ORIGINAL RESEARCH ARTICLE

Effect of Restriction Vegan Diet's on Muscle Mass, Oxidative Status and Myocytes Differentiation: a Pilot Study[†]

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Abstract

This study was conceived to evaluate the effects of three different diets on body composition, metabolic parameters and serum oxidative status. We enrolled three groups of healthy men (omnivores, vegetarians and vegans) with similar age, weight and BMI and we observed a significant decrease in muscle mass index and lean body mass in vegan compared to vegetarian and omnivore groups, and higher serum homocysteine levels in vegetarians and vegans compared to omnivores. We studied whether serum from omnivore, vegetarian and vegan subjects affected oxidative stress, growth and differentiation of both cardiomyoblast cell line H9c2 and H-H9c2 (H9c2 treated with H_2O_2 to induce oxidative damage). We demonstrated that vegan sera treatment of both H9c2 and H-H9c2 cells induced an increase of TBARS values and cell death and a decrease of free NO^{2-} compared to vegetarian and omnivorous sera. Afterwards, we investigated the protective effects of vegan, vegetarian and omnivore sera on the morphological changes induced by H_2O_2 in H9c2 cell line. We showed that the omnivorous sera had major antioxidant and differentiation properties compared to vegetarian and vegan sera. Finally, we evaluated the influence of the three different groups of sera on MAPKs pathway and our data suggested that ERK expression increased in H-H9c2 cells treated with vegetarian and vegan sera and could promote cell death. The results obtained in this study demonstrated that restrictive vegan diet could not prevent the onset of metabolic and cardiovascular diseases nor protect by oxidative damage.

Keywords: vegans diet, muscle mass, oxidative stress, myocyte differentiation

Introduction

Vegan and Vegetarian diets are associated with many health benefits such as a lower prevalence of hypercholesterolemia, diabetes mellitus, hypertension and lower percentage of ischemic heart disease compared to omnivorous diets (Craig et al., 2009; Huang et al., 2012; Crowe et al., 2013;). Currently, this restrictive diets is becoming increasingly popular for various reasons ranging from the protection of animals and the risk of disease to the use of growth stimulants and antibiotics for animal rearing (Fox et al., 2008; Rollin, 2003; Volpe 2005;). Vegans and Vegetarians eat more fruits and vegetables, grains and nuts than omnivores whereby they ingest large amounts of fibers and unsaturated fats and small amounts of total and saturated fatty acids. In this way, they show lower low density lipoprotein (LDL) (Chelchowska et al., 2003; Kelly and Sabate 2006; Mellen et al., 2008) levels and, consequently, a decreased risk of cardiovascular diseases (Chen et al., 2008; Fraser 2009). The vegan diet that completely excludes animal products such as meat, fish, dairy products, honey and eggs is very common among young people. This preference may arise from the increase of dairy allergies and intolerances in addition to ethical issues (Key 2006). Vegan diet contains less cholesterol and saturated fats and a large amount of cereals, legumes and dietary fibers compared to vegetarian diet. Therefore, vegans seem to have less proteins, saturated fats, retinol, vitamin D, calcium and zinc (Davey et al., 2006). When calcium and protein intake is respectively below 800 mg/day and less than 1.24g/protein/kg bodyweight, the risk of hip fracture significantly increases (Munger et al., 1999; Burckhardt 2013). Although some vegetables are rich in proteins and bioavailable calcium, vegetarian and vegan diets often contain minor amounts of these nutrients, therefore they might be the cause of osteoporosis. Studies by Haddad et al. (1999) assessed the nutritional status of adult vegans compared to omnivores about vitamin B12, iron, zinc and immune markers. They demonstrated that the protein content of vegan diet was significantly lower than the omnivorous one and that vegans had lower blood levels of saturated and monounsaturated fats as well as deficiency of iron, calcium, zinc, vitamin D, vitamin B12 and

amino acids; on the other hand, they showed higher levels of fibers and most nutrients, such as ascorbic acid, folic acid and (copper, magnesium and manganese). Moreover, vegans also showed lower blood levels of leukocytes, lymphocytes, platelets, complement factor 3 and urea nitrogen concentrations but an higher concentration of albumin. Finally, the vegans' body mass index was significantly lower compared to non-vegetarians (Haddad et al., 1999). Vitamin B12 is an essential cofactor for two enzymes, methylmalonyl-CoA mutase and methionine synthase. When its deficiency occurs, increased levels of hydrolyzed methylmalonyl-CoA lead to increased concentrations of methylmalonic acid, an important marker of vitamin B12 functional deficiency (Stabler et al., 1997).

