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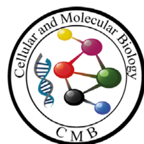

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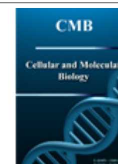

Original Article

Cellular toxicity of calcium propionate in human lymphocyte

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Abstract

Calcium propionate is the chemical substance added to food in order to prolong the shelf-life of factory made foods by inhibiting the development of bacteria, fungi and other microorganisms. The objective of this study was to investigate the ability of calcium propionate to induce cytotoxic and genotoxic effects in lymphocytes. Oxidative stress induction by calcium propionate was also studied. Four concentrations of calcium propionate (0.5, 1.0, 1.5 and 2.0 mg/ml) were applied in lymphocytes for 24 and 48 h treatment. It studied cytotoxic and genotoxic effects by MTT assay, chromosome culture technique, and micronucleus assay. Oxidative stress induction was studied by superoxide dismutase (SOD) activity assay. The results showed that lymphocyte viability was decreased significantly by calcium propionate at 1.5 and 2.0 mg/ml ($p < 0.05$). Calcium propionate induced chromosome aberration at 1.0, 1.5 and 2.0 mg/ml and sister chromatid exchange at 1.5 and 2.0 mg/ml ($p < 0.05$). It induced micronucleus formation at 0.5, 1.0, 1.5 and 2.0 mg/ml ($p < 0.05$). The calcium propionate concentrations of 0.5 - 1.0 mg/ml and 1.5 - 2.0 mg/ml could reduce SOD activity inhibition ($p < 0.05$). Calcium propionate induced oxidative stress in lymphocytes. It can be concluded that calcium propionate induces genotoxic risk and oxidative stress in lymphocytes. Based on this study and the positive results, consumers should be made aware that calcium propionate should be considered a genotoxic compound. The awareness of food preservative usage and the educational program must take place frequently for good human health in the community.

Keywords: Calcium propionate, Genotoxic effects, Oxidative stress, Lymphocyte.

1. Introduction

Food additives play a vital role in food supply. The food preservative is added to food in a scientifically controlled amount. These additions can be made during production, processing, treatment, packaging, transportation or storage of food. Preservatives are substances that are used to prolong the shelf-life of products by protecting them from deterioration caused by microorganisms. The increased consumption of food additives may cause toxic reactions [1].

Calcium propionate is the organic salt formed by the reaction of calcium hydroxide with propionic acid (also known as propanoic acid). Its chemical formula is $Ca(OOCCH_2CH_3)_2$. It is soluble in water and only very slightly soluble in alcohol. Metabolism of propionic acid begins with its conversion to propionyl coenzyme A. Propionic acid occurs naturally in some foods and acts as a preservative in those foods. Calcium propionate is used as a food preservative and is listed as E 282. It is used in breads and other baked goods. Calcium propionate is effective against both *Bacillus mesentericus* rope and mold. Its ability can inhibit the growth of molds and other microorganisms. Calcium propionate is not toxic to these organisms, but it prevents them from reproducing and posing a health risk to humans [2]. Calcium propionate can cause allergies, intolerances, eczema, migraine headaches and skin rashes [3].

Many in vitro test systems are allowing the genotoxic effect evaluation such as chromosomal aberration of food additive in human cells. The sister chromatid exchange assay (SCE assay) was based on the incorporation of the thymidine DNA base analog 5-bromodeoxyuridine (5-BrdU) into the DNA of the cells that replicate twice [4]. The micronucleus test is a test used in toxicological screening for potential genotoxic compounds. The assay is now recognized as one of the most successful and reliable assays for genotoxic carcinogens, i.e., carcinogens that act by causing genetic damage and it is the OECD guideline for the testing of chemicals [5]. Cytokinesis-block micronucleus (CBMN) method was reported, where Cyt-B, an inhibitor of the spindle assembly, was used to prevent cytokinesis occurred after nuclear division. CBMN method is used for the assessment of chromosomal loss, breakage, and associated apoptosis and necrosis induced by different mutagens [6].

