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# Prevention of "nitrosative stress" by a nutritional supplement (LaVita®) – a randomized placebo controlled double blind clinical trial with healthy volunteers

**Claus Muss [1], Wilhelm Mosgoeller [2], Thomas Endler [3]**

1 Assoc. Prof. DDr. med. Claus MUSS, Internationale Gesellschaft für angewandte Präventionsmedizin I-GAP, Währingerstrasse 63, A-1090 Wien. Privates Institut für klinische Ernärungs- und Präventionsmedizin PIKEM; Associated Professor Public Health St. Elisabeth University (SEU), Bratislava (SK),
2 Prof. Dr. Wilhelm Mosgoeller; Inst. f. Krebsforschung, KIM-1, Medizinische Universität A-1090 Wien, Austria.
3 Primar Doz. Dr. med. Thomas Endler, Labor Endler, Währingerstrasse 63, A-1090 Wien, Austria.

*Correspondence to:* Assoc. Prof. DDr. med. C. Muss, International Research Group for applied Preventive Medicine (I-GAP), Währingerstrasse 63, A- 1090 Wien, Austria.
Email: profmuss@gmail.com

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*Key words:* **Multivitamin; mineral and trace element supplement (LaVita®); nitrosative stress; surrogate parameter; nitrotyrosine; mitochondrial dysfunction; ubiquinone (Q10); L-Carnitine; Zinc; superoxiddismutase activity (SOD); silent inflammation**

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## Abstract

**BACKGROUND**: A common pathomechanism involved in many degenerative manifestations of non-communicable diseases is nitrosative stress, giving rise to a chronic insidious inflammation causing silent inflammation at a cellular level. The release of nitric oxide inhibits multiple enzyme reactions with reduced oxidative phosphorylation and mitochondrial ATP depletion.
**MATERIAL AND METHODS:** We hypothesized that enzyme-inhibition can be alleviated by micronutrient supply, and studied laboratory parameters associated with nitrosative stress (nitrotyrosine, mitochondrial activity) after a micronutrient supplementation (a multivitamin mineral and trace element formulation as verum: LaVita®) and a placebo in healthy volunteers (n=150) for six months.
**RESULTS:** Mean nitrotyrosine levels dropped significantly after 3 month in the verum and placebo group, whereas mitochondrial activity increased after three month in the verum group (p=0,087), but not in the placebo group (p=0,990). Ubiquinone – an essential ingredient for mitochondrial function– increased after six months in the verum group, but not after placebo consumption (p=0,001). Serum zinc and cellular zinc increased steadily after 3 and 6 month verum intake (p<0,001). As the enzyme superoxide dismutase (SOD) is mainly involved in the formation of nitrosative stress (peroxides) we measured the activity, and found significant differences in the placebo and verum group after 3 and 6 month (p=0,050 and p=0,003 respectively).
**CONCLUSION:** We conclude that a balanced combination of vital nutrients may reduce nitrosative stress and silent inflammation, and consequently the risk for various forms of degenerative diseases.

ORIGINAL ARTICLE

Abbreviations:

| | |
|---|---|
| CoQ | Coenzyme Q |
| CPT I | Carnitine palmitoyltransferase I |
| iNO | inducible nitric oxide |
| NCD | non communicable and degenerative desease |
| NO | nitric oxide |
| NOS | NO-synthase |
| Q10 | Ubiquinone, Coenzyme Q10 |
| ROS | reactive oxygen species |
| RNS | reactive nitrogen species |

## 1 INTRODUCTION

The prevalence of degenerative multisystem disorders, with multiple co-morbidities increases worldwide. A common pathology of the various forms is cellular nitrosative stress due to increased inducible nitric oxide (iNO). A continous over-production of nitric oxide (NO) leads to the formation of toxic peroxynitrite. Peroxynitrite itself is a highly reactive species which can target various cellular components like lipids, thiols, amino acid residues, DNA bases, and low-molecular weight antioxidants (O'Donnell *et al.* 1999). Furthermore, peroxynitrite reduces enzyme activity of iron-containing enzymes of the mitochondrial respiratory complexes. Since peroxynitrite nitrosates aromatic amino acids, and oxidizies enzymatic SH groups and other protective cellular antioxidants, important biological cell function may increasingly be influenced already before apparent obvious organ dysfunctions occur. Nitrosative stress influences mitochondrial enzymes involved in energy transfer and ATP production (Hiller *et al.* 2016).

