

Evaluation of Natural Substances' Protective Effects Against Oxidative Stress in a Newly Developed Canine Endothelial Cell-Based Assay and in Cell-Free Radical Scavenging Assays

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ABSTRACT

Oxidative stress has been shown to induce damage in a variety of canine and human cardiovascular cell types, and is implicated in the initiation and progression of various cardiac diseases. Various antioxidant agents used in the management of heart failure appear to confer cardio-protective benefits. In light of growing evidence for the inadequacy of a monodimensional antioxidant strategy, there is a demand for an integrated approach using a combination of antioxidant compounds with complementary or

interdependent effects. In addition there is a lack of specific data on the effects of natural antioxidant substances in canine endothelial cells. To address this topic, we developed a new canine aortic endothelial cell-based H₂O₂ oxidative stress assay. The antioxidant and cytoprotective effects of four substances, ie, L-carnitine, taurine, pomegranate extract, and soy isoflavone extract, alone or in combination, were investigated with the newly developed cell-based assay and two free radical scavenging assays. The results show that pomegranate extract, both alone and in combination with the other substances, possesses significant strong antioxidant and cytoprotective activities in canine endothelial cells. Data from this and other published studies suggest that these

natural substances could be of interest when developing a multi-dimensional dietary strategy to reduce the onset and progression of oxidative stress-induced canine disease and perhaps more specifically the endothelial degeneration involved in progressive valvular insufficiencies.

INTRODUCTION

Increased life expectancy of dogs is now common due to the many advances in canine nutrition, preventive health care, medicine, and surgery in recent decades. Cardiac diseases are considered to be the second most prevalent cause of death for dogs.¹ Valvular endocardiosis and dilated cardiomyopathy are the most common of these, and are known to have increased prevalence in aged dogs.² Oxidative damage leading to cell death may constitute one of the most proximal of the cascade of events that result in heart failure. Experimental studies by Ceselli et al have highlighted a major role for oxidative stress in the development of canine cardiovascular disease.³ Chronic mitral valvular insufficiency (CMVI), the major cause of heart failure in dogs, is usually caused by a progressive myxomatous degeneration of the atrioventricular valves that contributes to the pathogenesis of this disease,⁴ and to an increased cellular oxygen demand. Recent data suggest that oxidative stress may also play a significant role in the initiation and the regulation of cardiomyocyte apoptosis in a variety of cardiac diseases⁵ and that alterations in antioxidant reactivity in the blood of dogs can be related to the severity of the cardiac insufficiency.^{6,7}

Therefore, since oxidative stress plays a significant role in the development and progression of cardiac diseases, the possibility of regulating it with exogenous compounds has been further investigated. The benefits of the amelioration of oxidative stress found in conjunction with pathophysiological abnormalities have been shown in humans, and during the past decade considerable effort has been made to identify and characterize plant substances that can protect endothelial

cells from oxidative stress.^{8,9} L-carnitine and taurine, well known for their antioxidant activity, have been demonstrated to protect human endothelial cells from oxidative stress^{10,11} and to confer beneficial effects on certain dog cardiomyopathies.^{12,13} They have become two of the most widely used dietary supplements in dogs suffering from cardiac problems.¹⁴

Polyphenolic compounds such as soy isoflavones found in soybean extracts and tannins from pomegranate extracts have been widely investigated for their antioxidant activity and their potential beneficial effects on cardiovascular diseases.¹⁵ Indeed, several studies have reported soy isoflavones to have a scavenging effect on reactive oxygen species, inhibitory activity on lipid peroxidation and cytoprotective effects against oxidative stress.^{9,16} Pomegranate, thanks to its high content in polyphenols, possesses strong antioxidant and cytoprotective activities on human cells.^{8,17}

However it appears that the protective effects on endothelial cells conferred by antioxidant substances may be species-dependent and cannot be extrapolated from species to species.¹⁸ Thus, even if the antioxidant capacity of these four substances has been evaluated in several *in vitro* free radical scavenging assays (such as Oxygen Radical Absorbance Capacity, Ferric Ion Reducing Antioxidant Power, etc) and on human endothelial cells, their capability to protect specifically canine endothelial cells from an oxidative challenge remains uninvestigated.

To address this question, we have developed a canine endothelial cell-based model assay to allow the evaluation of natural substances for their cytoprotective effects. The effects of four natural substances, alone or in combination, have been tested with the cell-based assay and with two free radical scavenging assays (TEAC and DPPH) in order to determine their potential in developing a multimodal dietary strategy to reduce the onset and progression of canine oxidative stress-related diseases.