Superoxide dismutase (SOD, EC 1.15.1.1) is one of the most important antioxidative enzymes. Three forms of superoxide dismutase are present in humans, in all other mammals, and in most chordates. SOD1 is located in the cytoplasm, SOD2 is in the mitochondria, and SOD3 is

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extracellular. The first is a dimer (consists of two units), whereas the others are tetramers (four subunits). SOD1 and SOD3 contain copper and zinc, whereas SOD2, the mitochondrial enzyme, has manganese in its reactive center. The genes are located on chromosomes 21, 6, and 4, respectively. It alternately catalyzes the dismutation (or partitioning) of the superoxide (O_2^{-}) radical into either ordinary molecular oxygen (O_2) or hydrogen peroxide (H_2O_2). Superoxide is one of the main reactive oxygen species in the cell. It is produced as a by-product of oxygen metabolism and, if not regulated, causes many types of cell damage. Hydrogen peroxide is damaged and it is degraded by other enzymes such as catalase. SOD is an important antioxidant defense in nearly all living cells exposed to oxygen. As a consequence, SOD serves a key antioxidant role [7]. The physiological importance of SODs is illustrated by the severe pathologies evidently in mice genetically engineered to lack these enzymes [8]. SOD1 mutation can cause familial Amyotrophic Lateral Sclerosis (ALS). Mice lacking SOD1 develop a wide range of pathologies, including hepatocellular carcinoma, an acceleration of age-related muscle mass loss, an earlier incidence of cataracts and a reduced lifespan. In mice inactivation of SOD2 causes perinatal lethality. The extracellular superoxide dismutase (SOD3, ecSOD) contributes to the development of hypertension. Diminished SOD3 activity has been linked to lung diseases such as Acute Respiratory Distress Syndrome (ARDS) or Chronic Obstructive Pulmonary Disease (COPD) [9].

Many food preservatives are prevalent. Many people are concerned about the idea of preservatives that are used as ingredients in the foods they eat. The possible genotoxic effects of food preservatives such as calcium propionate are still unknown or their results are conflicting. The teratogenicity and reproductive toxicity test for calcium propionate was negative but there was evidence that it was a mutagen [10]. At present, there is no published data about toxicity of calcium propionate completely on cellular viability, chromosome aberrations, micronucleus induction and oxidative stress induction by SOD activity inhibition in human cells. The toxic effects of calcium propionate in human cells need further investigation. For this reason, this study aimed to examine the cytotoxic and genotoxic effects of calcium propionate in human lymphocytes by MTT assay, chromosome culture technique, sister chromatid exchange assay and micronucleus assay. The effect of calcium propionate was also studied on oxidative stress, especially SOD activity. This study will be useful for the consumers for prevention of calcium propionate toxicity. They may realize that calcium propionate is toxic in preservative food.

2. Materials and Methods

2.1. Chemicals and lymphocyte culture

Calcium propionate, 5-bromodeoxyuridine and cytochalasin B were obtained from Sigma-Aldrich, USA. Human lymphocyte cell line (ATCC® PCS-800-013TM) was cultured in RPMI medium (containing 10% fetal bovine serum (Invitrogen, USA.), antibiotics and phytohemagglutinin M). The human lymphocyte culture was incubated at 37 C in 5% CO_2 incubator.

Lymphocyte cultures were added with 0.5, 1.0, 1.5 and 2.0 mg/ml concentrations of calcium propionate for 24 h in cytotoxic study. Lymphocyte cultures were added with 0.5, 1.0, 1.5 and 2.0 mg/ml concentrations of calcium propionate for 24 and 48 h in both genotoxic study and SOD activity assay. The total cell culture incubation time of each experiment before cell harvesting was 72 h. There was no addition of calcium propionate in the control experiment. Each experiment in this study was repeated three times and results were expressed as mean±SD

2.2. Cytotoxic study of calcium propionate in lymphocytes

Lymphocytes (1x 10^6 cells/ml) were cultured and added with 0.5, 1.0, 1.5 and 2.0 mg/ml concentrations of calcium propionate for 24 h treatment. The viability of cells was assessed by MTT assay.