Mitochondrial impairment and the generation of reactive oxygen species (ROS) have been associated with 1) aging and 2) related to pathological conditions of degenerative disorders. Mitochondrial dysfunctions and lowered ATP production may play a role in the onset in various chronic non communicable and degenerative desease (NCD) with central metabolic abnormalities (Morris & Maes 2014). Biomolecular modifications mediated by reactive oxygen species (ROS) or reactive nitrogen species (RNS) are closely associated with the destruction of key macromolecules and inactivation of antioxidant enzymes, which compromises antioxidant defense approaches to expel ROS/RNS and to alleviate toxic oxidative/nitrosative stress. It therefore seems reasonable to investigate preventive strategies (Nemzer *et al.* 2014; Lee *et al.* 2016).

Recently, dietary antioxidants have gained significant attention as potential preventive and therapeutic agents against ROS-generated aging and pathological conditions. It was shown that direct alleviation of ROS by antioxidants, prevented mitochondria-mediated intracellular oxidation as they scavenged mitochondrial ROS and suppressed cell death (Maharjan *et al.* 2016). It is undisputed that mitochondria play a major role in chronic inflammation. Animal trials and observations in human clientel (Prangthip *et al.* 2016; Scialo *et al.* 2016) stimulated investigation of the anti-oxidant potential of Coenzyme Q (CoQ). It is a naturally occurring molecule located in the hydrophobic domain of the phospholipid bilayer of all biological membranes. It is now known, that all cells are able to synthesize functionally sufficient amounts of CoQ under normal physiological conditions (Varela-Lopez *et al.* 2016). CoQ is a molecule found in different dietary sources, which can be reabsorbed and incorporated into biological membranes. CoQ therapies alleviate the effects of aging.

A long array of late data described the beneficial effect of Ubiquinone (Q10) on mitochondrial respiration and nitrosative stress meanwhile. During the reduction of oxygen by oxydative phosphorylation 3 Mol ATP are generated. For this process electrons are transferred from NADPH to oxygen via six different redox systems. Ubiquinone is the less abundant redox system in the membrane of the mitochondria. Because of the low concentration it is the speed controlling redox-system in the energy metabolism. With growing age and exposure to sunlight Q10 is reduced to 50%.

Due to the high number of carbon-carbon double bonds it has a higher reducing capacity compared to vitamin C or vitamin E. Thereby Q10 is the first line of defense against free radicals, and it is an optimal stabilizer of the ion channels of cellular membranes.

L-Carnitine is also a cofactor required for the metabolism of fatty acids in mitochondria. Carnitine palmitoyltransferase I (CPT I) catalyzes the binding of acyl-CoA with carnitine to form acylcarnitine, which is shuttled across the inner mitochondrial membrane in exchange for free carnitine. Once acylcarnitine is inside the mitochondrial matrix, CPT I and acyl-CoA, is metabolized via beta-oxidation. Many disorders of intermediary metabolism, including those affecting electron transport, can result in carnitine deficiency. Under normal circumstances, about 25% of the necessary carnitine is synthesized in vivo and 75% is consumed in the diet. Carnitine deficiency can cause clinical myopathy or cardiomyopathy and lead to rhabdomyolysis or other symptoms of chronic mitochondrial deficiencies (Pons & De Vivo 1995). Supplemental carnitine therapy is accepted for those with proven carnitine deficiency, it remains an unproven but widely used treatment for those with mitochondrial disorders (Campos *et al.* 1993; Vorgerd & Deschauer 2013).

