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The Ω-3 fatty acid docosahexaenoic acid selectively induces apoptosis in tumor-derived cells and suppress tumor growth in gastric cancer

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ABSTRACT

Despite current achievements and innovations in cancer treatment, conventional chemotherapy has several limitations, such as unsatisfactory long-term survival, cancer drug resistance and toxicity against non-tumoral cells. In the search for safer therapeutic alternatives, docosahexaenoic acid (DHA) has shown promising effects inhibiting tumor growth without significant side effects in several types of cancer, but in gastric cancer (GC) its effects have not been completely described. In this study, we characterized the effects of DHA in GC using *in vivo* and *in vitro* models. Among all of the evaluated Ω-3 and Ω-6 fatty acids, DHA showed the highest antiproliferative potency and selectivity against the GC-derived cell line AGS. 10–100 μM DHA decreased AGS cell viability in a concentration-dependent manner but had no effect on non-tumoral GES-1 cells. To evaluate if the effects of DHA were due to apoptosis induction, cells were stained with Annexin V-PI, observing that 75 and 100 μM DHA increased apoptosis in AGS, but not in GES-1 cells. Additionally, levels of several proapoptotic and antiapoptotic regulators were assessed by qPCR, western blot and activity assays, showing similar results. In order to evaluate DHA efficacy *in vivo,* xenografts in an immunodeficient mouse model (BALB/cNOD-SCID) were used. In these experiments, DHA treatment for six weeks consistently reduced subcutaneous tumor size, ascitic fluid volume and liver metastasis. In summary, we found that DHA has a selective antiproliferative effect on GC, being this effect driven by apoptosis induction. Our investigation provides promising features for DHA as potential therapeutic agent in GC.

1. Introduction

Gastric cancer (GC) constitutes one of the most relevant types of cancer, with more than 700,000 annual deaths, being the fourth most prevalent cancer worldwide (Ferlay et al., 2015; Ferro et al., 2014; Kamangar et al., 2006). Generally, this disease is asymptomatic and diagnosed on advanced or terminal stages, when metastatic foci are already formed (Subhash et al., 2015). Despite improvements in diagnosis and therapy, overall prognosis remains poor (Nienhuser and Schmidt, 2018), and the molecular mechanisms underlying GC development remain unclear (Li et al., 2014). Although surgery is the preferred treatment, its use is largely associated with significant risks (Alkhaffaf et al., 2017). On the other hand, conventional cytotoxic therapies lead to unsatisfactory long-term survival, mainly related to drug resistance development by tumor cells and toxicity towards normal cells (D'Eliseo and Velotti, 2016; Tang et al., 2017). Consequently, new treatments are urgently required for GC treatment and prevention.

Natural products with antitumor activity and mild side effects are promising candidates to be used as anticancer agents (Haque et al., 2017; Zhang et al., 2015). Moreover, studies have shown that some natural compounds are able to increase the efficacy of conventional chemotherapeutic drugs (Ebrahimifar et al., 2017). Within natural compounds that have been studied as potential anti-cancer drugs, omega-3 (Ω-3) polyunsaturated fatty acids (PUFAs) have received

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special attention for their varied array of beneficial properties (Vaughan et al., 2013). PUFAs are essential fatty acids for human health because mammals lack the desaturases necessary to synthesize alpha linolenic acid (ALA; 18:3), precursor of eicosapentanoic acid (EPA; 20:5) and docosahexaenoic acid (DHA, 22:6) (Ostermann et al., 2017). Consequently, these Ω-3 PUFAs must be acquired almost exclusively from diet (Kaur et al., 2014), being found mostly on cold-water fish as EPA and DHA (Berquin et al., 2008), or consumed as dietary supplements (Vaughan et al., 2013). Epidemiological studies have linked fish oil consumption with a decrease in cancer incidence, attributing these effects to its content of EPA and DHA. However, DHA is often regarded as the most effective anticancer Ω-3 PUFA due to its unique effect altering membrane composition (Siddiqui et al., 2011). DHA supplementation has an effective adjuvant action, and it has emerged as a promising chemosensitizer (Siddiqui et al., 2011). As an antineoplastic agent, several effects for DHA have been reported, like inhibition of tumor cell proliferation and viability, apoptosis induction and reactive oxygen species generation (D'Eliseo and Velotti, 2016; Zhang et al., 2015).

According to this, in the present study we investigated the effects of DHA in GC using both *in vitro* and *in vivo* models. We found that DHA can efficiently inhibit cell viability on a gastric adenocarcinoma-derived cell line (AGS) by apoptosis induction, and also reduce tumor growth in a immunodeficient mice model. Altogether, these results portray DHA as a promising agent to be used on GC.

2. Materials and methods

2.1. Animal care

All procedures were performed according to the policies set out by the International Council on Animal Care and were approved by the Research Ethics Committees from the Faculty of Medicine at Universidad Católica del Norte (CECFAMED-UCN) and Fundación Ciencia y Vida.

2.2. Reagents

Ω-3 PUFAs docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and alpha linolenic acid (ALA), and Ω-6 PUFA arachidonic acid (AA) ethyl esters were purchased from Nu-Check (Nu-Chek-Prep Inc., USA). Geltrex matrix (ThermoFisher, USA) was used as basement membrane matrix gel for *in vivo* experiments.

2.3. Cell culture

In vitro experiments were performed using human cell lines derived from gastric adenocarcinoma (AGS; ATCC® CRL-1739), non-tumoral gastric epithelia (GES-1; cells kindly donated by Dr. Dawit Kidane from the University of Texas at Austin, USA) and embryonic kidney (HEK-293; ATCC® CRL-1573). Dulbecco's Modified Eagle Medium (DMEM) was used for maintenance of GES-1 and HEK-293 cells, and Kaighns' Modified F-12 (F12K) medium was employed for AGS. For both cases, media were supplemented with 10% fetal bovine serum (Biological Industries, Israel), 100 IU/ml penicillin G and 100 μg/ml streptomycin sulfate (Sigma-Aldrich, USA). Cell lines were cultured in an incubator at 37 °C with humidified atmosphere containing of 5.0% CO_2, and they were routinely subcultured after 4 days, performing every experiment between the 8th and 30th passage for each cell line.

