESULTS

Effect on cytotoxicity and viability: There was a significant reduction of the total number of EAC cells in the peritoneum after treatment with calcium chloride at 50 ($p<0.05$), 100 and 200 ($p<0.01$) mg kg^{-1} body weight. The mean percent viability was significantly ($p<0.05$) reduced at the higher doses (100 and 200 mg kg^{-1}., body weight) only, as compared to the values obtained in the positive control group. ADM treatment (group 2) also significantly affected the viability ($p<0.05$) and reduced the total number of EAC cells ($p<0.001$), as compared to the values obtained in the positive control group (Table 1).

Effect on the levels of nucleic acid and proteins: Treatment with calcium chloride significantly reduced the EAC-cell levels of proteins at 100 ($p<0.05$) and 200

Table 1: Effect of calcium chloride on cytotoxicity and viability of EAC cells of Swiss albino mice

Treatments	Dose (mg/kg/day)	Total cell counts ($\times 10^6$ mL)	Percent Viable cells
Positive control	Tap water	618.40±72.57	91.99±1.02
ADM	1	164.00±30.51***	76.51±4.90*
Calcium chloride	50	403.20±40.47*	88.84±2.72
Calcium chloride	100	286.60±35.72**	87.49±1.69*
Calcium chloride	200	258.00±30.36**	85.07±2.15*

Data are expressed as Mean±SE. Five mice were used in each group; Treatments were significantly different at $p<0.05$ (ANOVA) in post-hoc analysis; Groups 2, 3, 4 and 5 were statistically compared with group 1; *$p<0.05$; **$p<0.01$; ***$p<0.001$ (Student's t-test)

Table 2: Effect of calcium chloride on the levels of nucleic acids and proteins in EAC cells of Swiss albino mice

Treatments and dose (mg/kg/day)	Total protein (mg mL^{-1})	RNA (µg mL^{-1})	DNA (µg mL^{-1})
Positive control (tap water)	168.40±4.30	599.52±28.77	331.65±6.15
ADM (1)	129.55±5.75***	388.15±30.16***	202.12±10.17***
Calcium chloride (50)	162.80±5.13	512.90±23.56*	296.90±10.37*
Calcium chloride (100)	151.17±6.18*	442.30±16.90**	232.72±11.10***
Calcium chloride (200)	147.95±4.09**	406.70±21.75***	210.16±10.67***

Data are expressed as Mean±SE. Five mice were used in each group *p<0.05; **p<0.01; ***p<0.001 (Students' t-test) groups 2, 3, 4 and 5 were statistically compared with group 1

Table 3: Effect of calcium chloride on malondialdehyde concentrations in EAC cells of Swiss albino mice

Treatments	Dose (mg/kg/day)	MDA Concentrations (p mole mL^{-1} P.F)
Positive control	Tap water	839.40±20.25
ADM	1	658.90±27.16***
Calcium chloride	50	739.12±31.52*
Calcium chloride	100	742.79±21.10**
Calcium chloride	200	703.68±20.96***

Data are expressed as Mean±SE. Five mice were used in each group; Treatments were significantly different at p<0.05 (ANOVA) in post-hoc analysis; Groups 2, 3, 4 and 5 were statistically compared with group 1; *p<0.05; **p<0.01; ***p<0.001 (Student's t-test). PF: Peritoneal fluid

(p<0.01) mg/kg/day. The concentrations of RNA and DNA were significantly reduced at 50 (p<0.05) and 200 (p<0.001), whereas at 100 mg/kg/day, there was a significant reduction of RNA (p<0.01) and DNA (p<0.001). ADM treatment also caused a significant (p<0.001) reduction in protein and nucleic acid contents as compared with the positive control value (Table 2).

Estimation of MDA concentrations: The administration of calcium chloride resulted in a significant reduction of MDA levels in EAC cells at 50 (p<0.05), 100 (p<0.01) and 200 (p<0.001) mg/kg/day. ADM treatment was also found to significantly (p<0.001) affect the levels of MDA as compared with the value obtained in EAC cells in the positive control (Table 3).