Even the increase of serum homocysteine is not only a vitamin B12 but also folate deficiency marker (Snow 1999). This phenomenon is recurrent in a large part of vegetarians and may contribute to an increase of the atherosclerotic risk in these subjects. On the other side, the intake of plant based foodstuffs with multiple antioxidants leads to a reduction of the arteriosclerosis risk, stroke and coronary heart disease. The most likely hypothesis is that the healthier lifestyle of nonmeat-eaters could be reversed by an increase of homocysteine as result of vitamin B12 deficiency (Rauma et al., 1995; Mezzano et al., 1999; Herrmann et al. 2001). The main active antioxidants include vitamins such as α-tocopherol and ascorbic acid, flavonoids and carotenoids such as lycopene, lutein, β-carotene, cryptoxanthin and zeaxanthin. Their biological activity consists in reducing circulating total cholesterol levels and inhibiting the LDL cholesterol oxidation with an increase of HDL cholesterol (Grajek 2004). However, vegetarian nutrition provides adequate antioxidants which effectively prevent free radicals generation. Somannavar et al. revealed that serum malondialdehyde level was significantly increased in non-vegetarians compared to vegetarians: an increased lipid peroxidation and a low antioxidant level in non-vegetarians compared to vegetarians were detected (Somannavar and Kodliwadmath 2012). Differences in hypertension and mean systolic and diastolic blood pressures among meat-eaters and nonmeat-eaters occur. In particular, the meat-eaters had the highest values while vegans the lowest. This

could be attributed to differences in body mass index that were lower in nonmeat-eaters [Appleby et al. 2002). In some cases, high body mass indexes are independent of diet and can be associated to viral infections that induce alterations to adipose tissue distribution and biology. Recent studies (Rizzo et al, 2011; 2012) showed that infectious agents (such as C. Pneumoniae) induced the production of specific pro-inflammatory cytokines in the adipose tissue, with broad effects on hormone expression, lipid storage, and the composition of adipose-resident immune cell populations; these events are strictly related to the development of obesity .

The aim of this study was to establish whether omnivore, vegetarian and vegan diets affects anthropometric, metabolic and serum oxidative status of young men. We investigated the protective effects of vegan, vegetarian and omnivorous sera on hydrogen peroxide-induced morphological changes in H9c2 myoblast cells. For this purpose, we treated H9c2 cell line with vegan, vegetarian and omnivorous sera in order to prevent the effects of hydrogen peroxide (H_2O_2)-induced cellular damage on H9c2 differentiation.

Material and Methods

Subjects. We enrolled three groups of men (10 omnivores, 10 vegetarians and 10 vegans) living in Italy in Campania area. According to their answers to a questionnaire about the frequency of their physical activity, these men were sedentary or moderately physically active. We excluded subjects with a history of diseases or using antibiotics. The omnivorous men consumed all major types of food, the vegetarians consumed milk products and eggs, the vegans consumed no animal products and milk derivatives and eggs. In order to be included as a regular vegetarian or vegan, the subjects were required to have followed this diet for at least 2 years. The subjects were informed of the research and gave their consensus for use of serum samples.

*Anthropometry measurements***.** The participants height and body weight were measured with an automatic height/weight measurement system (DS-102; Jenix®, Seoul, Korea). BMI (kg/m^2) was calculated. Body fat was measured using a bioelectrical impedance analyzer (Jawon Medical, Seoul, Korea).

Collection of samples. Serum samples for this study were obtained from 30 volunteers normal weight young men (age range 20-30, mean). All participants were metabolically healthy. Twelve-hour fasting blood samples were drawn from the subjects in Vacutainers (Becton-Dickinson Vacutainer Systems) containing SST clot-activating gel ~~between 6:30 and 8:00 AM days~~. The serum was separated by centrifugation and stored at 80°C until analysis. Weight was measured by using a scale from Pennsylvania Medical Scales (model no. 7500). Height was measured with a stadiometer from Seca, attached to the wall. BMI was calculated as weight (kg)/height (m2). Baseline clinical characteristics of the study population are summarized in Table 1. Laboratory parameters such as AST, ALT, total cholesterol, triglycerides, GGT were carried out using standard clinical chemical methods.

2.3. Serum Evaluation of Oxidative Stress Markers

2.3.1 DPPH -scavenging assay.

The test was performed according to Brand-Williams, Cuvelier, and Berset (1995). 20 µl of serum were added to 3 mL of DPPH solution (6×10^{-5} mol/L) and the absorbance was determined at 515 nm every 5 min until the steady state.

