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Cobalt Decorporation at Intracellular Level Using Pentetate Calcium Trisodium

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Chelation therapy; Decorporation; Calcium-Diethylenetriaminepentaacetic acid; Cobalt chloride; Fura-2AM

Abbreviations:

PBMC: Peripheral blood mononuclear cells; Ca-DTPA: Calcium-Diethylenetriaminepentaacetic acid; $CoCl_2$: Cobalt chloride; Fura-2AM: Fura-2-acetoxymethyl ester; PBS: Phosphate buffered saline.

1. Abstract

1.1. Background: Some chemical agents have been studied for chelation therapy against heavy metal poisoning/ contamination. But none of them were found to be satisfactory for cobalt decorporation. Therefore, present mitigation study was performed to find out cobalt decorporation ability of 'Pentetate Calcium Trisodium' or Calcium-Diethylenetriaminepentaacetic acid (Ca-DTPA) against intracellular cobalt contamination.

1.2. Study Design: Ca-DTPA was examined as a prophylactic cobalt chelator in Peripheral Blood Mononuclear Cells (PBMC) exposed to high concentration of cobalt chloride. The effect of Ca-DTPA on cell viability was examined using Trypan blue cytotoxicity assay. Intracellular cobalt absorption and its chelation by Ca-DTPA was assessed using Fura-2AM fluorescent dye through fluorometer, and its distribution was observed through fluorescence imaging using fluorescent microscope.

1.3. Results: Ca-DTPA showed non-toxic effects on PBMC upto 100 mM concentration. Cell morphological study revealed protective chelating efficiency of Ca-DTPA. Significant ($P \leq 0.01$) decrease in intracellular cobalt deposition after Ca-DTPA treatment was observed (as compared to cobalt control).

1.4. Conclusions: Present study showed the potential of Ca-DTPA on preventing intracellular cobalt deposition by extracellular chelation of cobalt.

2. Introduction

Human activities are the major cause of different types of pollution. Among them, environmental pollution due to heavy metals and their subsequent effects on humans/ animals down the food chain creates potential health hazard. Not only that, heavy metals also produces deleterious effects on flora and fauna because of their persistent and non-biodegradable nature. They cannot be destroyed biologically as they do not get metabolized in the body and hence remain accumulated in the cells and tissues inside the body [1, 2]. Moreover, heavy metals from any anthropogenic disasters (viz., attacks through radioactive bombs, radionuclides contamination etc.) have different modes of internalization [3-5]. Their primary routes are inhalation, ingestion, skin absorption and wound absorption. They have the ability to alter body's cell defense mechanism at cellular level by penetrating themselves in the interior of the cell via calcium channels and by reacting with cell's surface structure as membrane forms the first facing target for the action of any heavy metal [6]. Nevertheless, it is well established that transition metal ions are essential nutrients to the life. Cobalt, Copper, Iron, Manganese, Nickel, and Zinc all have unique physico-chemical properties that make them attractive molecules for use in biological systems [7]. Among them, Cobalt is specifically required for the normal functioning of body organs as a source of vitamin B_{12} cobalamines [8, 9]. It acts as a co-enzyme to catalyze various metabolic reactions which participates in DNA synthesis, fatty acid synthesis and energy production [7, 10]. It is among one of the redox-active metal that maintains cellular redox homeostasis within its strict physiological limits at a concentration of 20µg/l in the blood [11, 12]. However, any disruption of Cobalt homeostasis due to its contamination by excessive exposure to toxic levels is a matter of concern [8, 9]. Due to its accumulation in the cell nucleus

and in perinuclear structure in the cytosol, it can induce DNA damage, gene mutation, sister chromatid exchanges, DNA cross-links and aneuploidy, which has been reported in *in-vitro* studies using animals and human cells [9, 13-17]. Therefore, removal of Cobalt from cell has been a subject of paramount importance.