In nitrosative stress NO acts in a concentration and redox-dependent manner to counteract oxidative stress either by directly acting as an antioxidant through scavenging ROS, such as superoxide anions, to form peroxynitrite or by acting as a signaling molecule, thereby altering gene expression. NO can interact also with different metal centres in proteins, such as heme-iron, zinc-sulfur clusters, iron-sulfur clusters, and copper, resulting in the formation of a stable metal-nitrosyl complex or production of varied biochemical signals, which ultimately leads to modification

of protein structure/function. The intracellular zinc availability stabilizies nitrosation and contributes to mitochodrial stability.

Defects in ATP production and the electron transport complex, in turn, are associated with an elevated production of superoxide and hydrogen peroxide in mitochondria creating adaptive and synergistic damage. Both preclinical and clinical studies revealed that NCD´s and neurological disorders may be associated with altered levels of oxidative stress markers and typically reduced concentrations of several endogenous antioxidant compounds, such as vitamin E, zinc and Q10, or enzymes, like superoxide dismutase, and with an impairment of the total antioxidant status (Siwek *et al.* 2013).

Late data from animal research has proven conditions of oxidative and nitrosative stress by experientally induced zinc defiency, being accompanied by inflammation and alterations in the expression of matrix extracellular proteins. Zinc deficiency here contributed to a strong immunopositive area of anti and pro-apoptotic proteins (Biaggio *et al.* 2014).

Further research data also suggest that NO helps to orchestrate gene expression, e.g. via posttranslational modifications of transcription factors essential for the production of cytokines and the cooordination of inflammatory agents. A prevalent DNA binding motif of transcription factors is the zinc finger structure with $Zn^{2+}$ tetrahedrally coordinated between a β-hairpin and a short α-helix, creating a small, functional and independently folded domain. In these zinc fingers cysteine thiols and histidine imidazole nitrogens serve as direct ligands for the zinc ion. NOS-nitrosates cysteines in metallothionein, mediating the release of $Zn^{2+}$ from this zinc-storing protein, and thereby inducing $Zn^{2+}$ release within cells and is able to inhibit zinc finger-dependent gene transcription (Kroncke *et al.* 1994; Klug & Schwabe 1995; Berendji *et al.* 1997; Berendji *et al.* 1999; Kroncke & Carlberg 2000; Berendji-Grun *et al.* 2001).

Evidently micronutrient zinc is important for maintenance and development of immune cells of both the innate and adaptive immune system. A disrupted zinc homeostasis affects immune related cells, leading to impaired formation, activation, and maturation of lymphocytes, disturbed intercellular communication via cytokines, and weakened innate host defense via phagocytosis and oxidative burst. In humans zinc defiency worsens inflammation (De Paula *et al.* 2014; Mertens *et al.* 2015) and results from animal research may also implicate effect on SOD activity and mitochondrial respiration leading to neurological disorders (Bahadorani *et al.* 2013).

A growing number of studies described a link between nitrotyrosine and neurological and other chronic diseases. Nitrotyrosine can be considered a marker of nitrosative stress. During an inflammatory processes, large amounts of NO are locally released from the aminogroup of L-arginines by the enzyme NO-synthase (NOS). In high concentrations NO reacts with superoxide to form peroxynitrite, a key oxidant and cytotoxic species in several pathologies. Peroxinitrite is highly reactive and shows a high affinity to aromatic amino acids, e.g. to the phenolic ring of tyrosine. The resulting nitrotyrosine is a stable product and might be seen as a correlate of peroxynitrite production, and its accumulation in cells and tissues is a marker of oxidative stress and nitrosative stress, respectively.

In this context, we investigated the effects of a vitamin mineral and trace element supplementation, enhanced with Q10 and L-carnitine and zinc on parameters associated with nitrosative stress.