2.3.2 Ferric reducing antioxidant power (FRAP) assay.

The FRAP assay was carried out by adding 2.5 mL of acetate buffer, pH 3.6, 0.25 mL of TPTZ solution (10 mM) in 40 mM HCl, 0.25 mL of $FeCl_3 \cdot 6H_2O$ solution (12 mM), and 150 µL of sample. After 30 min incubation at room temperature the absorbance of the product (ferrous

tripyridyltriazine complex) was read at 593 nm. The standard curve was linear between 20 and 800 μM of Trolox. Results were expressed as micromoles of Trolox equivalents (TE)/mL of serum.

2.3.3 Determination of total phenol content.

Total phenolics were determined by Folin–Ciocalteu assay with slight modifications. Briefly, Folin-Ciocalteu's phenol reagent (62.5 μL) and dd H_2O (250 μL) were added to sample (62.5μL). After 6 min, 7% Na_2CO_3 solution (62.5μL) and dd H_2O (500 μL) were added to the mixture, which was incubated for 90 min and the absorbance was read at 760 nm. The standard curve was obtained in the range of 0–70 μg/mL gallic acid. Total phenolic content of serum was expressed as mg of gallic acid equivalents (GAE)/mL of serum

2.3.4 ABTS scavenging assay.

ABTS method is based on the reduction of the ABTS•+ activity by the antioxidants contained in the sample. A solution of 7.4 mM ABTS•+ (5 mL) mixed with 140 mM $K_2S_2O_8$ (88 mL) was prepared, stabilized for 12 hours at 4°C and then mixed with ethanol (1:88, v/v). Subsequently, 100 μL of diluted serum were added to 1 mL of diluted ABTS•+, incubated for 2.5 min and the absorbance was read at 734 nm. The standard curve was linear between 0 and 20 mM Trolox. Results were expressed as micromoles of TE/mL of serum

2.4. In Vitro Cell Culture Studies.

Rat cardiomyocytes (H9c2) (ATCC, Manassas, VA) cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100U/mL of penicillin, and 100 lg/mL of streptomycin in 150 cm2 tissue culture flasks at 37°C in a humidified atmosphere of 5% CO_2. The cells were fed every 2-3 days and subcultured once they reached 70–80% of confluence. After 4 hours incubation, cells were washed with 1% PBS to remove unattached dead cells and treated for 30 minutes with 50 mM H_2O_2 (H-H9c2).The H9c2 and H-H9c2 cells were then incubated for 72 hours with media containing 10% of vegan, vegetarian and omnivorous ~~subjects~~ sera.

2.4.1 Cell proliferation assay.

The evaluation of cell proliferation was performed on H9c2 cell line after 72 hours incubation with media containing 10% of vegan, vegetarian and omnivorous ~~subjects~~ sera. The cells were seeded in 96-well plates in a number of 30×10^2 per well. The growth inhibition was assessed by MTT viability assay after 72 hours of treatment as previously described (De Maria et al., 2013). Then MTT assay was carried out by triplicate determination on at least three separate experiments. All data were expressed as mean ± SD.

2.4.2 Morphological evaluation of Cardiomyocytes by confocal microscopy.

After 72 hours incubation of cells with different sera, cells were fixed for 20 minutes with a 3% (w/v) paraformaldehyde (PFA) solution and permeabilized for 10 minutes with 0.1% (w/v) Triton X-100 in phosphate-buffered saline (PBS) at room temperature. To prevent nonspecific interactions of antibodies, cells were treated for 2 hours in 5% fetal bovine serum (FBS) in PBS, then cells were incubated with a specific mouse monoclonal antibody raised against vimentin (1:1,000 in blocking solution, 3% (w/w) BSA in TBS-Tween 0.1%, Sigma) for 2 hours at 37°C. After several washes, cells were incubated with a secondary IgG goat anti-mouse antibody (Alexa Fluor 488, Life Technologies, Carlsbad, CA) diluted 1:1,000 in blocking solution for 1 hour at room temperature. The slides were mounted on microscope slides by Mowiol. The analyses were performed with a Zeiss LSM 510 microscope equipped with a plan-apochromat objective X 63 (NA 1.4) in oil immersion. Vimentin fluorescence was collected in a multi-track mode.

