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Research Article

Micronutrient Supplementation Augments Adenosine Triphosphate (ATP) production in Caco-2 cells

Gaurav K. Jain [a]*, Pavitra Solanki [a], Parimal Mahajan [b], Gajanan Panchal [b], Maneesha Khalse [b], Kamlesh Patel [b]

[a] Centre for Advanced Formulation Technology, Department of Pharmaceutics, Delhi Pharmaceutical Sciences and Research University, Pushp Vihar, New Delhi 110017, India

[b] Medical services, Lupin Ltd. Kalpataru Inspire Off., Western Expressway, Highway, Santacruz East, Mumbai, Maharashtra 400055, India.

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*For Correspondence:

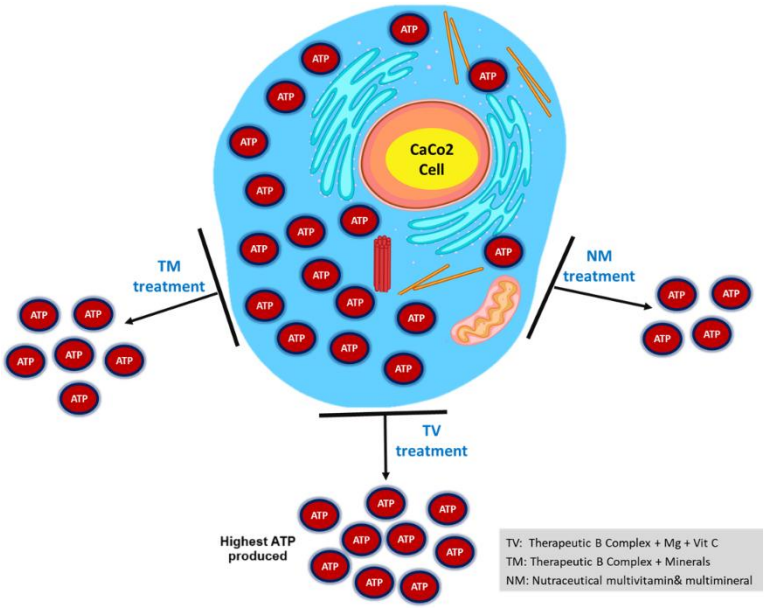
Gaurav K. Jain, DSIR-DPSRU-CRTDH, Centre for Advanced Formulation Technology, Department of Pharmaceutics, Delhi Pharmaceutical Sciences and Research University, Pushp Vihar, New Delhi 110017, India.

Abstract

Over-the-counter micronutrient supplements are widely used to alleviate symptoms of fatigue and tiredness. Owing to variable concentrations of vitamins and minerals in these supplements, their efficacy may vary significantly. The present study aimed at evaluation of ATP production efficacy of micronutrient supplements using Caco2 cells. Three supplements containing therapeutic vitamin B complex with magnesium and vitamin C (TV), therapeutic vitamin B complex with minerals (TM) and nutraceutical multivitamin and multimineral (NM) were selected. Caco2 cells were cultured and treated with varied concentration of supplements (12.5, 25, 50, 100, 200 µg/mL). The cells were incubated for varied time at 37°C in 5% CO_2 atmosphere. The cells were lysed and amount of ATP produced was determined by colorimetry at 570 nm. Cells without treatment (control) and cells treated with 5 µg/mL sodium lauryl sulphate for 30 min (negative control) were used for comparison. A dose-dependent increase in ATP production was observed for all the three supplements. The optimum level of ATP was obtained when cells were treated with 100 µg/mL of supplements for 1 h. Cells treated with supplements showed markedly higher ATP production compared to control and negative control cells. Further, cells treated with TV showed markedly higher ATP level compared to cells treated with TM and NM at both 50 and 100 µg/mL concentration. ATP level was nearly 1.7-fold and 1.2-fold higher in TV treated cells compared to NM and TM treatment, respectively. The decreasing order of ATP production in cells by supplement treatment is as follows: TV > TM > NM. Further, compared to control cells, the cells treated with TV produced nearly 3-fold higher ATP. In conclusion, higher ATP levels in micronutrient treated cells support the therapeutic role of micronutrient supplements in fatigue and tiredness. Further, the concentration of micronutrients is crucial for therapeutic efficacy.