## 2 MATERIAL AND METHODS

We used the multivitamin and mineral concentrate LaVita® as a biological source of antioxidants and micronutrients. The test substance is produced from fruits and vegetable fortified with vitamins, minerals and trace elements (*Table 1*). In this report we focus on the bioavailbilty of antioxidants essential for mitochondrial function. The bioavailbilty of other ingredients have been reported previously (Muss *et al.* 2015a).

### 2.1 Study design

The study design was randomized, prospective, double-blind, and placebo controlled. The study protocol was designed by a research-consulting company (SCIgenia, Vienna, Austria, www.scigenia.com). Study design and realisation including monitoring by this company complied with the guidelines for GCP (Good Clinical Practice). Medical physicians recruited healthy volunteers living a steady life, according to predefined inclusion and exclusion criteria over a time period of three years (2011 to 2013). The exclusion criteria eliminated participants with diseases potentially constituting a study bias (Muss *et al.* 2015b).

After recruitment and randomisation we assigned about 120 Volunteers to the verum group and about 40 tests persons to the placebo group. As indicated in the results section not all paramters were analysed in all test persons. From every volunteer and for every parameter we obtained baseline blood parameters, then they received the studybox containing the study documents and the substance for regular intake at home. They were recalled after three months (timepoint M3), for the second visit and the second blood sample. The intake of the study substance continued for another three month term. At the exit visit the subjects contributed the third and last blood sample 6 months after participation start (timepoint M6).

The blood samples were analysed by the accredited laboratories Endler (Vienna, Austria) and Biovis MVZ GmbH (Limburg, Germany) with standardized and certified laboratory methods.

**Table 1.** Ingredients in 10 ml; 2 x 10 ml was the recommended daily dose

| Ingredients | per 10 ml |
|---|---|
| ß Carotene | 4000 µg |
| Vitamin B1 | 3 mg |
| Vitamin B2 | 2,5 mg |
| Viamine B3 (Niacine) | 40 mg |
| Viamine B5 | 8 mg |
| Vitamin B6 | 4 mg |
| Vitamin B9 (Folic Acid) | 400 µg |
| Vitamin B12 | 5 µg |
| Vitamin C | 300 mg |
| Vitamin D | 5 µg |
| Vitamin E | 30 mg |
| Vitamin K | 30 µg |
| Vitamin H (Biotin) | 70 µg |
| Coenzym Q10 (Qu10) | 5 mg |
| Calcium | 7 mg |
| Chromium | 15 µg |
| Copper | 25 mg |
| Iodine | 25 µg |
| Iron | 4 mg |
| Magnesium | 30 mg |
| Mangan | 1 mg |
| Molybdenium | 30 µg |
| Potassium | 65 mg |
| Selenium | 35 µg |
| Zinc | 5 mg |
| L-carnitine | 30 mg |
| Tryptophane | not determined |
| Omega 3 fatty acidy | 30 mg |

### 2.2 Measurement of biomarkers relevant for nitrosative stress

For this study we determined relevant biomarkers associated with nitrosative stress (Teixeira *et al*. 2016). Nitrotyrosine was measured with an Enzyme-Linked-Immuno-Sorbent-Assay (ELISA) with two polyclonal antibodies against nitrated proteins. A peroxidase-conjugated polyclonal anti-nitrotyrosine antibody was added and a primary antibody – nitrated protein – peroxidase-conjugate was detected by tetramethylbenzidine as peroxidase substrate.

**Table 2**, Parameters which were obtained by standard analytical methods in a certified medical laboratory

| Parameter | Analysis methodology | |
|---|---|---|
| Zinc | ICP-MS | Inductively coupled plasma - Tandem mass spectrometry |
| L-carnitine | LCMS | Liquid chromatography – mass spectroscopy |
| Q 10 | HPLC | High performance liquid chromatography |
| Superoxid dismutase activity (SOD) | | Colorimetric enzyme activity test |

Mitochondrial activity was measured in a subcellular fraction assaying for mitochondrial-specific cytochrome C oxidase activity in soluble and membrane bound mitochondria samples (Cossarizza *et al*. 1993).