2.4.3. Thiobarbituric Acid-Reactive Species (TBARS) Assay.

Serum samples were incubated with 0.5 mL of 20% acetic acid, pH 3.5, and 0.5mL of 0.78% aqueous solution of thiobarbituric acid. After heating at 95◦C for 45 minutes, samples were centrifuged at 4000 r.p.m. for 5 minutes. The TBARS were quantified by spectrophotometry at 532

nm (Tenore et al. 2013). Results were expressed as TBARS μM/μg of serum proteins. Each data point is the average of triplicate measurements, with each experiment performed in triplicate.

2.4.4 Nitrite Levels.

Nitric oxide is rapidly converted into the stable end products nitrite and nitrate. Nitrite was measured by the Griess reaction. Briefly, 10 μL of serum was mixed with an equal volume of Griess reagent (0.5% sulfanilamide, 2.5% H_3PO_4, and 0.05% naphthylethylene diamine in H_2O) and incubated for 10 minutes at room temperature. Absorbance was assayed at 550 nm and compared with a standard curve obtained using sodium nitrite.

2.4.5 Western Blots.

Cardiac H9c2 cells were collected by centrifugation and then resuspended in ice cold 50mM potassium phosphate buffer (pH 7.4), containing 2 mM EDTA. The cells were sonicated for 10 seconds, followed by centrifugation at 13,000 g for 10 minutes at 4°C. The resulting supernatants were collected and kept on ice for immediate measurements, as described below. Protein expression was determined by Western blot. Briefly, H9c2 cells were cultured with different sera for 72 hours, and then cell pellets were lysed with 1 mL of lysis buffer. The lysates were centrifuged at 12,000 rpm for 10 minutes at 4°C. The supernatants were used to detect ERK, pERK. All Western blots were repeated for three times. Tubulin was used as internal control. To quantify the results, the relative amount of each protein was determined.

2.5. Statistical Analysis.

Statistical analyses were performed with GraphPad Prism version 4.0 for Windows, GraphPad Software (SanDiego, CA). The values were compared by matched-pair *t*-tests. All data were expressed as means ± SE unless otherwise noted. A *P* value of < 0.05 was considered statistically significant.

Results

Anthropometry measurements and Serum Metabolic Parameters of vegetarian, vegan and omnivorous men.

In Table 1 was reported the distribution of food energy in the three different diets: omnivore , vegan and vegetarian. Omnivores eat more red meat, white meat and dairy than cereals, fruit and vegetables. The Vegetarians switch away from beef and chicken to fruit and vegetables, while also reducing oils and snacks. The Vegans, compared to vegetarians, eliminate dairy and switch to cereals, fruits and vegetables. In table 2 were reported clinical characteristics of three volunteer groups (ages 29±5) enrolled for this study: vegetarians, n = 10; vegans, n = 10 and age-matched omnivores (n=10), both vegans and vegetarians do not have used nutritional supplements. All subjects were normal body weight men with BMI 21±1.8. However, we observed significant decrease in muscle mass index and lean body mass only between vegan *vs* omnivorous groups ($P<0.001$). The serum metabolic parameters of enrolled subjects (Table 2), were in the normal range except for the homocysteine values. In vegetarian and vegan homocysteine level was 12±1 and 13±0.9 micromol/l respectively, while the level in consuming traditional diet subjects was 10±0.7 micromol/l. The significance of vegetarian and vegan homocysteine values *vs.* omnivorous ones was $P <0.0001$.

Serum Oxidative status of vegetarian, vegan and omnivorous diet men.

Serum antioxidant activities of vegan, vegetarian and omnivorous groups (Table 3) were analyzed using three methods: DPPH and ABTS assays that measured radical scavenging activity and ferric-reducing antioxidant potential (FRAP) assay. Both DPPH and ABTS values showed no significant differences among three groups, while the FRAP values were increased in vegetarians and omnivores compared to vegans but the variation was significant only in vegetarians *vs* vegans with a $P=0.026$ values. Additionally, total phenolic content (folin assay) were also determined.

Surprisingly, sera total phenolic content was significant major in vegetarians and omnivores with a P value equal to 0.0014 and 0.001, respectively compared to vegan sera. We have also evaluated the serum oxidative status of three diet groups by nitric oxide (as NO^{2-}) quantification and lipid peroxidation marker (TBARs) [Stiuso P. et al., 2014;27]. We observed an increase of about two-fold of NO^{2-} in serum of vegans and vegetarians compared to omnivores, while TBARS levels increased of two-fold in vegans sera with a P value P<0.0001 compared to vegetarians and omnivores.

In vitro sera effects on survival and oxidative stress of H9c2 cardiomyocytes.

