EFFECT OF THE DIETARY SUPPLEMENT ELEVATP ON BLOOD ATP LEVEL: AN ACUTE PILOT CLINICAL STUDY

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Abstract: *Objectives:* Adenosine triphosphate (ATP) participates in a number of biological processes and its levels diminish during aging. We studied the effects of a proprietary combination of a plant-mineral-rich ancient peat material and a polyphenol-rich apple extract, marketed under the trade name elevATP™, on blood ATP levels. *Design:* Acute, placebo-controlled, prospective clinical trial. *Participants:* 18 generally healthy, adult human subjects. *Intervention:* A single, 150 mg dose of elevATP™ or 50 mg of encapsulated silica oxide (placebo). *Measurements:* Blood was collected prior to, and at 60, 90 and 120 minutes after treatment. We measured whole blood ATP, total mammalian target for rapamycin (mTOR), lactate, reactive oxygen species (ROS), and glucose. We also identified and quantified the mineral and bioactive components of elevATP™. *Results:* When compared to the placebo group, elevATP™ caused an acute increase in blood levels of ATP by 64% (P=0.02). ROS and lactate levels were unchanged by elevATP. Total mTOR levels in blood were modestly, but significantly, lower after treatment. *Conclusion:* Results show that treatment with a single dose of elevATP™ increased blood ATP levels without increasing ROS. Confirmation of these results in a larger study sample is needed. Trials in older individuals may be particularly informative.

Key words: Blood total ATP, blood ROS, blood lactate, blood total mTOR, healthy aging, micronutrients, polyphenols.

Introduction

elevATP™ is a blend of plant bio-inorganic trace minerals and polyphenol-rich apple extracts . The plant mineral portion has previously been reported by our research team to have potential to increase blood levels of ATP in human subjects. Additionally, dietary polyphenols are widely distributed in fruits, wine, tea, vegetables and fruits and possess many biological functions, (for review see (1)). Recently, polyphenols have been shown to play an important role in the functioning of mitochondria (2-6).

Mitochondria are the primary energy generating organelles of the cell, producing ATP through a chain of enzyme complexes. These enzymes require metals such as iron, copper and manganese for catalytic activities. However, mitochondria are highly sensitive to oxidative damage and must balance the availability of transition metals with the generation of reactive oxygen species (ROS) (7). ATP not only is an intracellular energy carrier and participates in hundreds of biochemical reactions (8), but also has a number of extracellular functions. ATP is typically released in response to various stimuli, such as mechanical pressure, or after treatment with agonists, such as serotonin and acetylcholine (9). Extracellular ATP is a requirement for several physiological processes, such as platelet aggregation, peripheral and central neurotransmission, clot formation, cell recognition and immune responses (10-15).

During the process of aging, intracellular ATP decreases and the ability to generate ATP is diminished (9, 16, 17). While this affects intracellular processes, it also suggests that the ability to release ATP to the extracellular milieu for regulatory processes might be limited in aged cells and tissues. Consequently, basic and clinical research has focused on ATP supplementation as means to promote muscle energy metabolism and healthy aging in humans (18).

Previous studies have described conflicting results regarding the use of exogenous ATP as a dietary supplement (19). It has been reported that chronic intake of exogenous ATP can cause alterations in blood oxygenation, peripheral blood flow and muscle metabolism (9). Also, an increase of ATP production is associated with an increase of intracellular ROS (20), which can compromise the integrity of cells by inducing oxidative stress and causing cellular dysfunction (21). Because of the limitations of direct ATP supplementation, some groups have turned to indirect approaches to

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increase physiological ATP production. Recent studies have shown that natural supplements such as polyphenols can enhance and increase the concentration of ATP, as well as lower the activity of lactate dehydrogenase (LDH) and creatine pyruvic kinase (CPK) (21). Our research team is investigating various types of natural products capable of increasing endogenous pools of intracellular ATP, without increasing the production of ROS (22, 23).