2.4. Cell functionality assays

In order to evaluate cell functionality, as an indicator of cell viability, in gastric cancer-derived and non-tumoral cell lines, MTT assays were performed in 96-well plates, seeding 5×10^3 cells per well, and incubating overnight to promote cell adhesion. After 24 h, medium was replaced with supplemented DMEM or F12K solutions containing DHA, EPA, AA or ALA at concentrations previously described for anti-proliferative assessments of these Ω-3 PUFAs on lung, ovarian, colorectal and glial carcinoma cell lines (Bai et al., 2019; Kim et al., 2018; Liu et al., 2018; Sam et al., 2018). After 24–48 h of incubation, an aliquot of 5 mg/ml 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich, USA) reagent was added to each well and plates were incubated at cell culture conditions for 1 h before absorbance measurement. After incubation, plates were shaken for 10 min and absorbance was measured at 490 nm using a NOVOstar® multiplate reader. Median inhibitory concentrations (IC_{50}) for DHA and EPA were calculated for every cell batch treated with these PUFAs that exhibited a concentration-dependent inhibition. Each functionality curve was adjusted to an exponential fit using GraphPad software, and final IC_{50} values were obtained calculating the mean ± S.E. of all the batches tested. Each assay was performed at least in triplicate.

2.5. Apoptosis assays

2.5.1. Cell membrane integrity

AGS and GES-1 cells were cultured in 6-well plates, seeding 2×10^4 cells per well. After an overnight incubation, each well was treated for 6 h with 0, 75 or 100 μM DHA, considering a condition with 50 μM cisplatin (CisP; Sigma, USA) as positive control for apoptosis. After treatment, cells were stained with Hoechst 33342, annexin V and propidium iodide (PI) (Invitrogen, USA) according to manufacturer instructions, and analyzed in a Zeiss LSM-800 laser scanning confocal microscope using a previously described protocol for image acquisition (Ramirez-Rivera et al., 2018).

2.5.2. Western blot

AGS and GES-1 cells were seeded into 60 mm cell culture plates at 70–80% confluence (4–5 × 10^5 cells/plate) and incubated for 24 h to promote cell adhesion. After that, each plate was treated for 6 h with a solution of supplemented culture medium plus 0, 100, 200 μM DHA or 50 μM CisP. After treatment, cells were harvested on ice using RIPA buffer, and total protein content was measured using the Pierce BCA Protein Assay kit (Thermo Scientific, USA). 30 μg of each protein extract was loaded on 10% SDS-polyacrylamide gels, which were transferred to PVDF membranes after electrophoresis. Membranes were blocked using 5% non-fat milk for 30 min, and then incubated overnight at 4 °C with one of the following primary antibodies: caspase-3 p17 (Santa Cruz Biotechnology, USA; sc-271028; 1:1000 dilution) or β-actin (Thermo Scientific, USA; PA-20536-1; 1:1000 dilution), being the later used as loading control. After primary antibody incubation, membranes were washed twice with TBS-T and incubated with a horseradish peroxidase (HRP)-conjugated anti-mouse IgG secondary antibody (Rockland Immunochemicals, USA; 610–1302; 1:5000 dilution) at room temperature for 1 h. Finally, membranes were washed twice with TBS-T at room temperature and treated with SuperSignal West Pico PLUS Chemiluminescent substrate solutions (Thermo Scientific, USA) for detection in a C-DiGit blot scanner.

2.5.3. Gene expression of proapoptotic and antiapoptotic regulators

To guarantee quality and reproducibility in our experiments, MIQE Guidelines were considered in every aspect of the following protocol (Bustin et al., 2009). In the same way as the cell cultures used for Western Blot, AGS and GES-1 cell lines were seeded on 60 mm culture plates in order to achieve a 70–80% confluence after a 24 h incubation (4–5 × 10^5 cells/plate). Then, cell were treated for 6 h with the following experimental conditions using supplemented culture medium as solvent: 0, 100, 200 μM DHA and 50 μM cisplatin. After treatment, mRNA was extracted using TRIzol® reagent (Invitrogen, USA) according to the manufacturer's protocols. Yield and purity ratios (260 nm/280 nm and 260 nm/230 nm) were established using a NanoDrop One® microvolume spectrophotometer (Thermo Scientific, USA). Reverse transcription was performed with an Affinity Script® qPCR cDNA

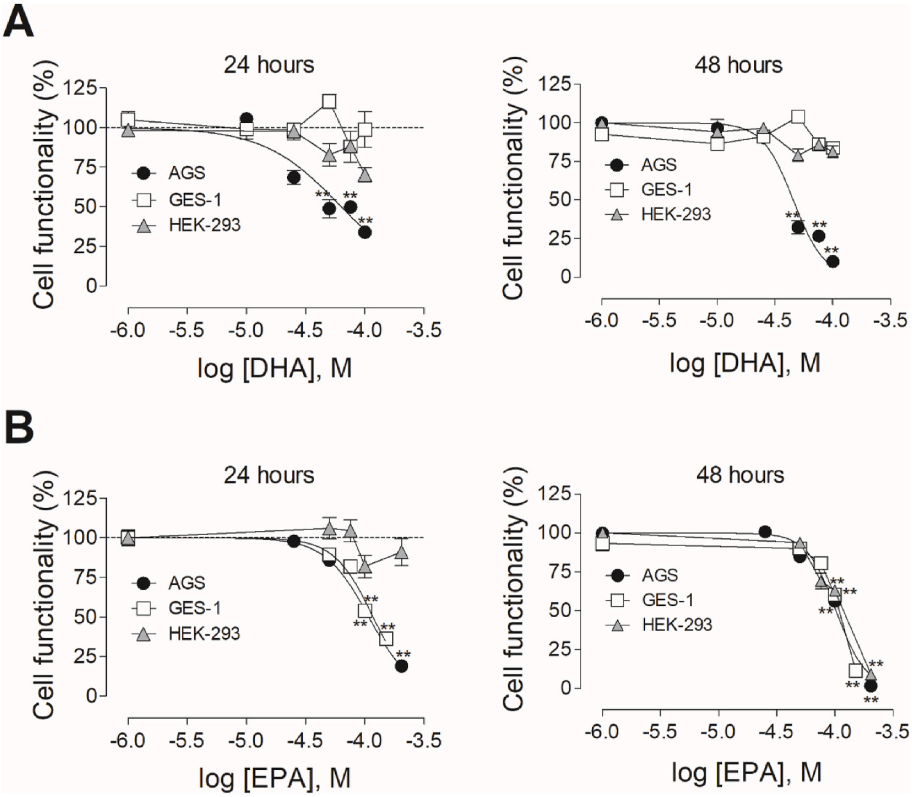

Fig. 1. DHA and EPA decreases AGS cell functionality. A and B. Ω-3 PUFAs DHA (**A**) or EPA (**B**) were incubated for 24 (left graphs) or 48 h (right graphs) in AGS (black circles), GES-1 (open squares) and HEK-293 cells (grey triangles). The estimated IC_{50}s for DHA in AGS cells (**A, closed circles**) were 63.2 ± 4.4 at 24 h (left graph) and 45.4 ± 3.9 μM at 48 h (right graph). In the case of EPA in AGS cells (**B, closed circles**), IC_{50}s were 104.2 ± 4.6 at 24 h (left graph) and 96.8 ± 2.9 μM at 48 h (right graph). EPA also inhibited GES-1 cell functionality (**B, open circles**), estimated IC_{50}s were 107.8 ± 2.9 μM and 105.5 ± 2.3 μM at 24 and 48 h, respectively. EPA also decreased HEK-293 cell functionality at 48 h with an IC_{50} of 107.8 ± 7.2 μM (B, grey triangles). Cell functionality was determined using MTT method, comparing the different treatments conditions to control (untreated) cells. For IC_{50} calculations, concentration-response curves were adjusted to an exponential function. **P < 0.01, Kruskal-Wallis and Dunn's tests, n = 11–25.