Keywords: Vitamins, Minerals, Fatigue, Caco2 cells, ATP assay

Graphical Abstract



INTRODUCTION

Fatigue, tiredness, exhaustion, and lack of energy is a very common problem for which thorough examination and laboratory tests do not provide a satisfactory explanation[1]. Fatigue and energy are interrelated and a common feature of fatigue is a 'sense of energy depletion', which can be related to an insufficient amount of cellular energy[2]. Adenosine triphosphate (ATP) produced through oxidative phosphorylation in mitochondria is the source of energy for cellular processes such as metabolic reactions, muscle contraction, active transport, and nerve function[3]. Recent research has dramatically increased our understanding of the biochemical processes of cellular energy generation and demonstrated that low cellular ATP have effect on cognitive and psychological processes and causes restlessness, tiredness, physical and mental fatigue[4]. The association of low cellular ATP with anxiety, depression, sleep apnea, anemia, insomnia, thyroid and in few cases with genetic disorders has also been established[5-8]. Recent studies suggest that limitation in energy production processes limit the rates of energy expenditure and hence performance. Thus, it is expected that a strategy for enhancing cellular ATP could have therapeutic prospects in overcoming physical and mental fatigue[9,10]. Micronutrient supplements play key role in a variety of metabolic pathways that support vital cellular and molecular functions[11]. It has been recognized that a lack of micronutrients may impair cellular energy production, resulting in symptoms of fatigue, tiredness, and lack of energy[12]. Theoretically, micronutrient supplement that could increase ATP could also enhance performance and overcome fatigue. Claims regarding the effects of micronutrients particularly B vitamins and minerals on fatigue, cognition and psychological functions are authorized in several reports[13-25] and shown in Figure 1. B vitamins are involved in at least one of the several steps of the energy-production system within the cell and their adequate supply is essential for functioning of the energy-production system. Vitamin B1 (thiamine) is involved in dehydrogenase reactions and helps in formation of acetyl-CoA[14]. Vitamin B2 (riboflavin) is an integral part of the coenzymes flavin mononucleotide and flavin adenine dinucleotide and is critical for production of acetyl-CoA via metabolism of proteins, fats and carbohydrates[15]. Vitamin B3 (niacin) act as precursor for NAD and NADH, which play important role in citric acid cycle and as electron donors during phosphorylation[16]. Vitamin B5 (pantothenic acid) is an essential precursor in the biosynthesis of Coenzyme A[17]. The metabolically active form of vitamin B6 (pyridoxine) act as cofactors of enzymes facilitating metabolism of amino acids and lipids, gluconeogenesis, synthesis of niacin and heme and hormone action[18]. Vitamin B7 (biotin) serves as a cofactor for several carboxylases enzymes that play key role in energy production and storage through synthesis of fatty acids, catabolism of amino acids and gluconeogenesis[19]. Vitamin B9 (folic acid) apart from playing a vital role in synthesis of DNA and RBC is crucial for the synthesis of thymidylate and purines, obligatory for production of cytosolic and mitochondrial ATP, total nucleotide triphosphate (NTP) and deoxy-NTP[20]. Vitamin B12 (cobalamin) is a cofactor of the methylmalonyl-CoA mutase enzyme which produces succinyl-CoA from methylmalonyl-CoA[21].

