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Acute Effect of MCRC on Selected Blood Parameters - A Placebo-controlled Acute Clinical Study

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Abstract

Acute clinical testing was performed on healthy human subjects to verify whether a single 150mg dose of a proprietary formula marketed under the trade name "Mitochroma^{TM}" (MCRC) can actually increase blood levels of ATP. Sera and blood were collected immediately prior to treatment and at times 30, 60, and 90 minutes after treatment to measure amounts of blood ATP, lactate, ROS, and pO_2. Additionally, whole blood was collected at 270 minutes after treatment to measure expression of selected cytokines and chemokines. In comparison to the placebo group, samples collected from subjects treated with MCRC showed increased levels of total blood ATP by 12.5% on average and reduced levels of lactate up to 13%. Blood levels of ROS and pO_2 were found unchanged under these experimental conditions. Analyses of blood collected at 270 minutes showed reduced levels of MCP-1 by up to 21%, and increased levels of Interferon-alpha up to 16%. In summary, collected data shows that treatment with a single dose of MCRC resulted in an acute increase in blood levels of total blood ATP. These results justify further clinical studies on MCRC in order to determine the effects on a more narrowly selected subject population with reduced blood levels of ATP and increased blood levels of MCP-1.

Keywords: blood total ATP, blood lactate, blood ROS, acute clinical testing, oxygen metabolism, serum MCP-1, IFN-α.

1. Introduction

The "Mitochroma^{Tm} complex (MCRC) is comprised of a proprietary cold-water extract of humic shale (alternatively referred to as "ancient peat") and a patent-pending fermented blend of alfalfa, whey and spirulina. In previous *ex vivo* testing, this material has shown a rapid stimulatory

effect on intracellular adenosine triphosphate (ATP) levels in freshly isolated human peripheral blood cells (PBMC) during the first 20-60 minutes post treatment. Surprisingly, this effect was associated with reduced levels of lactate and a non-significant reduction of reactive oxygen species (ROS) [1]. The latter effect was deemed to be particularly interesting since increased production of intracellular ATP is typically associated with increased production of ROS. Given these results and the fact that the pool of ATP and the ability to generate ATP in cells declines with aging [2], initial clinical testing of the MCRP formula as an inducer of endogenous ATP production was undertaken.

ATP is a ubiquitous, energy-rich compound found in all cells and tissues, and used for innumerable metabolic reactions and physiological functions. The presence of intracellular ATP was first described by Fiske and Subarrow in 1925 [3]. Since then, intracellular ATP has been shown to play an important role in a number of biochemical reactions inside cells. More recently, it was reported that cells can secrete ATP to the extracellular matrix as a part of specific intracellular or extracellular regulatory process [4]. ATP released from the cell represents less than 1% of the intracellular ATP pool, which indicates that extracellular ATP signaling can be achieved without compromising cellular metabolism or any other essential intracellular reactions. ATP released by cells is typically in response to various stimuli, such as mechanical pressure, or after treatment with agonists, such as serotonin and acetylcholine [5]. Extracellular ATP is a requirement for several physiological processes, such as neurotransmission, clot formation, cell recognition and expansion and immune responses [6].

In humans, high levels of ATP in tissues are associated with an increased energy level, whereas in circulating blood it is said to improve the immune responses by modifying the recruitment and function of inflammatory cell types [5]. However, during the process of aging, the amount of total ATP in cells is reduced and the ability to generate ATP is diminished [5]. This suggests that the ability to release ATP to the extracellular milieu for regulatory processes might be limited in aged cells and tissues. ATP dietary supplements have surfaced as an alternative, to promote muscle growth and healthy aging.

Previous studies have reported conflicting results regarding the use of exogenous ATP as a dietary supplement (such as peak ATP® [7]). Jordan *et al* [5] showed that chronic oral administration of exogenous ATP can cause alterations in blood oxygenation, peripheral blood flow and muscle metabolism. In a study by Agteresch, however, only minor effects on health and survival rates in cancer patients were observed following administration of exogenous ATP [8]. A more viable alternative is the use of supplements that enhance physiological ATP synthesis.

Previous studies have demonstrated that MCRC is capable of enhancing ATP synthesis in peripheral blood cells, *ex vivo* [1]. The present study was performed to verify whether MCRC is capable of acutely increasing total endogenous ATP levels in subjects treated with a single dose. For this purpose, 36 healthy volunteers were selected and divided into three groups. Blood was drawn before treatment and at 30, 60 and 90 minutes after MCRC was administered in capsules and dissolved in water. Additionally, whole blood was collected 270 minutes post-treatment to verify whether MCRC affects the expression of major cytokines and chemokines.

