Research Article

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IN VITRO PROLIFERATIVE EFFICACY OF AN INNOVATIVE NATURAL CALCIUM FORMULATION IN BONE AND MUSCLE CELLS

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ABSTRACT

Calcium is an essential macronutrient with a critical role in structural, functional and metabolic processes, particularly bone and muscle systems. Although dietary intake typically meets calcium needs, uneven food habits, anti-nutrients and various pathophysiological conditions can lead to deficiencies. Addressing this gap, Dabur India Limited developed an Innovative Natural Calcium Formulation (INCF) featuring natural organic calcium, plant-derived vitamin D3 and lemon juice. Two dosage forms providing 250 mg and 500 mg of calcium were developed and tested for efficacy using *in vitro* proliferation of Chondrocyte (C20A4 - Bone) and Muscle (L6 rat skeletal) cells at noncytotoxic concentrations. Results showed chondrocyte proliferation of 9.9% - 24.4% and skeletal muscle proliferation of 10.94% - 28.46% compared to untreated control. These findings indicate the potential of INCF as an effective calcium supplement.

Keywords: Calcium Deficiency, Calcium Supplement, Bone and Muscle Development.

INTRODUCTION

The skeletal framework provides shape, stability and locomotion to the human body. Bones are critical for their role in hematopoiesis, mineral metabolism and endocrine signaling[1], while muscles are crucial for blood flow, nerve signalling and mechanical movement[2]. Together, bone and muscles form the core structural and physiological framework of the body. Calcium is vital for bone development and is a regulatory molecule for muscle contraction and flexibility. The recommended dietary allowance (RDA) of calcium for the Indian population is 1000 mg/day (both men and women), as specified by the Indian Council of Medical Research (ICMR) - National Institute of Nutrition (NIN)[3]. Altered Ca^{2+} levels are linked to conditions like osteoporosis, nutritional rickets, retarded growth, dystrophies, Brody's disease and malignant hyperthermia[4]. Vitamin D is essential for bone and muscle physiology, calcium homeostasis[5] gene expression, cell-cycle regulations, cancer prevention and cardiovascular health[6,7].

Calcium deficiency is primarily caused by dietary defects and conditions such as lactation, post-menopause and ageing[8]. Indian populations have lower calcium intake than Western countries[9-12], often due to a preference for non-dairy products owing to their cost effectiveness[13]. Although many calcium supplements are commercially available, some have been reported for heavy metal toxicity depending on the source of calcium[14].

To address this gap, Dabur India Limited developed an Innovative Natural Calcium Formulation (INCF) – a natural calcium supplement designed with an aim to support bone and muscle cell development. Combined with plant-derived vitamin D3 and lemon juice, it enhances the potential for calcium absorption[15]. Two formulations of INCF, Test Product – 1 (TP1) and Test Product – 2 (TP2), for elemental calcium equivalent of 250 mg and 500 mg respectively, were used in this study.

MATERIALS AND METHODS

The test product (TP), Dabur Innovative Natural Calcium Formulation, was evaluated for its cell proliferative properties using bone and muscle cell lines. C20A4 human chondrocyte cell lines were used to study bone development, while L6 rat skeletal myoblast cell lines were used for muscle development. The C20A4 cell line was procured from MERCK, while the L6 cell line was obtained from the National Centre for Cell Science (NCCS), Pune, India. Cells were cultured as per the standard practices. Stock cultures were grown in 25 cm^2 culture flasks, and cytotoxicity assays were performed in 96-well plates using DMEM-HG medium supplemented with 2 % Fetal Bovine Serum (FBS) and antibiotics in a humidified atmosphere of 5% CO_2 at 37 °C.

Cytotoxicity assay

Before cell proliferation assays, the cytotoxicity of the test products was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay based on the methods of Denizot (1986), to observe the optimal cell viability. Trypsinized cell culture monolayers were washed with DPBS (Dulbecco's Phosphate-Buffered Saline) and test products (7.8 µg/ml – 1000 µg/ml) were added in the 96-well plates. Untreated cells served as the control group. The plate was incubated at 37 °C for 24 hours in a 5% CO_2 atmosphere. After incubation, the test solutions were discarded and 100 µl of MTT, diluted with DPBS, was added to each well. The plate was gently shaken and incubated for 3 hours at 37 °C in a 5% CO_2 atmosphere. The supernatant was removed, 100 µl of DMSO (Dimethylsulfoxide) was added, and the plate was gently shaken to solubilize the formed

formazan. Absorbance was measured using a microplate reader (Bioteck, USA) at 570 nm.

Cell Proliferation

The cell proliferative property of the test product was determined on the C20A4 cell line and L6 cell line using standard methods. Cells ($5x10^4$) were seeded per well into a 6-well plate and incubated for 24 hours at 37 °C. Test substances were added to the wells in triplicate at 500 µg/ml and 250 µg/ml concentrations. After 72 hours of incubation, supernatants were removed and 2 ml of MTT was added to each well, which was incubated for 3 hours at 37 °C. Then, 2 ml of DMSO was added to each well. The supernatants were transferred to separate tubes and centrifuged at 1500 rpm for 5 minutes at 4 °C. From each sample, 100 µl of supernatant were transferred to 96 well plates in triplicate, along with the control cells. Absorbance was measured at 570 nm using a microplate reader.

