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# 1 PUFA and Oxidative Stress. Differential Modulation of the Cell
# 2 Response by DHA

3 **Mattia Di Nunzio [1],\*, Veronica Valli [2], Alessandra Bordoni [1,2]**

4 [1]  Interdepartmental Centre for Industrial Agri-Food Research, University of Bologna, piazza

5  Goidanich, 60 Cesena (FC) - 47521, Italy; E-Mail: mattia.dinunzio@unibo.it (M.D.N.);

6  alessandra.bordoni@unibo.it (A.B.)

7 [2]  Department of Agri-Food Science and Technology, University of Bologna, piazza Goidanich, 60 -

8  47521 Cesena (FC) - 47521, Italy; E-Mails: veronica.valli9@unibo.it (V.V);

9  alessandra.bordoni@unibo.it (A.B.)

10 \*  Author to whom correspondence should be addressed; E-Mail: mattia.dinunzio@unibo.it;

11  Tel.: +39-0547-338957; Fax: +39-0547-382348.

12 **Abstract:**

13  Although an increased dietary intake of long-chain n-3 PUFA is considered an effective

14  preventive strategy, a theoretical concern related to the possible increase of lipid

15  peroxidation induced by a PUFA-rich diet still remains. In this study, the effects of

16  different PUFA (linoleic, α-linolenic, arachidonic, eicosapentaenoic, and docosahexaenoic

17  acid) on cytotoxicity, lipid oxidation, and modulation of antioxidant defenses were

18  evaluated in HepG2 cells submitted to an oxidative stress ($H_2O_2$). Results clearly evidenced

19  that all supplemented PUFA but DHA enhanced cell susceptibility to $H_2O_2$. Overall, our

20  results underline that PUFA cannot be considered as a single category but as individual

21  compounds, and research on mechanisms of action and preventive effects should deal with

22  the individual fatty acids, particularly in the case of DHA.

23  **Keywords:** HepG2 cells; hydrogen peroxide; cytotoxicity; antioxidant enzymes

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## 26 1. Introduction

27  There are converging opinions among experts, organizations and health professionals that a

28  recommendation for a daily individual consumption of 500 mg of eicosapentaenoic (EPA)/

29  docosahexaenoic (DHA) acids would provide health benefits (Salem & Eggersdorfer, 2015). An

30  increased dietary intake of n-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFA), EPA and DHA,

31  is considered an effective strategy in the prevention and modulation of cardiovascular (Ohnishi &

32  Saito, 2013; Cao et al., 2015) and neurological diseases (Patrick & Ames, 2015), for the optimization

33  of cognitive performance (Muldoon et al., 2014) and brain development (Brenna & Carlson, 2014).

34    Since DHA and other PUFA are highly oxidizable molecules (Miyashita, 2014), a theoretical
35    concern remains on their use at high concentration in the prevention of chronic diseases having the
36    oxidative stress as one of the underlying mechanisms.

37    Besides being prone to oxidation, PUFA have been reported to activate the stress-signaling pathway
38    controlling the genetic program used by cells to induce the synthesis of proteins responsible for
39    detoxification and counteraction of the oxidative stress (Ma, 2013). In this way, PUFA can modulate
40    the activity of antioxidant enzymes (Wang et al., 2004) to ensure survival and reduced status.

41    Supplementing cultured cells with individual PUFA we previously observed diverse effects on
42    oxidation and modulation of antioxidant defenses. The different effects were not simply related to the
43    length of the carbon chain, or to the number and position of double bonds in the fatty acid (Di Nunzio
44    et al., 2011). In particular, DHA supplementation did not cause any adverse effect, suggesting that its
45    use as preventive dietary strategy does not induce a higher sensitivity to oxidation. It is conceivable
46    that the overall effects of the different PUFA are related to their individual ability to increase the
47    intracellular concentration of pro- or antioxidants.