2. Materials and Methods

2.1 Materials

MCRC powder was provided by FutureCeuticals, Inc., Momence, IL USA. Dulbecco's phosphate buffered saline (PBS) and water were purchased from Sigma Chem. Co. (St Louis, MO, USA). Protein Low Binding microtubes were obtained from Eppendorf (Hauppauge, NY, USA) and RC DC Protein Assay Kit II was purchased from Bio-Rad (Palo Alto, CA, USA). ADP-kinase kits were from Promega (Madison, WI, USA), intracellular ROS and iso-PGF_2-α assay 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).

2.2 Nutritional composition of MCRC

Proximate Analysis. Moisture content of MCRC was determined according to USP loss-on-drying (LOD) method [9]. Samples were heated in a vacuum oven at 70^{o}C for 7 hours. Total protein based on nitrogen content; was determined by the Kjeldahl method [10, 11] (total protein content = N x 6.25). Available carbohydrates were calculated by deducting the sum of crude protein, crude fat, ash and moisture from 100% of the dried mass. Ash content was determined by igniting the sample at 550^{o}C in an electric furnace, AOAC 923.03 [12]. The RSD values for moisture, ash, and protein by Kjeldahl method were 1.0%, 5.0% and 1.4%, respectively.

Mineral Analysis. A 1.2 g sample test portion was dry ashed at 500 ^{o}C ± 50 ^{o}C for 8 hours and treated with HNO_3. The resultant ash was treated with concentrated hydrochloric acid (5%), dried, and redissolved in hydrochloric acid solution [12]. 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) [13]. 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%

Sugar Analysis. Sugars (sucrose, glucose, fructose, maltose, lactose, and galactose) were extracted from an accurately weighed sample with 80% ethanol by allowing it to stand for 24 hours with occasional mixing. Aliquots were dried under inert gas (Argon) and were reconstituted with a hydroxylamine hydrochloride solution in pyridine containing phenyl-β-D-glucoside as the internal standard. The resulting oximes were converted to silyl derivatives with hexamethyldisilazane (HMDS) and trifluoracetic acid (TFA) treatment and subsequently analyzed by gas chromatography (Agilent 6890) equipped with a flame ionization detector FID (Agilent Technology Inc., Palo Alto, CA) [14, 15]. The limit of quantitation was 0.1%. The RSD values for fructose, glucose, maltose, sucrose, galactose were 3.2%, 5.7%, 5.9%, 2.6%, 4.2%, respectively.

Total Dietary Fiber Analysis. Determination of total dietary fiber (TDF) was based on the methods of Lee, Prosky, & De Vries, 1992 and Prosky *et al*., 1988 [16-18]. Duplicate samples were cooked at ~100 °C with heat stable α-amylase to give gelatinization, and then digested with enzymes (protease and amyloglucosidase) in a phosphate buffer to break down starch and some protein. Ethanol was added to each sample to precipitate any soluble fiber. The samples were filtered, and residues were rinsed with ethanol and acetone to remove starch and protein degradation products and moisture. Protein content was determined on one of the duplicates; ash content was determined for the other. The total dietary fiber in the sample was calculated after adjustment for the protein and ash values. All of these analyses were performed twice with a RSD of 6.6%.

Amino Acid Analysis. A sample of MCRC was hydrolyzed in hydrochloric acid (HCl) and adjusted to pH 2.2 for determination of all amino acids except tryptophan. Samples used for analyses of tryptophan were hydrolyzed in sodium hydroxide and adjusted to pH 5.2. Individual amino acids were determined by comparison to authentic standards using an automated amino acid analyzer Hitachi L-8900 (Hitachi Ltd., Japan) [19]. The RSD value for amino acid analysis was 1.8%.