To investigate whether different diets could affect *in vitro* cardiomyocytes survival and oxidative stress, we cultured H9c2 and H-H9c2 (cell incubated for 30 minutes with 50μM H_2O_2) myoblasts, an alternative cellular model for cardiomyocytes, for 72 hours with 10% of pooled sera from vegan, vegetarian and omnivorous subjects. Both H9c2 and H-H9c2 cells number after 72 hours of treatment with sera were reported in figure 1. The vegan sera induced a significant decrease (P=0.0163) of cells number compared to omnivorous and vegetarian sera. Surprisingly, H-H9C2 cells number significantly decreased of 2-fold with vegan sera compared to omnivorous sera treated cells (*P* value=0.0022). The lipid peroxidation evaluated by TBARS levels significantly increased in vegan sera-treated H9c2 and H-H9c2 compared to vegetarian and omnivorous sera treated cells (*P*= 0.0044). Moreover we observed a significant decrease of NO^{2-} concentration in both vegetarian and vegan sera treated H-H9c2 (P<0.0001) compared to omnivorous sera treated cells. These results demonstrated that omnivorous sera better protected H9c2 cells from hydrogen peroxide-induced cytotoxicity compared to vegetarian and vegan sera.

In vitro effects of omnivorous, vegetarian and vegan sera on H9c2 cells morphology.

In Figure 2 we reported a morphology change in the cytoskeleton when H9c2 and H-H9c2 cells were treated for 72 hours with vegan, vegetarian and omnivorous sera, then fixed, stained with anti-

vimentin antibody and examined by confocal fluorescence microscopy. The omnivorous sera treated H9c2 cells showed a long fusiform shape and compact parallel morphology, compared to vegetarian sera treated H9c2 that had a disordered organization and a more fine form. The cellular morphology of H9c2 incubated with vegan sera showed a less elongated shape compared to omnivorous sera treated cells but a more compact organization compared to vegetarian sera treated cells. As shown in Figure 2 (panel D, E, F), H_2O_2 pre-treatment of H9c2 cells led to distinctive morphological changes in cells treated with different sera. The omnivorous sera treated H-H9c2 cells retained fine elongated shape compared to (Figure 2 panel A) both vegetarian and vegan sera treated H-H9c2 cells that had lost cell-to-cell contacts and showed a more rounded morphological shape.

In vitro effects of omnivorous, vegetarian and vegans sera on MAP kinases pathways.

In order to investigate the protective effects of omnivorous, vegetarian and vegan sera on hydrogen peroxide (H_2O_2)-induced cellular damage of H9c2 cells, we examined the expression of extracellular signal-regulated kinases (ERK) (Di Domenico et al, 2017), ERK1/2 and pERK1/2 and Caspase-3 by Western blot analysis. The hydrogen peroxide decreased the ERK1/2 and p-ERK expression in omnivorous sera treated H-H9c2 cells compared to vegetarian and vegan sera treated H-H9c2 (Figure 3 panel A). Caspase-3 is an executor of apoptosis and cleaved caspase-3 is a caspase-3 activation marker. Interestingly, H9c2 cells treated with vegan sera increased cleaved caspase-3 expression compared to the omnivorous and vegetarian sera treated H9c2 cells, while H_2O_2 pre-treatment ~~of H9c2 cells~~ increased the cleaved caspase-3 in all H9c2 cells treated with the three types of sera.

Discussion

The effects of foods on human health are likely due to the synergic interactions of multiple factors, including fibers, fatty acids, proteins and bioavailability of micronutrients (Jacobs and Tapsell

2007). The role of a correct diet is crucial in the prevention of obesity that represents one of the main risk factors for cancer, cardiovascular and metabolic diseases (Feola et al, 2017). Few studies have been carried out on benefits and disadvantages of omnivorous, vegetarian and vegan diets in diseases prevention (Lea et al. 2006). In this study we have enrolled omnivorous, vegetarian and vegan ~~diets of~~ healthy men with similar age (25±5), weight (65±5) and BMI (21.5±1) in order to evaluate the effects of three different diets on body composition, metabolic parameters and serum oxidative status. We observed a significant decrease in muscle mass and lean body mass ($P<0.0001$) only in subjects with a restrictive vegan diet compared to omnivorous and vegetarian men ~~diets~~. Essential amino acids, such as leucine, are muscle protein synthesis activators via protein kinase mTORC1 (mammalian target of rapamycin complex1). The minor quantity of skeletal muscle mass in vegan subjects could be due to low content of leucine in the foods. Both omnivorous and vegetarian diets include foods with high leucine content (cheese, soybeans, beef, chicken, pork, nuts, seeds, fish) compared to plus restrictive vegan diets. Mildly elevated plasmatic homocysteine levels are associated to vascular disease (Genest et al. 2006; Schnyder et al., 2001). To this purpose, we have detected a mild but significant increase of homocysteine values (Table 1) in serum of both vegan and vegetarian groups. These results may be due to dietary deficiency of folate, vitamin B12 or choline in subjects submitted to vegan or vegetarian diets. The antioxidant compounds, mainly found in fresh fruits and vegetables, should maintain low levels of serum oxidative stress of vegetarian and vegan subjects compared to omnivore subjects. We showed that both DDPH and Abts radical scavenging activity were homogeneous in sera of the three diet groups, whereas the FRAP value (total antioxidant status of plasma) was significantly lower in vegans compared to vegetarians ($P=0.026$) and omnivores ($P=0.0142$). Surprisingly, also the phenol serum concentration was significant higher in omnivores compared to vegetarian and vegan sera. Furthermore, lipid peroxidation evaluated by TBARS assay showed a strong increase only in vegan sera compared to vegetarian and omnivorous sera. These results could be due to the higher presence of indigestible dietary fibers, especially in vegan diet, which could have determined the low

