**4-Hydroxyhexenal and 4-hydroxynonenal are mediators of the anti-cachectic effect of n-3 and n-6 polyunsaturated fatty acids on human lung cancer cells**

# 4-hydroxyhexenal and 4-hydroxynonenal are mediators of the anti-cachectic effect of n-3 and n-6 polyunsaturated fatty acids on human lung cancer cells

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ABSTRACT

Cachexia, the most severe paraneoplastic syndrome, occurs in about 80% of patients with advanced cancer; it cannot be reverted by conventional, enteral, or parenteral nutrition. For this reason, nutritional interventions must be based on the use of substances possessing, alongside nutritional and energetic properties, the ability to modulate production of the pro-inflammatory factors responsible for the metabolic changes characterising cancer cachexia. In light of their nutritional and anti-inflammatory properties, polyunsaturated fatty acids (PUFAs), and in particular n-3, have been investigated for treating cachexia; however, the results have been contradictory.

Since both n-3 and n-6 PUFAs can affect cell functions in several ways, this research investigated the possibility that the effects of both n-3 and n-6 PUFAs could be mediated by their major aldehydic products of lipid peroxidation, 4-hydroxyhexenal (HHE) and 4-hydroxynonenal (HNE), and by their anti-inflammatory properties. An “in vitro” cancer cachexia model, consisting of human lung cancer cells (A427) and murine myoblasts (C2C12), was used.

The results showed that: 1) both n-3 and n-6 PUFAs reduced the growth of lung cancer cells without causing cell death, increased lipid peroxidation and Peroxisome Proliferator-Activated Receptor (PPAR)α, and decreased TNFα; 2) culture medium conditioned by A427 cells grown in the absence of PUFAs blocked myosin production and the differentiation of C2C12 muscle cells; conversely, muscle cells grown in culture medium conditioned by the same cells in the presence of PUFAs showed myosin expression and formed myotubes; 3) adding HHE or HNE directly to C2C12 cells maintained in culture medium conditioned by A427 cells in the absence of PUFAs stimulated myosin production and myotube formation; 4) putative consensus sequences for (PPARs) have been found in genes encoding fast isoforms of myosin heavy chain, by a bioinformatics approach.

The overall results show, first, the ability of both n-3 and n-6 PUFAs and their lipid peroxidation products to prevent the blocking of myosin expression and myotube formation caused in C2C12 cells by medium conditioned by human lung tumor cells. The C2C12 cell differentiation can be due

to direct effect of lipid peroxidation products, as evidenced by treating C2C12 cells with HHE and HNE, and to the decrease of pro-inflammatory TNFα in A427 cell culture medium. The presence of consensus sequences for PPARs in genes encoding the fast isoforms of myosin heavy chain suggests that the effects of PUFAs, HHE, and HNE are PPAR-mediated.

KEYWORDS: cachexia; lung cancer; muscle cells; n-3 PUFAs; n-6 PUFAs, lipid peroxidation; HHE; HNE; myosin; PPARs; PPRE; TNFα.

## INTRODUCTION

Cachexia is both the most common and the most severe paraneoplastic syndrome; it was recently defined as a multifactorial condition. It is chiefly characterised by loss of skeletal muscle mass, and negative protein and energy balance, driven by a variable combination of reduced food intake and abnormal metabolism that is not fully reversed by conventional nutrition. It leads to a reduction in the response and tolerance to therapy, and in the quality and duration of life [1]. Cachexia occurs in about 80% of patients with advanced cancer; its incidence and prevalence differ in relation to the type of cancer, the highest incidence being observed in pancreatic, gastric and lung cancer [2]. Lung cancer induces sarcopenia associated to malnutrition, with a relatively high frequency (above 50%). Depending on the cachexia classification, about 20% of patients with non small cell lung cancer (NSCLC) are diagnosed as cachectic, and about 25% as pre-cachectic [3-5]. Malnutrition, due in part to alterations in neurohormonal mechanisms controlling food intake, contributes to metabolic alterations leading to a negative energy balance. This, in association with altered protein turnover and inflammation, drives the muscle wasting characterizing cachexia to develop [6]. With regard to the metabolic modifications occurring in cachexia, several cytokines produced by both tumour and host (TNF-$\alpha$, IL-6, IL-1, INF-$\gamma$) or by tumour alone (proteolysis-inducing factor, PIF, lipid mobilizing factor, LMF) have been identified as molecular mediators of cachexia [7,8].

Due to its multifactorial pathogenesis, cachexia is unaffected by conventional dietary interventions, and limited benefits have been demonstrated by enteral and parenteral nutrition [9,10]. For these reasons, nutritional interventions focused on the use of substances with both nutritional and anti-inflammatory properties, with the aim of improving energy balance and reducing inflammatory status, have been proposed. In this perspective, and in light of their anti-inflammatory properties, fish-oil and its primary components, n-3 polyunsaturated fatty acids (PUFAs), have been proposed for treating cachexia. Moreover, in patients with cancer, a low concentration of n-3 PUFAs in the

plasma phospholipids has been shown at diagnosis, the value further declining during cancer progression [11-13].

Among n-3 PUFAs, eicosapentaenoic (EPA) and docosahexaenoic (DHA) acid have been shown to down-regulate production of pro-inflammatory cytokines and acute-phase proteins in cancer patient blood [14-18], although a recent systematic review on the role of fish oil in treating cachexia concluded that there is insufficient evidence of a net benefit of n-3 PUFAs in advanced cancer. However, the same study reported that n-3 PUFA treatment can be beneficial for some selected patient populations, acting as post-operative support able to improve wound healing and reduce complications [19].

Different mechanisms have been postulated to explain the effect of n-3 PUFA, including modulation of the activity of some transcription factors, such as NFkB and PPARs, leading to a decreased production of pro-inflammatory cytokines and acute-phase proteins. Moreover, EPA has been reported to inhibit the activation of the ubiquitin-proteasome pathway induced by proteolysis-inducing factor (PIF) and to decrease expression of the lipid mobilizing factor (LMF) [20,21]. More recently, EPA and DHA have been reported to affect the balance between anabolism and catabolism in muscles, through their effect on signalling transduction proteins and adipokines, in old and obese persons, thus suggesting another possible mechanism underlying n-3 PUFA-mediated anti-wasting effect [23,24].