The differences between the baseline laboratory parameters at start, after three months, and after 6 months (timepoints M0, M3, and M6) were analysed with the softwarepackage IBM-SPSS (Version 22). We used the students T-test for paired data (before/after), and tested group differeneces with the Analysis of Variance taking the baseline values (M0) as covariable (ANCOVA). Statistical significance was assumed if the alpha error was $p < 0.05$.

Besides parameters associated with nitrosative stress (nitrotyrosine, mitochondrial activity), we determined zinc, l-carnitine and Q 10 levels, aswell as superoxid dismutase activity (SOD) in the blood by standard medical laboratory methods (*Table 2*).

## 3 RESULTS

All participant were healthy and stayed healthy throughout the trial. No side effects, in particular no allergic reactions were reported neither in the placebo nor in the verum group. The drop out rate was below 10% in both groups, rendering the study adherence of the participants above average. In the following we describe the changes of parameters associated with nitrosative stress and the changes of essential antioxidants like ubiquinone, L-carnitine and zinc in the verum or control group after three and six month participation and dailysubstance intake.

Nitrotyrosine was measured in volunteers receiving verum (N=107) and placebo (N=36). There was no significant difference between the placebo and verum group concerning the developement of nitrotyrosine measurements after three (p=0,894) and six month (p=0,745) (*Figure 1*). We observed an insignificant rise of nitrotyrosine in the Verum group after three months

(M3, p=0,083). The values dropped however significancy after further three months of continous intake (M3-M6: p=0,002). There was no significant difference between participation start and final measurements in our verum group (p=0,369).

The parameter mitochondrial activity was included later in the study course and measured in volunteers receiving verum (N=20) and placebo (N=13). In the verum group we observed an insignificant increase of the mitochondrial activity after 3 months (M0-M3; p = 0.087), again followed by a decrease in the second three month period (M3-M6, p= 0.026). In the placebo group the mitochondrial activity remained stable with insignifanct changes of measurements over the first three months period (p = 0.990), and after six months of investigation (p = 0.130; Figure 2).

L-carnitine was measured only in volunteers receiving verum (N=29). Free L-Carnitine increased in the verum group after 3 month of continous intake significantly (p<0,001). However, this intitial raise was again reversed after 3 additional months with significant decrease of L-carnitine levels between visit 2 (timepoint M3) and visit 3 timepoint (M6) rendering no significant average changes between beginning and outcome after 6 month (p= 0,476).

Ubiquinone (Q10) was measured in volunteers receiving verum (N=107) and placebo (N=36). Q10 showed an significant increase in the verum group already after 3 months (M0-M3; p<0,001) and a further increase in the following 3 months. While after the first 3 months the verum and placebo group revealed similar levels, the difference between placebo and verum group was highly significant after 6 months (p=0,001, Figure 3).

The zinc-blood levels were measured only in volunteers receiving verum (N=29). After a slow increase in the first term (M0-M3) the increase during the second term (between M3-M6) and the resulting increase between the complete six months (M0-M6) were highly significant (p=0,001; *Figure 4*) for both zinc compartments blood cells and serum.

Superoxid dismutase (SOD) activity was measured in volunteers receiving verum (N=106) and placebo (N=36). In the verum group the activity of the superoxid dismutase remained constant without significant changes after 3 or 6 months (p=0,834 and p=0,697). However there was a significant difference of SOD activity between verum and placebo groups after 3 and 6 months ( p=0,05; p<0,003) due to increased activity in the placebo group (*Figure 5*).