Fatty Acid Profile Analysis. Fats and fatty acids were extracted from MCRC samples by a hydrolytic method according to AOAC official method 996.06 [20]. Pyrogallic acid was added to minimize oxidative degradation of fatty acids during analysis. A triglyceride, tri-undecanoin (C11:0), was added as the internal standard. Fat was extracted into ether, then methylated to form fatty acid methyl esters (FAMEs) using borontrifluoride in methanol. FAMEs were quantitatively measured by capillary gas chromatography (GC) Agilent 6890A (Agilent Technology, Inc., Palo Alto, CA) against a C11:0 internal standard. Total fat was calculated as the sum of individual fatty acids and expressed as triglyceride equivalents. Saturated and monounsaturated fats were calculated as sum of respective fatty acids. The limit of quantitation by this method was 0.01% and the RSD value for

total fatty acids was 3.6% and for saturated, monounsaturated, polyunsaturated and trans fatty acids the RSD values were 1.22%, 1.65%, 1.34% and 1.85%, respectively.

Cholesterol Analysis. The sample was saponified using ethanolic potassium hydroxide. The unsaponifiable fraction that contains cholesterol and other sterols has been extracted with toluene. The toluene is evaporated to dryness and the residue is dissolved in dimethylformamide (DMF). The samples are derivitized to form trimethylsilyl ethers. The derivitized cholesterol has been quantitatively determined by gas chromatography using 5 a-cholestane as an internal standard. The limit of quantitation was 1 mg/100g and RSD value was 3.2%

Vitamin Analysis. Vitamin C in the MCRC sample was extracted, oxidized, and reacted with o-phenylenediamine to produce a fluorophor [21]. Samples were weighed and extracted by homogenizing them in metaphosphoric acid-acetic acid solution (15 g HPO_3 and 40 ml HOAc in 500 ml H_2O). Filtered or centrifuged sample extracts were diluted appropriately to a final concentration of 10-100 mg of ascorbic acid/100 ml. The vitamin C content was determined by comparison of the sample extract fluorescence to the fluorescence of a known standard concentration with RSD value of 6.33%. Beta carotene analysis was performed by reverse-phase HPLC Agilent 1100 system equipped with UV detection (Agilent Technology Ins., Palo Alto, CA) and compared to a standard curve [22]. The RSD value was 7.4%.

Energy value calculation. The energy value was calculated from the concentration of proteins, fat and carbohydrates using the energy conversion factors reported in Southgate & Durnin, 1970 [23]. The protein concentration expressed as the total amino acids was taken for calculations.

2.3 Clinical study

Inclusion and Exclusion criteria. The pilot clinical study protocol (ABC-NCI-11-02-HH2O-2) was approved by the Institutional Review Board at Vita Clinical SA, Avenida Circunvalacion Norte #135, Guadalajara, JAL, Mexico 44270. Thirty six adult volunteers were recruited for this study. All study subjects were generally healthy and not using any type of medication or supplements for a period of 15 days prior to the start of the study. The criteria of selection included their age (>25 and <45 years) with a BMI >21 and <30 kg/m² (overweight and moderately obese), with a mean BMI of 26.43 (SD 3.035). Subjects were free of rhinitis, influenza and other infections, non-diabetic and generally free of allergies to dietary products. Subjects using anti-inflammatory, anti-pain medications, statins or anti-diabetic drugs, anti-allergic medicines, multivitamins or supplements rich in polyphenols were excluded. Subjects were fasted for 12h prior to the initial blood draw. After participants gave written consent, groups of 12 subjects (each containing six males and six females per group) were randomly selected from the pool to receive: a.) one encapsulated dose of placebo (empty capsules) (Group 1); or, b.) 150mg of encapsulated MCRC (group 2); or, c.) 150mg of MCRC in 100 mL of water (Group 3). Subjects in Groups 1 and 2 were administered 100ml water to match the amount of water ingested by subjects in Group 3. Body temperature and blood samples were taken prior to and after treatment. Blood samples were collected every thirty minutes after treatment in heparinized tubes and snap frozen to preserve ATP and ROS integrity. Lactate, partial pressure of oxygen (PO_2) and oxygen saturation (sO_2) were measured with a portable clinical blood analyzer unit (i-STAT Portable Clinical Blood Analyzer, Abbott Laboratories, NJ USA).

Blood Collection. Before samples were orally ingested, blood was collected at Time Zero (T0). For each participant two 9 mL blood samples were drawn from an antecubital vein: one tube contained lithium-heparin as an anticoagulant and the other was anticoagulant-free. Following Time Zero, blood was drawn at 30, 60 and 90 after MCRC was administered. One last blood sample was drawn 4.5 h after MCRC ingestion (T270). Throughout the protocol time course, volunteers were advised to rest. Water was permitted to be consumed *ad libitum*. Immediately after collection, blood samples were gently inverted, aliquoted, snap frozen and kept at -70°C until further use. Serum samples were collected upon clot formation after centrifugation. Serum was aliquoted, snap frozen and kept at -70°C until use.