Minerals such as magnesium has a predominant role in the ATP production and utilization. Magnesium binds to ATP to form biologically functional forms (Mg-ATP complex). In mitochondria Mg-ATP complex. Cellular ATP is present as and this complex act as cofactors for several kinases active during glycolysis help export mitochondrial ATP into cytosol and thus deliver energy within the cell[22]. Similarly, role of other minerals such as zinc, copper, selenium, chromium picolinate in ATP production through various mechanisms is well documented. Zinc being important component of RNA polymerase plays vital role in protein synthesis and ATP production. Recent in vitro studies showed that zinc is conducive to mitochondrial pyruvate transport, oxidative phosphorylation, lipid, carbohydrate and energy metabolism[23]. Copper is a component of cytochrome c oxidase, a vital enzyme in the electron transport chain that produces ATP[13]. Selenium, a component of glutathione peroxidase and selenoproteins, is shown to increase skeletal muscle mitochondrial volume density, mitochondrial biogenesis and metabolism leading to enhanced intracellular ATP[24]. Chromium picolinate plays a role in regulating carbohydrate, fat and protein metabolism and studies have demonstrated its usefulness in reducing muscle fatigue[25].

Though immense literature exists documenting the role of micronutrients in energy production but most of the reports are theoretical and lacks research data. Additionally, there is mounting evidence regarding toxic effects of long-term micronutrients use[26]. To date, a vast number of over-the-counter micronutrient supplements containing variable concentrations of vitamins and minerals are being utilized by patients to overcome symptoms of fatigue and tiredness. Therefore, it is crucial to assess the efficacy of ATP production by micronutrient supplements. In this study, we compared the ATP production efficacy of three micronutrient supplements using Caco2 cells, a model of human intestinal epithelial cells. In the past, Caco-2 cells have also been used to study the release and hydrolysis of ATP. [27, 28]