In this study, 18 healthy fasting subjects were given a single encapsulated dose of 150 mg of elevATP™ or placebo. We report that elevATP™ significantly increased blood ATP levels with respect to the baseline and versus the placebo, while reducing mammalian target for rapamycin (mTOR) levels and showing no statistically significant effect on serum level of lactate and ROS.

Materials and Methods

Materials

elevATP™ powder was provided by FutureCeuticals, Inc., Momence, IL USA. Dulbecco's phosphate buffered saline (PBS), phenyl methane-sulfonyl-fluoride (PMSF), dimethyl sulfoxide (DMSO), 200% Proof ethanol; leupeptin and water were purchased from Sigma Chem. Co. (St Louis, MO, USA). 5-O-Caffeoylquinic acid, Gallic acid and quercetin-3-glucoside were purchased from Sigma Aldrich (Poole, UK). (–)-Epicatechin and Phloretin-2'-O-glucoside were purchased from Extrasynthese, (Genay, France).

Methanol and acetonitrile were obtained from Rathburn Chemicals (Walkburn, Scotland). Formic acid was obtained from Fisher Scientific (Loughborough, UK). Protein Low Binding microtubes were obtained from Eppendorf (Hauppauge, NY, USA) and RC DC Protein Assay Kit II was from Bio-Rad (Palo Alto, CA, USA). Intracellular ROS kits were purchased from Cell Biolabs (San Diego, CA, USA). ATP-luciferase assays were obtained from Calbiochem (San Diego, CA, USA). Heparin and "dry" blood collection tubes were obtained from BD Vacutainer (Franklin Lakes, NJ, USA). Total mTOR ELISA kits were purchased from Cell Signaling Technology® (Danvers, MA, USA). Portable gas meter and CG4+ cartridges were from Abbott Laboratories (Abbott Park, IL, USA).

ElevATP™ Mineral Analysis

A 1.2 g sample test portion of ElevATP™ was dry-ashed at 500°C ± 50°C for 8 hours and treated with nitric acid. The resultant ash was treated with concentrated hydrochloric acid (5%), dried, and redissolved in hydrochloric acid solution (24). The amount of each element was determined by comparing the emission of the unknown sample against the emission of each element from standard solutions using Inductively Coupled Plasma Atomic Emission Spectroscopy (ICAP-61E-Trace, Thermo Jarrell-Ash) (25)or by mass spectrometry (USP <730>). All standard solutions used were obtained from Inorganic Ventures (Christiansburg, VA, USA) and were of analytical-reagent grade. The RSD for analysis of each element was 4.8%

Polyphenols Analysis

Polyphenol analysis was carried out on a Thermo Surveyor HPLC system comprising of an autosampler with sampler cooler maintained at 6 °C and a photodiode array detector scanning from 200-600 nm. Samples (5 or 10µl) were injected onto a 250 x 4.6mm C18 RP Polar Column (Phenomenex; Torrance, CA, USA) maintained at 40 °C and eluted with a 5-40% gradient of 0.1% formic acid and acetonitrile at 1 mL/min over 45 minutes. The eluted sample passed serially through an absorbance detector and then a fluorescence detector (Jasco, Japan; excitation λ 290 nm, emission λ 320 nm). Twenty percent of the sample was diverted to the electrospray interface of the mass spectrometer. All samples were run in negative ion mode using data-dependent MS-MS for compound identification. The scan range was from 150-1500 amu.

Samples of apple extract were also analyzed using a UHPLC system (Thermo Fisher Scientific, San Jose, CA, USA) with an Orbitrap Exactive mass spectrometer. In this system, a 2 mm version of the column described above was used with the same mobile phase gradient running at a reduced rate of 200 mcL/min. Identifications are based on co-chromatography with authentic standards and from comparison of exact mass or MS-MS spectra with previously published data (26). Quantification with authentic standards was carried out on both absorbance and fluorescence data. Quantification of catechins and procyanindins in the UHPLC system was carried out in the mass spectrometer.