MATERIALS AND METHODS

Chemicals and Cell Culture Reagents

Canine Aortic Endothelial Cells (CnAOEC) and Canine Endothelial Cell Growth Medium were purchased from Tebu-Bio (Le Perray-en-Yvelines, France). Trolox[®], ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid), DPPH (1,1-diphenyl-2-picrylhydrazyl), H₂O₂, MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were bought from Sigma-Aldrich (St Louis, MO, USA). Soy isoflavone extract is a standardized 40% isoflavone extract provided by ADM (Decatur, IL, USA). Pomegranate extract was bought from Polynat (Las Palmas, Spain) and is standardized to contain at least 40% of punicosides. Finally, L-Carnitine and Taurine were provided by Arnaud SAS (Paris, France). The sample concentrations to be tested in the different assays, ie, 1, 50 and 250 µg/ml., were estimated by reference to existing published studies using human endothelial cells or data reporting plasma concentrations in dogs or humans following oral supplementation,^{10, 12, 19-21} and the absence of cytotoxicity of the proposed levels substances on canine AOEC (data not shown).

Development of the Canine Endothelial Cell Model

For the development of the CnAOEC cell assay, several parameters were tested to define the optimum growing conditions for the cells, the selection of the appropriate method for cell viability measurement and suitable compounds to generate reproducible oxidative stress. Canine Aortic Endothelial Cells (CnAOEC) were thawed and cultured in Canine Endothelial Cell growth medium following the provider's instructions.

Thawed cells were inoculated in a coated flask in growth medium and left for 24 hours at 37°C, 5% CO₂, to allow adhesion. After 24 hours, cells were rinsed with Hank's buffered salt solution (HBSS) 1X and new medium was added. Once reaching 80% of confluency, cells were trypsinized at room temperature and re-seeded. When the sub-cultured cells formed a monolayer, the

same method was used to detach and re-seed the cells to create continuous passages. Cells were seeded at different densities from 2.10⁴ to 5.10⁴ cells/cm² in 96-well plates. They were monitored to establish if they demonstrated their normal cobblestone shaped appearance when they reached confluency. This was continued over several passages to ensure there was no alteration in behavior or appearance.

Measurement of Cell Viability by MTT Assay

The MTT assay was used to assess the viability of CnAOEC cultures exposed to cytotoxic treatments (Digitonin, 60µM or DMSO at a range of concentrations from 0.1 to 20%) or to increasing concentrations (ranging from 0.5 to 16mM) of H₂O₂ for 24 hr in order to induce an oxidative stress. In this assay, cell damage is assessed 24 hr after challenge with the cytotoxic substance by incubating for 4 hr with MTT at 0.5mg/ml, 37°C, 5% CO₂. Plates are centrifuged, the formazan resulting from the reaction with MTT is solubilized in DMSO and absorbance is measured at 570 nm, with a correction at 690 nm.

Evaluation of the Potential of Natural Substances to Protect CnAOEC from Oxidative Damage

The ability of natural substances to protect cells from the negative effects of oxidative stress was evaluated in CnAOEC. The different samples were diluted in culture medium, and no solubilization issue was observed with any of the tested substances. The cells were then pretreated with increasing concentrations (1, 50, 250µg/ml) of soy isoflavone extract (S), pomegranate extract (P), L-carnitine (C), taurine (T), and SPCT mixture (mix of the four substances where each is present at 0.25, 12.5, 62.5 µg/ml respectively to give the same final sample concentrations of 1, 50, 250 µg/ml for 8 hr. After the pretreatment period, cells were submitted to an oxidative stress (H₂O₂ at 2mM) for 24 hr and cell viability was assessed by the MTT assay as previously described.

Table 1: Analysis of CnAOEC Cell Viability assessed by MTT assay (A) in presence of Digitonin and different concentrations of DMSO and (B) after the exposure with different concentrations of hydrogen peroxide. Data are mean values of 4 (A) or 3 (B) independent experiments \pm SD.

A

<i>Treatment</i>		<i>% of viability</i>	<i>SD</i>	<i>p value</i> <i>(t-test vs control)</i>
Control		100	12	
Digitonin (μ M)	60	27	10	0.000
DMSO (%)	0.1	61	3	0.000
	0.5	77	8	0.005
	1	75	4	0.002
	5	75	21	0.000
	10	50	11	0.000
	20	46	12	0.000

B

<i>Treatment</i>		<i>% of viability</i>	<i>SD</i>	<i>p value</i> <i>(t-test vs control)</i>
Control		100	12	
Digitonin (μ M)	60	27	10	0.000
H2O2 (mM)	0.5	117	18	0.000
	1	126	18	0.000
	1.5	99	31	0.905
	2	51	12	0.000
	4	38	1	0.000
	8	43	4	0.000
	16	44	4	0.000

Trolox Equivalent Antioxidant Capacity (TEAC) Assay

This method is based on the ability of antioxidant molecules to quench the long-lived radical ABTS^{•+}, a blue-green chromophore with characteristic absorption at 734 nm, compared with Trolox, a water-soluble vitamin E analog, as a positive control. Samples of the test substances and Trolox are diluted in PBS buffer in order to obtain the selected concentrations. Then 200 μ l of ABTS^{•+} at 7 mM concentration is added to 50 μ l of sample or Trolox solution in 96-well plates. Absorbance of the different samples is read every 2 minutes for 6 minutes (incuba-

tion time) at room temperature. Results are presented as percentage of ABTS radical inhibition, and in Trolox equivalent (μ M).