48    In this light, the concomitant oxidative status of the cell could have a role in its response to PUFA
49    supplementation, and the differential effect of DHA than other PUFA needs to be confirmed in cells
50    submitted to an oxidative insult. To evaluate it, in the present work, we supplemented HepG2 cells
51    with linoleic acid (18:2n-6, LA), α-linolenic acid (18:3n-3, ALA), arachidonic acid (20:4n-6, ARA),
52    eicosapentaenoic acid (20:5n-3, EPA), and docosahexaenoic acid (22:6n-3, DHA), then a mild
53    oxidative stress reflecting physiological condition was induced (Halliwell et al., 2000; González-
54    Flecha et al., 1993; Khassaf et al., 2001). HepG2 cells were used as model system since liver is a major
55    organ attacked by reactive oxygen species (ROS) (Sànchez-Valle et al.,  2012), and parenchymal cells
56    are primary cells subjected to oxidative stress induced injury in the liver. Cells were supplemented
57    with physiological concentration (60 µM) of n-6 and n-3 PUFA, and cell survival, lipid oxidation, and
58    antioxidant defenses were evaluated after exposure to hydrogen peroxide.

59    **2. Experimental Section**

60    *2.1. Chemical*

61    Dulbecco's modified Eagle's medium (DMEM), and Dulbecco's phosphate-buffered saline (DPBS)
62    were purchased from Lonza (Milan, Italy). All chemicals and solvents were of the highest analytical
63    grade and were purchased from Sigma-Aldrich (Milan, Italy).

64    *2.2. HepG2 cells tissue culture*

65    HepG2 human hepatoma cells were maintained at 37°C, 95% air, 5% $CO_2$ in DMEM supplemented
66    with 10% (v/v) fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin (Di Nunzio et al.,
67    2010). Once a week cells were split 1:20 into a new 75cm$^2$ flask, and medium was refreshed after 72 h.

68    *2.3. Fatty acid supplementation and oxidative stress*

69    Cells were seeded in 6-well plates, and after 24 hours, at 75-80% confluence, they were incubated
70    for 21 h with the different fatty acids at 60 µM concentration. Free fatty acids were dissolved in 100%

isopropanol, and complexed to bovine serum albumin (BSA). Fatty acid-BSA complexes were prepared fresh each time, and BSA final concentration was 0.5% in serum free DMEM. Unsupplemented (US) cells received corresponding amounts of BSA and isopropanol, which final concentration in the media was kept below 1% (v/v). LA, ALA, ARA, EPA, and DHA were used for cell supplementation. After 21 h incubation cells were washed twice with warm DPBS, and then exposed for 20 or 60 min to 0.2 mM $H_2O_2$ in Earle's Balanced Salt Solution (EBSS) (116 mM NaCl, 5.4 mM KCl, 0.8 mM $NaH_2PO_4$, 26 mM $NaHCO_3$, 2.38 mM $CaCl_2$, 0.39 mM $MgSO_4$). Some unsupplemented cells received EBSS only (control cells). Then EBSS medium was collected, centrifuged at 400 g for 3 min, and used to evaluate LDH release and TBARS concentration. Cells were washed twice with warm DPBS, and processed for further analysis.

## 2.4. Lactate dehydrogenase (LDH) release

LDH activity in the EBSS was assessed following spectrophotometrically for 1 min the rate of NADH oxidation at 340 nm (Di Nunzio et al., 2013), and enzyme activity was expressed as mUnits/ml.

## 2.5. Thiobarbituric acid reactive substances (TBARS) concentration

TBARS, the end products of lipid peroxidation, were assayed in EBSS as previously reported by Valli *et al.* (Valli et al., 2012), and their concentration was expressed as relative fluorescence units (RFU) normalized for mg protein in the corresponding well.

## 2.6. Cell viability

Cell viability was evaluated by the 3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, based on the cleavage of the yellow tetrazolium salt MTT to purple formazan crystal by metabolic active cells (Danesi et al., 2011). Results were expressed as percent of control cell viability, assigned as 100%.

## 2.7. Conjugated diene (CD) level

Cellular level of CD containing lipids was assayed as reported by Di Nunzio *et al.* (Di Nunzio et al., 2011), and expressed as percent of the value detected in control cells, assigned to 100%.

## 2.8. Cytoplasmic total antioxidant activity (TAA)

Cytoplasmic TAA was measured as reported by Di Nunzio *et al.* (Di Nunzio et al., 2013), based on the ability of the antioxidant molecules in the sample to reduce the radical cation of ABTS. Values were normalized for protein content in the sample, and expressed as micromoles of trolox equivalent (TE)/mg protein.