2.3. Genotoxic study of calcium propionate in lymphocytes

For chromosomal culture technique and sister chromatid exchange assay, lymphocytes (1x 10^6 cells/ml) were cultured in RPMI medium supplemented with 10 µg/ml 5-bromodeoxyuridine. The cells were added with 0.5, 1.0, 1.5 and 2.0 mg/ml concentrations of calcium propionate for 24 and 48 h treatment. 0.06 µg/ml colchicine was added 1 h prior to the harvesting of the culture. For cell collection, the cultures were centrifuged (216 g for 15 min), added with hypotonic 0.075 M KCl solution for 30 min at 37 ˚C and then fixed in methanol and acetic acid in a 3: 1 ratio for 20 min, at room temperature and centrifuged. Cells were treated with a fixative, repeated twice. Finally, metaphase spreads were prepared by dropping the concentrated cell suspension onto slides. The slides were stained with 5% Giemsa (pH = 6.8) prepared in Sorensen buffer solution, for 20-25 min, washed with distilled water, dried at room temperature and mount the slides. The chromosomal aberrations were scored from 100 well-spread metaphases for each calcium propionate concentration. The mean frequency of abnormal cells and the number of chromosomal aberrations per cell were calculated.

For sister chromatid exchange assay, the slides were stained with Giemsa following sister chromatid exchange technique [11]. The number of SCEs was scored from a total of 50 cells in the second metaphase stage for each treatment.

For micronucleus assay, lymphocytes (1x 10^6 cells/ml) were cultured and added with 0.5, 1.0, 1.5 and 2.0 mg/ml concentrations of calcium propionate for 24 and 48 h treatment. Cytochalasin B (conc. 5.2 µg/ml) was added to arrest cytokinesis at 44 h after the start of culture. Cells were harvested at 28 h later, then added with a hypotonic solution (0.075 M KCl) and fixed with methanol and glacial acetic acid in a 3:1 ratio v/v, supplemented with formaldehyde, according to Palus et al (2003) with some modifications. The slides were air-dried and stained with 5% Giemsa. Micronucleus was scored from 1000 binucleated cells per calcium propionate concentration [12]. The chromosomal aberrations, chromosome break, sister chromatid exchange and micronuclei were observed under microscope.

2.4. SOD activity assay of calcium propionate in lymphocytes

Lymphocytes (1x 10^6 cells/ml) were cultured and added with calcium propionate concentrations of 0.5,

1.0, 1.5 and 2.0 mg/ml for 24 and 48 h treatment. After treatment, the SOD activity in cells was determined using superoxide dismutase activity assay kit (BioVision) provided by the manufacturer.

2.5. Statistical analysis

For the percentage of abnormal cells, chromosomal aberrations, and micronucleus cells, the results were expressed as mean±SD. SOD activity was also expressed as mean±SD. The statistical significance by comparing data among the treated groups and the control group was analyzed using ANOVA and $p < 0.05$ was regarded as statistically significant.

3. Results

3.1. Cytotoxic effect of calcium propionate in lymphocytes

The calcium propionate concentrations of 1.5 and 2.0 mg/ml could reduce lymphocyte viability ($p < 0.05$) for 24 h treatment compared with the control group (Figure 1).

3.2. Genotoxic effects of calcium propionate in lymphocytes

The results of chromosome aberrations in this study indicated that the concentrations of calcium propionate significantly increased chromosomal aberrations at 1.0, 1.5 and 2.0 mg/ml for 24 h treatment (Figure 2) and 48 h treatment (Figure 3) compared with the control group ($p < 0.05$). The effect was concentration-dependent in both 24 and 48 h treatments. Abnormal structural chromosomes such as chromosome gap, and sister chromatid separation were recorded. Polyploidy was recorded as numerical chromosomal aberration in this study.

For sister chromatid exchange analysis (SCE), calcium propionate induced a concentration dependent increase in the number of SCEs/cell at 1.5 and 2.0 mg/ml compared with the control group ($p < 0.05$) in both 24 and 48 h treatments. Figure 4 was shown about sister chromatid exchange caused by calcium propionate at 48 h treatment.