Synthesis Kit (Agilent, USA) using 0.5 μg of extracted mRNA, considering for every sample a no-RT control (-RT). For the qPCR reactions, Brilliant II SYBR® Green QPCR Master Mix (Agilent, USA) was used considering 50 ng of cDNA, 300 nM as final concentration for every pair of primers, and a no-template control (NTC) to each one of the genes studied. For each experimental condition, Bcl-2, Bcl-XL, Bax and Smac/DIABLO were established as objective genes, and B2M was used as referential gene. Primers for B2M were designed using Primer-BLAST (forward: 5′-AAG TGG GAT CGA GAC ATG TAA GCA-3′; reverse: 5′-GGA ATT CAT CCA ATC CAA ATG CGG C-3′), and for the objective genes we used primers previously designed by our group (Ramirez-Rivera et al., 2018). The following thermocycling protocol was applied: initial denaturation at 95 °C for 10 min, and 40 amplification cycles at 95 °C for 30s and 60 °C for 60s.

To determine relative gene expression, efficiency curves were obtained for each gene, choosing the most proper calculation method between the one proposed by Livak and Schimittgen or Pfaffl according to the differences in the amplification efficiency obtained for each one of the analyzed genes (Livak and Schmittgen, 2001; Pfaffl, 2001). All these experiments were done and analyzed using 0 μM DHA condition as basal expression control.

2.5.4. Caspase 3/7 activity assay

AGS and GES-1 cells were seeded into white opaque 96-well plates at a density of 5×10^3 cells/well and, after an overnight incubation for cell adhesion, plates were treated with 0, 50, 100, 200 μM DHA, or 50 μM CisP. Each condition was incubated for 6 or 24 h, and then treated with Caspase-Glo 3/7® reagent (Promega, USA) according to manufacturer's guidelines. After an incubation of 3 h, caspase 3/7 activity was determined as luminescent signal in a NovoStar® multiplate reader. Each assay was performed in triplicate, and caspase 3/7 activity was determined by a ratio of relative light units (RLU) readings obtained from the different experimental conditions and a basal cell proliferation control consisting in cells without treatment.

2.6. Preliminary DHA single dose toxicity assessments

In order to establish DHA acute toxicity *in vivo*, and prior to tumor growth inhibition experiments, 6-week non-tumoral male BALB/c mice were used to evaluate single dose DHA toxicity. Animals were injected intraperitoneally with 100 μl of a DHA solution prepared according to Trepanier et al. (2014). Doses contained 1.0 g/Kg DHA and 100 mg/ml bovine serum albumin (BSA). As control condition, animals were injected with 100 μl of a mixture of 100 mg/ml BSA and saline. After injection, animals were observed for 15 days, and their main features were described according to the parameters proposed by Morton and Griffiths (1985). In parallel to animal observation, blood samples were collected to evaluate hematologic and biochemical profiles using a LaserCyte Dx analyzer. Animals were euthanized after toxicological evaluation.

2.7. In vivo tumor growth inhibition

Xenotransplantation was performed in BALB/c NOD mice with severe combined immunodeficiency (SCID), in which the development of T and B lymphocytes is suppressed, but their innate immunity is preserved, allowing them to be used as a model to assess cancer cell lines tumorigenic potential *in vivo*. For this purpose, we applied an ectopic injection of AGS/Geltrex into subcutaneous space at the right flank region of female and male BALB/c NOD/SCID mice (weight = 20 ± 3 g) from Jackson Laboratories. A total of 18 mice were used and randomly divided into three groups. All mice were injected subcutaneously with 5×10^6 AGS cells (1:1 Geltrex-gel in 100 μl) to induce gastric tumorigenesis. Parallelly, and prior to the treatment period beginning, animals were treated with a 0.1 g/ml DHA solution in BSA prepared according to Trepanier et al. (2014). Two groups of mice were treated with DHA at a dosage of 1.0 g/Kg for 4 or 6 weeks (named as T4 and T6 groups, respectively), and a third group served as control without DHA treatment. DHA dosage was selected according to previous *in vivo* investigations using breast and prostate tumor models, and had proven to

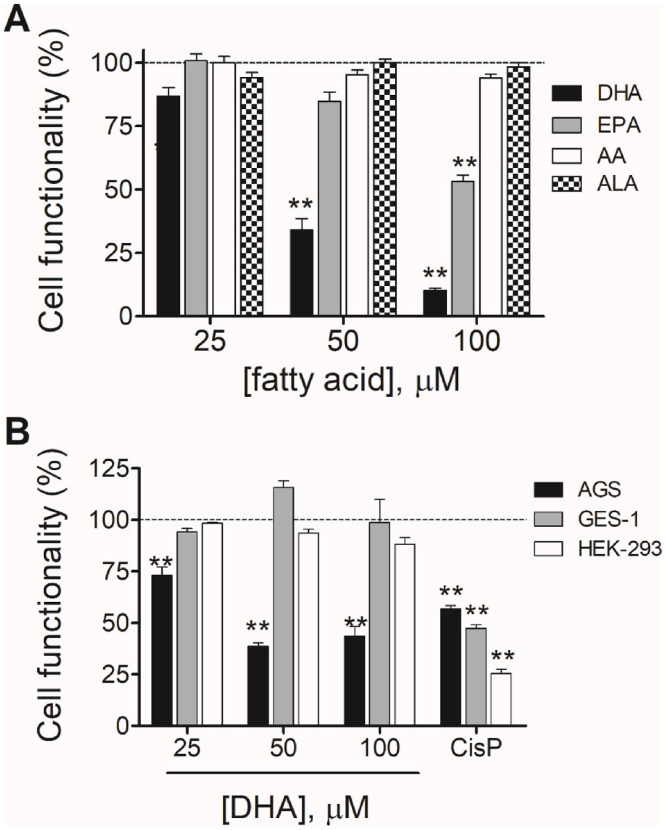

Fig. 2. DHA is a potent and selective inhibitor of AGS cell functionality. A. Effects of the fatty acids DHA (black bars), EPA (grey bars), arachidonic acid (AA, open bars) and alpha linoleic acid (ALA, striped bars) on AGS cell functionality after a 48 h incubation. **B.** Effects of a 48 h treatment with 25, 50 and 100 μM DHA, or 50 μM cisplatin (CisP) on AGS (black bars), GES-1 (grey bars) or HEK-293 (open bars) cell proliferation. **P < 0.01, as compared to basal functionality in untreated cells (represented by the dotted line). Kruskal-Wallis and Dunn's tests, n = 8–25.