DPPH Antioxidant Assay

This antiradical activity assay is based on the ability of antioxidant molecules to reduce the stable purple DPPH radical. The test was performed in 96 well-plates where 190 μ l of DPPH solution (150 μ M) in ethanol were mixed with 10 μ l of sample solution prepared in PBS buffer at the selected concentrations. The absorbance of the mixture is read at 540 nm every 5 minutes for 30 minutes at room temperature. Upon reduction, the color of the solution fades (violet

Table 2: Data from TEAC assays of natural substances. Data are the mean of three independent experiments. Data not sharing the same superscript are significantly different ($P < 0.05$).

TEAC ASSAY					
		ABTS ^{o+} inhibition		Trolox equivalent	
Sample		%	SD	%	SD
Control		0.00	4.09	0.000	0.33
Soy Isoflavones ($\mu\text{g}/\text{ml}$)	1	9.04	1.94	3.54 [†]	1.33
	50	83.44	5.42	33.43	0.36
	250	91.62	2.99	36.68 [†]	11.32
Pomegranate ($\mu\text{g}/\text{ml}$)	1	27.84	4.12	11.11	9.80
	50	97.37	2.75	38.99 [□]	66.17
	250	97.38	2.61	38.99 [□]	73.94
L-Carnitine ($\mu\text{g}/\text{ml}$)	1	0.45	4.95	0.14 [§]	2.35
	50	0.37	4.59	0.11 [§]	1.16
	250	0.92	4.19	0.32 [§]	1.85
Taurine ($\mu\text{g}/\text{ml}$)	1	0.00	4.02	0.00 [§]	1.08
	50	0.00	4.42	0.00 [§]	0.43
	250	0.00	4.66	0.00 [§]	2.01
SPLT.Mix ($\mu\text{g}/\text{ml}$)	1	8.91	3.57	3.52 [†]	3.42
	50	97.16	2.66	38.90 [□]	50.62
	250	96.79	2.82	38.76 [□]	65.44
Trolox (μM)	5	12.67	4.29	-	-
	10	25.45	3.48	-	-
	15	37.29	3.66	-	-
	20	50.59	3.27	-	-
	25	62.32	2.48	-	-
	30	75.23	2.17	-	-
	40	97.81	2.54	-	-

color to pale yellow color). The percentage of reduction (referred to as “inhibition” or “quenching”), reflects the free radical scavenging capacity of the sample tested. Trolox is again used as a positive control. Results are presented as percentage of inhibition of the DPPH radical. It is noteworthy that with the pomegranate extract at the concentration of 250 $\mu\text{g}/\text{ml}$, a precipitate was observed after the incubation with DPPH, preventing a relevant reading of the absorbance.

STATISTICAL ANALYSIS

Experimental data from the cell-free systems

were expressed as mean \pm SD and analyzed using Student’s t-test with commercial software (SYSTAT, SPSS Inc, Chicago, Ill). Data obtained with the natural substances in the canine aortic endothelial cell-based assay were statistically analyzed by one-way ANOVA. Differences were considered significant at $P < 0.05$.

RESULTS

Development of the CnAOEC Cell Based Assay

At 5.10^4 cells/ cm^2 cells demonstrated their typical cobblestone shaped appearance and

reached 100% confluency after 7 days of culture (Fig. 1). Because the behavior and appearance of CnAOEC remain unchanged between passage 1 to 14 (data not shown), cells were used until passage 14 at a maximum. The MTT assay was selected to measure cell viability based on the data obtained following the treatment of the cells with digitonin at 60 μ M (73% reduction \pm 10%) and with DMSO at 10 and 20% (Table 1A). Free radical injury to the CnAOEC was induced with a H₂O₂ treatment at a concentration range of 2 to 16 mM. MTT assay results indicated that cell viability was significantly decreased after 24 hr of exposure (Table 1B).

These conditions were selected in order to perform the screening of the selected natural substances.