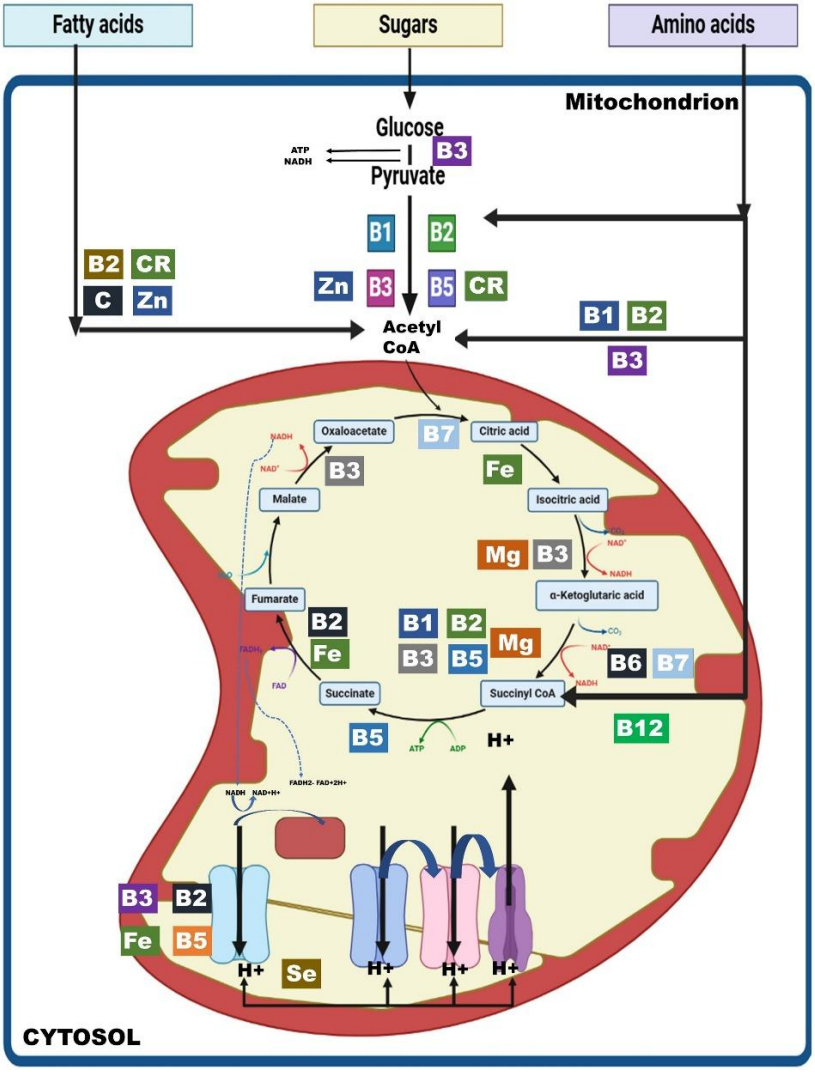


Figure 1: Schematic representation of role of micronutrients in ATP production

MATERIALS AND METHODS

Materials

Caco2 cells were purchased from National Centre for Cell Science (Pune, India). Basal cell culture medium, fetal bovine serum (FBS) and phosphate buffer saline (PBS) were purchased from Himedia (Thane, India). Centrifuge tubes (50 ml and 1.5 ml) and 6-well plates were purchased from Tarson (Noida, India). ATP colorimetric assay kit (ab83355, Abcam) was provided by Life Technologies (New Delhi, India).

Micronutrient Supplements

Three micronutrient supplements were selected to evaluate their effect on cellular ATP production. First supplement (TV), has therapeutic B complex with magnesium and vit C. Second supplement (TM) has therapeutic B complex with minerals. Third supplement (NM) is a nutraceutical multivitamin and multimineral. The detailed composition of TV, TM and NM is shown in Table 1. All the supplements were provided by Lupin Limited (Mumbai, India).

Table 1: Composition of commercial micronutrient supplements used in the study

Supplements	Micronutrient Composition		
	TV	TM	NM
Vit B1 (mg)	10	0	1.1
Vit B2 (mg)	10	0	1.3
Vit B3 (mg)	100	100	14
Vit B5 (mg)	5	0	4
Vit B6 (mg)	3	3	1.5
Vit B7 (mcg)	260	0	30
Vit B12 (mcg)	15	15	1
Vit C (mg)	100	0	40
Vit E (mg)	0	0	10
Folic acid (mg)	1.5	1.5	0.117
Mg (mg)	32.4	0	2
Zn sulphate (mg)	0	22.5	10
Selenious acid (mcg)	0	100	30
Copper (mg)	0	0	1.7
Iodine (mcg)	0	0	140
Chromium (mcg)	0	250	30

Reagents and Standards

ATP assay buffer and ATP probe in DMSO was used as provided. ATP standard was reconstituted in 100 µL of double distilled water to generate a 10 mM ATP standard stock solution. ATP converter enzyme and developer mix was dissolved in 220 µL assay buffer before use. All the reagents were freshly prepared and kept on ice during the assay. ATP standard stock solution (10 µl) was diluted with dH2O (90 µl) to generate 1 mM ATP standard. The 0, 2, 4, 6, 8, 10 µl of this solution was added into series of wells and volume adjusted to 50 µl/well with assay buffer to generate 0, 2, 4, 6, 8, 10 nmol/well of standard.

Cell culture and treatment

Caco2 cells were cultured in media with 10% FBS and penicillin-streptomycin at 37°C in 5% CO_2 atmosphere (Memmert, Germany). Once the cells were 80% confluent, they were trypsinized and used for the experiments. Cells (0.25 x 10^6 cells/ mL) were seeded in a 6-well plate and were allowed to grow overnight. Micronutrient supplements at different concentrations (12.5, 25, 50, 100, 200 µg/mL) were added to respective wells. The plates were incubated for different time (treatment time, 30 min, 1 h, 2h, 4 h) at 37°C in 5% CO_2 atmosphere. Well plate without supplement treatment were used as control (CO), whereas cells treated with sodium lauryl sulphate (5 µg/mL) for 30 min were used as negative control (NO).

Following treatment and incubation, the spent media was removed and the cells were washed with PBS. The cells were resuspended in 100 µL of assay buffer and quickly homogenized by pipetting up and down a few times. The cells were then centrifuged for 5 min at 4°C at 13,000 g in a cold microcentrifuge (Remi, India) to remove insoluble material. The enzymes, if any, were removed from the sample by deproteinizing. The supernatant was collected and transferred to a new tube and kept on ice.