In this context, chelation therapy could be considered as an effective treatment to induce decorporation burden at cellular level. Chelation therapy is a medical procedure that involves administration of chelating agents to remove heavy metals from the body. This uses special drugs that bind to metals in the body. It is used for treatment of heavy metal poisoning of iron, Hg, As, Pb. However, till date, no potent chelator for cobalt is known. Only oral D-penicillamine is tried for chelation of such redox active metal [18]. But due to its adverse effects on health like anorexia, nausea, vomiting, risk of causing anaphylactic reaction in penicillin allergic patients, induction of several cutaneous lesions, dermatomyosites, dryness and adverse effects on collagen etc., its uses are restricted [19, 20]. Therefore, a frantic search for better chelating agent for cobalt decorporation is still on.

Diethylenetriamine Pentaacetate (DTPA) is a member of a family of polyaminocarboxylic acid which is classified in categories of Extra Cellular Fluids Complexing Agents (ECFCA). Due to its negative charges, it does not have the accessibility to cross cell membrane. It gets distributed throughout the extracellular fluid and undergoes chelation extracellularly [21-23]. The calcium salt of DTPA is known as 'pentetate calcium trisodium' and is commonly referred to as Ca-DTPA. This calcium bound ligand enhances the excretion of transuranium elements from the body by exchanging calcium for a metal of greater binding capacity, and the complex is rapidly excreted out via glomerular filtration into the urine [22, 24]. It is an FDA approved chelating agent and best known for elimination of known or suspected internal contamination of Plutonium (Pu), Thorium (Th), Americium (Am), and Curium (Cm) [25]. Therefore, our research is focusing on the mitigating effects of Ca-DTPA against intracellular cobalt deposition in human peripheral blood mononuclear cells (PBMCs). Thus, the present study is comprised of three parameters: (i) Trypan blue cell survival assay to study cytotoxity of Ca-DTPA; (ii) *in-vitro* qualitative analysis of intracellular cobalt absorption and chelation capacity of Ca-DTPA using fluorescent dye Fura-2-acetoxymethyl ester (Fura-2AM) through Fluorometer, and (iii) indirect analysis of cobalt distribution alone and after Ca-DTPA treatment through fluorescent imaging.

3. Materials and Methods

3.1. Reagents

RPMI-1640 culture media (with L-glutamine and sodium bicarbonate), Histopaque-1077, Penicillin-Streptomycin, Di-methyl Sulfoxide (DMSO), Diethylenetriamine penta-acetic acid calcium trisodium salt (Ca-DTPA), and Fura-2AM (in 1mg/ml DMSO) were purchased from Sigma-Aldrich Co., St. Louis, MO, USA. Ammonium chloride, Potassium Hydrogen carbonate, EDTA, Fetal Bovine Serum (FBS), Trypan blue and all reagents of buffers were procured from Hi-Media Laboratory Ltd., Mumbai, India. HEPES (1M) solution from Hyclone and cobalt (II) chloride hexahydrate were obtained from Tokyo Chemical Industry Co. Ltd., Japan. All other chemicals used were of analytical grade.

3.2. Peripheral Blood Mononuclear Cell (PBMC) Isolation:

Human peripheral blood was drawn from healthy donors by venopuncture procedure in EDTA-coated vacutainers (Vacuette, Greiner Bio-One, Austria) with their informed consent to donate blood for this study. PBMC were isolated by density gradient centrifugation using Histopaque-1077. Blood samples (2ml/donor) were diluted with Phosphate Buffered Saline (PBS) (1:1 ratio) and carefully layered on top of 1ml Histopaque-1077 in a 15ml Falcon tube (Tarson Products Pvt. Ltd., Kolkata, India) for separation of lymphocytes. The tube was carefully centrifuged at 600g for 20min at 4^{o}C. After centrifugation, buffy coat containing mononuclear cells were pipetted out from the interphase and washed with PBS (2ml). The cell suspension was centrifuged at 600g for 10min at 4^{o}C. Then the pellet was suspended in 3ml of RBC lysis buffer (0.15M NH_4Cl, 1mM $KHCO_3$, 0.1mM EDTA) and incubated for 15min in dark at room temperature for removal of RBCs. After incubation, cell suspension was centrifuged at 600g for 20min at 4^{o}C. The supernatant was discarded and the pellet was suspended in 2ml PBS and centrifuged again at 600g for 20min at 4^{o}C. Cells were washed twice and pellet was suspended in 1ml complete RPMI-1640 medium containing L-glutamine and sodium bicarbonate, 10% FBS, HEPES buffer (1M), 10,000 U/ml Penicillin and 10mg/ml Streptomycin, and maintained on ice.