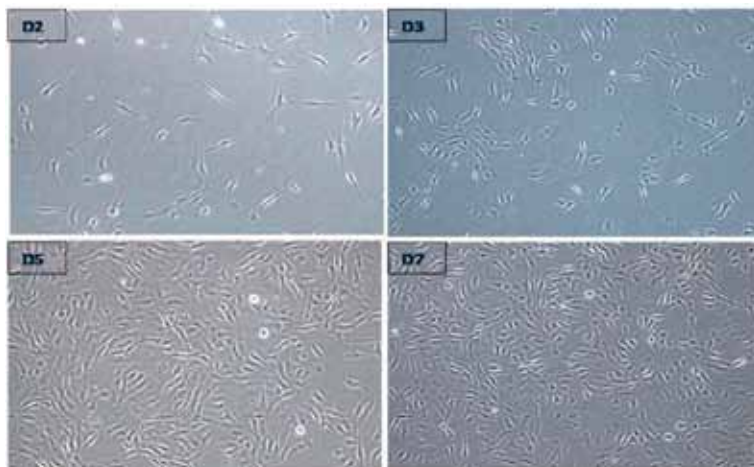
Anti-oxidant Protection of Natural Substances in CnAOEC

The protective effect of pre-treatment with natural substances on cell viability in H₂O₂-treated cells was evaluated using the MTT assay. The data showed that between 1 μ g/ml and 250 μ g/ml, taurine, L-carnitine, and soy isoflavones extract were not able to maintain CnAOEC viability during a 2 mM H₂O₂ oxidative stress challenge (Fig. 2a). However pomegranate extract showed a strong significant protective effect on CnAOEC

Table 3: Data from DPPH assays of natural substances. Data are mean of three independent experiments. NC= Not calculated because of a precipitate formation

DPPH ASSAY			
Sample		% DPPH quenching	SD
Control		0.00	3.74
Soy Isoflavones (μ g/ml)	1	1.65	4.93
	50	2.35	4.62
	250	10.98	4.56
Pomegranate (μ g/ml)	1	11.53	7.02
	50	55.26	3.39
	250	NC	NC
L-Carnitine (μ g/ml)	1	6.74	5.45
	50	4.97	5.8
	250	14.94	5.09
Taurine (μ g/ml)	1	4.95	6.03
	50	10.84	5.12
	250	25.09	4.8
SPLT.Mix (μ g/ml)	1	5.26	5.29
	50	44.49	1.88
	250	50.98	5.01
Trolox (μ M)	5	4.21	3.62
	10	9.91	2.75
	15	17.00	2.83
	20	23.49	3.79
	25	28.6	2.75
	30	33.06	2.37
	40	41.18	1.48

Figure 1: Morphological observation of CnAOEC seeded at 5.104 cells/cm² After 2, 3, 5 and 7 days of culture



viability against H₂O₂ induced cell damage and in a dose-dependent manner. Indeed, at 50 and 250 µg/ml, pomegranate extract improved the cells' viability following challenge to 35% and 82% respectively, which is equivalent to an increase in viability of approximately 2.2-fold and 5.1-fold respectively when compared to the unprotected oxidatively stressed cells (Fig. 2a). Moreover, this protective effect was still observed when pomegranate extract was mixed with the other substances. Indeed, as shown in Figure 2b, the mix of the 4 substances at 50 µg/ml and 250 µg/ml (corresponding to a concentration of 12.5 and 62.5 µg/ml for each ingredient respectively) improves CnAOEC viability to 41% and 51 % respectively (equivalent to a 2.6-fold and 3.2-fold increase in viability respectively when compared to the unprotected oxidatively stressed cells). Surprisingly, the level of cell protection induced by the mix of substances is similar to the protection induced by pomegranate extract alone.

Radical scavenging activity assessment of natural substances in vitro

In the TEAC assay (Table 2), pomegranate, and soy isoflavone extracts showed a strong antioxidant activity. Indeed, pomegranate extract almost completely quenched ABTS radical at 50 and 250 µg/ml. ABTS radical was also inhibited, in a dose-dependent

manner, by 50 and 250 µg/ml of soy isoflavones (83 and 91% quenching respectively).

On the other hand, L-carnitine and taurine showed no effect on inhibition of ABTS radical. ABTS radical was also almost completely inhibited with 50 and 250 µg/ml of the mix (respectively 12.5 and 62.5 µg/ml of each ingredient).

This percentage of inhibition is equivalent to the one obtained with 50 µg/ml of pomegranate alone. In the DPPH assay (Table 3), both pomegranate extract and SPCT mix at 50 µg/ml showed a significant scavenging effect on DPPH free radical (around 50 %) that is similar to the activity obtained with the Trolox control at 40 µM. Soy isoflavones extract, L-carnitine, and taurine present an inhibitory effect on DPPH radical quenching of 11%, 15%, and 24 % respectively at 250 µg/ml.