bioaccessibility and bioavailability of antioxidant molecules such as polyphenols in the small intestine, and a consequently increased of oxidative status sera only in vegan subjects (Palafox-Carlos et al., 2011). In vitro studies have highlighted the protective activity of plant food constituents such as phenols and mixtures and their preventive effects against oxidative stress induced by cell death (Saberi-karimian M, 2017; D'angelo S, 2012). The balance between oxidation and anti-oxidation is critical in maintaining a redox-homeostasis and a consequently healthy biological system (Bouayed and Bohn 2010).

In literature it was reported that the treatment with serum from patients with different diseases could have effects on proliferation, apoptosis and angiogenesis of cultured cells (Stiuso et al. 2014; Valgimigli et al., 2003; Pannella et al., 2016). In this study, we have used this approach to show the effects of sera from subjects with three dietary approaches on oxidative stress, growth and differentiation of both H9c2 and H-H9c2 cardiomyoblast cells (H9c2 treated for 30 minutes with 50 μM H_2O_2). The H9c2 cell line is used to mimic *in vitro* both skeletal and cardiac muscle, for its biochemical morphological and electrical/hormonal signaling properties (Sardão et al., 2007). On the other hand, this cell line has been used as a model to investigate the protective effect of several compounds against hydrogen peroxide-induced cardiotoxicity (Kim et al., 2014). Furthermore the H9c2 cell line can be inducted to differentiate when cultured in particular condition and to show an elongated shape and a parallel fashion morphology (Branco et al., 2015).

Our data demonstrated that the vegan sera treatment of both H9c2 and H-H9c2 cells induced an increase of TBARS value, markers of lipid peroxidation compared to vegetarian and omnivorous sera. Increased lipid peroxidation may be due to high doses of antioxidant molecules, vitamin C, vitamin E, and carotenoids present in the vegan diet, and may cause toxicity and also show pro-oxidant activity (Bouayed and Bohn 2010). Moreover, we observed a significant decrease of free nitric oxide in the medium of vegan and vegetarian sera treated H-H9c2 cells with a concomitant increase of cell death compared to omnivorous sera. Under cellular oxidative stress conditions, the

amount of diffusible free NO decreased because it was transformed in peroxynitrite ($ONOO^-$), when it reacted with superoxide radicals.

The peroxynitrite damages lipids, proteins and nucleic acids by inducing apoptotic cell death (Chung et al., 2001). Studies by Levrand (2007) demonstrated that homocysteine induces cell death in H9c2 cells through peroxynitrite generation. According to this report we supposed a potential involvement of the homocysteine in regulating the balance between NO and peroxynitrite, since it was increased in both vegan and vegetarian serum compared to cells treated with omnivorous serum. Moreover, we have evaluated the ability of three different sera to induce H9c2 differentiation by confocal microscopy. The image of omnivorous sera treated H9c2 cells showed an elongated morphological organization, compared to vegetarian and vegan sera treated cells with a plus fine and minus elongated form. Furthermore, the hydrogen peroxide pre-treatment of H9c2 cells induced: 1) a fine fibers organization in omnivorous sera treated cells; 2) reduction in size and rounding up of vegan and vegetarian treated cells. These data demonstrated that the omnivorous sera have major antioxidant and differentiation properties compared to vegetarian and vegan sera. Finally, the influence of the omnivorous, vegetarian and vegan sera on MAPKs pathway was evaluated. MAPKs are serine/threonine protein kinases involved in cellular proliferation, differentiation and death (Di Domenico et al., 2017). Activation of ERK1/2 in response to H_2O_2 is mediated through Ras/Raf1/Mek pathway and may promote inflammation and result in cellular necrosis (Cagnol and Chambard 2010). The ERK expression and its active form (pERK) increased in vegetarian and vegan sera treated H-H9c2 cells. A growing number of studies suggests that several compounds present in foods consumed by vegetarians and vegans, such as resveratrol and quercetin, can promote cell death by ERK activation (Shih et al., 2002; Kim et al., 2008).