Clinical Blood Analysis. For the determination of blood gases, finger blood samples were analyzed with a clinical blood gas analyzer (i-STAT Portable Clinical Blood Analyzer). Aliquots (100 ul) of blood were taken by finger puncture and collected in 100 uL heparin-sulfate capillary tubes (Fisher Scientific). Blood was loaded in CG4+ Cartridges (Abbot Laboratories, NJ, USA) for pH, PCO_2, PO_2, TCO_2, HCO_3, BEecf, sO_2 and lactate determination. Blood was collected at baseline (T0) and subsequent samples were collected every 30 min (T30, T60, T90) post treatment. A blood sample was also collected after 4.5 h (T270) for cytokine/chemokine analysis.

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. 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 ATP standard curve.

Reactive Oxygen Species (ROS) detection. Reactive oxygen species (ROS) were detected by using a cell-based ROS assay kit (Cell Biolabs, San Diego, CA, USA) which is designed to detect ROS in media through cell leakage. However, with modifications to the original method, ROS were detected in lysed human blood. Briefly, 10 µL of diluted whole blood (1:100 in PBS) was mixed with 100 µL 2', 7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) 1X in PBS 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 45 min at 2 min intervals. ROS concentration was determined by comparison to a 2', 7'-Dichlorodihydrofluorescein (DCF) Standard Curve. A non-treated blood sample was used as control.

Table 1. Nutritional characterization of MCRC.

Nutrient	Units	Content
Energy value	Cal/100g	106
Energy value from fat	Cal/100g	4.12
Cholesterol	mg/100g	<1.00
Total Carbohydrates	g/100g	23.5
Total Dietary Fiber	g/100g	19.5
Sugar Profile		
Fructose	g/100g	0.19
Glucose	g/100g	0.49
Sucrose	g/100g	0.12
Lactose	g/100g	5.22
Maltose	g/100g	0.11
Galactose	g/100g	0.21
Total Sugars	g/100g	6.34
Protein (N x 6.25) Kjeldahl Method	g/100g	2.01
Fatty Acid Profile		
Saturated Fatty Acids	g/100g	1.26
Monounsaturated Fatty Acids	g/100g	0.10
Polyunsaturated Fatty Acids	g/100g	0.06
Trans Fatty Acids	g/100g	0.08
Total Fatty Acids	g/100g	0.46
Vitamin A	IU/100g	<100
Vitamin C	mg/100g	3.07
Ash	g/100g	67.44
Moisture	g/100g	6.63

Serum cytokine measurements. Serum samples were measured for Monocyte chemo-attractant protein-1 (MCP-1) and Interferon-alpha (IFN-α) at 0, 60 and 270 minute with a multiplex bead-based human cytokine magnetic 30-plex assay panel (Invitrogen, Carlsbad, CA, Catalog no. LHC6003M). Measurements were performed on a Luminex 200 instrument (Luminex, Austin, TX); according to the manufacturer's recommendations. The samples were assayed using the antibody magnetic bead mix with a biotinylated detection antibody followed by streptavidin-phycoerythrin. The raw data (mean fluorescent intensity) were processed on Xponent (Luminex, Austin, TX) to obtain concentration values by comparison with a standard curve according to the manufacturer's instructions.

3. Results

The "Mitochroma^{Tm} complex (MCRC) is comprised of a proprietary cold-water extract of humic shale (alternatively referred to as "ancient peat") and a patent-pending fermented blend of alfalfa, whey and spirulina. MCRC was analyzed for minerals, sugars, total dietary fiber, fatty acids, and vitamins contents Table 1). Also, mineral composition (Table 2) and amino acid composition (Table 3) were also obtained.

Table 2. Minerals composition of MCRC

Mineral	Concentration in mg/100g
Iron	570
Sodium	1444
Magnesium	7469
Calcium	829
Selenium	0.27
Manganese	171.18
Chromium	0.72
Copper	1.53
Zinc	91.17

Results presented in Table 2 show that this material is a good source of daily value (DV) of certain nutritional minerals based on a 100 gram serving size: Ca – 82.9 %DV; Mg – 1867 %DV; Na – 60.2 %DV; Fe – 3166 %DV; Zn – 607.8 %DV; Se – 385.7 %DV; Cu – 76.5 %DV; Mn – 8560 %DV; Cr – 600 %DV.