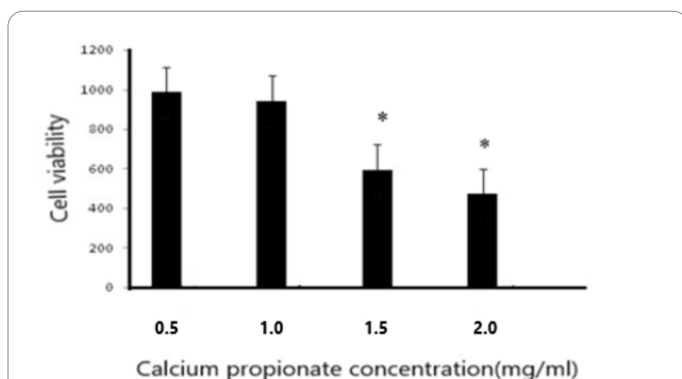

Fig. 1. The effect of 24 h treatment with different concentrations of calcium propionate (0.5, 1.0, 1.5 and 2.0 mg/ml) on cell viability. *Calcium propionate significantly reduced. lymphocytes viability at 1.5 and 2.0 mg/ml compared with the control group ($p < 0.05$).

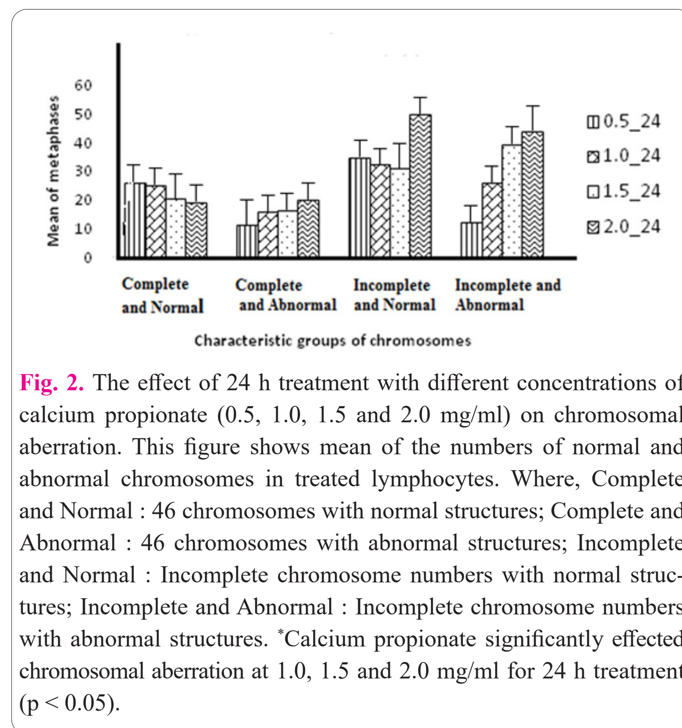

Fig. 2. The effect of 24 h treatment with different concentrations of calcium propionate (0.5, 1.0, 1.5 and 2.0 mg/ml) on chromosomal aberration. This figure shows mean of the numbers of normal and abnormal chromosomes in treated lymphocytes. Where, Complete and Normal : 46 chromosomes with normal structures; Complete and Abnormal : 46 chromosomes with abnormal structures; Incomplete and Normal : Incomplete chromosome numbers with normal structures; Incomplete and Abnormal : Incomplete chromosome numbers with abnormal structures. *Calcium propionate significantly effected chromosomal aberration at 1.0, 1.5 and 2.0 mg/ml for 24 h treatment ($p < 0.05$).