Our study in vitro demonstrates the beneficial effect of omnivorous sera treatment on: 1) cellular proliferation; 2) lipid peroxidation; 3) extracellular free NO production; 4) morphological organization of both H9c2 and H-H9c2 cells. Relying on these results, we concluded that the long-term consumption of restrictive vegan diet, if not adequately integrate with intake of necessary and

protective nutrients, can't neither protect and prevent oxidative damage nor can't inhibit chronic diseases and maintain an healthy biological system.

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Food	Type of diet	Kcal/daily	
	Omnivore	Vegetarian	Vegan
Beef, Lamb	312	0	0
Chicken, fish, Pork	260	0	0
Dairy	338	208	0
Cereals, breads	416	936	1118
Vegetables	78	338	364
Fruit	26	130	156
Oils, spreads	702	624	598
Snacks, sugar	220	208	208
Drinks	208	156	156

Table 1. Food energy distribution in omnivorous, vegetarian and vegan diets

All the three diets have been based on an average of 2,600 Kcal of food consumed per day.

Table 2. Anthropometric status and serum metabolic parameters in omnivorous, vegetarian and vegan subjects.

	Omnivorous (n=10)	Vegetarian (n=10)	Vegan (n=10)
BMI	**23±0.4**	**21±2.3**	**20.5±0.5**
FBM	**13,8±1,5**	**12,5±3,3**	**13,2±0,35**
LBM	**55,4±0,3**	**54,7±6,3**	**44,3±0,3**
MM	**32,1±0.81**	**32,8±1,4**	**27,3±1,2**
Serum Metabolic Parameters			
Total-C, mg/dl	154±28	**159±23**	**148±10**
LDL-C, mg/dl	**79±23**	**82±21**	**81±20**
HDL-C, mg/dl	**47±11**	**52±18**	**48±4**
TG, mg/dl	**67±33**	**81±35**	**91±23**
Homo, μmol/l	**10±1.0**	**12±0.8**	**13±1**
AST-GOT	**19±3.5**	**19±2.7**	**15.5±3.5**
ALT-GOT	**15±6**	**13±5**	**10±1.4**
LDL/HDL	**1.6±0.17**	**1.57±0.15**	**1.7±0.13**

All data are expressed as means ±SE (*n =10* subjects for group). BMI, body mass index; MM, Muscle Mass; lean body mass, LBM; Fat Body Mass, FBM, Total-C, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; Homo, Homocysteine; TBARS, thiobarbituric acid reactive substance; NO, nitric oxide.

Table 3. Antioxidant and oxidative status parameters and total phenols evaluation in serum of omnivores, vegetarians and vegans

	Omnivorous (n=10)	Vegetarians (n=10)	Vegans (n=10)
Antioxidant parameters			
DPPH mmol/mL	**0.0018±0.0002**	**0.0016±0.0003**	**0.0017±0.0002**
Phenol mg/mL	**1.35±0.02**	**1.25±0.02**	**1.164±0.006**
Abts mmol/mL	**0.027±0.004**	**0.027±0.003**	**0.025±0.0036**
Frap mmol/mL	**0.00031±0.00002**	**0.00037±0.00003**	**0.00025±0.000015**
Oxidative parameters			
TBARS µM/µg protein	**0.0022±0.00039**	**0.0028±0.0004**	**0.0046±0.0005**

All data are expressed as means ±SE (*n =10* subjectsfor group). The DPPP, Phenol, Abts, Frap value was normalized for ml of serum.