Clinical Study

Inclusion and Exclusion Criteria

This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Institutional Review Board at Vita Clinical SA, Avenida Circunvalacion Norte #135, Guadalajara, JAL, Mexico 44270 (Study protocol ABC-NCI-12-14-ATP). Eighteen study participants were selected. They were generally healthy, not using any type of medication or supplements for a period of at least 15 days prior to the start of the study, with ages between 21 and 55 and a BMI between 21 and 30 kg/m² (SD ±5.88). Participants were

excluded if they self-reported symptoms or carried an active diagnosis of rhinitis, influenza, other acute infections, or diabetes mellitus. Subjects were also excluded if they reported allergies to dietary products. Subjects were excluded upon the use of anti-inflammatories, analgesics, statins, diabetic drugs, anti-allergy medicines, multivitamins or supplements rich in polyphenols.

Blood Collection

Enrolled participants were instructed not to eat for 12h prior to the initial blood draw. Body temperature and blood samples were taken prior to and during treatment. After participants gave written consent, subjects were randomly assigned to either the treatment or placebo group with similar characteristics for age and weight in both groups. The placebo group took 50 mg of encapsulated silica oxide, while the treatment group ingested 150 mg of encapsulated elevATP™. Participants in both groups received 200 ml water to swallow with the test capsule.

Four hundred microliters of blood were collected by finger puncture and placed in Safe-T-Fill® Capillary blood collection tubes (Ram Scientific Inc. Yonkers, NY) or 100 µL heparin-sulfate capillary tubes (Fisher Scientific). Samples were collected at each of four time points: immediately prior to test capsule administration (T0) and at 60, 90 and 120 minutes. Immediately after collection, blood samples were either snap frozen for ATP and ROS assays or further processed for total mTOR assays. Participants remained at rest during testing.

ATP Detection and Quantification

ATP concentration was determined using an ATP Assay Kit (Calbiochem, San Diego, CA, USA) with a modification to the original method, as previously described (23). Briefly, 10 µL of lysed blood and 100 μL ATP nucleotide-releasing buffer containing 1 μL luciferase enzyme mix were added to a white plate and immediately placed on a luminometer (LMaX, Molecular Devices; Sunnyvale CA, USA). A kinetic assay was read at 470 nm for 15 min at 3 min intervals. Relative Light Units (RLU) were recorded and ATP concentrations determined in comparison to an standard curve for ATP.

ROS Detection

ROS were detected by using a cell based ROS assay kit (Cell Biolabs, San Diego, CA, USA) with modifications to the original method, as previously described (23). Briefly, 10 µL of diluted whole blood (1:100 in water) was mixed with 100 µL 2′, 7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) 1X in water in a clear bottom black plate

(Rochester, NY USA). This mixture was immediately placed in a fluorescence spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) and a kinetic assay was run, recording Excitation/Emission (Ex/Em) at 480/530 nm for 60 min at 5 min intervals. ROS concentration was determined by comparison to a 2′, 7′-Dichlorodihydrofluorescein (DCF) Standard Curve.

Lactate Detection

For the determination of lactate levels, finger blood samples were analyzed with an i-STAT clinical blood gas analyzer (Abbott Laboratories, Abbott Park, IL, USA). 100μL of blood were loaded in CG4+ Cartridges (Abbot Laboratories, NJ, USA) and tested for lactate.

Total mTOR Detection

For Total mTOR analysis, cell lysates were prepared according the instructions included in the kit. Briefly, 100 µL of whole blood were added to 900uL of 1X Cell Lysis Buffer, containing 1 mM PMSF into a 1.5 ml tube. Samples were placed in an ice bath and sonicated for 5 minutes. Afterwards, cell lysates were centrifuged at 14,000 x g for 10 minutes at 4 °C. The supernatant was used for Total mTOR determination, according to the manufacturer's instructions.