be safe both for humans and mice (Calviello et al., 1999; Fasano et al., 2017; Kuriki et al., 2003; Tomaszewski et al., 2020), without effects in other cell parameters such as membrane fatty acid content (Abbott et al., 2012) All the animals were euthanized at the sixth week post AGS/-Geltrex inoculation and tumors were collected, sized and weighed, and analyzed for signs of spontaneous metastasis. Tumor growth was monitored by palpation, and its length, width and height was measured using a digital caliper. Tumor volume (V) was calculated using the following equation, where w = width, l = length, and h = height:

$$V = \frac{\pi}{6} * l * w * h$$

2.8. Spontaneous metastasis evaluation

In order to confirm metastatic nodules formation on euthanized mice, tissue analysis was performed on every necropsy. Formation of macro-metastatic nodules were identified as changes on normal tissue structure, signs of parenchymal damage, and distinctive coloration changes in the analyzed organs.

2.9. Statistical analysis

To evaluate cell viability, Kruskal-Wallis nonparametric test and Dunn post-test were used. All *in vitro* experiments were repeated at least three times, using their respective mean as final result. For *in vivo* evaluations, comparisons were made between each DHA-treated mice group and their respective control condition using an unpaired Student's t-test. GraphPad Prism Software v5.0 was used for data analysis.

3. Results

3.1. DHA and EPA decreases cell viability in GC-derived cells

In order to analyze the effects DHA and EPA, we performed concentration-response experiments on AGS (human gastric adenocarcinoma), GES-1 (human non-tumoral gastric mucosa) and HEK-293 (human embryonic kidney) cell lines using the MTT assay. According to these results, DHA was the most effective and selective Ω-3 PUFA, inhibiting AGS cell functionality in a concentration-dependent manner in the range of 1–100 μM DHA, at both 24 and 48 h of incubation (Fig. 1A). On the other hand, no significant effects of DHA were observed on GES-1 and HEK-293 cells functionality at both 24 and 48 h of incubation (Fig. 1A). The estimated IC_{50} for DHA were 63.2 ± 4.4 at 24 h, and 45.4 ± 3.9 μM at 48 h of incubation. On the other hand, EPA showed less potency and selectivity (Fig. 1B) than DHA for the same cell lines. IC_{50} for AGS cells treated with EPA were similar at 24 and 48 h incubation (104.2 ± 4.6 and 96.8 ± 2.9 μM, respectively), but EPA also inhibited GES-1 cell functionality at 24 and 48 h (IC_{50} 24 h = 107.8 ± 2.9 μM and IC_{50} 48 h = 105.5 ± 2.3 μM, Fig. 1B), and also inhibited HEK-293 cell functionality at 48 h of application (IC_{50} = 107.8 ± 7.2 μM, Fig. 1B), therefore exhibiting less selectivity for cancer-derived cells than DHA. Next, we assessed AGS cell functionality in order to observe if the effects of DHA and EPA were specific, or they can be achieved using Ω-3 and Ω-6 PUFAs metabolic precursors like alpha linolenic acid (ALA) and arachidonic acid (AA), respectively. After a 48 h incubation, we found that neither ALA nor AA had any effect on AGS cell functionality (Fig. 2A). Since our results showed that DHA was the most potent Ω-3 PUFA inhibiting AGS cell functionality, we tested its selectivity and compared it against cisplatin (CisP), a conventional antineoplastic agent. After a 48 h incubation, DHA proved to have an important selectivity, with notorious effects on the GC-derived cell line AGS without affecting the non-tumoral cell lines GES-1 and HEK-293. Effects of CisP were non selective, inhibiting cell functionality in all the evaluated cell lines (Fig. 2B).

3.2. DHA inhibits AGS cells functionality by apoptosis induction

After we determined that DHA is a potent and selective agent for AGS cells, we evaluated if these effects were due to apoptosis induction. As a first approach, we used Annexin V-PI staining in AGS and GES-1 cells after a 6 h treatment with different DHA concentrations. We observed an important increase of apoptotic cells in AGS treated with 75 and 100 μM DHA, but no changes on GES-1 cells under the same conditions (Fig. 3B and C and 3F-G, respectively). In contrast, cisplatin induced apoptosis in both cell lines (Fig. 3D and H). Next, we evaluated changes in protein levels of procaspase 3, zymogen of caspase 3, an essential protein in the execution phase of apoptosis. When apoptosis is induced, procaspase 3 levels diminish because of its cleavage and subsequent dimerization of its small and large subunits, conforming the active enzyme caspase 3. To evaluate this, we incubated AGS and GES-1 cells with 100 and 200 μM DHA, or 50 μM cisplatin for 6 h, and evaluated procaspase 3 protein levels by Western blot. The 200 μM DHA condition was included in the experiments of Figs. 4 and 5 in order to compare our results to a previous study that measured the effects of DHA in the activity apoptosis mediators in a breast cancer model (Xue et al., 2017). In AGS cells, we observed a decrease in procaspase 3 protein levels using 100 and 200 μM DHA. This effects were not observed in GES-1, where DHA treatment had no effect on procaspase 3 protein levels. In contrast, cisplatin decreased procaspase 3 protein levels in both cell lines (Fig. 4A and B). Finally, after observing a decrease on procaspase 3 protein levels, we performed a caspase 3/7 activity assay in order to prove apoptosis induction via executioner caspases. For these experiments, we found