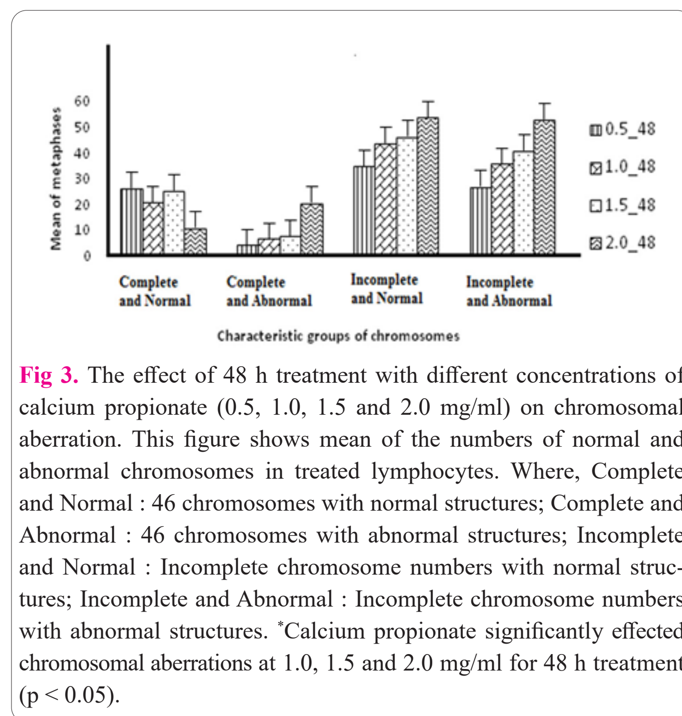

Fig 3. The effect of 48 h treatment with different concentrations of calcium propionate (0.5, 1.0, 1.5 and 2.0 mg/ml) on chromosomal aberration. This figure shows mean of the numbers of normal and abnormal chromosomes in treated lymphocytes. Where, Complete and Normal : 46 chromosomes with normal structures; Complete and Abnormal : 46 chromosomes with abnormal structures; Incomplete and Normal : Incomplete chromosome numbers with normal structures; Incomplete and Abnormal : Incomplete chromosome numbers with abnormal structures. *Calcium propionate significantly effected chromosomal aberrations at 1.0, 1.5 and 2.0 mg/ml for 48 h treatment ($p < 0.05$).

Table 1. Effect of calcium propionate on micronucleus formation in lymphocytes.

Calcium propionate conc. (mg/ml)	No. of micronucleus±SD (cells/1000 binucleated cells) in 24 h treatment	No. of micronucleus±SD (cells/1000 binucleated cells) in 48 h treatment
0	10±4.2	10±5.3
0.5	32±22.5*	36±14.4*
1.0	33±18.5*	49±12.7*
1.5	37±5.0*	51±8.0*
2.0	39±13.6*	53±42.3*

*Calcium propionate significantly increased micronuclei at 0.5, 1.0, 1.5 and 2.0 mg/ml in 24 and 48 h treatments compared with the control group ($p < 0.05$).

The micronucles was observed in this study. Calcium propionate significantly increased micronuclei at 0.5,1.0, 1.5 and 2.0 mg/ml for 24 and 48 h treatments compared with the control group ($p < 0.05$) (Table 1)

3.3. Calcium propionate effects on SOD activity

At 24 and 48 h treatments, the concentrations of 0.5 - 1.0 mg/ml and 1.5 - 2.0 mg/ml calcium propionate could reduce the percentage inhibition of SOD activity ($p < 0.05$). It was detected that these calcium propionate concentrations affected SOD activity in lymphocytes (Figure 5).

The results of all experiments presented that the calcium propionate concentrations of 1.5 and 2.0 mg/ml affected lymphocyte viability, chromosome aberrations, sister chromatid exchanges, micronucleus formation and SOD activity inhibition ($p < 0.05$). The results showed that calcium propionate had a cytotoxic effect on human lymphocytes and had genotoxic effects on human chromosomes and micronucleus in vitro. Calcium propionate affected oxidative stress by reducing percentage inhibition of SOD activity.