Quantification of NP-SH levels: Calcium chloride treatment caused a significant and dose-dependent reduction in the NP-SH concentration in EAC cells in mice peritoneum at 50 (p<0.05), 100 (p<0.01) and 200 (p<0.001) mg/kg/day, as compared with the positive control value. ADM treatment also significantly (p<0.001) reduced the glutathione levels of EAC cells as compared with the values obtained in the positive control group (Table 4).

Table 4: Effect of Calcium chloride on the NP-SH levels in EAC cells of Swiss albino mice

Treatments	Dose (mg/kg/day)	NP-SH Concentrations (p mole mL^{-1} P.F)
Positive control	Tap water	1292.78±20.95
ADM	1	1092.55±30.47***
Calcium chloride	50	1227.13±18.95*
Calcium chloride	100	1168.30±17.10**
Calcium chloride	200	1069.21±22.65***

Data are expressed as Mean±SE. Five mice were used in each group; Treatments were significantly different at p<0.05 (ANOVA) in post-hoc analysis groups; Groups 2, 3, 4 and 5 were statistically compared with group 1; *p<0.05; **p<0.01; ***p<0.001 (Student's t-test). PF: Peritoneal fluid

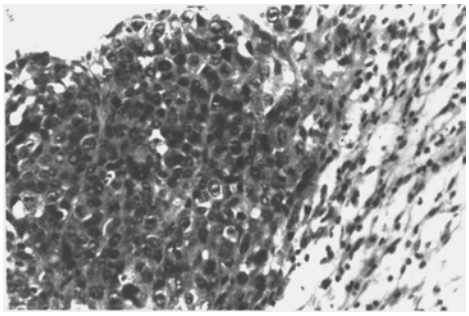

Fig. 1: Microphotograph from an EAC-cell inoculated mice (positive control) showing mixed inflammatory reaction predominantly lymphocytes in fast growing tumor with many mitotic figures (H and E x200)

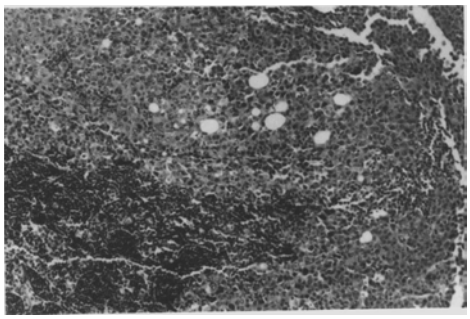

Fig. 2: Microphotograph from an EAC-cell inoculated mice (positive control) showing the white mass, tumor growth and lymphocytes reaction (H and E x40)

Effect on histopathological changes: The EAC cells were found to induce intramuscular tumors at the point of inoculation. These tumors were prominent and revealed fast growth with mixed inflammatory reaction predominantly lymphocytes (Fig. 1), white mass and lymphocytic reaction (Fig. 2), indicating a continuous proliferation activity in the positive control group. Calcium chloride treatment (50 mg/kg/day) reduced

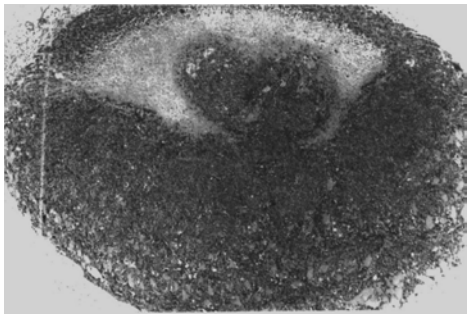

Fig. 3: Section through tumor from an EAC-cell inoculated mice treated with Calcium chloride (50 mg/kg/day) showing a big tumor with about 20% necrosis (H and E x40)