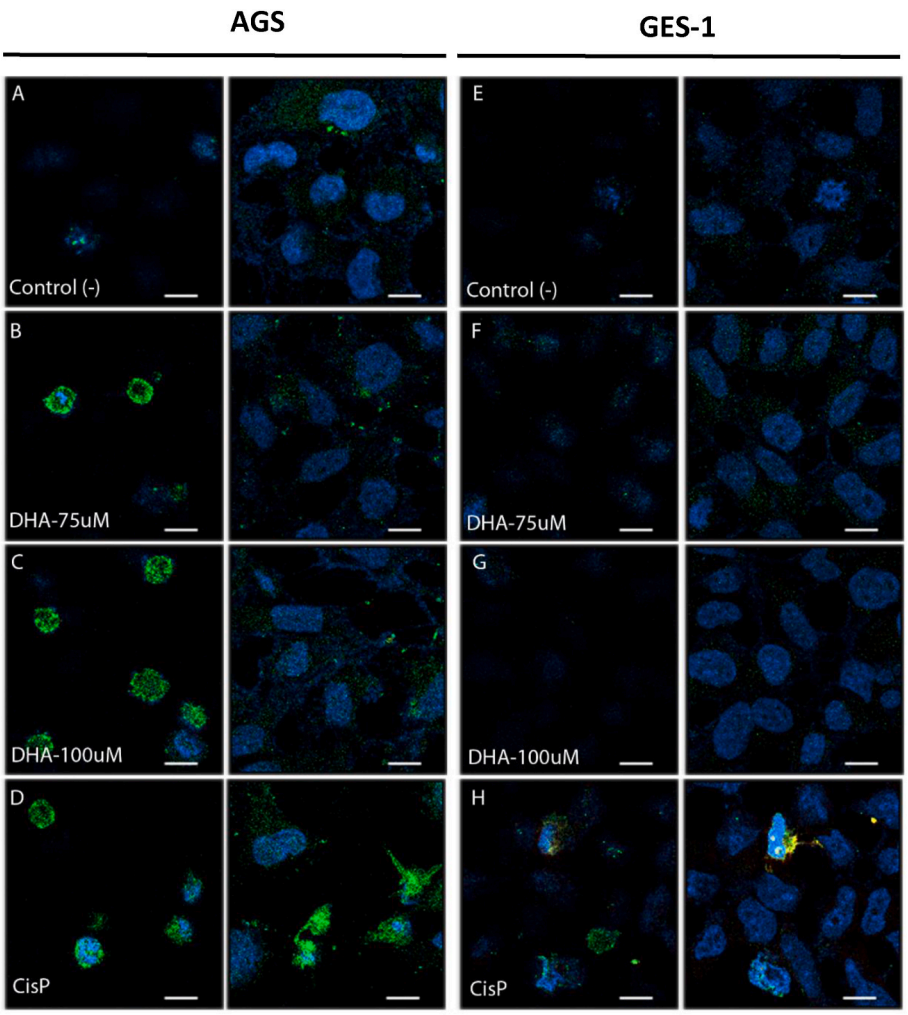

Fig. 3. DHA induces apoptosis in AGS cells. Cells were fixed and stained with Hoechst 33342 (blue, nucleus), annexin V (green, apoptotic cells) and PI (red, necrotic cells) in AGS (**A to D**) and GES-1 (**E to H**) dells. Representative images of control (**A and E**) cells, or treated with 75 (**B and F**) or 100 μM DHA (**C and G**). Note that annexin V staining is evident in AGS but not in GES-1 cells. **D and H.** Representative images of cells treated with 50 μM cisplatin (CisP), note that in both cell lines annexin V staining is observed. Images are representative of at least three separate experiments.

caspase 3/7 activity patterns consequent with the ones observed for procaspase 3 protein levels: a significant DHA-induced increase of caspase activity in AGS, minimal caspase 3/7 activity on DHA-treated GES-1 cells, and increased caspase activity in both cell lines after treatment with cisplatin (Fig. 4C and D). Next, we further investigated the effects of DHA on apoptosis, evaluating changes in mRNA levels of antiapoptotic (Bcl-2 and Bcl-XL) and proapoptotic (Bax and Smac/-DIABLO) mediators in order to analyze them from the perspective of mitochondrial integrity. To evaluate this, we incubated AGS and GES-1 cells with DHA, or 50 μM cisplatin for 6 h, and evaluated Bcl-2, Bcl-XL, Bax, Smac/DIABLO mRNA levels by qPCR, using B2M as reference gene and $2^{-\Delta\Delta Cq}$ as quantification method. In AGS cells, we observed a decrease on Bcl-2 and Bcl-XL mRNA levels, and an increase on Bax and Smac/DIABLO, being this effect similar between both 100 and 200 μM DHA and 50 μM cisplatin (Fig. 5A). However, DHA treatment on GES-1 cells did not changed Bcl-2, Bcl-XL and Bax mRNA levels, and showed a mild reduction of Smac/DIABLO levels (Fig. 5B). However, cisplatin was able to induce changes in the proapoptotic/antiapoptotic balance in GES-1 cells, indicating again a lack of selectivity of this compound (Fig. 5B).

3.3. DHA does not induce side effects on mice

Before *in vivo* tumorigenesis experiments, we evaluated potential secondary effects of DHA administration on BALB/c mice. Single intraperitoneal injection of DHA (1.0 g/Kg) did not induced signs of acute toxicity after 15 evaluation days (n = 4). An observation battery taken from Morton and Griffiths' Guidelines (Morton and Griffiths, 1985) was applied, and we did not observe any changes in the following parameters: general appearance (including opaque or closing eyes), decreased food/water intake, dehydration, weight loss, hypoactivity, restlessness or abnormal aggression, vocalizations or respiratory distress, cranial deformity or neurological damage signs, coarse or shaggy hair, stooped posture lordosis or kyphosis, skin pathologies, mobility restrictions, changes in stool/urine, and ocular and nasal discharges. In addition, we obtained blood samples from the DHA-injected mice and performed hematological (Table 1) and biochemical (Table 2) profiles. In general, we did not observed any substantial alteration in hematological profiles after DHA administration, with the exception of a diminished platelet count in every experimental group (Table 1), including control condition. This situation is probably due to technical limitations associated to the automated analyzers used for this measurement. These devices normally underestimate platelet counts in mice due to both the small size of mice platelets and their propensity to aggregate. This underestimation was partially corrected by DHA treatment, which ameliorates part of the underestimation seen in control group due to its well-known effect as platelet aggregation inhibitor (O'Connell et al., 2015). We also observed a decrease in lymphocytes percentage, and an increase in monocytes and eosinophils percentages in both control and DHA-treated groups. These changes could be explained as typical hematological responses to the stress conditions associated with animal manipulation, because DHA treatment group showed similar values to those observed for the control group, proving that this alterations are not related with DHA effects. According to the biochemical profile, most of the evaluated