DISCUSSION

The development of cardiovascular disease is a function of numerous environmental and genetic factors, and data from human epidemiological studies indicate that a diet rich in fruits and vegetables has a protective effect.²² Several experimental studies have demonstrated the beneficial effects of antioxidants in animal models of heart failure. In particular, antioxidants can inhibit cardiac myocyte hypertrophy, apoptosis, and the transition of hypertrophy to heart failure and are able, additionally, to attenuate cardiac dysfunction.^{12,13}

The objective of this study was to evaluate the protection against H₂O₂-induced oxidative stress in canine endothelial cells and the free radical scavenging properties of natural anti-oxidant substances. Prior to

our study, no compounds were described for their *in vitro* effects against oxidative stress in canine endothelial cells. Moreover, according to Ram & Hiebert, it seems that in some cases the protective effect observed on aortic endothelial cells with natural antioxidant molecules can be species-dependent.¹⁸ For this purpose, and since no canine cell-based model was available to date, we developed an assay based on the measurement of cell viability following an oxidative stress using aortic endothelial cells from dogs. We have shown that H₂O₂ was able to generate oxidative damage that led to CnAOEC death at concentrations above 2 mM. At concentrations below 2 mM, a slight increase in metabolic activity was observed.

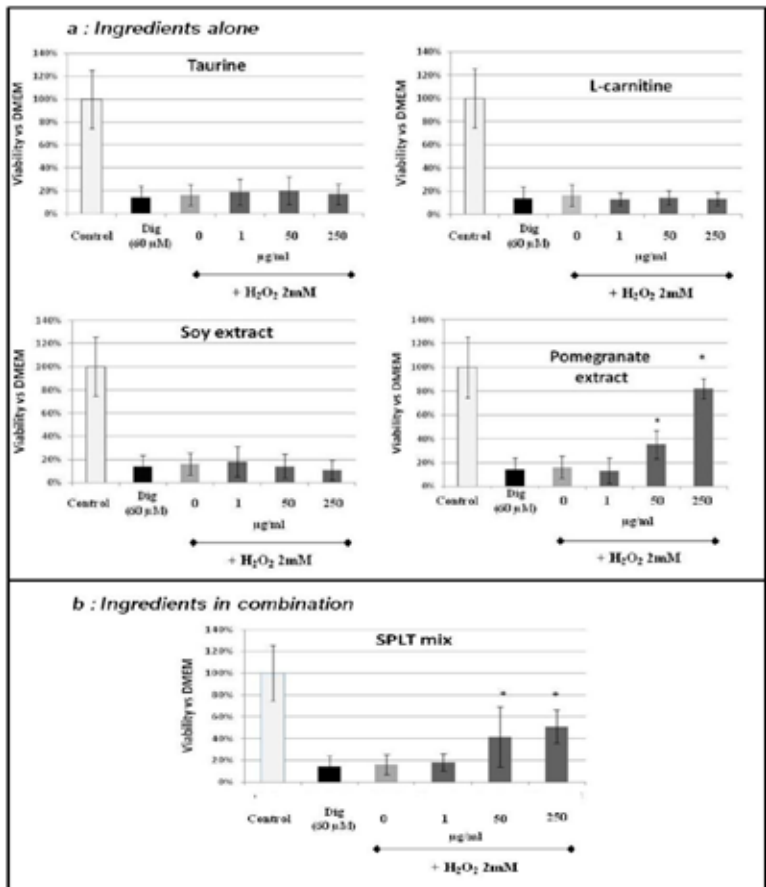
It has already been reported that, in some cases, cells surviving H₂O₂ induced oxidative stress develop a hypertrophy linked with the activation of different metabolic pathways.²³

Following the development of the cell based assay, the antioxidant potential of four substances, ie, L-carnitine, taurine, pomegranate, and soy isoflavones extracts that were individually previously associated with beneficial effects against oxidative stress in human endothelial cells^{9-11, 17} have been investigated. The choice of the individual components was based on scientific studies and was also

influenced by the need for complementary biological effects other than just antioxidant actions. For the evaluation of the antioxidant and cytoprotective effects of the selected natural substances, different concentrations between 1 and 250 µg/ml have been selected according to the data available in the literature.^{12, 19, 20}

The different samples have been evaluated for their cytoprotective effect against H₂O₂ induced oxidative stress in the newly developed assay. Pomegranate extract alone or in combination was able to show a cytoprotective effect. This effect of pomegranate observed on oxidatively injured CnAOEC is in accordance with previous results on human endothelial cells and macrophages

Figure 2: Effect of pre-treatment with different natural substances of CnAOEC exposed to H₂O₂ 2mM on cell viability, assessed by MTT. Data are mean of 8 independent experiments ± SD. * significantly different (P<0.05). One way ANOVA student test.



submitted to H₂O₂ or LDLox, showing that pomegranate extract exerts a cytoprotective effect in addition to the antioxidant activity seen in cell-free assays.⁸ Soy isoflavones extract, L-carnitine and taurine used alone did not show a protective effect on oxidative-stressed CnAOEC in this model.