In the group of amino acids (Table 3), the highest concentration was found for glutamic acid followed by aspartic acid. The percentage of essential amino acids to the total amino acids was 44.6%.

Table 3. Amino acids composition for MCRC

Amino acid	Content in mg/100g
Aspartic Acid	138.0
Theonine	63.9
Serine	50.1
Glutamic Acid	180.0
Proline	88.3
Glycine	30.9
Alanine	60.0
Valine	64.4
Isoleucine	60.0
Leucine	93.1
Tyrosine	30.3
Phenylalanine	35.6
Lysine	79.2
Histidine	16.6
Arginine	30.4
Cystine	19.3
Methionine	19.4
Tryptophan	17.4
Total	1076.9

Thirty-six subjects were selected for the clinical study. Subjects were randomly assigned to the three treatment groups. Group 1 (placebo) was given an empty capsule, and groups 2 and 3 were treated with encapsulated MCRC and powder dissolved in water, respectively. Clinical results are presented as the percentage above baseline (T0), and T1 corresponds to the average of the sum of T30 and T60. T2 corresponds to the data collected from T90 only. As can be observed in Table 4, treatment with placebo (Group 1) and with encapsulated dose of MCRC (Group 2) did not change the total amount of ATP in whole blood during 90 minutes after the treatment. In comparison, treatment with MCRC dissolved in water (Group 3) resulted in an increase of total ATP up to 12.5% over T0.

Table 4. Effect of single dose treatment with MCRC on whole blood ATP.

Total ATP in whole blood						
Parameter	Group 1		Group 2		Group 3	
	T1	T2	T1	T2	T1	T2
Mean	97.89	96.00	96.66	97.3	112.52	107.8
SD	12.11	14.02	11.82	10.56	23.16	14.11
P value	0.45	0.43	0.24	0.43	0.03	0.09
Percent increase vs. placebo	0	0	-	-	14.63%	12.29%

*Vein blood was collected at time 0, 30, 60 and 90 minutes, diluted in nucleotide releasing buffer and assayed for total ATP. Concentration was estimated based on a Standard Curve and calculated as % change over time 0 (T0). T1 represents average values of T30 and T60 (early acute effect) and T2 represents T90 (late effect)

Table 5. Effect of Placebo, MCRC in capsules (G2) or MCRC in water (G3) on blood total lactate levels.

Blood lactate results						
Parameter	Group 1		Group 2		Group 3	
	T1	T2	T1	T2	T1	T2
Mean	102.9	99.16	99.40	92.36	92.50	87.16
SD	18.48	16.84	24.53	35.82	18.31	28.06
P value	0.47	0.82	0.91	0.32	0.06	0.05
Percent decrease relative to control	0	0	-	6.7%	10.1%	12.1%

*Lactate was calculated using Standard i-STAT CG4+ cartridges. Data was calculated as the % change over T0 (baseline). T1 represents the average values of T30 and T60 (acute effect) and T2 represents blood lactate levels after 90 minutes (late effect).

Fresh finger blood was also collected to determine lactate, PO_2 and SO_2 using an i-STAT portable clinical blood analyzer. As shown in Table 5, blood lactate levels remain statistically unchanged during 30-90 minutes over T0 in groups 1 and 2. Interestingly, treatment with MCRC in water (group 3) resulted in a significant reduction of blood lactate levels of up to 13% ($p<0.09$).

Reactive oxygen species (ROS) were also determined in blood samples collected at 0, 30, 60 and 90 minutes after the treatment. Samples were thawed on ice, vortexed and diluted 1:100 before mixing with the DCFH-DA substrate. Samples were analyzed as the percent (%) change over T0 and collected data is presented in Table 6. These results show no statistical significance between groups.

For cytokine and chemokine analysis, blood collected at T0, T60 and T270 minutes after treatment was loaded onto a Luminex 200® instrument and detection was determined based on a standard curve according to the protocol provided with the kit. Collected results show that MCP-1 levels detected in blood were reduced in Group 1 (Placebo) and Group 2, but not in Group 3.

Table 6. Effect of placebo or MCRC on total blood ROS.