ATP Assay

ATP reaction mix was prepared by mixing 88% assay buffer, 4% ATP probe, 4% converter enzyme and 4% developer mix. The control was also prepared in same manner; however, 4% converter enzyme was replaced with assay buffer. In the absence of converter enzyme, the assay detects only endogenous glycerol phosphate but not ATP. Briefly, reaction mix (50 µL) was added into each standard, sample, and control wells and the well plates were incubated at room temperature for 30 min protected from light. The readings were measured on a microplate reader (ELX800, Biotek) at 570 nm optical density. All assays were performed in duplicate and controls were run with all the samples. The correction in the absorbance were made by subtracting mean value of the blank from all sample readings. The corrected values for each standard were plotted as a function of the final concentration of ATP. The formula for straight line was used to calculate ATP concentration in the wells.

Statistical Analysis

All the results were presented as the mean ± standard deviation (SD). Data between the groups was compared using the student t-test. The $p < 0.05$ was considered statistically significant. All statistical calculations were done using SPSS V26.0 (SPSS Inc., Chicago, USA).

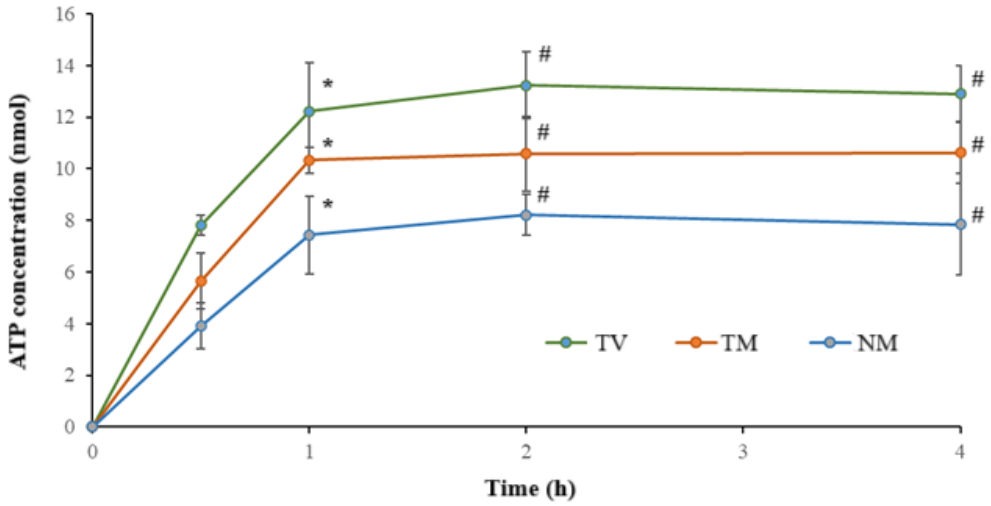
RESULTS

Effect of treatment time

The amount of ATP produced following treatment of cells with supplements for 30 min, 1 h, 2 h and 4 h is shown in Figure 2. With increase in treatment time from 30 min to 1 h, a significant ($p< 0.05$) increase in ATP production was observed. Thereafter, increasing the treatment time to 2 h and 4 h, resulted in small but insignificant ($p>0.05$) increase in ATP. Similar results were obtained for all the three supplements tested and therefore treatment time of 1 h was selected for further experiments.