Isolated lymphocytes were categorized into four different groups: Group I- Normal control (untreated cells); Group II- Cobalt control ($CoCl_2$ treated cells); Group III- Drug control (Ca-DTPA treated cells) and Group IV- Experimental Treatment (Ca-DTPA treated cells + $CoCl_2$ exposure). Solutions of drug and cobalt chloride were prepared by dissolving them separately in sterile water, filtered then used for treating cells. Research protocol was performed as per the published procedure [26].

3.3. Determination of Cytotoxicity (cell viability) of Ca-DTPA

In order to determine the cytotoxicity of Ca-DTPA, the cells were incubated with various concentrations of Ca-DTPA (10, 50, 100, 500, 1000, 1250, 1500, 2000 mM) at 37^{o}C for 2hrs in a CO_2 incubator. Sufficient number of cells ($2x10^5$ cells/ml) was stained with Trypan blue solution (0.4%). Trypan blue solution was mixed with one part of cell suspension (10µl) in an eppendorf tube and the mixture was kept for 2-3min at room temperature. Following this, the cell suspension was mixed properly and approximately 10µl of it was loaded onto a Hemocytometer and cells were counted under binocular microscope. Counting of cells was done within 5min of

mixing with Trypan blue to avoid reduction in viable cell count. Cell viability was determined by counting the number of live as well as dead cells and percentage viability was calculated based on the fraction of viable cells with respect to the total cell count.

3.4. Fura-2AM loading and measurement of Fura-2AM fluorescence

In each categorized group, 10µl cells were seeded in each well of 96 well plate and incubated at 37°C for 30mins with 5% CO_2 in a CO_2 incubator. Then cells were treated with different concentrations of 100µl Ca-DTPA for 10min at room temperature (prior to exposure to cobalt chloride). Following 10 mins, they were subjected to incubation with 100µl of 5mM $CoCl_2$ for 30 mins. Subsequently, plated cells were loaded with 2 µM of Fura-2AM and incubated further for another 45min in a CO_2 incubator in dark. Following incubation, cells were washed twice with PBS and centrifuged at 600g for 5min at 4°C. Then pellets were suspended in buffer and Fura-2AM fluorescence was measured at 510 nm emission after excitation at 340 nm and 380 nm using Fluorometer (Synergy Hi microplate reader; BioTek, Instruments, Inc., Winooski, USA) at room temperature. The fluorescence changes were noted after calculating the fluorescence dye ratio.

3.5. Cobalt Imaging after Fura-2AM loading

After isolation of PBMCs, 10µl cells were seeded in each well of 96 well plate at a density of $5x10^5$cell/ml and proceeded according to the aforementioned treatment. Fura-2AM loaded glass slides and coverslips were washed with PBS. Slides were prepared with a drop of cell suspension according to their categorized groups. Slides were assembled inverted into microscope holder and the cell morphological changes according to fluorescence ratio was observed (excitation 340 and 380nm, emission 510nm) at a magnification of 40X (Olympus IX51 Inverted Fluorescence Microscope, Japan). Cells treated in different conditions were screened and images were captured.

3.6. Statistical Evaluation

All assays were performed in triplicates for statistical evaluation and validity of results.