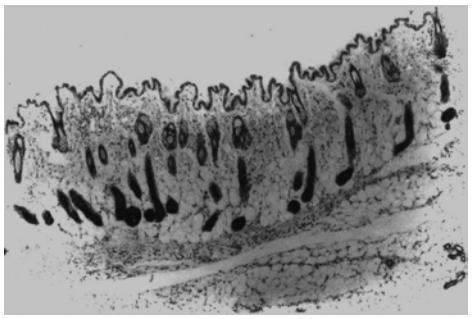

Fig. 4: Microphotograph from an EAC-cell inoculated mice treated with Calcium chloride (50 mg/kg/day) showing no effect on skin as revealed by normal hair follicles and skin (H and E x40)

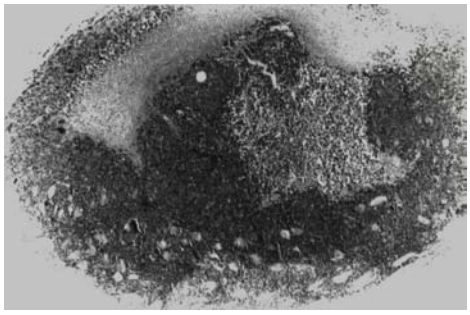

Fig. 5: Microphotograph from an EAC-cell inoculated mice treated with Calcium chloride (100 mg/kg/day) showing the growing tumor with necrosis (H and E x40)

tumor size with about 20% necrosis (Fig. 3). However, there was no effect on skin as revealed by normal hair follicles and skin (Fig. 4). On increasing the dose

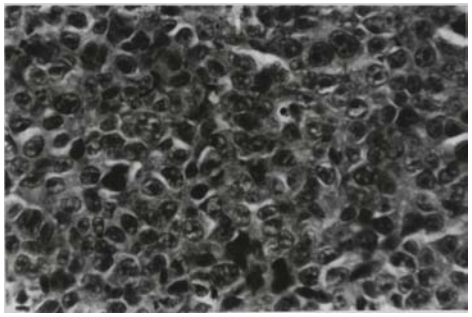

Fig. 6: Microphotograph from an EAC-cell inoculated mice treated with Calcium chloride (100 mg/kg/day) showing marked reaction in mitosis indicating slow growth of tumor (H and E x400)

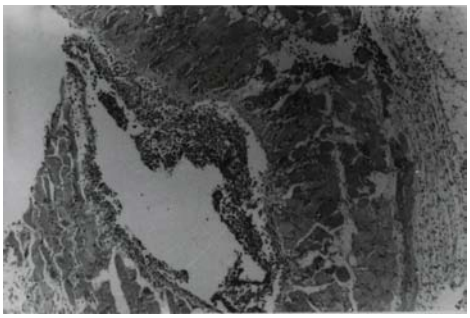

Fig. 7: Microphotograph from an EAC-cell inoculated mice treated with Calcium chloride (200 mg/kg/day) showing marked reduction in tumor size. The tumor is not continuous and appears growing very slow and broken into parts (H and E x40)

Table 5: Effect of Calcium chloride on survival (days) of mice implanted with EAC cells

Treatments	Dose (mg/kg/day)	Survival (No. of days)
Positive control	Tap water	16.71
ADM	1	25.29
Calcium chloride	50	22.50
Calcium chloride	100	16.29
Calcium chloride	200	14.86

(100 mg/kg/day), the necrosis was increased (Fig. 5) and there was marked reduction in mitosis, indicating slow growth (Fig. 6). There was marked reduction in tumor size after treatment with calcium chloride at 200 mg/kg/day, the tumor was found to be discontinuous and appeared growing slow and broken into parts (Fig. 7). At the same dose there was marked reduction in mitosis (Fig. 8). The treatment with ADM at 1 mg/kg/day showed marked reduction of the tumor size with extended necrosis (Fig. 9) and a marked reduction in the incidence of mitosis (Fig. 10).