The present research investigated the possibility that anti-cachectic properties of n-3 PUFAs could be mediated by the aldehydic products derived from their lipid peroxidation. 4-hydroxyhexenal (HHE) is the principal aldehyde generated by the non-enzymatic peroxidation of n-3 PUFAs. Due to its electrophilicity, HHE interacts strongly with cellular nucleophilic molecules, thus positively or negatively affecting several cell functions [22]. HHE-induced cytotoxicity has been reported in different cell types, including rat neurons and muscle cells, and human lens and renal epithelial cells. [25-29]. Moreover, the HHE concentration has been shown to be significantly higher in the hippocampus/parahippocampal gyrus of patients with preclinical and late-stage Alzheimer's disease,

than in that of normal subjects [30]. Conversely, it has recently been shown that, in some cases, lipoxidation of proteins can cause a gain of function/activity, suggesting that the formation of covalent protein/α,β-unsaturated aldehyde adducts, derived from lipid peroxidation, might be a step in a redox signalling pathway of physiological significance [31]. The present study was also based on a previous finding that, at a concentration found in many normal tissues and plasma, 4-hydroxynonenal (HNE), the major lipid peroxidation product of n-6 PUFA, induced the differentiation of murine erythroleukemia MEL cells and human promyelocytic HL-60 cells, by modulating the expression of several genes involved in cell cycle control [32-34]. Moreover in some tumours, arachidonic acid (AA), a fatty acid belonging to n-6 PUFA, was shown to be present in a lower percentage in total fatty acids in comparison with corresponding normal tissues [35].

In this light, the research investigated the effect of both n-3 and n-6 PUFAs and their lipid peroxidation products (HHE and HNE) on muscle cell differentiation, using an "in vitro" cancer cachexia model, consisting of human lung cancer cells (A427) and murine myoblasts (C2C12).

Moreover, to evidence the mechanisms underlying the effect of PUFAs on C2C12 cell differentiation, the expression of peroxisome proliferator activated receptor (PPAR) α and the release of TNFα were also evaluated in A427 cells and in their culture medium, respectively. Human lung cancer cells were chosen since, in lung cancer patients, cachexia is the main cause of death despite the improvement in anticancer therapies. Moreover, little is known about the effect of PUFAs on lung-cancer-induced cachexia.

## MATERIALS AND METHODS

### Treatment of A427cells with PUFAs

Human lung adenocarcinoma cells A427 (ATCC, MD, USA) were seeded (20,000 cells/cm$^2$) in DMEM/F12 medium with 2 mM glutamine, 1% antibiotic/antimycotic solution and 10% FBS (medium A). Twenty-four hours after seeding, medium A was replaced with medium B, containing DMEM/F12 medium, 2 mM glutamine, 1% antibiotic/antimycotic solution, 2% horse serum (HS),

and n-3 (EPA plus DHA) or n-6 PUFAs (AA) prepared in HS. EPA and DHA were administered simultaneously at a ratio of 1.5:1. For all treatments, the final concentrations were 10 or 50 μM. In control cells, a quantity of HS equivalent to the highest dose administered of PUFAs was added to the culture medium, in addition to the 2% HS already present in medium B.

Twenty-four hours after addition of PUFAs or HS, culture media were collected and centrifuged at 2800 g for 10 min (centrifuge J6B Beckman, CA, USA) at room temperature. The collected media were used to determine lactate dehydrogenase (LDH) release, and as conditioned medium to culture C2C12 cells. After removing media, A427 cells were detached with trypsin/EDTA (0.25%/0.03 mM), centrifuged at 900 g for 10 min (centrifuge J6B Beckman, CA, USA) and used for the assays listed below.

C2C12 cell culture conditions

Murine muscle C2C12 cells (ATCC, MD, USA) were seeded at 6,000 cells/cm$^2$ in medium A for 4 days. After this time, to induce differentiation into myotubes, medium A was replaced with medium B, in which C2C12 cells were maintained for 3 further days. At the end of this period, medium B was replaced with medium conditioned by A427 cells, grown for 24 hours in the presence or absence of PUFAs. Cells were analyzed after a further 4 days. C2C12 cells maintained in medium B for a further 4 days were labelled “Tdiff”, and were taken as positive control.

Treatment of C2C12 cells with HHE and HNE

In these experiments, after removal of medium B (after 3 days), C2C12 cells were cultured in medium conditioned by A427 cells grown in the absence of PUFAs, and fortified with HNE or HHE at a concentration of 1 μM or 5 μM. The addition of 1 μM aldehyde was repeated every 45 minutes up to 10 treatments (total amount 10 μM); in the case of 5 μM aldehyde, it was added as a single dose. C2C12 cells cultured in medium conditioned by A427 cells for 24 hours, in the absence of PUFAs, were used as control cells. C2C12 cells were analyzed after a further 4 days.

161

162 Cell growth and viability

163 The numbers of A427 cells present in the monolayer and in the culture medium were evaluated by
164 counting cells in a Burker chamber, and are expressed as cells/$cm^2$. Cell viability was also evaluated
165 in culture medium, as LDH release [36], and as DNA content after propidium iodide staining by
166 using flow cytometry [37]. The viability of C2C12 cells during the different treatments was
167 evaluated as LDH release.

168

169 Immunofluorescence staining

170 To evaluate the production of myosin and myotube formation, at the different experimental times
171 C2C12 cells were washed with PBS, fixed with acetone/methanol (1:1) at -20°C for 20 minutes, and
172 kept at 4°C until use. Cells were then treated and viewed as in [38].

173

174 Western blot analysis

175 A427 and C2C12 cells were analyzed for ALDH3A1 expression using Western blot analysis [38].
176 Polyclonal anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or monoclonal anti-aldehyde
177 dehydrogenase (ALDH) 3A1 antibodies were used.

178

179 ELISA analysis

180 TNF-α content was evaluated in the culture medium of A427 cells by the Enzyme Linked Immuno
181 Sorbent Assay (Invitrogen Corporation, Frederick, MD, USA).

182

183 Real time PCR analysis

184 PPARα mRNA content was examined in A427cells treated (sample) or not (control) with PUFAs
185 for 24 hours.

Total RNA was extracted by using the TRI reagent (Sigma-Aldrich, St. Louis, MO, USA). Real-time PCR was performed with single-stranded cDNA prepared from total RNA (1 μg) using a High-Capacity cDNA Archive kit (Applied Bio Systems, Foster City, CA).

The forward and reverse primers were designed using Beacon Designer® software (Bio-Rad, Hercules, CA). Twenty-five microliters of a PCR mixture containing cDNA template equivalent 40 ng of total RNA, 5 pmoles each of forward and reverse primers, and 2× IQ SYBR Green SuperMix (Bio-Rad, Hercules, CA) were amplified using an iCycler PCR instrument (Bio-Rad, Hercules, CA) with an initial denaturation at 95°C for 3 min, followed by 35-40 cycles at 95°C for 30 s, annealing at 52°C for 40 s, extension at 72°C for 40 s. Each sample was tested in duplicate, and threshold cycle (Ct) values were averaged from each reaction. The change in expression was defined as that detected in the A427 cells treated with PUFAs versus that detected in the control cells, calculated as $2^{-\Delta\Delta Ct}$, where $\Delta Ct = Ct_{sample} - Ct_{GAPDH}$ and $\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{control}$.

Lipid peroxidation in A427 cells

Malondialdehyde was measured in HPLC according to the method of Nielsen et al [39], with slight modifications. Briefly, aliquots of cell medium were mixed (volume/volume) with 0.6% (w/v) aqueous solution of thiobarbituric acid (TBA). The mixture was acidified with 1/20 volume of 100% (w/v) trichloroacetic acid and heated at 100°C for 1 hour. The samples were then cooled in ice and centrifuged at 13000 g for 5 minutes. Aliquots of 50 μl of the supernatant were injected into the HPLC system.