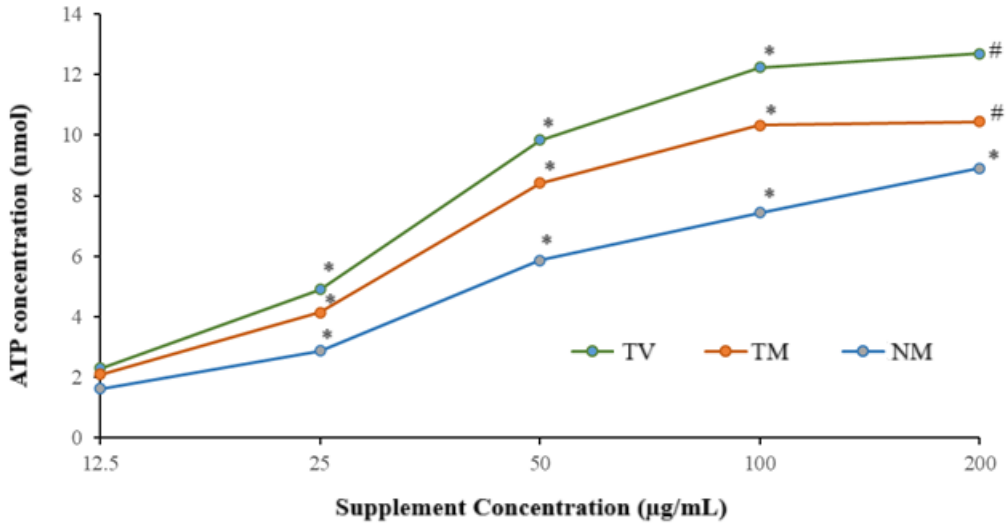
Effect of supplement concentration

The effect of supplement concentration on ATP production was determined. Caco2 cells were treated with supplements at concentration of 12.5, 25, 50, 100, 200 µg/mL. The results are presented in Figure 3. A dose-dependent increase in ATP production was observed for all the supplements. The saturated concentration for TV and TM supplements was 100 µg/mL. Though at 200 µg/mL concentration the ATP production was higher, but the difference was not significant for TV and TM supplements. For NM supplement the ATP production was maximum at 200 µg/mL and the values were significantly higher than those observed at 100 µg/mL. For comparison of supplements the concentration of 50 and 100 µg/mL was selected.



Time (h)	ATP concentration (nmol)		
	TV	TM	TN
0.5	7.8±0.38	5.7±1.1	3.9±0.9
1	12.2±1.89	10.3±0.5	7.4±1.5
2	13.2±1.3	10.6±1.45	8.2±0.8
4	12.9±1.1	10.6±1.2	7.9±1.98

Figure 2: Effect of treatment time of cells with supplements on ATP production. * p< 0.05, 1h vs 0.5 h; # p>0.05, 2h and 4h vs 1h



Supplement conc. (µg/mL)	ATP concentration (nmol)		
	TV	TM	TN
12.5	2.3	2.1	1.62
25	4.9	4.15	2.88
50	9.83	8.42	5.87
100	12.23	10.33	7.43
200	12.68	10.45	8.9

Figure 3: Effect of supplement concentration on ATP production. * p< 0.05 vs previous concentration and # p>0.05 vs previous concentration

Comparison of micronutrient supplements

Micronutrient supplements (TV, TM and NM) at concentration of 50 and 100 µg/mL were compared for their efficacy to produce cellular ATP Caco2 cells. Control group without treatment and negative control group with SLS treatment was also used for comparison. The amount of ATP produced following treatment as determined by colorimetry is shown in Figure 4. Cells treated with supplements showed markedly higher ATP production compared to control and negative control cells. ATP level in control cells was significantly higher ($p<0.05$) than negative control cells. Cells treated with TV showed markedly higher ATP level compared to cells treated with TM and NM at both the concentration tested. At 50 µg/mL, ATP level was 1.7-fold and 1.2-fold higher in TV treated cells compared to NM and TM treatment, respectively. Similar results were observed at 100 µg/mL and ATP level was higher in TV treated cells. The decreasing order of ATP production in cells by supplement treatment is as follows: TV > TM > NM. Further, compared to control cells, the cells treated with TV produced nearly 3-fold higher ATP.