Table 6: Effect of Calcium chloride treatment on body weight changes in mice implanted with EAC-cells

	Treatment and dose (mg kg^{-1} body weight)				
Days	Positive control (Tap water)	Adriamycin (1)	Calcium chloride (50)	Calcium chloride (100)	Calcium chloride (200)
0	26.66±0.75	26.42±0.92	23.85±0.82	25.23±0.68	26.63±0.56
5	28.76±0.62	27.12±0.78	24.45±0.68	27.12±0.78	27.71±0.76
10	34.10 ±1.17***	28.56±1.23	25.32±1.31	28.56±1.23	29.02±0.45
15	39.64±1.39***	28.01±0.95	28.23±2.25	29.50±1.31	32.70±1.35*
20	40.62±1.75***	28.40±0.83	27.64±2.00	33.52±2.24*	31.90±0.80**
23	41.23±1.68***	29.20±1.12	28.10±2.51	33.82±2.50*	-
30	-	29.31±0.91	30.85±3.90	-	-
35	-	30.57±1.32	31.85±3.05*	-	-
40	-	31.60±2.60	35.72±2.92**	-	-
45	-	32.57±4.59	-	-	-
50	-	33.60±6.60	-	-	-
51	-	34.60±5.40	-	-	-

Data are expressed as Mean±SE. Fifteen mice were used in each group; Treatments were significantly different at $p<0.05$ (ANOVA) in post-hoc analysis; Groups 2-11 were statistically compared to group 1. *$p<0.05$; **$p<0.01$; ***$p<0.00$ 1 (Student's t-test)

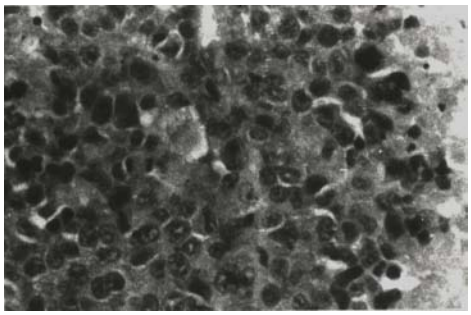

Fig. 8: Microphotograph from an EAC-cell inoculated mice treated with Calcium chloride (200 mg/kg/day) showing marked reduction in mitosis (H and E x400)

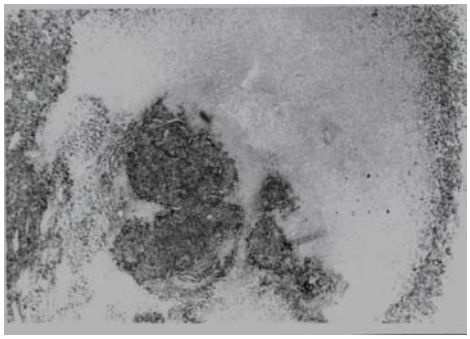

Fig. 9: Microphotograph from an EAC-cell inoculated mice treated with Adriamycin (1 mg/kg/day) showing inhibition of tumor growth and marked reduction in mitosis (H and E x100)

Effect on survival: The mean duration of survival of EAC cell-bearing mice (Positive control) was 16.71 days. No animal in this group survived beyond the 23rd day. Calcium chloride treatment (50 mg/kg/day) increased the mean survival period to 22.50 days with no animal alive beyond day 40. The mean duration of survival in the higher dose range of calcium chloride was 16.29 days and 14.86 at 100 and 200 mg/kg/day, respectively. The respective period of maximum survival was 23 days and 20 days at the medium and high doses. ADM treatment increased the survival period up to 25.21 days. No animal in this group lived beyond day 51 (Table 5).

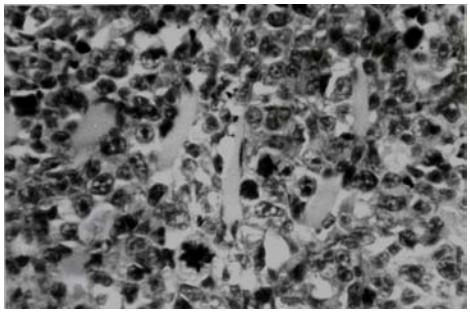

Fig. 10: Microphotograph from an EAC-cell inoculated mice treated with Adriamycin (1 mg/kg/day) showing inhibition of tumor growth and marked reduction in mitosis (H and E x400)