Results

Chemical Analysis

We determined the mineral (Table 1) and bio-active compound (Table 2) content of elevATP™. A total of 66 chemical elements were simultaneously assayed after acid mineralization using both ICP-OES and ICP-MS (Table 1). The total element content was 450,235 mg/kg, as determined by adding the concentrations of each element. The ICP-OES was used to determine 33 elements, while the remaining 33 elements were determined by using ICP-MS. The total amount of six macro-nutrient minerals (Ca, P, Na, K, Mg and S) was 424,087 mg/kg and the total amount of ten micro-nutrient minerals (B, Co, Cr, Cu, I, Fe, Mn, Mo, Se, and Zn) was 26,148 mg/kg.

The main plant phenolic component of elevATP™ by weight; was chlorogenic acid (5-O-caffeoylquinic acid), having a concentration of 201±11 mg / 100g. Procyanidins were the second most abundant phenolics with concentrations of dimers and trimers of 127 ± 1 mg / 100 g and 30 ± 0 mg / 100 g, respectively. The other major phenolic compounds were hydroxycinnamic acids, specifically chlorogenic and coumaric acids. ElevATP™ contained two catechins, (+)catechin and (-) epicatechin. Flavonol (quercetin) and dihydrochalcones (phloretin

and phloridzin) were detected in trace amounts of 39±4 mg / 100 g and 18±0 mg / 100 g, respectively.

Table 1
Mineral composition of elevATP™

Mineral	Concentration (mg/kg)	Mineral	Concentration (mg/kg)
Aluminum	16,463	Mercury	<0.01
Antimony	0.05	Molybdenum	0.07
Arsenic	0.58	Neodymium	1.37
Barium	15.44	Nickel	78.03
Beryllium	5.22	Niobium	0.91
Bismuth	2.04	Osmium	0.01
Boron	27.65	Palladium	0.05
Bromine	7.08	Phosphorus	224.41
Cadmium	2.13	Potassium	1,402
Calcium	11,831	Praseodymium	3.38
Cerium	4.76	Rhenium	0.02
Cesium	0.23	Rhodium	0.01
Chromium	19.73	Rubidium	7.79
Cobalt	38.12	Ruthenium	0.03
Copper	6.46	Samarium	2.78
Dysprosium	4.58	Scandium	1.15
Erbium	2.66	Selenium	2.56
Europium	0.86	Silicom	741.51
Gadolinium	4.66	Silver	0.12
Gallium	23.36	Sodium	36,820
Germanium	30.39	Strontium	63.64
Gold	3.73	Sulfur	249,100
Hafnium	0.96	Tantalum	0.31
Holmium	0.24	Terbium	0.29
Indium	0.11	Thorium	1.78
Iron	6,240	Thulium	0.21
Iodine	2.82	Tin	0.04
Lantharum	5.87	Tungsten	1.35
Lead	<0.05	Vanadium	0.05
Lithium	235.01	Ytterbium	2.26
Lutetium	0.31	Yttrium	23.25
Magnesium	124,710	Zinc	391.72
Manganese	1,674	Zirconium	1.67

Table 2
Bioactive compounds in elevATP™

| Analyte	Concentration in mg/100 g
5-O-caffeoylquinic acid	201 ± 11
Procyanidin dimers	127 ± 1
Procyanidin trimers	30 ± 0
Catechin	27 ± 2
Epicatechin	50 ± 1
4-O-p Coumaric acid	25 ± 2
Phloretin xyloglucoside	8 ± 0
Phloretin glucoside	8 ± 0
Quercetin	39 ± 4
Phloretin	2 ± 0

Effect of elevATP™ in Humans

Eighteen subjects were included in this clinical study. Participants (10 male and 8 female) with ages >19 and <59 had a mean BMI of 26.43 (SD 5.88). Participants were randomly assigned, four female and five male per group to two groups. The placebo group (n = 9) received 50 mg of silica oxide and the test group (n= 9) received 150 mg of elevATP™. ATP levels obtained from samples collected at 60, 90, and 120 minutes were averaged and compared the effect of elevATP™ to placebo. Blood ATP levels in the elevATP™ treatment group increased by 64% (Figure 1), which was statistically significant (p=0.016) when compared to the placebo. Blood ATP levels did not increase significantly in the placebo group.