The HPLC system was equipped with a Novapak C18 4um 3.9x150 mm column. The elution was isocratic. The mobile phase consisted of a mixture of a 10 mM potassium dihydrogen phosphate solution adjusted at pH 6.8 with 1M KOH and methanol in ratio 60/40. The flow rate was 1 ml/min.

Detection was performed by a spectrofluorometer at 532 nm ex/553 nm em. The peak of the MDA-TBA adduct was well resolved at baseline and its retention time in our conditions was 4.8 min.

Identification and quantification of MDA concentration in the samples was performed by comparison to the chromatograms of various concentrations of standard MDA sodium salt, synthesized by Dr Bruno Tasso, according to Nair et al [40], treated in the same way as the samples. The lowest tested concentration of standard that was still quantifiable was 0.125 μM.

Detection of putative peroxisome proliferator response element (PPRE)

The presence of the putative PPRE sequence in genes encoding fast isoforms of myosin heavy chain (MyHC) was evaluated with the NHR SCAN software package.

Statistical analysis

All data are expressed as means ± S.D. Differences between means were assessed by analysis of variance followed by the post-hoc Newman-Keuls test.

## RESULTS

The effect of PUFAs on growth and viability of lung cancer cells is reported in Figure 1. Exposing A427 cells to PUFAs caused a dose-dependent decrease in cell growth that was more marked for n-3 PUFAs (panel A): 10 or 50 μM EPA plus DHA reduced cell numbers by 22% and 38%, respectively, whereas AA only affected A427 cell numbers significantly (-20%) at the highest concentration used. Neither n-3 nor n-6 PUFAs significantly induced either necrosis (panel B) or apoptosis (panel C).

Lipid peroxidation (Figure 2) was measured in the culture medium of A427 cells exposed to PUFAs and expressed as the production of MDA. A similar and significant increase was observed in cells

234 exposed to EPA plus DHA or to AA (about +40% or +70%, in the presence of 10 or 50 μM PUFAs,
235 respectively).
236 Figure 3 panel A shows that both concentrations of EPA+DHA decreased TNFα content in the
237 culture medium of A427 cells, whereas only the highest concentration of AA decreased this
238 cytokine. Figure 3 panel B reports that PPARα mRNA was increased in A427 cells treated with
239 both 50 μM PUFAs, being the highest increase in A427 cells treated with EPA+DHA.
240 Culture medium conditioned for 24 h by A427 cells, in the presence or absence of PUFAs, was used
241 to grow murine muscle C2C12 cells, with the aim of investigating its effect on muscle cell
242 differentiation. No significant change in C2C12 viability was evidenced by analyzing LDH activity
243 in the culture medium, at any of the experimental conditions investigated (data not shown).
244 Figure 4 shows that C2C12 cells grown in medium conditioned by A427 cells, in the absence of
245 PUFAs, showed no production of myosin and no myotube formation, unlike cells maintained in
246 differentiation medium (Tdiff). Conversely, in C2C12 cells grown in medium conditioned by A427
247 cells in the presence of both n-3 and n-6 PUFAs, myosin production and myotube formation both
248 occurred, the most significant effect being evident in cells grown in medium conditioned in the
249 presence of 50 μM EPA plus DHA.
250 To investigate the role of lipid peroxidation products in reversing the anti-differentiation effect of
251 lung cancer cells on C2C12 cells, HHE and HNE were directly added to C2C12 cells grown in
252 medium conditioned by A427 cells, in the absence of n-3/n-6 PUFAs. Figure 5 shows the effect of
253 aldehyde administration on C2C12 cell viability: only HHE, at either of the concentrations used,
254 significantly increased LDH release.
255 Figure 6 shows that both myosin content and myotubes in C1C12 increased in a dose-dependent
256 manner, in C2C12 cells exposed for 4 days to aldehydes, the strongest effect being observed in cells
257 exposed to 5 μM HNE.

Western blot analysis of ALDH3A1 protein content showed that neither A427 nor C2C12 cells express this enzyme, which is significant in metabolizing aldehydes derived from lipid peroxidation (Figure 7).

Table 1 reports the analysis of the putative PPRE motif in fast isoforms of myosin heavy chain (MyHC). The NHR SCAN software evidenced the putative presence of the PPRE consensus sequence in both exons /introns of IIa, IIb, IId/x genes, but not in the promoter regions.

## DISCUSSION

In this research the anti-cachectic effect of both n-3 and n-6 PUFAs was investigated alongside with the possibility that this effect can be mediated by their major aldehydic products of lipid peroxidation, HHE and HNE, and by their anti-inflammatory properties. Aldehyde products derived from the lipid peroxidation of PUFAs have been shown to exert both regulatory and detrimental effects on several cell functions, mainly depending on their cellular concentration. When produced at physiological or low levels, lipidic aldehydes can act as signalling molecules targeting specific pathways, including tyrosine kinase signalling and the adaptive response mediated by Nrf2, AP-1, PPARs and NFkB [41]. Conversely, high intracellular levels of lipidic aldehydes are responsible for cytotoxic or cytostatic effects [42,43].

Among aldehydes derived from lipid peroxidation, α,β-unsaturated aldehydes are those that have been studied in most depth; they are the most important molecular mediators of the effects of both n-3 and n-6 PUFAs.

The “in vitro” model of cancer cachexia used in this research enabled both the effect of PUFA administration on pro-cachectic activity of human lung cancer cells, and the effects of culture medium conditioned by cancer cells in the presence or not of PUFAs on muscle cell differentiation, to be investigated.

The most important finding of the study is that both PUFA families, n-3 and n-6, decreased the proliferation of human lung cancer cells, and were able to prevent the inhibition of muscle cell

differentiation induced by lung cancer cells. The observation that AA decreased cancer cell proliferation disagrees with the results of certain other studies [44-46], but confirms previous studies by the present research group evidencing that this n-6 PUFA suppresses the growth of well differentiated human lung tumour cells A549 and of rat hepatocarcinoma cell lines [47-50]. The inhibitory effect of AA on cancer cell proliferation has been inversely related to the ability of cells to metabolize aldehydic products of lipid peroxidation, in particular to the expression of aldehyde dehydrogenase 3A1 (ALDH3A1) [49]. For this reason, in this research the protein content of ALDH3A1 was evaluated in A427 and C2C12 cells, neither of which expresses this enzyme. The lack of this enzyme in A427 cells could be important in maintaining the high level of lipid peroxidation products observed after n-3 or n-6 PUFA supplementation, and, in consequence, might be the explanation for their effect on both cell proliferation and pro-cachectic properties of A427 cells. At the same time, the poor ability of C2C12 cells to catabolise aldehydes might be crucial in maintaining aldehyde levels that are able to induce muscle cell differentiation. In fact, unlike free radicals, which have a very short half-life, HNE and other aldehydes deriving from lipid peroxidation can diffuse from the site where they are produced and modulate signalling pathways in other cells.