Effect on body weight changes: The EAC cell-bearing mice (Positive control) showed a significant ($p<0.01$, $p<0.001$) increase in the body weight on days 10-23, as compared with the initial body weight. Treatment with calcium chloride failed to significantly increase the body weight up to 30 days (50 mg kg^{-1} body weight) 15 days (100 mg kg^{-1} body weight) and 10 days (200 mg kg^{-1} body weight). There were no significant changes in the body weight of EAC cell-bearing mice up to the last day of survival (51 days) after treatment with ADM (Table 6).

DISCUSSION

Results obtained in the present study revealed that the treatment with calcium chloride caused significant

reduction in the number of EAC cells and their viability at both low and higher doses. ADM, a cytotoxic drug was also found to reduce the number of EAC cells, which was much lower than the calcium chloride treated groups. There are no parallel studies on the cytotoxic potentials of calcium chloride. However, literature reports suggest that cytotoxicity, in general, can be mediated by mechanisms which are dependent on calcium ions (Orrenius *et al*., 1989). Increased intracellular content of calcium ions has been shown to cause cell death and cytotoxicity (Dhawan *et al*., 1991). Although, there is no direct literature on cytotoxic activity of calcium chloride, it has been shown to protect against the carcinogenicity and toxicity of other compounds. Nishikawa *et al.* (1992) found calcium chloride to inhibit the post-initiation phase of two-stage glandular stomach carcinogenesis in rats induced by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and sodium chloride. Addition of calcium chloride to rat liver metabolic activation system (S9) increased the cytotoxic activity of dimethylnitrosamine in Chinese hamster ovary cell/Hypoxanthine-guamine phosphoribosyl transferase (CHO/HGPRT) system (Tan and Hsie, 1981). The exact mode of action of calcium chloride-induced cytotoxicity is not known, however; it might be related with the increase of endogenous calcium ions. The increase of calcium is known to cause disruption of calcium homeostasis, which has been associated with the onset of cytotoxicity characterized by disruption of cytoskeleton, DNA adducts or breaks and extensive damage to other sub-cellular components leading to cell death (Khanna *et al*., 1991; Cullinane *et al*., 2000). These reports confirm the observation in present study on reduction of nucleic acids and proteins in the EAC cells of the same animals. These results are in agreement with the observation of Furihata and Matsushima (1990) who found calcium chloride to inhibit sodium chloride-induced replication of DNA synthesis in pyloric mucosa of F344 rats. ADM treatment also inhibited the contents of nucleic acids and proteins in EAC cells, which might have contributed to the observed cytotoxicity of ADM, in addition to the production of free radicals (Singal *et al*., 2000; Gewirtz, 1999).

Antineoplastic drugs in general are known to increase the load of free radicals and stimulate lipid peroxidation (MDA) in the body (Halliwell and Gutteridge, 1987). MDA is a compound that increases oncogenic potentials (Borek, 1988; Surapaneni and Venkata, 2006) and hence any rise in the levels of MDA is deemed to be associated with cancer. In the present study, the higher concentration of MDA present in the EAC cells was found to be reduced after treatment with calcium chloride. This reduction in the levels of MDA might be related to the antioxidative potentials of calcium chloride. These data confirm observation of a previous report, which showed calcium chloride to reduce the MNNG/sodium chloride-induced increase in the levels of MDA in gastric mucosa of rats (Nishikawa *et al*., 1992). These results and the observation on reduction of MDA do not correspond with the data of Kim and Sharma (2004), who found the influence of calcium ions, on ROS induction, by heavy metals. The discrepancy between these reports and the results obtained in the present study might be related with reduction of MDA to be associated with the reduced number of EAC cells caused by calcium chloride. This is also confirmed by the ADM-induced reduction in the levels of MDA in the reduced number of EAC-cells.