4. Discussion

This study evaluated the mutagenicity of calcium propionate which is the food additive commercially and commonly used foods by genetic testing in vitro. The analysis of chromosomal aberrations, SCEs, and micronucleus are popular biomarkers of genetic, carcinogenic and mutagenic effects. Chromosomal aberrations can trigger the development of cancer [13]. SCEs are often regarded as a parameter to assess genotoxicity. A micronucleus is either an acentric chromosome fragment or a whole chromosome left behind during mitotic cell division, appearing as a small additional nucleus in the cytoplasm of the interphase cells. This assay detects both chromosome breakage and chromosome lagging due to dysfunction of mitotic apparatus [14,15]. In this study, the genetic effects of calcium propionate on cultured lymphocytes were evaluated at the cytogenetic level, by monitoring with chromosomal aberrations, SCEs and micronucleus. The results clearly revealed the genotoxic effects of calcium propionate. Calcium propionate significantly increased the frequency of chromosomal aberrations at 1.5 and 2.0 mg/ml concentrations for the 24 h treatment and 48 h treatment. Calcium propionate also could induce SCEs according to these concentrations. Calcium propionate induced micronucleus in this study. The numbers of micronucleus were increased when the concentrations of calcium propionate increased. These data presented that calcium propionate is a genotoxic agent. MTT assay was studied for cellular proliferation toxicity by calcium propionate and it showed that this preservative could reduce cellular viability. Calcium propionate was reported that it may decrease dry matter intake (DMI) and insulin sensitivity in steers [16]. It evoked immune suppressive effects by promoting inflammatory reactions, lymphocyte depletion, hemorrhage, and necrosis in spleen and affected the upregulation of IFNγ, TNF-α, IL-6, IL-1β, and IL-10 in the exposed rat [17]. Calcium propionate affected the hepatic tissues in rat, including vacuolar degeneration of hepatocytes with pyknotic nuclei and kupffer cell growth. It provoked hepatic and renal inflammatory reactions via Toll-like receptors/nuclear factor kappa B signaling pathway. It has a negative impact on liver and kidney function. The negative effects could be mediated via oxidative stress induction, inflammatory reactions, and cytokine production. AST and ALT enzyme activity were excessive utilization in inactivating the free radicals generated by calcium propionate. The enhanced lipid peroxidation was linked to the effect of increased reactive oxygen species (ROS) formation caused by calcium propionate [18]. The acute effect of calcium propionate on glucose metabolism in human was also investigated [19]. The mechanism of calcium propionate-induced DNA damage is presently unknown. Alkylation of DNA bases has been postulated for inducing DNA da-

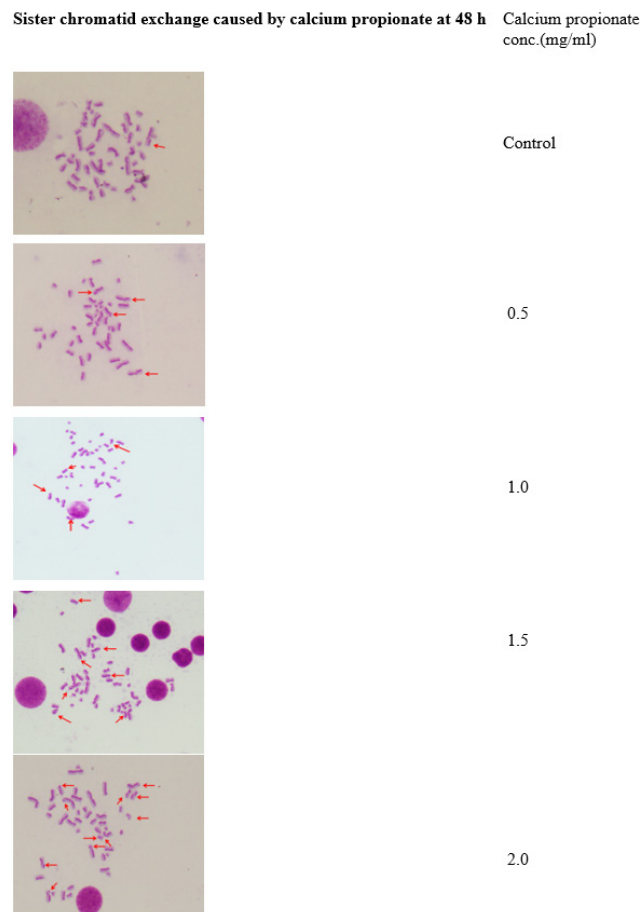

Fig. 4. The effect of 48 h treatment with different concentrations of calcium propionate (0.5, 1.0, 1.5 and 2.0 mg/ml) on sister chromatid exchange. *Calcium propionate at 1.5 and 2.0 mg/ml caused sister chromatid exchange in treated lymphocytes ($p < 0.05$).