HNE has been reported to induce cell differentiation in human leukemic HL-60 cells and in murine erythroleukemia MEL cells [32], the pathways proposed being different: translocation of PKC, formation of adducts with Heat Shock 60 kDa Protein 1 [51], and inhibition of telomerase activity [52]. In the light of these observations, the supposed involvement of lipid peroxidation products in mediating the anti-cachectic properties of lung cancer cells was investigated. Exposing C2C12 muscle cells, maintained in medium conditioned by A427 cells in the absence of PUFAs, to HNE or to HHE induced the expression of myosin and the formation of myotubes, indicating the ability of both lipid peroxidation products to stimulate muscle cell differentiation, counteracting the inhibitory effect of medium conditioned by lung cancer cells. These results show, firstly, that HHE also possesses differentiation properties, and secondly that both the major lipid peroxidation products of

n-3 and n-6 PUFAs can block the anti-differentiation effect of lung cancer cells and induce expression of myosin in murine muscle cells.

The lipid peroxidation products can induce muscle cell differentiation indirectly by blocking the anti-differentiation effect of TNFα produced and released by cancer cells, and directly by affecting myosin expression, as evidenced by treatment of C2C12 cells with HHE and HNE. The first mechanism contributing to C2C12 cell differentiation, i.e. the decrease TNFα content in culture medium of A427 cells, can be explained by the increased PPARα expression, induced by PUFAs or their lipid peroxidation products, including HHE and HNE. This statement is in agreement with our previous researches, showing that PUFAs were able to induce PPARs, and, as consequence, to decrease tumour cell proliferation and cytokine content in cell culture medium [49,53,54].

It has been shown that HNE acts as an intracellular agonist of peroxisome proliferator-activated receptors (PPARs) and that it is thus involved in regulating several intracellular pathways [55,56]. PPARs are nuclear transcription factors that, after ligand binding, heterodimerize with RXRα (PPARα/RXRα) and bind to the consensus sequence named PPRE (Peroxisome proliferator response element) in the target genes.

The PPRE motif consist of 2 half-sites (AGGTCA N AGGTCA) with an interspacing nucleotide (DR-1, direct repeat 1) [57]. Based on these observations, the presence of putative PPRE sequences in genes encoding fast isoforms of myosin heavy chain (MyHC) was investigated. The bioinformatic approach used in this preliminary analysis evidenced the presence of putative PPRE in both exons and intron of genes encoding fast myosin heavy chains, but not in the promoter region. This suggests that the increased production of myosin observed in C2C12 cells, grown in medium conditioned by A427 cells in the presence of PUFAs, and in the same cells directly exposed to HHE or HNE, could be PPAR-dependent.

Taken as a whole, these results demonstrate the ability of both n-3 and n-6 PUFAs, and of their aldehydic lipid peroxidation products HHE and HNE, to decrease TNFα release and to prevent the

decrease of myosin expression and myotube formation that is determined by culturing C2C12 muscle cells in the presence of medium conditioned by lung tumour cells. The lower induction of differentiation observed in C2C12 cells treated with HHE in comparison with C2C12 cells grown in culture medium of A427 cells treated with EPA+DHA can be due to the fact that the treatment with n-3 PUFAs caused, other that the production of lipid aldehydes, a decrease of TNFα. This decrease was higher than in the case of A427 cells treated with AA, and this difference could explain the higher differentiation effect induced by EPA+DHA respect to AA.

This finding could represent an important starting point to investigate in greater depth the possibility of designing therapeutic protocols using natural dietary substances, such as n-3 and n-6 PUFAs, to both directly reduce the growth of cancer cells, and reduce their ability to decrease myosin synthesis and differentiation in muscle cells, which occurs in cancer cachexia.

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FIGURE CAPTIONS

Figure 1. Effect of n-3 or n-6 PUFAs on growth and viability of human lung cancer cells A427.
Data are means ± S.D. from 4 experiments. For each panel, means with different letters are significantly different from one another ($p<0.05$) as determined by analysis of variance followed by post-hoc Newman-Keuls analysis.
C, control cells; E + D 10, cells treated with 10 µM EPA + DHA (1.5:1 ratio); E + D 50, cells treated with 50 µM EPA + DHA (1.5:1 ratio); AA 10; cells treated with 10 µM AA; AA 50; cells treated with 50 µM AA.

Figure 2. Lipid peroxidation in culture medium of human lung cancer cells A427 treated or not with n-3 or n-6 PUFAs for 24 hours.
Data are means ± S.D. from 4 experiments and are expressed as percentage of control value taken as 100. Means with different letters are significantly different from one another ($p<0.05$) as determined by analysis of variance followed by post-hoc Newman-Keuls analysis.
MDA, malondialdehyde; C, control cells; E + D 10, cells treated with 10 µM EPA + DHA (1.5:1 ratio); E + D 50, cells treated with 50 µM EPA + DHA (1.5:1 ratio); AA 10; cells treated with 10 µM AA; AA 50; cells treated with 50 µM AA.

Figure 3. TNFα (panel A) and PPARα (panel B) in culture medium of A427 cells treated or not with n-3 or n-6 PUFAs for 24 hours and in A427 cells, respectively.
Data are means ± S.D. from 4 experiments and are expressed as percentage of control value taken as 100. For each panel, means with different letters are significantly different from one another ($p<0.05$) as determined by analysis of variance followed by post-hoc Newman-Keuls analysis.

552 C, control cells; E + D 10, cells treated with 10 μM EPA + DHA (1.5:1 ratio); E + D 50, cells
553 treated with 50 μM EPA + DHA (1.5:1 ratio); AA 10; cells treated with 10 μM AA; AA 50; cells
554 treated with 50 μM AA.
555
556 Figure 4. Myosin content evidenced by immunofluorescence in C2C12 murine myoblasts grown in
557 culture medium conditioned by human lung cancer cells A427, in the presence or absence of
558 PUFAs.
559 Tdiff, cells maintained in medium B; none, cells grown in culture medium conditioned by A427
560 cells in the absence of PUFAs; E + D 10, cells treated with 10 μM EPA + DHA (1.5:1 ratio); E + D
561 50, cells treated with 50 μM EPA + DHA (1.5:1 ratio); AA 10; cells treated with 10 μM AA; AA
562 50; cells treated with 50 μM AA.
563
564 Figure 5. Effect of HHE and HNE on viability of C2C12 murine myoblasts grown in culture
565 medium conditioned by human lung cancer cells A427 in the absence of PUFAs.
566 Data are means ± S.D. from 4 experiments. Means with different letters are significantly different
567 from one another ($p<0.05$) as determined by analysis of variance followed by post-hoc Newman-
568 Keuls analysis.
569 Tdiff, cells maintained in medium B; none, cells grown in culture medium conditioned by A427
570 cells in the absence of PUFAs; HHE 1, cells grown in culture medium conditioned by A427 cells in
571 the absence of PUFAs and treated with 1 μM HHE; HHE 5, cells grown in culture medium
572 conditioned by A427 cells in the absence of PUFAs and treated with 5 μM HHE; HNE 1, cells
573 grown in culture medium conditioned by A427 cells in the absence of PUFAs and treated with 1
574 μM HNE; HNE 5, cells grown in culture medium conditioned by A427 cells in the absence of
575 PUFAs and treated with 5 μM HNE (for treatment details see Materials and Methods).
576