## 2.9. Reduced glutathione (GSH) content

103    Cytoplasmic GSH content was determined as previously described (Valli et al., 2012), normalized
104    for protein content in the sample, and expressed as nmoles of GSH/mg protein.

105    *2.10. Antioxidant enzymes activity*

106    Cytoplasmic superoxide dismutase (SOD) activity was assayed using a commercial kit (Cayman
107    Chemical, Michigan, USA) following the manufacture instructions. SOD activity (Units) was then
108    normalized for mg of protein in the sample. Cytoplasmic catalase (CAT) activity was assayed
109    spectrophotometrically at 240 nm following the extinction of $H_2O_2$ (Danesi et al., 2006), normalized
110    for protein content in the sample, and expressed as Units/mg protein. Cytoplasmic glutathione-S-
111    transferase (GST) activity was assayed as previously reported by Di Nunzio *et al.* (Di Nunzio et al.,
112    2011). GST activity (nmol/min) was then normalized for protein content in the sample. Cytoplasmic
113    glutathione peroxidase (GPx) activity was assayed using a commercial kit (Cayman Chemical,
114    Michigan, USA) following the manufacture instructions. GPx activity (nmol/min) was then normalized
115    for protein content in the sample.

116    *2.11. Protein content*

117    Protein content was determined according to Bradford (Bradford, 1976), using BSA as standard.

118    *2.12. Statistical analysis*

119    Data are reported as mean ± SD of at least six samples derived from three independent cell cultures.
120    Statistical analysis was by the one way ANOVA with Dunnett as post-test.

121    **3. Results**

122    To evidence the detrimental effect of $H_2O_2$ addition, control cells were compared to stressed cells,
123    both US and fatty acid supplemented. Then a statistical comparison was performed among stressed
124    cells, to verify the differential effect of the single fatty acids, and to evidence whether the diverse
125    supplementation could protect or exacerbate the outcomes of the oxidative stress.

126    *3.1. $H_2O_2$-induced cell oxidative damage*

127    The $H_2O_2$-induced cell damage was evaluated by measuring cell viability, LDH release, TBARS
128    level and CD content (figure 1). As shown in figure 1A, in all cells $H_2O_2$ exposure caused a significant
129    reduction of viability with respect to controls. Among stressed cells, viability decreased in all
130    supplemented cells, but DHA.
131    The oxidative stress caused a significant increase in LDH activity and TBARS concentration in the
132    medium of all cells except the US and DHA supplemented ones (figure 1B and 1C, respectively).
133    Notwithstanding the increase of TBARS in almost all stressed cells, the level of CD, the first products
134    of lipid peroxidation, increased in LA and ARA supplemented cells only (figure 1D).
135
136    *3.2. Modulation of antioxidant defenses*

137    To evaluate the effect of PUFA supplementation on the cell response to an oxidative stress,
138    cytosolic TAA and GSH content (figure 2), and the activity of SOD, CAT, GST and GPx enzymes
139    (figure 3) were determined.

140    Compared to controls, $H_2O_2$ exposure caused a significant decrease of cytosolic TAA in US and LA
141    supplemented cells only (figure 2A), and no modification in GSH content apart from a significant
142    increase in DHA supplemented cells (figure 2B). Comparing stressed cells, TAA appeared higher in
143    ARA and DHA supplemented hepatocytes, the latter also showing a higher GSH concentration.

144    Compared to controls, upon $H_2O_2$ exposure a significant increase of SOD activity was detected in
145    LA and n-3 PUFA supplemented cells (figure 3A), and CAT activity increased in all cells (figure 3B).
146    GST activity was not modified in US cells, while it significantly increased in all supplemented cells
147    but DHA ones (figure 3C). Furthermore, a significant increase in GPx activity was detected in LA
148    supplemented cells only (figure 3D). Comparing stressed cells, a differential influence of the
149    supplemented fatty acids on enzyme activities was evidenced. SOD activity being higher in EPA and
150    DHA supplemented, CAT in ARA and EPA supplemented, GST in ARA supplemented, and GPx in
151    LA supplemented cells than in US ones.

152    Since cellular oxidative status may be influenced by nonlethal oxidative stress exposure in a time
153    depending manner (Brunk et al., 1995), SOD, CAT, GST and GPx activities were also evaluated after
154    20 min $H_2O_2$ exposure (figure 4A, 4B, 4C and 4D respectively).