4. Results

4.1. Determination of Cytotoxicity of Ca-DTPA

To determine the optimal dose of Ca-DTPA for further experiments, various concentrations viz., 10, 50, 100, 500, 1000, 1250, 1500, 2000 mM of Ca-DTPA were used in the cytotoxicity assay employing Trypan blue staining. In comparison to the normal control (untreated cells), no significant cytotoxicity was found with 10, 50 and 100 mM Ca-DTPA treatment (Table 1). However, significant ($p \leq 0.01$) decrease in viability was observed with rest of the higher concentrations in a dose-dependent manner viz., 15% (500 mM), 40% (1000 mM), 50% (1250 mM), 62% (1500 mM) and 0% (2000 mM), respectively. Based on these results, 10-100 mM concentrations of Ca-DTPA were chosen for further experiments to determine its chelating efficiency.

Table 1: Determination of Cytotoxicity of Ca-DTPA on Cell Viability

Test	Concentration of Ca -DTPA (mM)	Cell Viability (%)
Normal control (Untreated cells)	Nil	100
Experimental 1	10	100
Experimental 2	50	100
Experimental 3	100	100
Experimental 4	500	85
Experimental 5	1000	60
Experimental 6	1250	50
Experimental 7	1500	38
Experimental 8	2000	0

Each concentrations of Ca-DTPA were tested in triplicates (n = 3) to ascertain its cytotoxicity on PBMC using Cell Viability assay (Trypan Blue assay). Data presented are the mean percentage cell viability relative to the normal control (untreated cells, Group-I). *$P \leq 0.01$ compared to the normal control (untreated cells).

4.2. Cell Morphology

Fluorescence images after Fura-2AM treatment showed the control cells (Group-I) with normal intact cell surface along with pan nuclear structure and low fluorescence ratio (Figure 1a). After Co^{2+} exposure (Group-II, Cobalt control), the cells presented punctuated cell surface with shrinked nucleus and high fluorescence ratio (Figure 1b). On the other hand, Ca-DTPA treatment alone (Group-III, Drug control) showed similar morphological effects as compared to that of normal control (Group-I, untreated cells) (Figure 1c); whereas, cells treated with Ca-DTPA prior to metal exposure (Group-IV, Experimental treatment) showed comparative modulatory effect of Ca-DTPA as compared to that of cobalt control (Group-II) (Figure 1b and 1d).

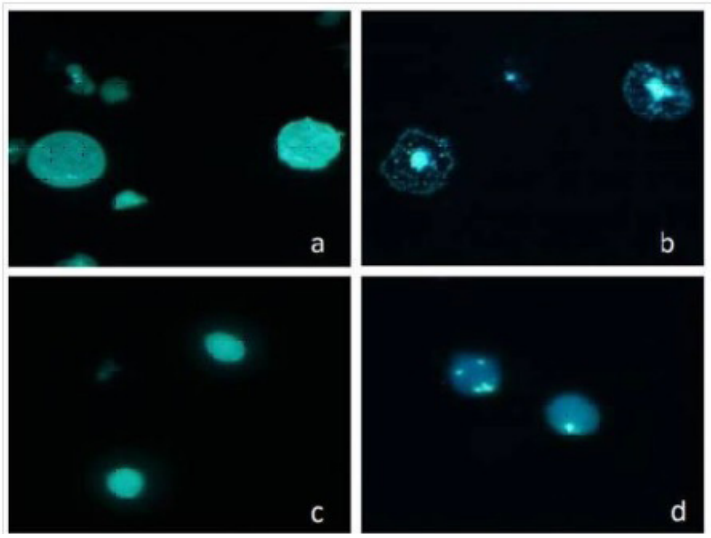

Figure 1: Fluorescent microscopic images of PBMC. (a) Normal control (Untreated cells); (b) Cobalt control (incubated with 5 mM $CoCl_2$); (c) Drug control (incubated with 100 mM Ca-DTPA); (d) Experimental Treatment (incubated with 100 mM Ca-DTPA prior to 5mM $CoCl_2$ exposure).