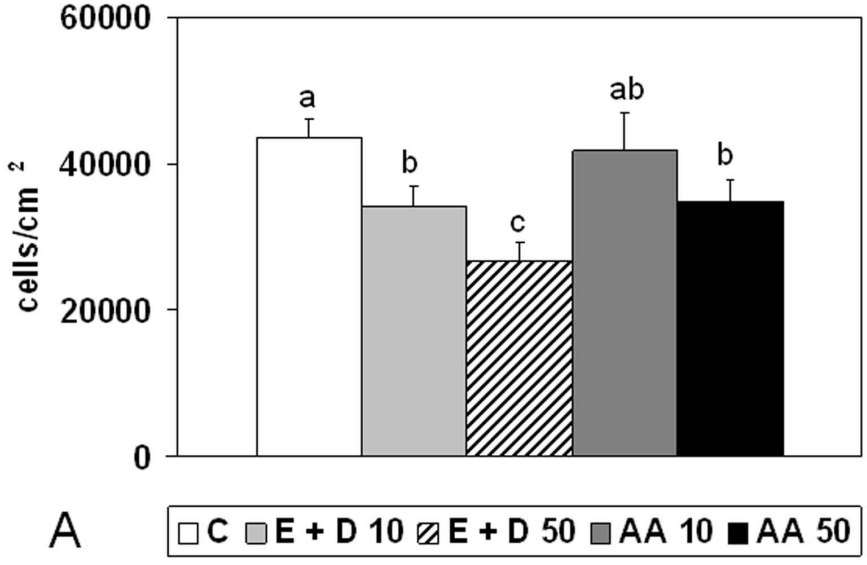
577 Figure 6. Myosin content evidenced by immunofluorescence in C2C12 murine myoblasts grown in
578 culture medium conditioned by human lung cancer cells A427 in the absence of PUFAs and treated
579 with HHE and HNE.
580 Tdiff, cells maintained in medium B; none, cells grown in culture medium conditioned by A427
581 cells in the absence of PUFAs; HHE 1, cells grown in culture medium conditioned by A427 cells in
582 the absence of PUFAs and treated with 1 μM HHE; HHE 5, cells grown in culture medium
583 conditioned by A427 cells in the absence of PUFAs and treated with 5 μM HHE; HNE 1, cells
584 grown in culture medium conditioned by A427 cells in the absence of PUFAs and treated with 1
585 μM HNE; HNE 5, cells grown in culture medium conditioned by A427 cells in the absence of
586 PUFAs and treated with 5 μM HNE (for treatment details see see Materials and Methods).
587
588 Figure 7. Aldehyde dehydrogenase 3A1 protein content in human lung cancer cells A427 and in
589 C2C12 murine myoblasts.
590 Lane 1, A427 cells; lane 2, C2C12 cells; C+, positive control.

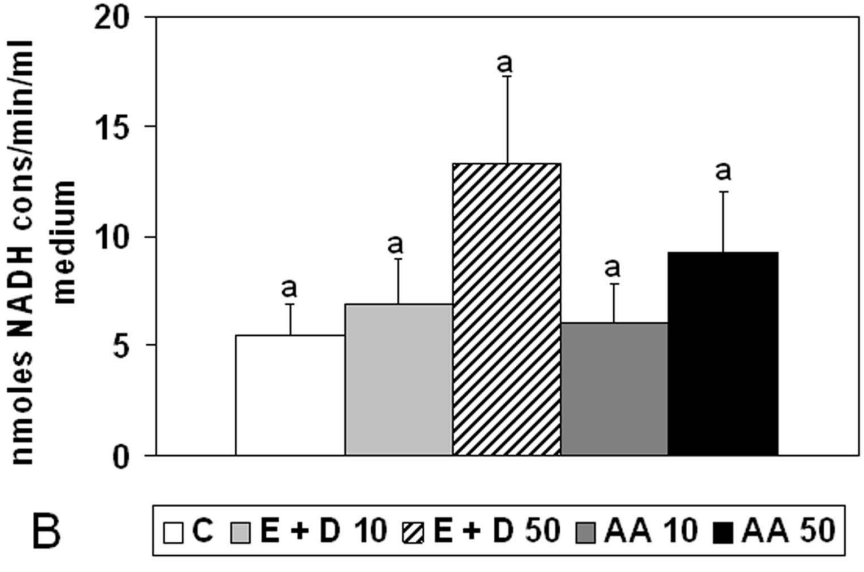
**TABLE 1** –PUTATIVE PPRE MOTIF IN GENES ENCODING FAST ISOFORMS OF MYOSIN HEAVY CHAIN

| **MyHC** | **Gene I.D.** | **PUTATIVE PPRE MOTIF** | | |
|---|---|---|---|---|
| | | **Promoter region** | **Exons / Introns** | |
| | | | | **bp** |
| IIa | 17882 | Not present | 1 DR1 (intron 3)<br>1 DR1 (exon 4)<br>1 DR1 (intron 16)<br>1 DR1 (exon 19) | 3543 -3555<br>4032 - 4044<br>11674 - 11686<br>14111 - 14123 |
| | | | | |
| IIb | 17884 | Not present | 1 DR1 (intron 1)<br>1 DR1 (intron 4)<br>1 DR1 (intron 15)<br>1 DR1 (exon 27) | 229 - 241<br>3890 - 3902<br>10814 - 10826<br>16059 - 16071 |
| | | | | |
| IId/x | 17879 | Not present | 1 DR1 (intron 5)<br>1 DR1 (intron 9)<br>2 DR1 (intron 15) | 2502 - 2514<br>5309 - 5321<br>7975 - 7987<br>8606 - 8618 |

MyHC, myosin heavy chain

Figure 1


60000
40000
20000
0
cells/cm 2
a
b
c
ab
b
A
C
E + D 10
E + D 50
AA 10
AA 50


20
15
10
5
0
nmoles NADH cons/min/ml medium
a
a
a
a
a
B
C
E + D 10
E + D 50
AA 10
AA 50