## 4 DISCUSSION

Our study on healthy probands was primarily set to investigate the preventive potential of chronic disease originating from mitochondrial disorders. In our healthy clients without any symptoms we found only little changes of nitrosative stress. This result was antecipated, because typically nitrosatve stress appears under stronger chronic stress conditions only. Nitrosative stress can be linked to the initiation of mitochondrial dysfunction (Perez-Gallardo *et al*. 2014; Sharipov *et al*. 2014).

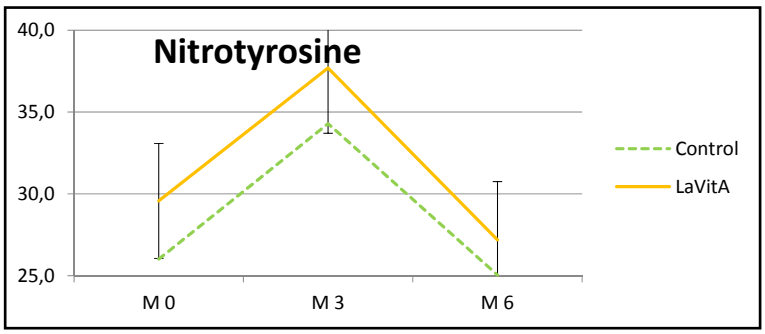


**Figure 1:** Nitrotyrosine measurements in verum and control group, at baseline (M0) after three (M3) and six month (M6) of continous consumption

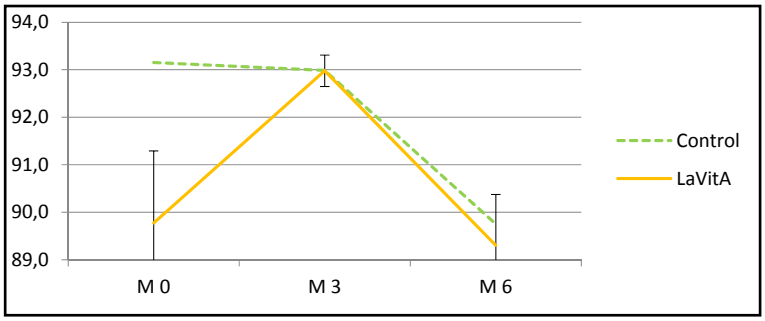


**Figure 2**: Average measurements of mitochondrial activity in placebo and verum group at baseline (M0), after three (M3) and six month (M6) of continous consumption.

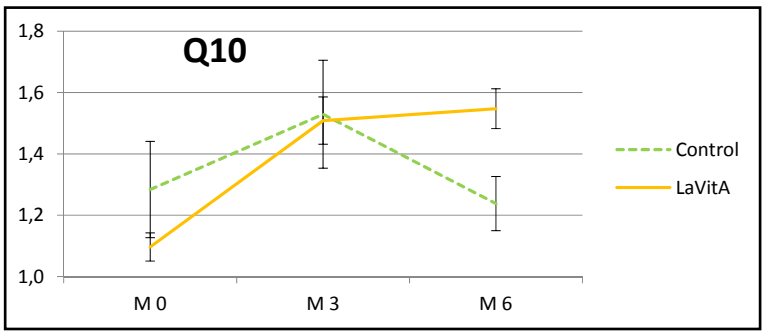


**Figure 3**: Measurements of Ubiqinone (Q 10) in placebo (control) and verum group. At participation start (M 0) the groups did not differ significantly, after six month of continous intake the benefit in the verum group was significant (p < 0,001).