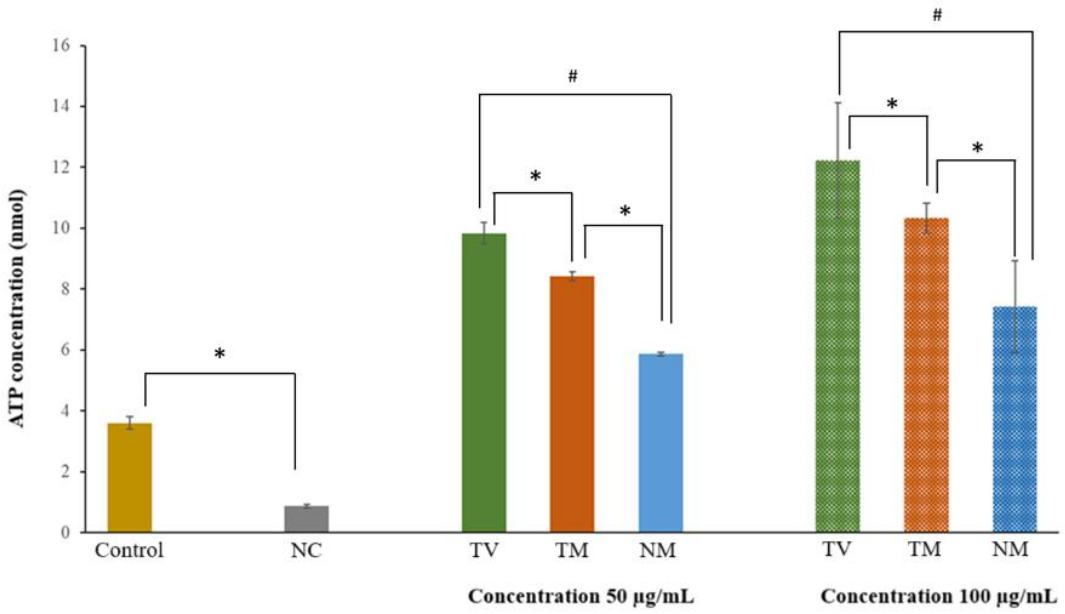


Figure 4: Comparative ATP produced following treatment of cells with control, negative control (NC), and micronutrient supplements (TV, TM and NM) at concentration of 50 and 100 µg/mL and at treatment time of 1 h. * $p<0.05$, # $p<0.001$.

DISCUSSION

Micronutrient supplements are widely available commercially as over the counter products. Most of these micronutrient supplements contains varied concentrations, ratios and proportions of vitamins and minerals, with B-vitamins and minerals being most prominent. The role of micronutrient supplements in cellular ATP production has been well reviewed, however, proof-of-concept studies to validate the same are limited[13]. Though, few studies describe the role of single micronutrient in ATP production[14-18] but the data is poorly elucidated. Thus, we conducted an in vitro studies using Caco2 cells to demonstrate the efficacy of micronutrient supplements in ATP production. Caco2 cells have been used in our study since they are easy to handle and they have been used in past for studying ATP release and hydrolysis[27, 28]. Further, caco-2 cells are human intestinal epithelial cells widely used for oral absorption and permeability studies[29] and are best suited for efficacy study of oral micronutrient supplements. Cells were grown in basal cell medium with limited micronutrients. Use of basal cell medium allows production of ATP by micronutrient treatment which could be distinguishable and comparable. Since no similar study has been reported previously therefore to optimize maximum ATP production multiple experiments were conducted with varied treatment time (30 min to 4 h) and micronutrient concentration (6.5 µg/mL to 200 µg/mL). Our results showed that maximum ATP level was achieved when cells were treated with micronutrients for 1 h. This also reveal that following oral administration the effect of micronutrient supplement will be apparent within 1 h. Interestingly, micronutrient supplements dose-dependently increased ATP level, with maximal intracellular ATP level observed at 100 µg/mL. Our results were in agreement with previous reports where dose-dependent increase in intracellular ATP was observed following treatment with pyruvate[30] and kaempferol[31]. Notably, the ATP levels in cells treated with 100 µg/mL and 200 µg/mL of TV and TM were not significantly different. This showed that the process of ATP production is saturable. This could be plausible explanation for harmful effects observed at very high doses of vitamins and minerals[26]. Several reports demonstrated neuropathy and ataxia with high doses (>200 mg/ day) of vitamin B6 and hepatotoxic and hypoglycemic effects at high doses (>3000 mg/ day) of vitamin B3. Minerals such as magnesium can cause diarrhea at doses greater than 400 mg/day and high