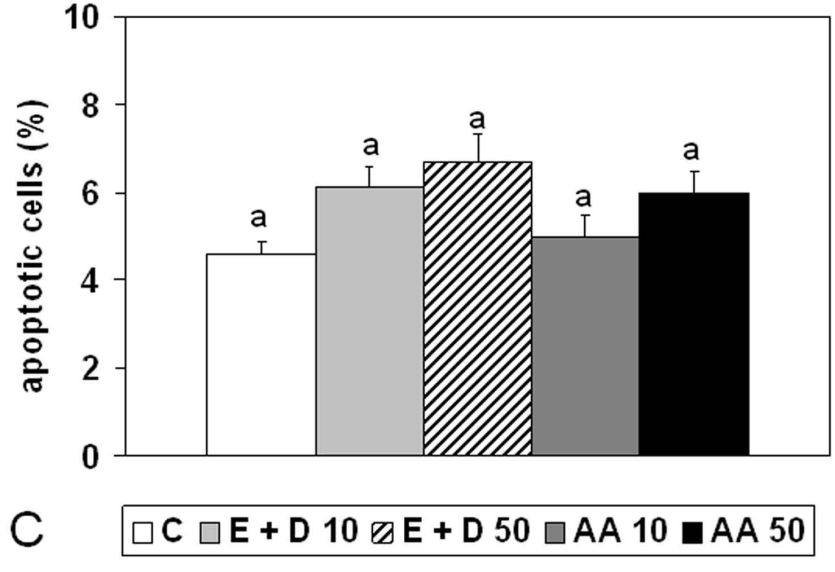

10
8
6
4
2
0
apoptotic cells (%)
a
a
a
a
a
C
E + D 10
E + D 50
AA 10
AA 50

Figure 2

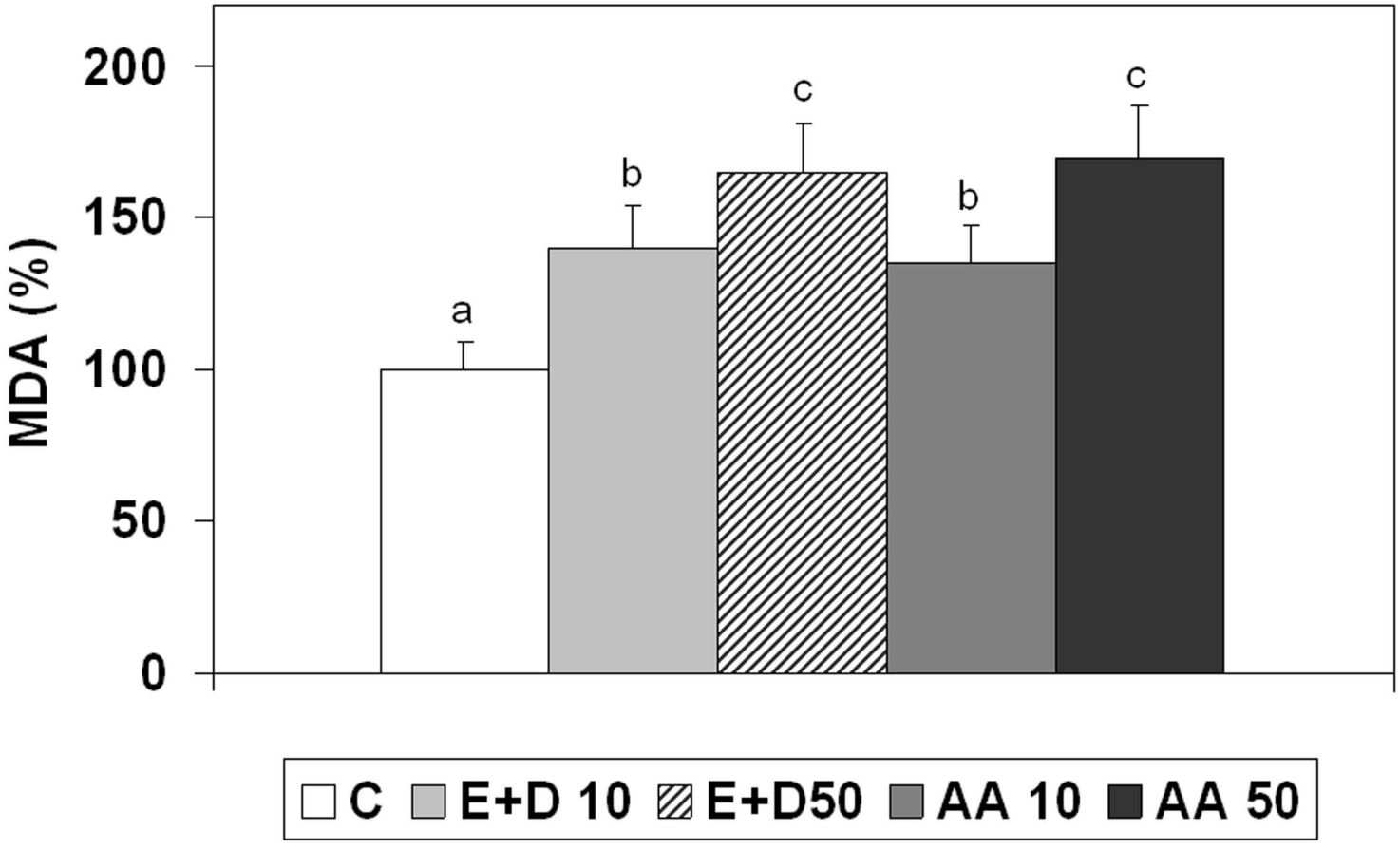


Figure 3

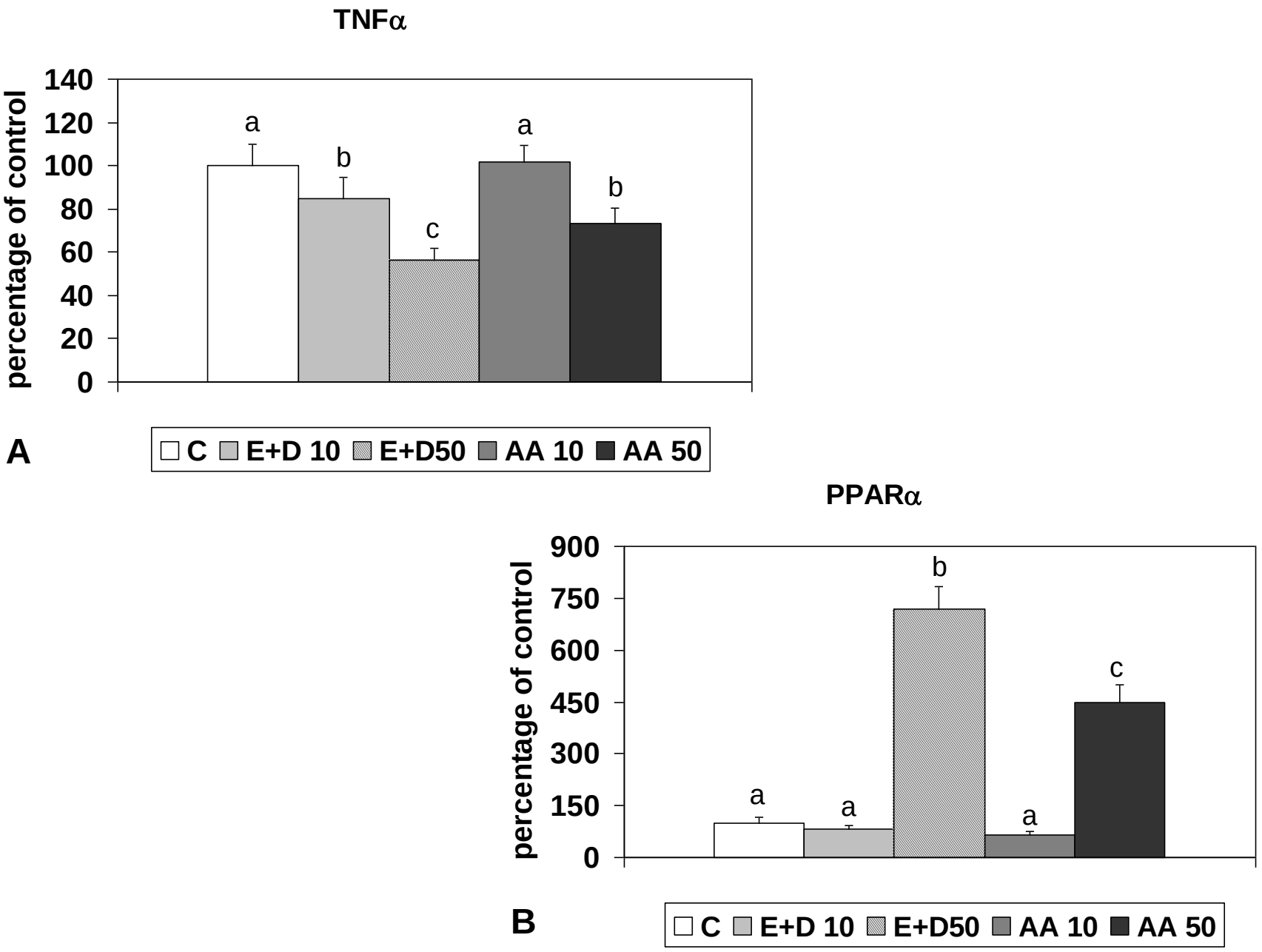

TNFα
percentage of control
140
120
100
80
60
40
20
0
a
b
c
a
b
A
C
E+D 10
E+D50
AA 10
AA 50
PPARα
percentage of control
900
750
600
450
300
150
0
a
a
b
a
c
B
C
E+D 10
E+D50
AA 10
AA 50