Since higher levels of ATP have been associated with an increase in free radicals, we measured ROS in blood. Samples were normalized as the percent change over baseline at time zero. The elevATP™ treated group had 10% lower ROS levels than the silica-treated group, a difference that was statistically significant (Student's t-test; p=0.011). It is important to note that ROS levels were 105% of baseline in the placebo group and 95% of baseline in the treatment group, both of which reflected insignificant changes from baseline (Figure 2).

Figure 1
Effect of elevATP™ on blood ATP levels. Whole blood was collected from placebo-treated or elevATP™-treated subjects at T0 (baseline), T60, T90 and T120. ATP was detected by using a luciferase-based assay on 10μl of lysed whole blood. Data from T60, T90 and T120 were compared to baseline and pooled for comparison between treatment groups. ATP was significantly higher after treatment with elevATP™ (p=0.016). Data are presented as Mean +/- SE, n=9
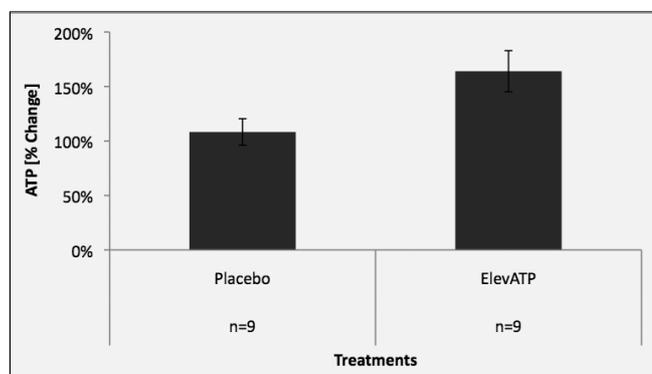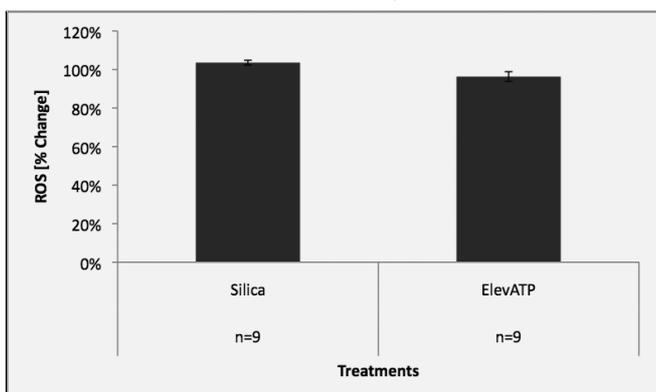

Lactate levels were 11% higher than baseline in the treatment group, while levels in the placebo group were 9% lower (Figure 3). The differences between groups were not statistically significant (p=0.081). The mammalian target of rapamycin (mTOR) was also measured, since it can act as an ATP sensor. As shown in Figure 4, total mTOR remained unchanged in the both groups compared to baseline. The placebo group had a non-significant increase of 5% compared to the baseline and the elevATP™ group showed a slight decrease of 3%. However, when compared with each other, differences were statistically significant (p=0.021). When compared with placebo group, the treated group showed no statistically significant difference in blood glucose levels

(p=0.898). In both groups, glucose levels remained stable, as can be observed in Figure 5.