Interestingly, it has been recently reported that the soy-derived isoflavones genistein and daidzein significantly protect human vascular endothelial cells against H₂O₂-induced oxidative stress injury, and that this protective effect against oxidative stress in vascular endothelial cells is likely to be mediated by multiple mechanisms of action involving the regulation of the survival signaling pathway of the Bcl-2 family, phosphoinositide 3-kinase, and the regulation of estrogen receptors.⁹ Based on this observation, and on the low activity of the soy extract in our study, it should be of interest to further investigate if this mechanism of action can be effective in a canine endothelial cell model. The lack of cytoprotective effect of L-carnitine and taurine suggests that the described protective activity of these compounds might be related to different mechanisms of action. Indeed, it has been reported that taurine significantly attenuated the increase in LDH activity and the decrease in the level of NO in human endothelial cells submitted to an ox-LDL stress.¹¹ The mechanisms by which L-carnitine exerts its antioxidant properties are still under consideration, although it has been shown that this substance prevents lipid peroxidation of phospholipid membranes and increases protein and gene expressions resulting in antioxidant enzymes and NO.²⁴ We would not expect such effects to be visible in our model.

The combination of natural substances (each at equivalent dose) showed a significant protective effect of CnAOEC against oxidative stress, increasing cell viability. This effect seems to be due to pomegranate antioxidant properties or potential complementary effects of the substances. Importantly, it seems that the presence of the other

substances in the mix did not antagonize the pomegranate activity. When we consider that taurine and carnitine are commonly used in dogs with cardiac diseases, it is important to establish that there is not an inhibitory effect on other antioxidant's actions when they are combined. Indeed in this study, there may even be a trend towards a better effect of the combination, as in the 50 µg/ml of SPCT mixture, pomegranate is only at 12.5µg/ml and this mix led to a 41% increase in viability comparable to the value (ie, 35 %) of pomegranate alone at 50 µg/ml.

In the free radical scavenging assay, the pomegranate extract was the only substance to show high scavenging activity both on ABTS and DPPH radicals. These results are in accordance with the results from previous studies on pomegranate extract. Preparations (extracts or juices) from different parts (arils, peel, or whole fruit) of pomegranate extract have revealed diverse antioxidant potency, assessed on several radical scavenging methods.^{8, 25-27} Pomegranate extract showed a high antioxidant activity in a DPPH model system, in addition to a significant ability to scavenge superoxide, hydrogen peroxide, hydroxyl radicals, and nitric oxide and to inhibit the oxidation of lipids and proteins *in vitro*.²⁸ It appears that the antioxidant activity of pomegranate extract is related to a high content of punicosides (≥40%).^{25, 29} Soy isoflavones demonstrated a direct scavenging activity only with the TEAC assay and a weak effect on DPPH assays. Previous studies have shown that the antioxidant activity of isoflavones in DPPH assays may differ according to their chemical structure³⁰ and it seems that they are more efficient in assays involving hydrogen atom transfer reactions such as LDL oxidation assay.^{31, 32}

Finally, L-carnitine and taurine show no scavenging activity with the ABTS but a weak (carnitine) to mild (taurine) effect on DPPH assay. These data confirm the results obtained during previous works showing that taurine's antioxidant activity is mainly linked with a direct scavenging effect on

reactive oxygen species, such as superoxide anion, hydroxyl radical, and hydrogen peroxide.³³ L-carnitine has been described recently to be an effective antioxidant in different in vitro assays including hydrogen peroxide scavenging, metal chelating activity, superoxide anion radical scavenging, and also in DPPH scavenging.³⁴

In prevention and treatment of cardiovascular disease in dogs, taurine and carnitine are often used in combination. Additionally there are increasing amounts of data suggesting the need for a multi-dimensional approach to antioxidant management. Thus, we have also decided to evaluate the radical scavenging activity effect of the mixture of the 4 substances. The results show a strong scavenging activity with both assays. It is likely that in the TEAC assay, the positive result is mainly related to the activity of pomegranate and soy isoflavone extracts, since L-carnitine and taurine alone do not demonstrate an effect in this particular assay. With the DPPH radical assay, the reducing capacity appears to be mainly related to the activity of pomegranate extract. It has been shown that isoflavones-containing soy extract and a mixture of the isoflavones genistein and daidzein similarly inhibit apoptosis in human endothelial cells.³⁵ Moreover, according to that study, soy isoflavones also failed to have any substantial activity in classical in vitro testing of ROS-scavenging potential at the same concentrations that we used. It was suggested that the alteration by the isoflavones of cellular levels of proteins involved in the apoptotic pathway is perhaps more important than their antioxidants effects.