Figure 4

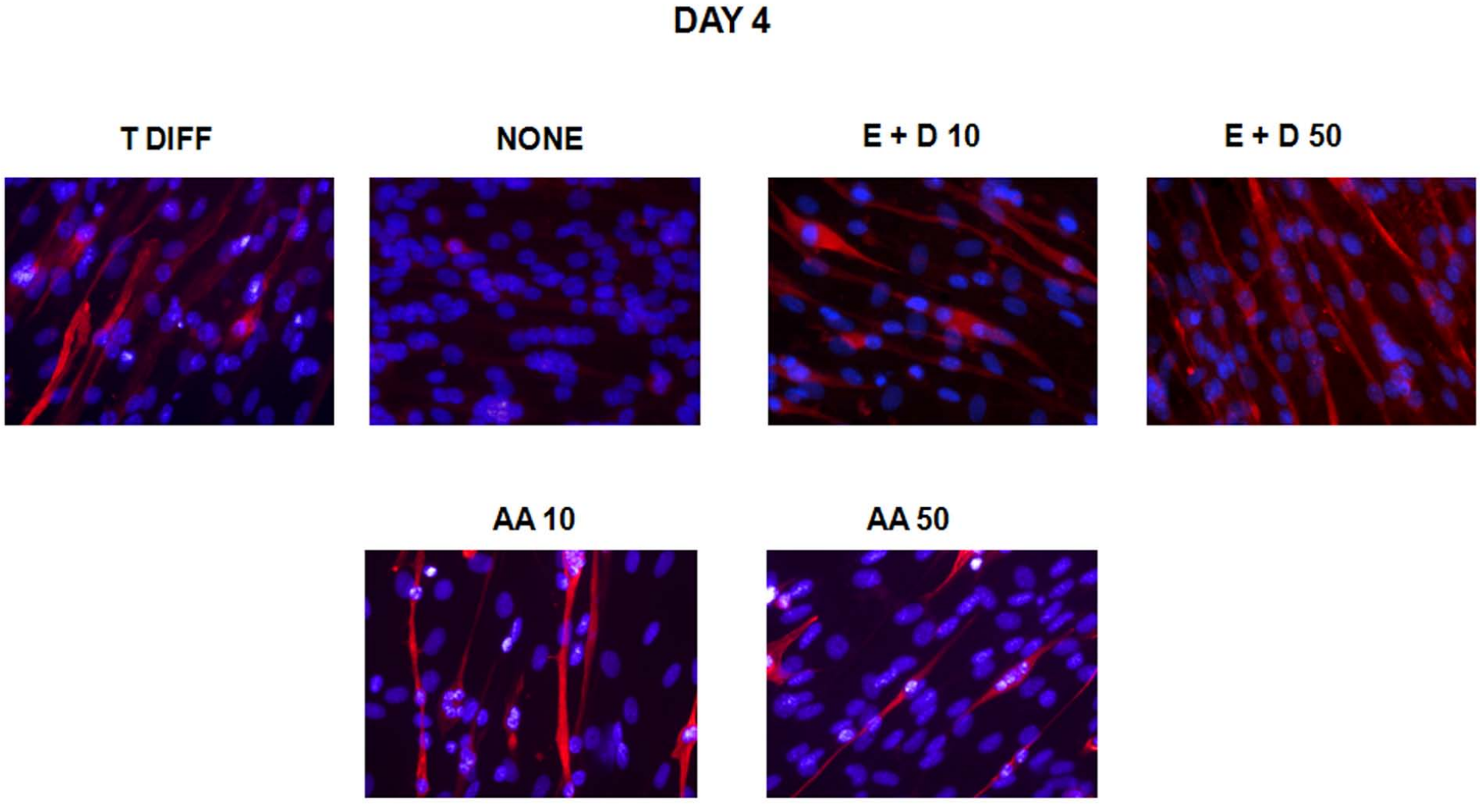


Figure 5

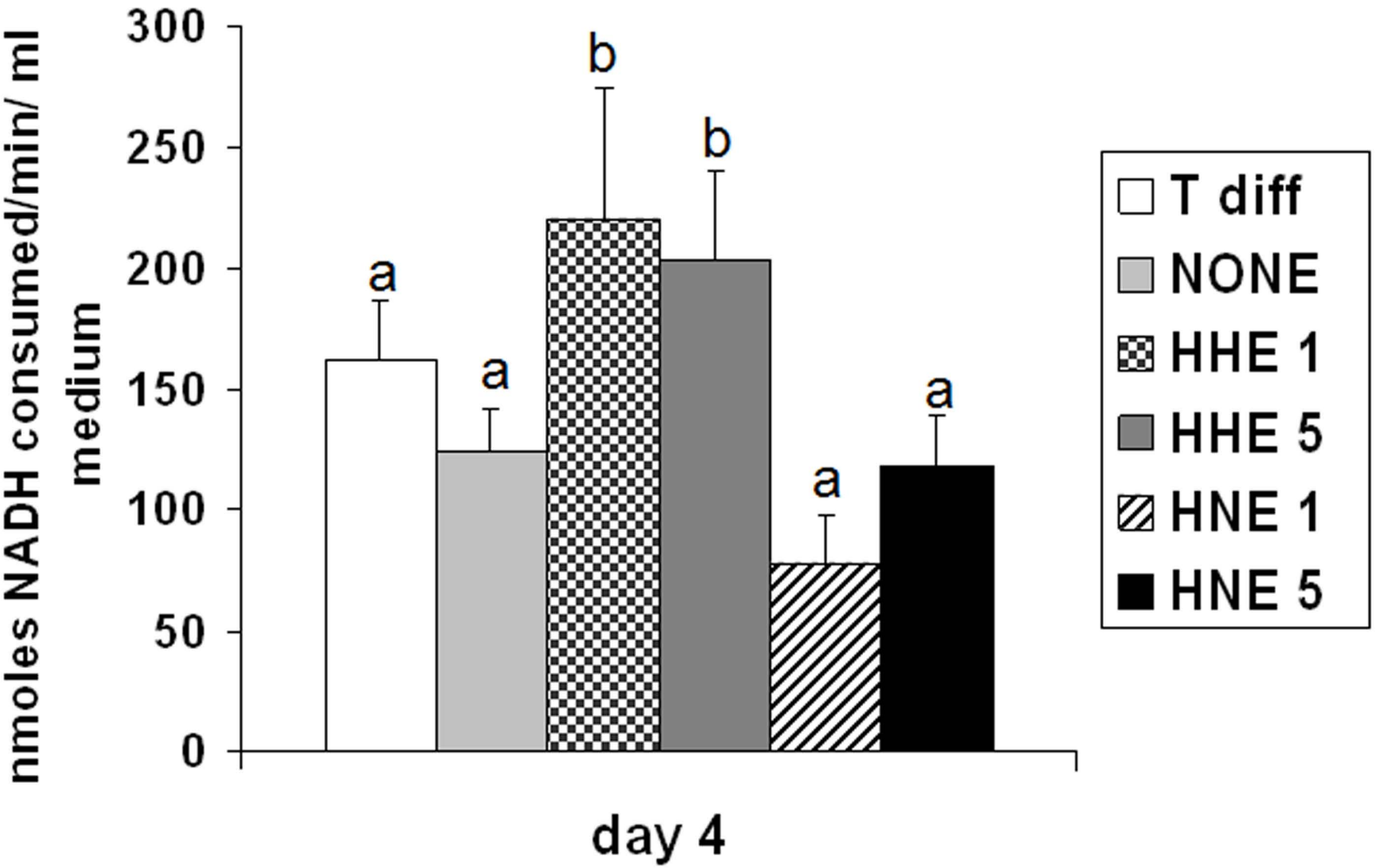

300
250
200
150
100
50
0