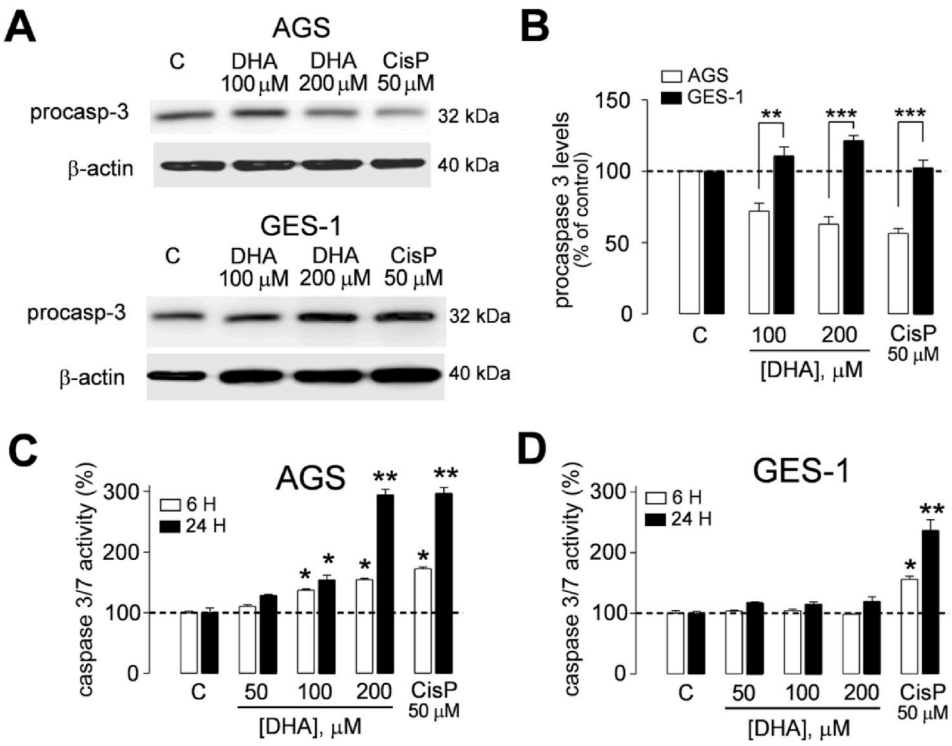

Fig. 4. DHA induces caspase 3 and 7 activation in AGS cells. A. Representative blots showing procaspase 3 and β-actin protein levels in the absence or presence of 100 and 200 μM DHA or 50 μM cisplatin (CisP) in AGS (upper blot) and GES-1 (lower blot) cells. **B.** Summary of the experiments showing changes in procaspase 3 protein levels after DHA or CisP treatment. **P < 0.01, ***P < 0.001, unpaired t-test performed comparing each treatment condition with its respective untreated control. n = 5. **C and D.** Summary of caspase 3 and 7 activity experiments in the absence or presence of 50, 100 and 200 μM DHA, or 50 μM CisP in AGS (**C**) and GES-1 (**D**) cells. *P < 0.05; **P < 0.01, One-way ANNOVA and Dunnett's tests, n = 3.

parameters stayed at physiological ranges after DHA treatment, except for calcium and serum albumin levels, which were below their referential values in DHA-treated animals (Table 2). Serum calcium reduction can be explained by the decrease of parathyroid hormone levels that occur after DHA administration (Plas et al., 2019). On the other hand, lower levels of serum albumin in DHA-injected animals compared to control animals can be explained by the additional albumin present in the vehicle injected to control animals that, in the case of DHA-treated animals, is complexed with DHA and therefore it enters to cell's cytoplasm.

3.4. DHA reduces tumor growth and suppress metastasis in xenografted mice

In a final set of experiments, we tested if DHA treatment was able to suppress tumor growth and metastasis in BALB/c NOD/SCID mice, which were subcutaneously injected with AGS cells in order to induce ectopic tumor formation. Then, animals were routinely treated by intraperitoneal administration every Monday, Wednesday and Friday with either vehicle (control group) or with 1.0 g/Kg DHA during four (T4 group) or six (T6 group) weeks. This DHA concentration was chosen considering that: i) intraperitoneal and oral bioavailabilities are similar for this compound (Turner et al., 2011); ii) absorption rate of DHA as ethyl ester is around 21% (Lawson and Hughes, 1988), making necessary the use of lipid additives like alpha-tocopherol, triacylglycerol or synthetic phospholipids in order to improve DHA absorption (Hachem et al., 2020; Libinaki and Gavin, 2017). In all cases, tumor growth was monitored until day 52, when animals were euthanized (Fig. 6A). During these experiments, we continuously monitored tumor growth using a digital caliper. With this data, we generated tumor growth curves for each animal from the three experimental groups (Fig. 6B). In control group (black circles), we observed a sustained tumor growth in all the animals tested. In T4 group (Fig. 6B, grey circles), we also observed a sustained tumor growth, but in a smaller degree as compared to control group. Also, tumors in T4 group started to grow at day 35, later than control group. In T6 group (Fig. 6B, open circles), tumor growth was significantly reduced comparing with the other groups, and one specimen barely developed a 10 g tumor (not shown). In order to compare these results between experimental and control groups, we used mean tumor volumes from the last 10 and 15 experiment days. This time range was established to eliminate the variation of the first days of treatment, when tumor growth did not follow a sustained pattern. Fig. 5C shows that DHA treatment for 6 weeks (T6 group) significantly reduced tumor volume in the last 10 and 15 days of experiment in comparison with control group. For T4 group we found a significant reduction only at the last 15 days of experiment (Fig. 6C). In both analyses (last 15 and 10 days), mean body weight of mice did not change in any of the experimental groups, indicating that DHA treatment induced specific effects on tumor growth (Fig. 6D).

As mentioned before, animals were euthanized after day 52 (see Fig. 6A) and tumor characteristics and possible metastatic nodules were analyzed. Fig. 7 shows features of tumor development and metastasis after AGS cells xenograft in all three experimental groups. All of the xenografted mice developed tumors: a compact, highly demarked mass was formed, especially in T6 group (Fig. 7A). In addition, we observed a significant difference between tumor sizes, as can be deducted from representative images (Fig. 7A), being the tumors from the T6 group evidently smaller as compared to other groups. Next, we analyzed the volume of ascitic fluid present in the peritoneal cavity. In control group, 3 of 5 animals presented a significant volume of ascitic fluid, whereas no increase was observed in T4 or T6 groups (Fig. 7B). Consonant with this, the same 3 animals from control group that presented an increased ascitic fluid volume also showed an increased number of malignant cells (Fig. 7B). On the contrary, no malignant cells were found in T4 and T6 groups (Fig. 7B). Then, we analyzed in every experimental group the occurrence of spontaneous liver metastasis. In control condition, 3 of 5 animals developed metastasis having several tumor-like formations in their livers (Fig. 7C). Liver metastasis on DHA-treated conditions were noticeable on 1 of 6 mice in the T4 group, but none of the animals from T6 group (Fig. 7C). According with these results, we observed significant differences in liver masses from the control group as compared to T4 and T6 (Fig. 7D).