Reactive Oxygen Species (ROS) Analysis						
Parameter	Group 1		Group 2		Group 3	
	T1	T2	T1	T2	T1	T2
Mean	98.66	104.83	97.04	95.66	100.66	101.91
SD	6.87	16.72	9.66	12.94	12.58	16.99
P value	0.34	0.23	0.14	0.10	0.79	0.57

*ROS was calculated from diluted blood samples and calculated from a DCF standard curve. Data was calculated as % change over T0. T1 is presented as the average of the sum of T30 and T60 values (early acute effect) and T2 represents 90 min after treatment. Results indicate that % change of total ROS in blood is not significant.

Table 7. Effect of an acute single dose of MCRC on MCP-1 and IFN-α.

Interferon-alpha (IFN-α) and MCP-1 Results						
Parameter	Group 1		Group 2		Group 3	
	IFN-α	MCP-1	IFN-α	MCP-1	IFN-α	MCP-1
Mean	101.03	90.1	116.07	79.06	106.08	98.8
SD	20.23	16.53	13.1	11.69	17.11	13.38
P value	0.8775	0.0785	0.001146	0.0003073	0.02249	0.7799

* Treatment with MCRC showed a reduction of MCP-1 and an increase of IFN-α. Data are expressed as % change over T0.

Since blood levels of MCP-1 were also reduced in group 1, additional t-test analysis was performed to confirm statistical differences in Group 1 and Group 2. Results show that reduction of MCP-1 in group 2 over group 1 is statistically significant ($p<0.093$). Reduction of blood MCP-1 level in group 3 was not significant over T0 or over Placebo group.

Also, IFN-α, showed a statistically significant increase of up to 16% in Group 2 over T0 ($p<0.00012$). In group 3, the results showed a 6% increase over T0 ($p<0.025$). In group 1 there was no change in IFN-α at 270 minutes after the treatment if compared to T0 (Table 7).
Other cytokines and chemokines included in the panel did not show significant changes over T0 or were below detection levels in all groups (data not shown). Samples collected at T0 and T270 were analyzed for blood chemistry and collected results did not indicate any irregularities due to the treatment and procedures undertaken during this acute study (data not shown).

4. Discussion

Red blood cells (RBC) are the most abundant cell type in blood. RBC contain ATP in millimolar concentrations [24-32] . The main function of RBC *in vivo* is to deliver oxygen to various organs and tissue. However, there are studies demonstrating that RBC are involved in other functions of the body, besides gas exchange [5]. Indirectly, RBC can affect vasodilation, platelet function, and functioning of endothelial and smooth muscle cells mainly by releasing ATP to extracellular milieu [25, 33]. Also, it has been reported that extracellular ATP may exert pronounced effects on a variety of biological processes including neurotransmission, muscle contraction, cardiac function, immune cell function, platelet function and vasodilatation [25, 34]. Interestingly, the flow of ATP released from RBC determines plasma levels of extracellular ATP [34] and this process may depend on the intracellular amount of ATP in RBC. This is particularly important since the average amount of total ATP in human blood and tissues is reduced up to 50% during the process of aging [30, 35-37]. Several health conditions that are ATP-dependent can become evident with aging. Some of these conditions have been previously described, e.g., cancer [26-28, 34, 38, 39], systemic lupus erythromatosus [40] and type II diabetes [29].

It has been suggested that administration of exogenous ATP through nutritional supplements can slow the aging process [7]. However, ATP molecules are highly unstable and the ingestion of exogenous ATP may not deliver expected beneficial health effects [5]. Recent studies based on the bioavailability of exogenous ATP did not validate such an approach [41]. Since clinical standards for a nutraceutical ATP product have not been met to date, nutritional agents that can maintain and increase production of endogenous ATP instead of supplying exogenous ATP may constitute a more practical approach. The results of this study indicate that ingestion of MCRC, a plant derived material, is capable of increasing blood pool of endogenous ATP. MCRC does not contain ATP *per se* (data not shown) but it may increase amount of total ATP in whole blood up to 12.52% during the first 60 minutes after treatment (Table 4) when delivered in water Group 3). Since ATP concentration in RBC is in milli-molar quantities [42], the reported 12.52% increase indicates that a significant amount of ATP could be generated during the first hour of treatment with MCRC. Interestingly, as blood total ATP was increased after MCRC ingestion, total blood lactate was reduced by close to 13% in Group 3 whilst this effect was not observed in Group 2 (Table 5). These results suggest that encapsulated material takes longer to be absorbed and has a later effect, since . ATP and lactate shifts were not detected during 30-90 minutes after ingestion.
Short-term fasting does not result in an increase of blood ROS level as observed in Group 1 (Placebo). However, it has been reported that extended periods of fasting tend to decrease ROS production [43]. In this study, treatment with MCRC in water, resulted in an increased total pool of ATP and reduction of blood lactate amount without any effect on blood level of ROS.