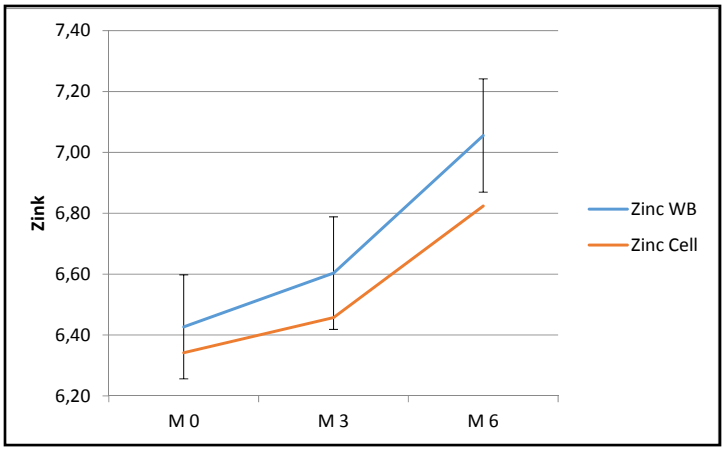


**Figure 4**: Zinc levels at start (M0), after three month (M3) and after 6 month (M6) of continous intake. The increase of both, cellular Zinc and whole blood (WB) Zinc was highly significant (p<0,001).

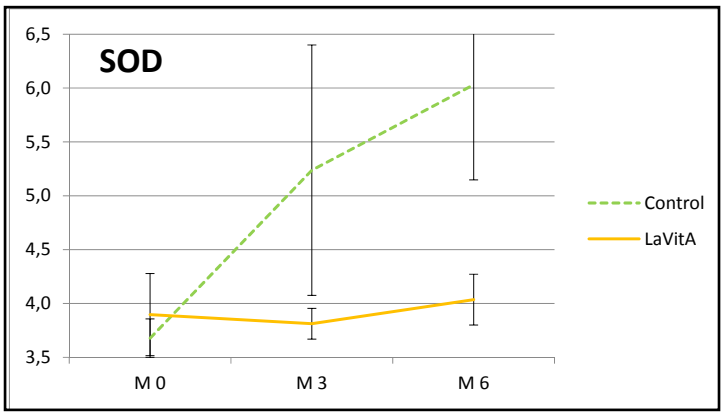


**Figure 5:** Measurements of SOD activity in placebo and verum group, at baseline (M0), after three (M3) and six (M6) month of continous consumption

Mitochondrial dysfunction plays a yet underestimated role in the pathology of various non communicable chronic disease (NCD). The importance to prevent or reduce nitrosative stress and to care for intact mitochondrial function has been discussed extensively (Jha *et al.* 2016; Sinha *et al.* 2016; Wada & Nakatsuka 2016).

Basically, in nitrosative stress overproduction of nitrite oxide and its reaction with superoxid leads to excessive peroxid production which inhibits mitochondrial enzymes and declines essential engery metabolism (Diers *et al.* 2013).

Although only marginally, the results in healthy subjects corroborate the preventive effect of continuous antioxidant supply with respect to nitrosative stress in humans. Evidently the production of nitrotyrosine is triggered by chronic dysregulation of the redox system. Equivalent supplementation for longterm intervention may influence the appearance of this parameter.

We also measured the mitochondrial activity of our particpants. In the verum group we observed the raise of mitochondrial activity above the baseline values, which was not observable in the placebo group. In the controlls, the average measurements of mitochondrial activity remained constant over the observation period.

### 4.1 Role of tissue reservoirs and metabolism

During the 6 month intake periode, many serum values were subject of continous exchange between blood and tissue reservoir. Most probably phenomena like "tissue saturation first" influenced the parameter dynamics, and prevented a linear rise of the serum parameters attributeable to the bioavailabale ingredients, or may have pevented linear metabolic changes due to continous intake.[24]

The continous exchange between blood reservoir and tissue, and the continuous filling up of the tissues can explain why we did not observe a continous trend in nitrosative parameters. Nevertheless those ingredients to support mitochondrial function showed sufficient bioavailbilty. With reference to the supply of L-carnitin and the observed increase of Q10 in the verum group in M0-M3 (Figure 3, p>0,001) we anticipate a protective impact of the study substance against nitrosative stress and consequently on the mitochondrial activity.(Ogasahara *et al.* 1985; Goda *et al.* 1987; Bresolin *et al.* 1988; Campos *et al.* 1993; Pons & De Vivo 1995). This conclusion is corroborated by the observation of positive effects of a combination therapy of ubiquinone and antioxidants on oxidative stress markers in various disease (Bresolin *et al.* 1988; Johansson *et al.* 2013; Rodriguez-Carrizalez *et al.* 2016).