Our data show that the antioxidant capacity of natural substances in cell-free assays does not necessarily correlate with activities in cell based models. The combination of easy to use and affordable in vitro assays and cell-based assays should be recommended as it reveals more information on the potential benefits of the substances tested.

In conclusion, the results provided by

cell-free and cell-based systems, in addition to already published data, suggest that taurine, L-carnitine, pomegranate extract, and soy isoflavones exert antioxidant and cytoprotective activities through different mechanisms of action. Multi-component antioxidant treatment directed to counteract pivotal ROS-activated pathways appears as a rational intervention in heart failure. Studies such as the Lyon Diet Heart Study suggest promise in the use of a multifaceted antioxidant approach and imply that agents with varied 'antioxidant' properties may prove most beneficial³⁶ and can be of interest to develop a multimodal dietary strategy to reduce the onset and progression of canine oxidative stress-related diseases such as heart failure following CMVI and different forms of cardiomyopathies.

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REFERENCES

1. Eichelberg, H. & Seine, R. [Life expectancy and cause of death in dogs. I. The situation in mixed breeds and various dog breeds]. *Berl Munch Tierarztl Wochenschr* 109, 292-303 (1996).
2. Guglielmini, C. Cardiovascular diseases in the ageing dog: diagnostic and therapeutic problems. *Vet Res Commun* 27 Suppl 1, 555-60 (2003).
3. Cesselli, D. et al. Oxidative stress-mediated cardiac cell death is a major determinant of ventricular dysfunction and failure in dog dilated cardiomyopathy. *Circ Res* 89, 279-86 (2001).
4. Corcoran, B.M. et al. Identification of surface morphologic changes in the mitral valve leaflets and chordae tendineae of dogs with myxomatous degeneration. *Am J Vet Res* 65, 198-206 (2004).
5. Kumar, D. & Jugdutt, B.I. Apoptosis and oxidants in the heart. *J Lab Clin Med* 142, 288-97 (2003).
6. Freeman, L.M., Rush, J.E., Milbury, P.E. & Blumberg, J.B. Antioxidant status and biomarkers of oxidative stress in dogs with congestive heart failure. *J Vet Intern Med* 19, 537-41 (2005).