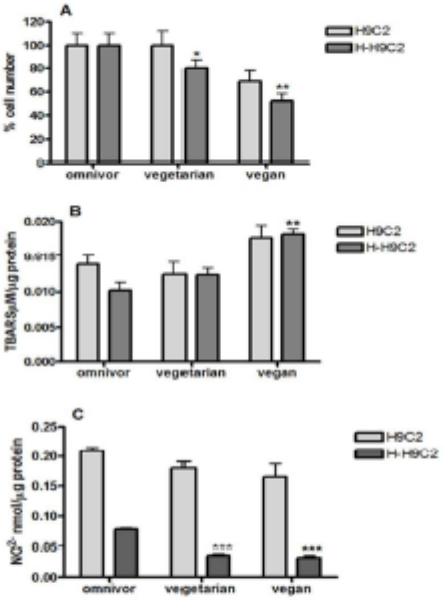

Figure 1. Effect on cells number and oxidative stress after 72 hours treatment of H9c2 and H-H9c2 cells with omnivorous, vegetarian and vegan sera. H9c2 and H-H9c2 cells were growth with 10% of omnivorous, vegetarian and vegan sera. A) The growth inhibition was assessed by MTT viability assay after 72 hours of treatment as described in "Material and methods" section. B) TBARS were quantified by spectrophotometry at 532 nm. Results were expressed as TBARS μM/μg of serum proteins. C) Nitrite was measured by the Griess reaction. Absorbance was assayed at 550 nm and compared with a standard curve obtained using sodium nitrite. Each data point is the average of triplicate measurements, with each experiment performed in triplicate.

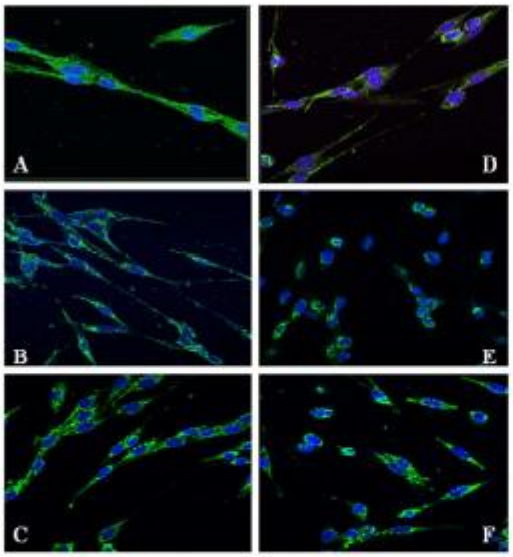

Figure 2. Morphologic characterization by confocal microscopy of vegan, vegetarian and omnivorous sera treated H9c2 and H-H9c2 cells. Both H9c2 and H-H9c2 were treated for 72 h with 10% vegan, vegetarian and omnivorous sera, then fixed, stained with anti-vimentin antibody and examined by confocal fluorescence microscopy. A, D) Images of H9c2 and H-H9c2 cells treated with omnivorous sera. B, E) Images of H9c2 and H-H9c2 cells treated with vegetarian sera. C, F) Images of H9c2 and H-H9c2 cells treated with vegan sera. Green=vimentin and Blue=DAPI. The analyses were performed with a Zeiss LSM 510 microscope equipped with a plan-apochromat objective X 63 (NA 1.4) in oil immersion. Vimentin fluorescence was collected in a multi-track mode.

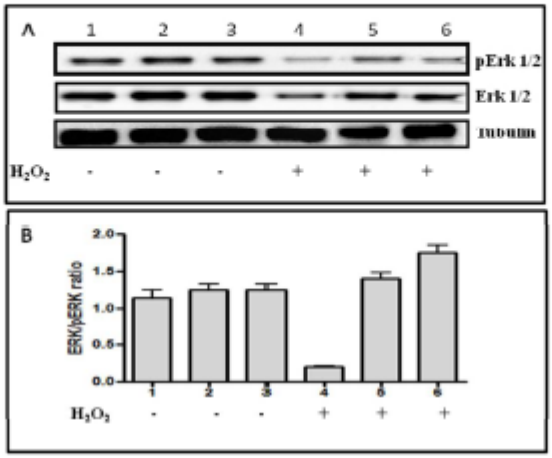

Figure 3. H9c2 cells were cultured with different sera for 72 hours, and then cell pellets were lysed with 1 mL of lysis buffer. The lysates were centrifuged at 12,000 rpm for 10 minutes at 4◦C. The supernatants were used to detect ERK, pERK. All Western blots were repeated for three times. Tubulin was used as internal control. To quantify the results, the relative amount of each protein was determined (A): Western blot analysis of omnivorous, vegetarian and vegan sera treated H9c2 cells (lane 1,2,3) and omnivorous , vegetarian and vegan sera treated H-H9c2 cells (lane4,5, 6). (B) The ERK/pERK ratio was calculated by dividing pERK expression levels for total ERK expression levels measured as arbitrary units with imageJ software. . The mean ± standard error values for at least three independent experiments are shown, along with representative Western blots.