L-Carnitine – acting as a donor of acetyl groups and facilitating the transfer of fatty acids from cytosol to mitochondria during beta-oxidation – plays an essential role in intermediary metabolism of mitochondrial respiration. Dietary supplementation of l-carnitine exerts neuroprotective, neurotrophic, antidepressive and analgesic effects in painful neuropathies and has been considered meanwhile an essential part of so called mitochondrial nutrients improving mitochondrial activity under clinical conditions (Pagano *et al.* 2014; Traina 2016). The increased supply of L-carnitine may have contributed to the improvement of the stress profile and mitochondrial functions in our verum group.

The regular intake of LaVita continuosly increased intracellular zinc levels throughout the test period (*Figure 4*), which correlates well with the SOD activity in the same periode (M0-M6 p<0,001). As SOD activity is the key enzyme function to degrade nitric oxid molecules, this observation corroborates – on a biochemical level – the beneficial effect of our test substance (Salat *et al.* 2013).

### 4.2 Superoxid dismutase activity (SOD)

SOD activity varied between placebo and verum groups significantly in the course of time. While we observed an incline of activity levels in the placebo group, there was a significant difference of SOD activity between verum and placebo groups after 3 and 6 month (Figure 5, p=0,05; p<0,003 respectively).

In humans three genes for superoxide dismutase (SOD) are known to occur in mitochondria, the cell's cytoplasm and outside the cells. The three enzymes can be derived from two precursor enzymes that require either copper and zinc as a cofactor or manganese or iron. We were not able to differentiate the origin on a cellular level of this enzyme activity and therefore enzyme activity analysis with regard to our hypothesis of nitrosative prevention has to remain speculative. However, SOD is well kown to be an extremely important enzyme for the prevention of oxidative stress. In mitochondrial superoxide dismutase is localized in the mitochondrial membrane and converts O2 to H2O2, which can relatively well pass through the membrane – out of the mitochondrion. The activity of SOD enzymes appears therefore to correlate with the age of an organism. According to our understanding in vivo measurements of SOD activity in human trials are not consistent to the aspect of longevity. Human cancer cells often exhibit only low activity of manganese superoxide dismutase (MnSOD). Some cancers are even directly attributable to the fact that in these patients the production of this enzyme greatly reduced or the enzyme itself is impaired by a mutation in its function. SOD enzyme activity is expressed with the purpose to reduce oxidative stress in the intracellular compartment as superoxid anions react together with nitrite oxide (NO) in the formation of aggressive peroxynitrite. SOD activity variations are therefore a product of persisting oxidatve stress. Overproduction of reactive oxygen species (ROS) intermediates above the functional capability of cellular antioxidants may result in instability of important macromolecules and represents the molecular basis of many diseases including inflammation processes, cardiovascular alterations, cancer (Zelen *et al*. 2010).

Overall data from literature corroborate that a decrease of SOD activity may enhance the accumulation of both intracellular and plasma pro-oxidants with a concomitant risk for chronic decrease (Gheddouchi *et al*. 2015). We therefore conclude about a redox saving effect of our test substance indicating that such an antioxidant (vitamin) rich drink may reduce the risk for nitrosative stress in humans.

## 5 CONCLUSION

We were able to show the preventive potential of a continous antioxidant supply with respect to nitrosative stress. As such, we observed a slight decrease of nitrotyrosine levels in our verum group after long term administration even in a healthy clientel. During our trial in the verum group L-carnitine, ubiquinone and zinc levels increased signifcantly. Therefore our results prove a redox saving longterm effect of LaVita® which cooroborates its preventative impact under the condition of continous consumption.

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