Glutathione (NP-SH) is a known scavenger of hydroxyl radicals and singlet oxygen. In the present investigation, the level of glutathione in EAC cells was significantly reduced after treatment with calcium chloride. This reduction was close to that affected by ADM. The simultaneous reduction of MDA and Glutathione is a rare possibility, especially, when calcium chloride is claimed to possess antioxidant potentials. However, it is possible that the simultaneous reduction of MDA and glutathione observed in the present study might be an endogenous interaction involving the depletion of glutathione in blocking the production of MDA (Terao and Niki, 1987). The other possibility is the reduced number of EAC cells, which might have been the cause of reduced levels of both glutathione and MDA. The results of the present study are further supported by the calcium chloride-related reduction of the tumor size, mitoses and necrosis in the EAC-cell bearing untreated mice. The improvement in the changes by calcium chloride was close to those caused by ADM treatment. These results support the observation of Berridge *et al.* (1998), who found that changes in the intensity of calcium are related with necrosis or disintegration of cells through the activity of calcium-sensitive protein-digesting enzymes.

Thus, the calcium chloride-induced cytotoxic activity may be related to the reduction of nucleic acids, MDA and glutathione, in addition to histopathological changes. Furthermore, the reduction of EAC cells may also be due to phagocytosis by peritoneal polymorph nuclear leucocytes and macrophages activated by depletion of glutathione (Halliwell and Gutteridge, 1987). Nevertheless, the present study on cytotoxicity, biochemical evaluation and histopathology investigation are not in agreement the observation on survival and body weight after treatment with calcium chloride. This part of the present study on EAC cell-bearing mice revealed that calcium chloride treatment checked the increase in body weight and prolonged the span of life in these animals only at the low dose. These changes point to the antitumor activity of calcium chloride, which is comparable to the effects

caused by ADM, a proven cytotoxic drug. On the other hand, the higher doses of calcium chloride increased the body weight much earlier and restricted the life span, which was close to mice in the positive control group. The discrepancy between the results of body weight and survival and the cytotoxic activity, biochemical study and histopathological observation might be due to the difference in the time of observation. While the cytotoxic activity, biochemical and histopathological changes were observed 10 and 5 days after the implantation of EAC cells and treatment respectively, the observations for body weight and mortality were continued until death of all the animals or up to a maximum of 50 days. The present investigation demonstrate that long term effects of the treatment of calcium chloride at the higher doses were toxic and reduced the duration of life.

The exact mechanism of toxicity caused by calcium chloride is not known. However, it might be attributed to calcium ions. Literature reports suggest that intracellular increase of calcium ions cause degradation of calcium homeostasis, which cause mitochondrial damage, accumulation of endogenous oxidants, resulting in cell death and increasing mortality (Zoetewij *et al*., 1993; Chen *et al*., 1994). The reason of increased mortality might be due to hypercalcemia induced co-morbidity-related to different disease states, including cardiotoxicity, hepatotoxicity, hyperparathyroidism and malignancy (Lafferty, 1991; Al-Nasser, 1998). Furthermore, the increased levels of calcium ions are reported to open a non-specific pore in the inner mitochondrial membrane, which renders mitochondria to be leaky causing uncoupling of energy transduction and the release of free calcium. Thus high cellular calcium and the corresponding impairment of mitochondrial ATP production might be an important contributory factor in calcium-related toxicity and subsequent mortality (Al-Nasser, 1998).

Taken together, on 11th day after inoculation with EAC cells, the 5 day treatment with calcium chloride was found to cause cytotoxicity that was substantiated by biochemical and histopathological changes. The results of survival and changes in the body weight revealed that the treatment with calcium chloride at the low dose showed an increased life span and restricted body weight, as compared to the higher doses, which failed to sustain the anti-cancer activity, as revealed by an increase of body weight and reduction of life span. The mortality observed at the higher doses might be related to hypercalcemia-related diseases and co-morbidity. Further experiments are warranted on the use of a sufficient number of lower doses of calcium chloride to determine a pharmacologically effective and non-toxic dose.

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