Figure 2

Effect of elevATP™ on concentration of ROS in whole bloo. Reactive oxygen species (ROS) were also detected after treatment with placebo or elevATP™. The placebo group showed a slight increase in ROS (5% over baseline) and the elevATP™ group showed a decrease (5% below baseline). Although a statistical significance was observed (p=0.011) when comparing the placebo to the elevATP™ group, when comparing these differences against the baseline, they are quite insignificant. Data are presented as Mean +/- SE, n=9

Figure 3

Plasma lactate levels after treatment with elevATP™. Lactate levels were also detected in plasma after treatment with elevATP™ and placebo. Although lactate levels were higher than baseline in the elevATP™ group by 11%, while levels in the placebo group were lower by 9%, the differences between groups were not statistically significant (p=0.081). Data are presented as Mean +/- SE,n=9
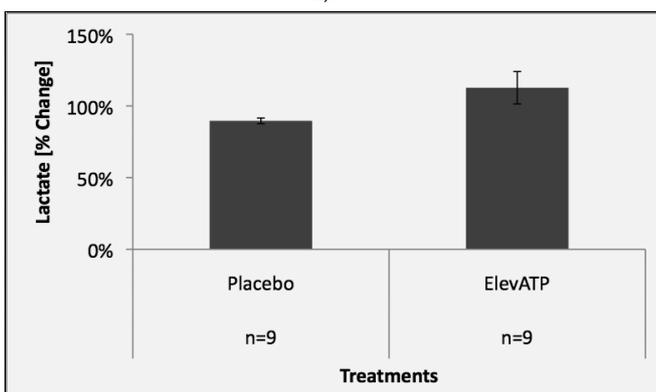

Discussion

Mitochondria are the primary location for the production of ATP molecules in most cells, carried out by enzymatic reactions. Although these enzymes require transition metals such as iron, copper and manganese for

their performance, they are highly sensitive to oxidative damage (7). Recently, polyphenols have been described as important role-playing molecules in the functioning of mitochondria (3, 27, 28) possessing excellent antioxidant potency (21). Selected targets included blood ATP, ROS, lactate, mTOR and glucose. Most of the ATP in blood is confined to the red blood cells (17, 29, 30, 31, #82, 32, 33). However, extracellular concentration of ATP has been also detected (34-38).

Figure 4

Total mTOR after treatment with elevATP™. Total mammalian target of rapamycin (Total mTOR) remained unaffected in both groups compared to baseline. The placebo showed a non-significant increase of 5% compared to the baseline and the elevATP™ group showed a slight decrease of 3%. However, when compared with each other, the decrease in mTOR in the elevATP™ groupwas statistically significant (p=0.021). Data are presented as Mean +/- SE, n=9
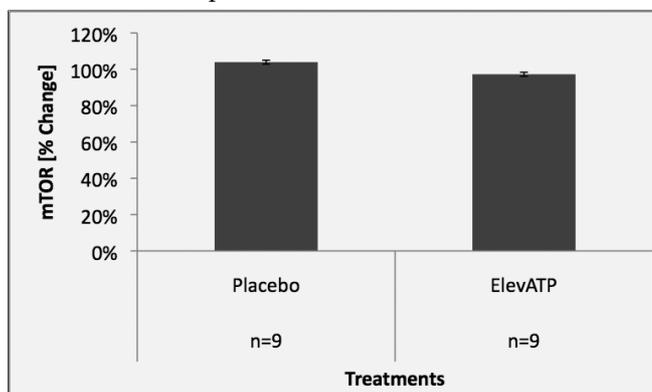
Figure 5

Glucose after treatment with elevATP™. Blood glucose was monitored over the duration of the study. In both groups, glucose levels remained stable and the differences between the placebo and elevATP™ treated groups were not significant (p=0.898). Data are presented as Mean +/- SE, n=9
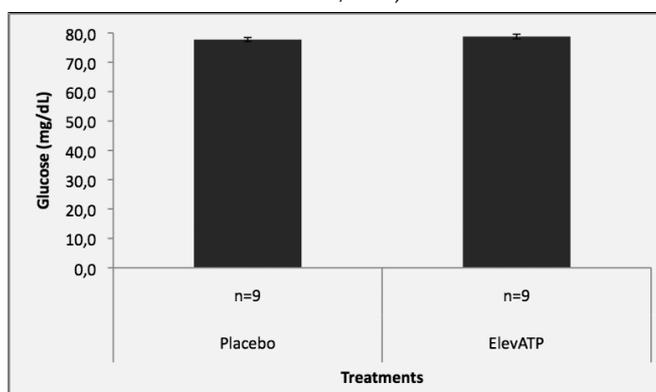
In this study, total ATP was measured in whole blood immediately after collection, as previously described (22). Blood collected from subjects treated with elevATP™