4.3. Determination of fluorescence after Cobalt exposure and Ca-DTPA treatment

Figure 2 showed the changes in the Fura- 2AM fluorescence ratio in PBMC by stimulation with cobalt chloride. Significant (P≤0.01) increase was observed in the fluorescence ratio after 30min of 5mM cobalt chloride exposure to the cells (Group-II) as compared to normal control (untreated cells, Group-I). Since, we observed an evanescent elevation of the Fura-2AM ratio, induced by $CoCl_2$, we examined the modulatory effect of Ca-DTPA to overcome this increase towards the basel level in a dose-dependent manner (20, 40, 60, 80, 100 mM). However, Ca-DTPA treatment of cells before cobalt exposure, counteracted the cobalt induced increment in Fura-2AM ratio, significantly. It was noted to be 100% at 80 mM Ca-DTPA concentration as compared to normal control (Group-I). A significant (P≤0.01) dose-dependent decrease by 5% (40 mM), 7% (60 mM), 12% (80 mM) and 11% (100 mM), was observed as compared to cobalt control (Group-II).

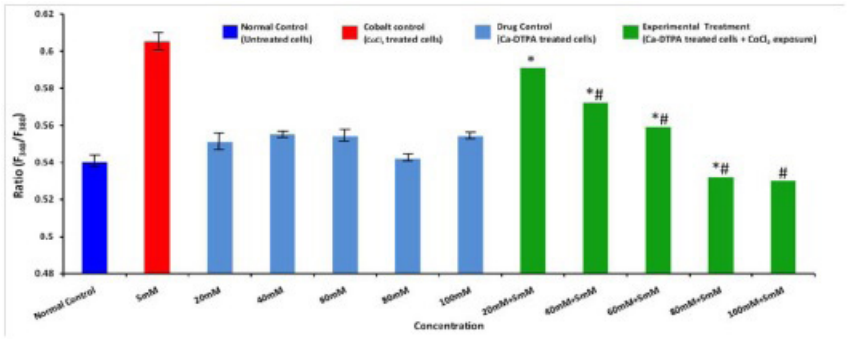

Figure 2: Fura-2AM fluorescence changes induced by cobalt content and its dose-dependent decrease (alleviation/ mitigation) following treatment with 20-100 mM Ca-DTPA. The values are expressed as mean ±standard deviation (n=3). *P≤0.01, compared to normal control; #P≤0.01, Ca-DTPA + cobalt treatment compared to cobalt control.

4.4. Statistical Evaluation

The data presented are expressed as standard deviation (S.D.) of triplicates. Differences between means were evaluated by applying Student's t-test and P≤0.01 was considered significant.

5. Discussion

In the present study, we focussed on the prevention of intracellular distribution of cobalt in PBMC using Ca-DTPA as a prophylactic agent. This study was proposed because cobalt is an essential trace metal which is considered to be an important nutrient for performing normal body functions by maintaining cellular redox homeostasis. But its excessive intake (lethal dose) due to any anthropogenic disasters can disrupt the normal cellular functions because of its intracellular deposition. Therefore, it becomes indispensable to study chelation of cobalt in PBMC as blood acts as a common compartment for transport and distribution after internal cobalt contamination and its accumulation could result in DNA damage, gene mutations etc. [8, 9]. Therefore, an FDA approved decorporative/ chelating agent called Ca-DTPA [25] has been studied in the present investigation because of (i) its great retention capacity in blood as blood provides its prime site of action [27]; and (ii) it

is an extracellular complexing agent because of its limitations to cross cellular membrane. Thus, present investigation was designed to study mitigating effects of Ca-DTPA in preventing cobalt distribution at intracellular level.