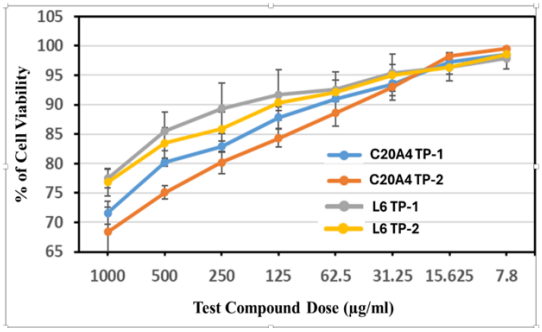

Figure 1: Cytotoxicity Assay Results for Chondrocyte C20A4 Cells and Rat Skeletal Muscle (L6) Cells

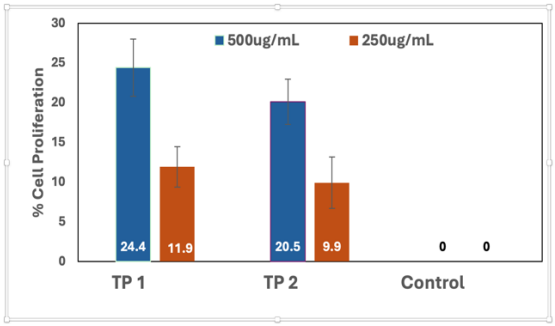

Figure 2: Cell Proliferation Assay Results for C20A4 Human Chondrocyte Cells

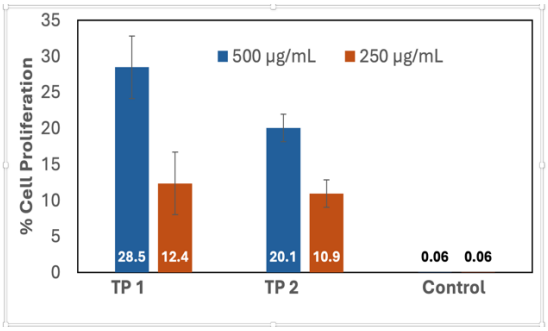

Figure 3: Cell Proliferation Assay Results for L6 Rat Skeletal Muscle Cells

RESULTS

Cytotoxicity

The cell viability of C20A4 and L6 Cells with two formulations, TP1 and TP2, were tested at concentrations ranging from 7.8 µg/ml to 1000 µg/ml (Figure 1). Results indicate that the CTC_{50} of both the formulations in the two cell lines is higher than 1000 µg/ml. Therefore, the proliferation assay was carried out at 250 µg/ml and 500 µg/ml, wherein 75% - 90% of cells remained viable at these concentrations.

Cell Proliferation

Cell proliferation assays on bone and muscle cell lines exhibit increased growth compared to control cells. The test products (TP1 and TP2) enhanced cell proliferation at the tested doses.

With TP1 (Figure 2), C20A4 cells showed 24.4% and 11.9% proliferation at doses of 500 µg/ml and 250 µg/ml, respectively. Similarly, on treatment with TP2, C20A4 cells showed proliferation of 20.1% and 9.9% at doses of 500 µg/ml and 250 µg/ml, respectively.

For L6 cell lines (Figure 3), treatment with TP1 resulted in 28.5% proliferation at 500 µg/ml and 12.4% proliferation at 250 µg /ml. Treatment with TP2 led to a 20.1% proliferation rate at 500 µg/ml and 10.9% at 250 µg/ml compared to the control.

DISCUSSION

Calcium depletion is a natural metabolic process but can be exacerbated by physiological, environmental, dietary and genetic factors. Deficiency may be attributed to limited nutrient awareness, irregular/uneven diet and economic challenges in developing populations. In this scenario, artificial calcium supplementation has evolved as a safe and effective alternative for providing the growth systems of the human body[16] with adequate calcium levels for continuous functioning.

Calcium supplements are vital for pregnant and lactating women, growing children and adults with high blood pressure and the elderly[17,18]. Calcium supplements are also essential in treating fractures, osteoporosis, chronic musculoskeletal pain, etc.[19] They have a key role in bone healing and recovery of orthopaedic patients.

Limited sunlight exposure due to indoor living conditions has led to decreased vitamin D3[16], which INCF can address. Additionally, lemon juice contains high concentrations of citric acid (1.44 and 1.38 g/oz) at about 8% of the dry weight of the fruit[20]. This is a critical advantage, as citric acid has increased calcium absorption and bone formation[15]. Consisting of naturally derived calcium, the INCF also avoids the risk of heavy metal toxicity, as reported in various studies with other supplements[21,22].

Innovative Natural Calcium Formulation tablets by Dabur India Limited offer a robust organic calcium supplement to address the lacunae in *denovo* calcium availability of the body. These tablets, with combined plant-derived vitamin D3 and lemon juice to enhance calcium absorption, show 9.9 – 24.4 % proliferation in bone cells (C20A4 cell lines) and 10.9 – 28.5 % in muscle cells (L6 cell lines) compared to controls. Moreover, in the cytotoxicity assessment, CTC_{50} values for TP1 and TP2 at 1000 µg/ml in both bone and muscle cells were more than 70%, indicating safety and non-cytotoxicity of the doses at 250 µg/ml and 500 µg/ml. This innovative product is a naturally derived nutraceutical formulation with the potential for effectively treating long-term calcium deficiency disorders.

CONCLUSION

The results suggest that Dabur Innovative Natural Calcium Formulation promotes the proliferation of chondrocytes and muscle cells. Its unique combination of calcium with vitamin D3 and lemon juice could be an ideal calcium supplement for optimal function and growth.

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