A previous *ex vivo* study performed on freshly isolated human peripheral blood cells showed that MCRC can increase intracellular ATP while decreasing intracellular lactate and keeping ROS levels unchanged. As previously reported, during *ex vivo* treatments, MCRC was shown to increase oxygen consumption rate (OCR) as was measured using extracellular flux technology (Seahorse Bioscience, North Billerica, MA, USA) [1]. In the present study, Changes in blood oxygen were measured using a portable gas meter. I-STAT® is designed for measurement of relatively large changes in whole blood sO_2 and pO_2. Even though

the I-STAT® has been well-validated for use in emergency rooms and the results are rapid and reliable, the authors learned that I-STAT® is not practical for large groups of subjects and for testing the comparatively small oxygen level changes in blood cells. Consequently, although the ATP, lactate, and ROS results of this present study suggest the possibility of associated increased oxygen consumption, a future clinical study will likely require a different approach to confirm the effect of MCRC on oxygen consumption in whole blood.

Collected blood was subjected to cytokine and chemokine measurements for two reasons; first, blood ATP may modulate T cell activity [40] and it can also stimulate anti-inflammatory responses [44]; second; MCRC is based on fermented alfalfa and spirulina, materials which may trigger immunological responses [45-51] . In order to verify whether MCRC affects blood levels of cytokines and chemokines, blood samples collected at T0, T60 and T270 minutes after the treatment were analyzed. Results showed that only MCP-1 and IFN-α were affected. As presented in Table 7, treatment with MCRC in capsule form (Group 2) resulted in a statistically significant reduction of blood levels of MCP-1 and an increase in blood levels of IFN-α. These results also showed that extended fasting as such may reduce blood levels of MCP-1 (Table 7); as previously reported [52-54]. However, this effect was potentiated by treatment with MCRC (group 2 data, Table 7). As previously mentioned, absorption rate of active components in MCRC could be different in Group 2 and Group 3. It would be expected that MCRC dissolved in water would be more bioavailable than MCRC in capsules. In support of this assumption, MCRC affected blood levels of ATP and lactate in Group 3 but not in Group 2 during the 30-90 minutes of blood collection time. These results are similar to what was previously observed *ex vivo* in PBMC, in which ATP increases were observed after 20 minutes of incubation with MCRC [1]. It is possible that ingestion of encapsulated MCRC may require more time to produce an increase of blood levels of ATP. On the other hand, serum levels of cytokines and chemokines were measured at T0 and T270 and collected results show that treatment with MCRC in capsulated form (Group 2) resulted in more significant effects on blood levels of MCP-1 and IFN-α than treatment with MCRC dissolved in water (Group 3). These results support the notion that MCRC dissolved in water is rapidly absorbed and that the encapsulated form takes longer to be released and metabolized. The previous results justify further clinical investigation to verify how both formulations of MCRC affect blood levels of total and plasma ATP, lactate and cytokines profile during a longer time period.

MCP-1 plays a significant anti-inflammation role and reduction of blood levels of this cytokine is considered beneficial [55]. In addition, MCP-1 contributes to development of cardiovascular conditions [56] including heart failure [57]. Similarly, IFN-α is important in cardiovascular conditions [58, 59] and also plays an important role in anti-viral defense, especially against Hepatitis virus type C and B [60-62], it contributes to the modulation of inflammatory conditions [58], has an anti-cancer effect [63, 64] and plays an important role in the influenza infection process [65].

In conclusion, acute treatment of healthy subjects with MCRC dissolved in water at a dose of 150 mg increased total blood ATP and reduced blood lactate during first 30-60 minutes post treatment without modifying blood levels of ROS. These results resemble the effect of activated Uncoupling Protein-2 (UCP2) previously mentioned [1]. However, this possibility needs to be verified in a separate set of experiments. Furthermore, reduced levels of MCP-1 and increased levels of IFN-α in blood were observed at 270 minutes after the MCRC ingestion. Based on the observations described above, these results justify further clinical investigation of MCRC on larger subject populations and with narrower intake criteria; for example, on aged subjects with minor cardiovascular conditions and elevated blood levels of MCP-1.

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