155    Compared to controls, upon the shorter $H_2O_2$ exposure time SOD activity increased in ARA and
156    EPA supplemented cells only, and no significant modification in CAT and GPx activity was detected.
157    GST activity was reduced in all supplemented cells except DHA and US ones. Comparing stressed
158    cells a differential effect of the supplementation on SOD, CAT and GST activities was still detected,
159    while no differences were evidenced in GPx activity.

160    **4. Discussion**

161    n-6 and n-3 PUFA are well known for their physiological functions and preventive actions (Liu et
162    al., 2015; Pelliccia et al., 2013) that are ascribable to different mechanisms including regulation of
163    membrane functionality, cell signaling, and gene expression (Robichaud & Surette, 2015; Righi et al.,
164    2011; Bordoni et al., 2007). In contrast to their health effects, PUFAs are known to be prone to rapid
165    peroxidation, which has been shown to impair membrane functions and inactivate proteins and
166    enzymes, eventually leading to various disorders and diseases (Csala et al., 2015). To explain this
167    apparent oxymoron, it has been speculated that the lipid peroxidation occurring in PUFA-rich cells
168    causes a low level, sub-toxic stress that could induce a protective adaptations over time (i.e. hormesis),
169    contributing to PUFA health effects. Actually, n-3 PUFA have been shown to up-regulate the
170    antioxidant systems in various cells (Giordano & Visioli, 2014), so counteracting the oxidative stress
171    (Zanatta et al., 2014; Tourtas et al., 2012). Although different PUFA share common chemical
172    characteristics, in a previous work we evidenced that they differently modulate the activity of
173    antioxidant enzymes, and have a different impact on cell oxidative status (Di Nunzio et al., 2011). A
174    concomitant oxidative stress could have an impact on the effects of PUFA, this representing an
175    important issue since a higher intake of PUFA, particularly n-3 PUFA, is suggested for the
176    prevention/modulation of a wide range of diseases associated with an increased production of ROS

177 (Fischer & Maier, 2015; Reuter et al., 2010; Pashkow, 2011; Salomone et al., 2016). To further
178 elucidate it, in the present work we have supplemented different PUFA to HepG2 cells, which were
179 then submitted to a mild oxidative stress.

180     Results herein reported clearly evidence that the cell response to a subsequent oxidative stress can
181 be modulated by a preceding PUFA supplementation.

182     All supplemented fatty acids except DHA enhance cell susceptibility to $H_2O_2$. This could be due to
183 an increased lipid peroxidation inducing disturbance of fine structures, alteration of integrity, fluidity,
184 and permeability, and functional loss of biomembranes, and generates potentially toxic products
185 (Ayala et al., 2014), as indicated by the increase in TBARS concentration in the medium of all
186 supplemented cells but DHA supplemented ones. Since CD containing lipids, the first products of lipid
187 peroxidation, are usually quickly cleaved to their corresponding breakdown products (Yin et al., 2011),
188 the higher CD concentration in n-6 PUFA than n-3 PUFA supplemented hepatocytes could indicate a
189 longer time lasting lipoperoxidation.

190     Although we already observed a PUFA sensitizing effect in basal condition (Di Nunzio et al.,
191 2011), results herein reported cannot be simply considered as its consequence but as an additive effect
192 due to the concomitant presence of PUFA and $H_2O_2$. It can be clearly evidenced comparing present
193 and previous data; as example, in basal condition LDH release was significantly higher in ARA
194 supplemented cells only (Di Nunzio et al., 2011), while it is significantly increased in all supplemented
195 cells except DHA ones after $H_2O_2$ addition, the increase being higher in ARA supplemented cells. It is
196 worth noting that DHA supplementation has no adverse effect either in basal condition (Di Nunzio et
197 al., 2011) or after the oxidative insult.