In the present study, cytotoxicity of Ca-DTPA has been determined using Trypan blue dye exclusion assay. This assay is based on the principle that live cells possess intact cell membrane thus causes exclusion of Trypan blue dye while dead cells become compromised with their membrane integrity and take up the dye and appear blue in color. Trypan blue dye exclusion assay was used to determine the cytotoxicity of Ca-DTPA using lymphocytes. Our findings recorded no significant detrimental effect of Ca-DTPA on cellular viability at concentrations 10-100 mM, which concluded negligible toxicity profile of Ca-DTPA in this range (10-100 mM). However, viability of cells decreased significantly above 100mM concentration (viz., 500, 1000, 1250, 1500, and 2000mM) in a dose-dependant manner. This indicated the cytotoxicity of Ca-DTPA above 100mM concentrations. This could be due to activation of the intrinsic mitochondrial death pathway which might be inducing cell apoptosis with increasing dose of Ca-DTPA above 100mM [28].

Fura-2AM is a cell-permeant, ester form, UV-excited radiometric fluorescent dye, used as a fluorescent calcium indicator for measuring intracellular calcium concentration. However, there are some heavy metals which can bind to this fluorescent dye depending on their binding affinity (higher than Ca^{2+}) and can change their fluorescent excitation spectra. Since, Cobalt (Co^{2+}) is known to quench the fluorescent signal of Fura-2AM because of its high affinity than that of Calcium [29], so we've used to measure its fluorescence activity to accomplish our investigation. We studied cell morphology by monitoring cellular fluorescence in cobalt treated lymphocytes after Fura-2AM tagging. Punctuated vacuoles were observed in the perinuclear region of the cells, indicating the presence of Co^{2+} induced oxidative stress/ disruption in the mononuclear cells at 5 mM $CoCl_2$ concentration. The present findings are in congruence with the previously published reports [30, 31]. On the other hand, prior treatment with Ca-DTPA revealed protective chelating effect in cells with reduced cellular destruction as compared to cobalt control (Group-II). Ca-DTPA itself showed no morphological changes in cells because of safe administrative dose (100 mM), already proved with cell survival or cytotoxicity studies. As our study was designed to measure intracellular cobalt deposition before or after Ca-DTPA treatment, we chose this cell permeant dye, which would bind to intracellularly distributed Co^{2+} after cleavage of AM group by esterase enzyme inside the cell to leave Fura-2 free acid [32]. Had the Fura-2 been extracellular, then it would have been chelated by Ca-DTPA itself [33].

It has been reported that Co^{2+} is known to quench the fluorescence signal of Fura-2AM because of its similar excitation spectrum to that of Ca^{2+} complex [29]. That is why we conducted present study for qualitative analysis of intracellular cobalt deposition using Fura-2AM. Upon binding of Fura-2 to Co^{2+} resulted in significant changes in the fluorescence intensity in PBMC. An evanescent increase was observed in the fluorescence ratio (F_{340}/F_{380}) in comparison to normal control (untreated cells, Group-I). On the other hand, upon drug treatment, the increase in ratio by stimulation with 5 mM cobalt chloride was decreased significantly at intracellular levels in a dose-dependent manner to the level of normal control (untreated, Group-I) as well as in comparison to cobalt control (Group-II). These findings suggested that the increase in the fluorescence was depending on Co^{2+} cation but not on intracelluar Ca^{2+} due to high affinity of Fura-2 for Co^{2+} relative to Ca^{2+} [29]. While the decrease in the fluorescence could be due to extracellular Co^{2+} chelation by Ca-DTPA, resulted in decreased intracellular Co^{2+} deposition.

6. Conclusion and Future Directions

Present study has exhibited the successful use of Ca-DTPA as an extracellular decorporative agent for preventing cobalt deposition intracellulary in the PBMC. In this context, Fura-2AM has helped to measure Co^{2+} uptake and to image intracellular Co^{2+} deposition in lymphocytes. However, for establishing Ca-DTPA a better decorporation agent with enhanced mitigation abilities, a rigorous preclinical investigations encompassing few of its modifications and their pharmacodynamic and pharmacokinetic studies are warranted.

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