doses of zinc are immunosuppressive and can also cause nausea and vomiting. Selenium at dose in excess of 0.91 mg/ day can cause brittle nails and hair, and peripheral neuropathies[26].

We compared three micronutrient supplements with varied composition and concentration. TV supplement has therapeutic B complex with magnesium and vit C, TM has therapeutic B complex with mineral and NM contains nutraceutical multivitamins and multiminerals. These different compositions allowed comparison of efficacy of therapeutic and nutraceutical concentrations of vitamins and minerals. No treatment group was considered as control whereas treatment with SLS was considered as negative control. The ATP level in control group was significantly higher ($p<0.05$) than negative control group. This could be due to presence of basal medium that supports cell growth and ATP production. Further, in negative control group, SLS treatment may have caused cell death and thus significantly low production of cellular ATP[32]. Higher ATP level in supplement treated cells substantiated the therapeutic use of supplements in overcoming fatigue and tiredness. Cells treated with TV showed 1.2-fold higher ATP compared to TM treatment signifying that vitamins are comparatively more effective than minerals. The findings were in agreement with previous reports[13, 33].

Nevertheless, higher ATP levels in TM treated cells compared to NM and control, substantiates the role of minerals in ATP production. Interestingly, TM supplement showed ATP rise even in the absence of magnesium, which is considered as very important factor for ATP generation since ATP in cells in bound to magnesium ions[22]. It is hypothesized that chromium picolinate or selenious acid or both in TM supplement have performed activity similar to magnesium and resulted in ATP production in cells. Markedly higher ATP level in cells treated with TV and TM compared to NM provide scientific evidence that support the role of micronutrient concentration in ATP production. Further, low ATP level in cells treated with NM might be due to low vitamins and minerals in nutraceutical composition. This also supports significantly higher ATP level in cell treated with 200 µg/mL NM compared to 100 µg/mL. In contrast, cells treated with TV and TM showed saturation at 100 µg/mL concentration. Interestingly, despite 10-fold lower micronutrients in NM supplement, the ATP levels were only about 1.7-fold lower. This underscores the possibility of synergistic activity of vitamin and minerals. The decreasing order of ATP levels in cells is as followed: TV > TM > NM>CO>NC.

The present in vitro study has several limitations. Firstly, the study evaluated the efficacy of supplement composition rather than individual components. It was speculated that determining efficacy of individual component would be difficult. This is because cell growth requires micronutrients and in presence of other micronutrient in media it would be difficult to analyze the effect of individual component. Secondly, study was conducted on Caco2 cells, whereas previously NSCLC A549 cells have also been used. Nevertheless, use of ATP release and hydrolysis has been studied with Caco2 cells previously[27, 28] and NSCLC A549 cells have been deployed previously for studying eATP with regard to drug resistance mechanisms and cancer metabolism and hence not used in this study. In conclusion, our studies provide the scientific evidence that support the therapeutic role of micronutrient supplements in fatigue, tiredness and lethargy or any other disease caused due to cellular energy depletion. Further, the composition and concentration of vitamins and minerals present in the supplements are crucial for optimal efficacy. Therapeutic B complex with magnesium and vitamin C or with minerals performed better than nutraceutical multivitamin and multimineral.

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Conflict of Interest: The co-authors Parimal Mahajan, Gajanan Panchal, Maneesha Khalse, Kamlesh Patel are the employees of Lupin Limited, Mumbai, India.

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Ethical approval: Not applicable.

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