198     This differential modulation of the cell response to the oxidative stress is in part ascribable to the
199 modulation of the antioxidant enzymes activities. The effect of PUFA on the antioxidant enzymes
200 status have been extensively studied leading to controversial results. To the Authors knowledge,
201 previous studies have been performed using fish oil (Avramovic et al., 2012; Romieu et al., 2008) or
202 other oils (Umesha & Naidu, 2015; Taranu at al., 2014; Haggag et al., 2014) containing different
203 combinations of PUFA, this not allowing to extrapolate the effect of the single fatty acid. Results
204 herein reported clearly evidence that each fatty acid has specific modulatory effects on antioxidant
205 enzymes, and promotes a specific profile of antioxidant enzyme activities that cannot be predicted
206 using oils containing different proportion of PUFA. The importance of the proportion of PUFA in a
207 mixture has been also shown by Molinar-Toribio *et al.* (Molinar-Toribio et al., 2015) who evidenced in
208 rat supplemented with EPA plus DHA mixtures that the activity of antioxidant enzymes increases with
209 magnitudes depending on the EPA:DHA ratio. Notably, the highest DHA concentration reduced the
210 oxidative stress.

211     Our study confirms that exposure time to $H_2O_2$ modulates anti-oxidant enzymes activity, as
212 previously reported by Shull *et al.* (Shull et al., 1991) and Franco *et al.* (Franco et al., 1999). In
213 addition, our results evidence that PUFA supplementation exacerbates this time-depending
214 modulation. Assuming that the observed increased activity of antioxidant enzymes is based on an
215 increased transcription of the corresponding encoding genes and an increased synthesis of the
216 enzymatic proteins, a lag time between the insult and the cell response is conceivable. This can explain
217 the less evident changes in antioxidant enzymes activities after 20 than 60 min $H_2O_2$ exposure. In
218 addition, the weaker cell response after shorter time of exposure could be due to the lower intracellular

219 concentration of $H_2O_2$. In fact, $H_2O_2$ diffusion across the plasma membrane is low during the first 10
220 minutes of exposure (Alexandre et al., 2006).

221 Oxidative stress underlies a complex array of metabolic alterations. Under this condition,
222 derangement of cell biochemistry and function is known to involve misregulation of key components
223 responsible for cell signaling, such as membrane receptors, kinases, phosphatases, and transcription
224 factors (Leonarduzzi et al., 2011; Brigelius-Flohé et al., 2011). In this view, the overall differential
225 effect of supplemented DHA could rely on different mechanisms. Among them, a different extent of
226 incorporation/metabolism of the supplemented fatty acid inside the cell. Although all supplemented
227 PUFA are readily incorporated by HepG2 cells at the expense of monounsaturated fatty acids, in a
228 previous work we observed different conversion rate (Di Nunzio et al., 2010). LA and ALA are further
229 metabolized upon cellular uptake, and EPA is partially desaturated/elongated to DHA. On the contrary,
230 no DHA retro-conversion is detected. Therefore the effects of all PUFA except DHA could be
231 ascribable to both the supplemented PUFA and its derivatives. The differential metabolism of
232 supplemented PUFA could also impact on the modification of membrane composition, and
233 consequently functionality, since the majority of PUFA is incorporated in the cell membrane
234 phospholipids (Abbott et al., 2012). The oxidative stress may selectively impair cell PUFA
235 concentration, which in turn may modulate the effect of the oxidative stress (Zaloga et al., 2006).
236 Oxidative condition modifies fatty acid composition in neonatal rat cardiomyocytes (Bordoni et al.,
237 2005), and a slight increase in the saturated and a decrease in monounsaturated and PUFA content has
238 been detected in the liver of pro-oxidant pesticide-treated rats (Nakbi et al., 2010). At membrane level,
239 exposure to an oxidative stress leads not only to fatty acid oxidation but also to phospholipid
240 breakdown due to activation of phospholipase $A_2$ (Nalbone et al., 1990). DHA released from
241 phospholipids is the substrate for the formation of resolvins and protectins, that act through distinct
242 receptors to unfold biological effects (Calder, 2015) including oxidation preventing effects (Liu et al.,
243 2014; Tian et al., 2015).

244 A higher DHA cellular content may play an important role in the protection from oxidative damage
245 because 4-hydroxyhexenal, an aldehyde derived from DHA peroxidation, up-regulates Nrf2-HO-1
246 antioxidant signaling pathway, which induces antioxidant effects (Gao et al., 2007).