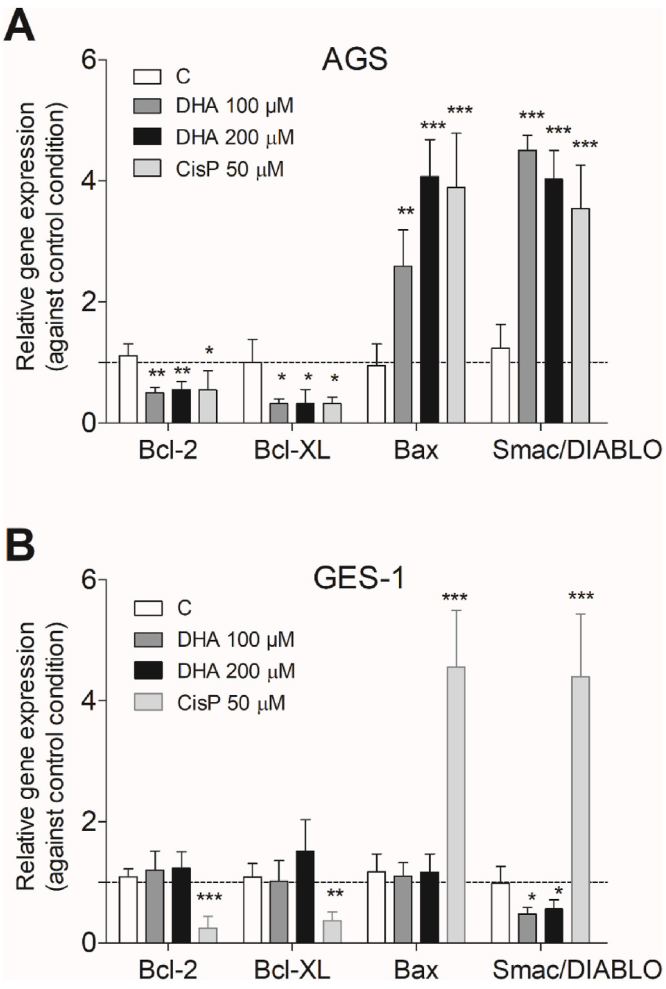

Fig. 5. Changes in mRNA levels of antiapoptotic and proapoptotic mediators. A and **B.** Relative gene expression of Bcl-2, Bcl-XL, Bax and Smac/DIABLO in AGS (**A**) and GES-1 (B) cells in control conditions (open bars), or treated by 6 h with 100 μM DHA (grey bars), 200 μM DHA (black bars), or 50 μM CisP (light grey bars). **P < 0.01; ***P < 0.001, Student's t-test, n = 4.

Table 1
Hematological profile for control and DHA-treated BALB/c mice.

Parameter	Range	Control	DHA injected
RBC (K/μl)	5.6–12.5	5.6 ± 0.6	6.1 ± 0.3
HTC (%)	29.0–43.0	27.5 ± 3.1	29.9 ± 1.3
HGB (mg/dl)	9.3–13.7	8.7 ± 1.0	10.4 ± 0.8
MCV (fl)	31.0–61.0	49.3 ± 0.2	49.0 ± 0.1
MHC (pg)	14.5–16.9	15.8 ± 0.2	15.9 ± 0.3
MCHC (g/dl)	30.5–32.9	32.2 ± 0.1	31.50 ± 0.3
PLT (K/μl)	900-1600	10.5 ± 2.2	410.0 ± 20.2
WBC (K/μl)	2.0–10.0	1.6 ± 0.2	2.3 ± 0.3
Neutrophils (%)	20.0–30.0	19.2 ± 3.1	25.6 ± 6.1
Lymphocytes (%)	70.0–80.0	55.0 ± 4.7	42.4 ± 5.2
Monocytes (%)	0.0–2.0	18.3 ± 7.4	19.7 ± 8.4
Eosinophils (%)	0.0–7.0	6.6 ± 2.9	5.1 ± 1.6
Basophils (%)	0.0–1.0	0.5 ± 0.2	0.9 ± 0.1

Analyzed parameters in the hematological profile were: hematocrit (HTC), red blood cells count (RBC), mean cell volume (MCV), hemoglobin (HGB), mean cell hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelets (PLT), white blood cells (WBC) and neutrophils, lymphocytes, monocytes, eosinophils and basophils percentages. Reference values were taken from (O'Connell et al., 2015; Restell et al., 2014; Zaias et al., 2009).

Table 2
Biochemical profile for control and DHA-treated mice.

Parameter	Range	Control	DHA injected
Glucose (mg/dL)	92–232	134.7 ± 8.9	111.0 ± 10.1
BUN (mg/dL)	11–17	20.0 ± 2.1	15.0 ± 0.6
Phosphorus (mg/dL)	7.8–14.7	8.5 ± 0.6	6.5 ± 3.2
Calcium (mg/dL)	5.9–12.5	5.2 ± 0.9	2.3 ± 1.2
Total proteins (g/dl)	4.8–8.7	3.4 ± 0.6	2.9 ± 0.7
Serum albumin (g/dl)	1.2–2.7	1.1 ± 0.1	0.5 ± 0.3
Globulins (g/dl)	1.3–2.5	2.0 ± 0.6	2.4 ± 0.5
Amylase (U/l)	420–2300	922 ± 90	444 ± 114

Analyzed parameters in the biochemical profile were: glucose, blood urea nitrogen (BUN), phosphorus, calcium, total protein, serum albumin, globulins and amylase. Reference values were taken from (Stechman et al., 2010; Zaias et al., 2009) and Charles Rivers Laboratories (https://animalab.eu/sites/all/pliki/produkty-dopobrania/balb_c_Mouse_clinical_pathology_data.pdf).

4. Discussion

Gastric cancer (GC) is a relevant disease, being the fourth most frequent cancer type, and the second cause of cancer-induced deaths worldwide. However, and despite all the advances achieved, it remains as a complex disease to diagnose and treat (Dai et al., 2013). Conventional cytotoxic therapies often lead to unsatisfactory long-term survival, mainly related to drug resistance development by tumor cells, and toxicity towards normal cells (D'Eliseo and Velotti, 2016). In this context, Ω-3 PUFAs, and particularly DHA, have gained attention of the scientific community due to their numerous beneficial effects on human health. Many clinical and epidemiological studies have shown positive roles for Ω-3 long-chain fatty acids in infant development, cancer, cardiovascular diseases, and, more recently, in various mental illnesses (Riediger et al., 2009). Ω-3 long-chain fatty acids and their mediators can inhibit inflammation, angiogenesis and cancer via multiple mechanisms, including reduced release of arachidonic acid from cell membranes, inhibition of enzymatic activities, and direct competition with arachidonic acid for enzymatic conversions (Gu et al., 2015). Among Ω-3 long-chain fatty acids, docosahexaenoic acid (DHA) has received particular attention for its anti-inflammatory, antiproliferative, proapoptotic, antiangiogenetic, anti-invasion, and antimetastatic properties (Merendino et al., 2013). Specifically in the case of cancer, several studies have demonstrated the role of DHA in prevention and treatment of various types of cancer (Park et al., 2013), including liver (Sun et al., 2013), colon (Yang et al., 2013), bladder (Parada et al., 2013), breast (Xue et al., 2014), and lung cancer (Yao et al., 2014). In gastric cancer there is also evidence about the beneficial effects of DHA (Sheng et al., 2014). In our study, we evaluated in more detail the effects of DHA on GC-derived cell lines, as well as its effects *in vivo* on xenografted mice.