7. Heteyey, C.S. et al. Plasma antioxidant capacity in dogs with naturally occurring heart diseases. *J Vet Med A Physiol Pathol Clin Med* 54, 36-9 (2007).
8. Sestili, P. et al. Cytoprotective effect of preparations from various parts of *Punica granatum* L. fruits in oxidatively injured mammalian cells in comparison with their antioxidant capacity in cell free systems. *Pharmacol Res* 56, 18-26 (2007).
9. Xu, S.Z. et al. Multiple mechanisms of soy isoflavones against oxidative stress-induced endothelium injury. *Free Radic Biol Med* 47, 167-75 (2009).
10. Calo, L.A. et al. Antioxidant effect of L-carnitine and its short chain esters: relevance for the protection from oxidative stress related cardiovascular damage. *Int J Cardiol* 107, 54-60 (2006).
11. Tan, B. et al. Taurine protects against low-density lipoprotein-induced endothelial dysfunction by the DDAH/ADMA pathway. *Vascul Pharmacol* 46, 338-45 (2007).
12. Kittleson, M.D., Keene, B., Pion, P.D. & Loyer, C.G. Results of the multicenter spaniel trial (MUST): taurine- and carnitine-responsive dilated cardiomyopathy in American cocker spaniels with decreased plasma taurine concentration. *J Vet Intern Med* 11, 204-11 (1997).
13. Sanderson, S.L. Taurine and carnitine in canine cardiomyopathy. *Vet Clin North Am Small Anim Pract* 36, 1325-43, vii-viii (2006).
14. Freeman, L.M., Rush, J.E., Cahalane, A.K., Kaplan, P.M. & Markwell, P.J. Evaluation of dietary patterns in dogs with cardiac disease. *J Am Vet Med Assoc* 223, 1301-5 (2003).
15. Nicholson, S.K., Tucker, G.A. & Brameld, J.M. Effects of dietary polyphenols on gene expression in human vascular endothelial cells. *Proc Nutr Soc* 67, 42-7 (2008).
16. Rimbach, G. et al. Antioxidant and free radical scavenging activity of isoflavone metabolites. *Xenobiotica* 33, 913-25 (2003).
17. de Nigris, F. et al. Effects of a pomegranate fruit extract rich in punicalagin on oxidation-sensitive genes and eNOS activity at sites of perturbed shear stress and atherogenesis. *Cardiovasc Res* 73, 414-23 (2007).
18. Ram, J.I. & Hiebert, L.M. Vitamin E protects porcine but not bovine cultured aortic endothelial cells from oxygen-derived free radical injury due to hydrogen peroxide. *Cell Biol Toxicol* 20, 55-67 (2004).
19. McClain, R.M., Wolz, E., Davidovich, A., Pfannkuch, F. & Bausch, J. Subchronic and chronic safety studies with genistein in dogs. *Food Chem Toxicol* 43, 1461-82 (2005).
20. Seeram, N.P., Lee, R. & Heber, D. Bioavailability of ellagic acid in human plasma after consumption of ellagitannins from pomegranate (*Punica granatum* L.) juice. *Clin Chim Acta* 348, 63-8 (2004).
21. Rathel T.R., Leikert J.F., Vollmar A.M., Dirsch V.M. The soy isoflavone genistein induces a late but sustained activation of the endothelial nitric oxide-synthase system in vitro. *Br J Pharmacol* 144(3), 394-399 (2005).
22. Hertog, M.G. et al. Flavonoid intake and long-term risk of coronary heart disease and cancer in the seven countries study. *Arch Intern Med* 155, 381-6 (1995).
23. Chen, Q.M., Tu, V.C., Wu, Y. & Bahl, J.J. Hydrogen peroxide dose dependent induction of cell death or hypertrophy in cardiomyocytes. *Arch Biochem Biophys* 373, 242-8 (2000).
24. Reznick, A.Z. et al. Antiradical effects in L-propionyl carnitine protection of the heart against ischemia-reperfusion injury: the possible role of iron chelation. *Arch Biochem Biophys* 296, 394-401 (1992).
25. Gil, M.I., Tomas-Barberan, F.A., Hess-Pierce, B., Holcroft, D.M. & Kader, A.A. Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. *J Agric Food Chem* 48, 4581-9 (2000).
26. Seeram, N.P. et al. In vitro antiproliferative, apoptotic and antioxidant activities of punicalagin, ellagic acid and a total pomegranate tannin extract are enhanced in combination with other polyphenols as found in pomegranate juice. *J Nutr Biochem* 16, 360-7 (2005).
27. Singh, R.P., Chidambara Murthy, K.N. & Jayaprakasha, G.K. Studies on the antioxidant activity of pomegranate (*Punica granatum*) peel and seed extracts using in vitro models. *J Agric Food Chem* 50, 81-6 (2002).
28. Kaur, G., Jabbar, Z., Athar, M. & Alam, M.S. *Punica granatum* (pomegranate) flower extract possesses potent antioxidant activity and abrogates Fe-NTA induced hepatotoxicity in mice. *Food Chem Toxicol* 44, 984-93 (2006).

29. Cerda, B., Espin, J.C., Parra, S., Martinez, P. & Tomas-Barberan, F.A. The potent in vitro antioxidant ellagitannins from pomegranate juice are metabolised into bioavailable but poor antioxidant hydroxy-6H-dibenzopyran-6-one derivatives by the colonic microflora of healthy humans. *Eur J Nutr* 43, 205-20 (2004).
30. Chen, Y.C. et al. DPPH radical-scavenging compounds from dou-chi, a soybean fermented food. *Biosci Biotechnol Biochem* 69, 999-1006 (2005).
31. Hwang, J., Wang, J., Morazzoni, P., Hodis, H.N. & Sevanian, A. The phytoestrogen equol increases nitric oxide availability by inhibiting superoxide production: an antioxidant mechanism for cell-mediated LDL modification. *Free Radic Biol Med* 34, 1271-82 (2003).
32. Meng, Q.H., Lewis, P., Wahala, K., Adlercreutz, H. & Tikkanen, M.J. Incorporation of esterified soybean isoflavones with antioxidant activity into low density lipoprotein. *Biochim Biophys Acta* 1438, 369-76 (1999).
33. Aruoma, O.I., Halliwell, B., Hoey, B.M. & Butler, J. The antioxidant action of taurine, hypotaurine and their metabolic precursors. *Biochem J* 256, 251-5 (1988).
34. Gulcin, I. Antioxidant and antiradical activities of *L-carnitine*. *Life Sci* 78, 803-11 (2006).
35. Fuchs, D. et al. Proteome analysis for identification of target proteins of genistein in primary human endothelial cells stressed with oxidized LDL or homocysteine. *Eur J Nutr* 44, 95-104 (2005).
36. de Lorgeril, M. et al. Mediterranean diet, traditional risk factors, and the rate of cardiovascular complications after myocardial infarction: final report of the Lyon Diet Heart Study. *Circulation* 99, 779-85 (1999).