247 Many studies indicate that PUFA, particularly EPA and DHA, control gene expression also by
248 directly governing the transcription/activity of peroxisome proliferator-activated receptor-alpha
249 (PPARα) (Di Nunzio et al., 2009; Georgiadi et al., 2012), whose activation may result in enhanced
250 expression/activity of antioxidant enzymes such as CAT and SOD (Toyama et al., 2004; Inoue et al.,
251 2001). PPARs exert their effect on gene transcription by dimerization with the 9-cis retinoic acid
252 receptors (RXRs) (Feige et al., 2005). RXR is also able to bind PUFA, mainly DHA (Crawford et al.,
253 2003). Other PUFAs such as EPA or ARA can activate RXR but with lower efficiency (Egea et al.,
254 2002). Thus, DHA can be considered a more efficient activator of PPARα (Bordoni et al., 2006).

255 In conclusion, based on reported results each PUFA seems to possess specific pro- and antioxidative
256 characteristics, not predictable on chemical basis such as the length of the carbon chain or the
257 unsaturation degree. According to many *in vivo* (Mas et al., 2010; Patten et al., 2013) and *in vitro*
258 (González-Périz et al., 2006; Mukherjee et al., 2004) studies, DHA appears the only fatty acid not
259 increasing cell susceptibility to $H_2O_2$, although its effect is not ascribable to a stronger induction of
260 antioxidant enzymes activity.

Overall, results herein reported clearly evidence that fatty acid supplementation can modify the cell response to an oxidative stress, and the response is dependent on the supplemented fatty acid. We are beginning to understand the physiology and molecular basis of the effects of n-3 and n-6 fatty acids, and thereby gradually encompass the full biological potential of these compounds. Our results underline that PUFA cannot be considered as a single category, but as individual compounds. It appears particularly evident for DHA, and although observed results must be confirmed *in vivo*, this apparent "DHA paradox" represents an important step ahead in the use of DHA supplementation as preventive strategy in the clinical practice.

## Disclosure statement

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## Funding statement

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457  **Figure 1. Cell viability A), LDH activity B), TBARS concentration C) and CD level D) in basal**

458  **condition and after 1h $H_2O_2$ exposure.**

459  *Data are means ± SD of at least six samples in each group, coming from three independent cell*

460  *cultures. Statistical analysis was by the one-way ANOVA p<0.001) using Dunnett's post-test to*

461  *compare: 1) stressed cells both US and supplemented) to controls \*p<0.05; \*\*p<0.01; \*\*\*p<0.001);*

462  *and 2) supplemented stressed cells to US stressed cells °p<0.05; °°°p<0.001).*

463

464  **Figure 2. TAA A) and GSH level B) in basal condition and after 1h $H_2O_2$ exposure.**

465  *Data are means ± SD of at least six samples in each group, coming from three independent cell*

466  *cultures. Statistical analysis was performed by the one-way ANOVA p<0.01) using Dunnett's as post-*

467  *test to compare: 1) stressed cells both US and supplemented) to controls \*p<0.05); and 2)*

468  *supplemented stressed cells to US stressed cells °p<0.05; °°p<0.01).*

469

470  **Figure 3. SOD A), CAT B), GST C) and GPx D) activity in basal condition and after 1h $H_2O_2$**

471  **exposure.**

472  *Data are means ± SD of at least six samples in each group, coming from three independent cell*

473  *cultures. Statistical analysis was performed by the one-way ANOVA A, B and C p<0.001; D p<0.01)*

474  *using Dunnett's as post-test to compare: 1) stressed cells both US and supplemented) to controls*

475  *\*p<0.05; \*\*p<0.01; \*\*\*p<0.001); and 2) supplemented stressed cells to US stressed cells °p<0.05;*

476  *°°p<0.01; °°°p<0.001).*

477

478  **Figure 4 SOD A), CAT B), GST C) and GPx D) activity in basal condition and after 20 min $H_2O_2$**

479  **exposure.**

480  *Data are means ± SD of at least six samples in each group, coming from three independent cell*

481  *cultures. Statistical analysis was performed by the one-way ANOVA A and B p<0.01; C p<0.001)*

482  *using Dunnett's as post-test to compare: 1) stressed cells both US and supplemented) to controls*

483     *p<0.05; **p<0.01; ***p<0.001); and 2) supplemented stressed cells to US stressed cells °p<0.05;

484     °°p<0.01; °°°p<0.001).