nmoles NADH consumed/min/ ml medium
a
a
b
b
a
a
day 4
T diff
NONE
HHE 1
HHE 5
HNE 1
HNE 5

Figure 6

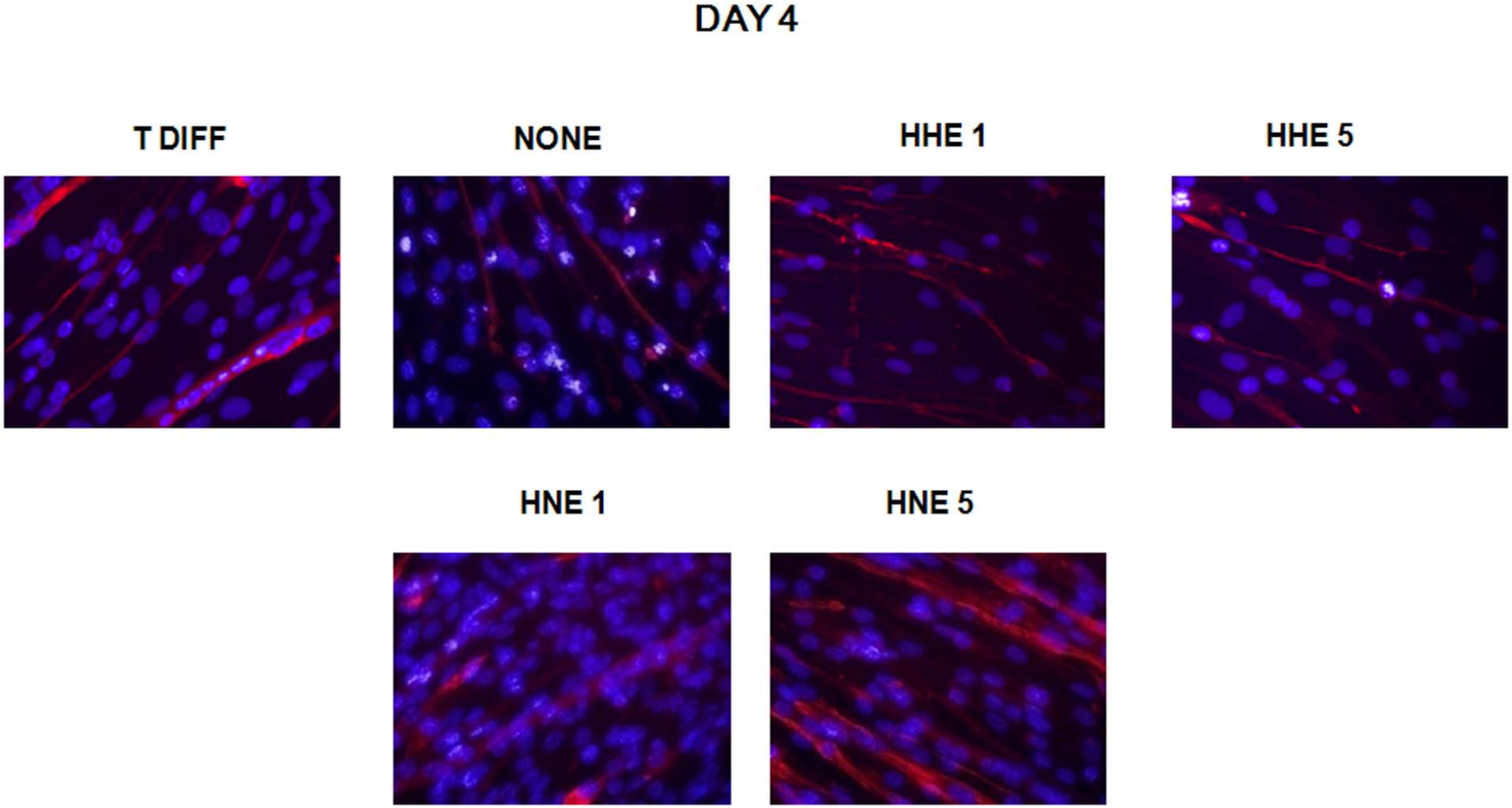


Figure 7