showed an increase in blood ATP (up to 45% compared to baseline). However, this was not observed in the group treated with placebo (Fig 1). This result suggests that elevATP™ could positively affect the process of ATP generation in whole blood, assuming that the chemical components present in elevATP™ are delivered quickly to blood stream. Hypothetically, elevATP™ could be used for stimulation of ATP in blood cells and possibly in other tissues and organs. However, a larger study is required to confirm the preliminary results of this clinical pilot study, which could identify possible mechanisms of action and verify whether ATP is increased in other tissues, such as skin or adipose tissues.

Studies of ATP levels in various states such as cancer (29, 39-41), systemic lupus (42), diabetes type II (17), and exercise performance (9, 21 , 43) broadly suggest that increased ATP levels correlate with health and performance. Likewise, ATP-producing ability of organs and tissues diminishes considerably with age (32). Growing evidence suggests that endogenous oxidants, such as hydroxyl radicals and hydrogen peroxide (HO-), superoxide (O2-) and singlet oxygen (1O2), accelerate the aging process by damaging cell macromolecules such as proteins, DNA and lipids (16). As the main source of ATP production switches from carbohydrate sources to fatty acids, the amount of free radicals generated increases (16) in all tissues, including blood (44). Moreover, the high oxygen tension in blood and iron in heme is a net oxidative environment, from both non-enzymatic and enzymatic pathways, despite lacking mitochondria. Mitochondria are the main source of oxidants in most non-blood cells and their integrity declines with age. With a loss of mitochondrial integrity, ATP synthesis is impaired while reactive oxygen species levels increase (45).

In our study, ROS were lower in the elevATP™ treated group compared to their baseline level. ROS levels in the placebo group were unchanged. These results suggest that the increase of ATP observed (Fig 1) does not result in a concomitant increase in ROS. This result has two possible explanations. It could be that elevATP™ increases blood ATP levels in a manner that is unrelated to ROS production. On the other hand, elevATP™ contains a number of potent compounds which could be directly scavenging and reducing ROS, thereby lowering their levels overall. Indeed both processes could be taking place. In any case, previous studies with ATP supplementation have resulted in undesirable increases in ROS. Therefore, elevATP™ may be a good candidate to enhance blood ATP levels without causing a concomitant increase in ROS.

mTOR is a member of the phosphoinositide kinase-related kinase (PIKK) family that functions as a central element in a signaling pathway involved in the control of many processes, including protein synthesis and autophagy (46). It has been described as a sensor of energy levels in the cell (47) and its activity increases in diseases such as cancer and diabetes. In certain cellular and animals systems, it also correlates with the speed of aging (48). mTOR activity can be affected by dietary microelements (49, 50). It has also been reported that dietary polyphenols and microelements can promote healthy levels of mTOR and enhance protein synthesis (51, 52). Our results show that elevATP™ reduced total mTOR in blood, an effect not seen in the placebo group (Fig 4). While this reduction was modest, it was statistically significant. These results suggest that the use of elevATP™ as a nutritional supplement may result in reducing mTOR levels. These results suggest that , a single treatment of elevATP™ at a serving of 150 mg resulted in a significant increase of blood level of total ATP; with no concomitant increase in blood ROS or serum lactate and a reduction of total mTOR levels in blood under these experimental conditions. These results should be considered preliminary and should be confirmed in larger clinical testing.

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