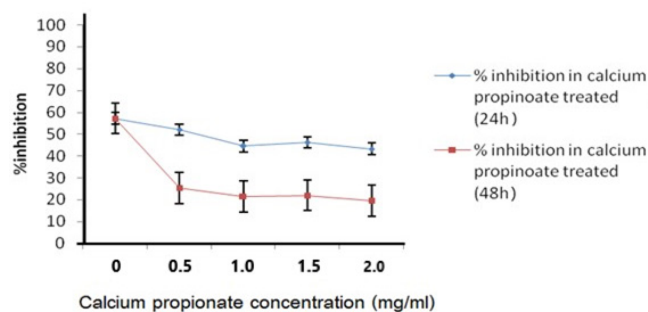

Fig. 5. The different concentrations of calcium propionate (0.5, 1.0, 1.5 and 2.0 mg/ml) treated in lymphocytes for 24 and 48 h affected SOD activity. *The concentrations of 0.5 - 1.0 mg/ml and 1.5 - 2.0 mg/ml calcium propionate could reduce the percentage inhibition of SOD activity ($p < 0.05$).

mage [20]. Chemically, oxidative stress is associated with increased production of oxidizing species or a significant decrease in the effectiveness of antioxidant defenses, such as SOD. The effects of oxidative stress depend upon the size of these changes, with a cell being able to overcome small perturbations and regain its original state [21]. Oxidative stress is suspected to be important in neurodegenerative diseases including Parkinson's disease and Alzheimer's disease. Oxidative stress is likely to be involved in age related development of cancer. The reactive species produced in oxidative stress can cause direct damage to the DNA and are therefore mutagenic, and it may also suppress apoptosis and promote proliferation, invasiveness and metastasis. Oxidative stress is important in neurodegenerative diseases and propanoic acid has neurobiological effects. When propanoic acid is infused directly into rodents' brains, it produces reversible behavior changes (e.g. hyperactivity, dystonia) and brain changes (e.g. innate neuroinflammation, glutathione depletion) that may be used as a model of human autism in rats [22]. This substance affected antioxidant levels in a rodent model and lycopene could reduce oxidative damage by participating in multiple biological activities [23]. Calcium propionate affected oxidative stress by reducing percentage inhibition of SOD activity in this study.

As a results, calcium propionate induced chromosome aberrations (both structural and numerical abnormalities), SCEs and the formation of micronuclei in lymphocytes. It induced cytotoxic effect on lymphocyte viability and most probably posed a genotoxic risk. Calcium propionate is involved in oxidative stress by inhibiting SOD activity. Calcium propionate may be involved in genotoxic and neurobiological effects in human. Calcium propionate should be considered as a genotoxic compound. Based on this study and the positive results, it is necessary to be careful when using calcium propionate as food preservative. Potassium sorbate is a food preservative and it also induced oxidative stress and genotoxicity in human lymphocytes from our previous study [24]. The usage of food preservatives demands more awareness and surveillance for human health. Educational programs and food quality control must take place frequently.

5. Conclusions

Calcium propionate induced chromosome aberration, sister chromatid exchange, micronucleus formation and SOD activity inhibition in lymphocytes. It induced oxidative stress in lymphocytes.

Conflict of interests

The authors have no conflicts with any step of the article preparation.

Consent for publications

The authors read and approved the final manuscript for publication.

Ethics approval and consent to participate

No human or animals were used in the present research.

Informed Consent

The authors declare that no patients were used in this study.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions

KP designed the study, performed the experiments, collected and analysed the data, wrote manuscript; MP designed the study, performed the experiments, analysed the data and wrote manuscript. All authors read and approved the final manuscripts.

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