In vitro cell functionality, apoptosis, procaspase 3 protein levels and caspase 3/7 activity assessments were done on AGS and GES-1 cells in order to evaluate DHA effects on GC. MTT assays were used to obtain IC_{50}, in order to describe potency and selectivity of the antiproliferative effects, and optimal incubation times of DHA and EPA. These studies revealed that DHA has a more potent antiproliferative effect than EPA and, more importantly, these effects appear to be more specific towards GC-derived cells instead of their non-tumoral counterparts. On the other hand, precursor molecules, such as arachidonic acid (AA) and alpha linoleic acid (ALA) did not show any effect on the afore mentioned gastric cell lines. These effects can be reflected by the IC_{50} values observed for AGS cells treated with AA and ALA (IC_{50} > 100 μM), EPA (IC_{50} ~ 100 μM) and DHA (IC_{50} = 45–60 μM) for 24 and 48 h, showing that only EPA and DHA are capable to reduce GC-derived cell functionality. For the case of non-tumoral cell lines (GES-1 and HEK-293 cells), DHA treatment showed no effect on them, but EPA showed antiproliferative effects, in some cases, with similar affinities to those observed on AGS cells. The lack of effects of 1–100 μM DHA treatment observed on non-tumoral cells allows the potential use of this molecule

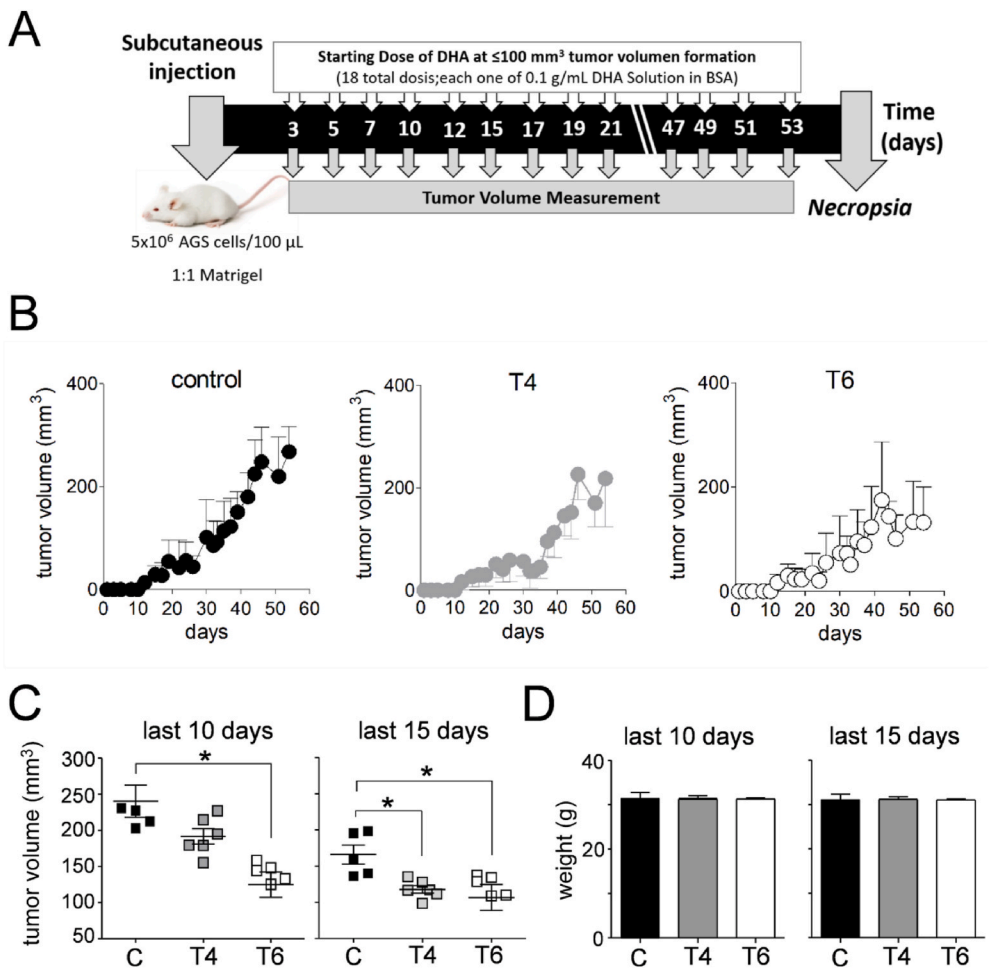

Fig. 6. Tumor growth in control and DHA-treated animals. A. Diagram showing the temporal course for the protocols used for tumor induction and DHA treatment in BALB/c NOD/SCID mice. **B.** Tumor growth curves from control (black circles, n = 5), T4 (grey circles, n = 6) and T6 (open circles, n = 6) groups. Tumor volume was measured three days a week using a digital caliper. **C.** Summary of mean tumor volumes in the last 10 and 15 days of animals from the control (black bars), T4 (grey bars) or T6 (open bars) groups. *P < 0.05, unpaired t-test performed comparing values obtained for each experimental condition (T4 and T6) with control group. n = 5–6. **D.** Summary of whole animal mean weight in the last 10 and 15 experiment days.

at high doses without affecting normal cells, contributing to a highly effective cancer treatment. On the other hand EPA exhibited an antiproliferative activity 50% weaker than DHA in AGS cells, but also their effects were observed in both tumoral and non-tumoral cells. These results are similar to those obtained by other investigators (D'Eliseo and Velotti, 2016), sustaining DHA promising characteristics for being a potential anticancer molecule, since it seems to be capable of causing cytotoxicity in cancer cells with little or no toxicity in normal cells. As a particular case, Dai et al. indicated that DHA, at a concentration of 180 µM, exerted little effect on GES-1 cells while inhibiting cell growth on mucinous gastric adenocarcinoma and papillomavirus-related endocervical adenocarcinoma cell lines (MGC-803 and SGC-7901 cells, respectively) (Dai et al., 2013). It is important to emphasize that the concentration range in which DHA exerted its effects in this study is in complete agreement with other works, that tested the effects of DHA on several types of cancer-derived cell lines (Hannafon et al., 2015; Jakobsen et al., 2008; Sam et al., 2018).

Comparing the effects of DHA and cisplatin in all the cell lines, we observed that low doses of DHA (25 µM) already produce a selective antiproliferative effect on AGS, without affecting GES-1 and HEK-293 cells. This antiproliferative effect of DHA at concentration ranging 50–100 µM is more important than those observed for cisplatin at all the cell models. According to our results, DHA had a more selective antiproliferative effect than cisplatin, a widely used chemotherapeutic agent. These results are supported by other studies, where authors used DHA as an adjuvant to improve the efficacy of traditional antineoplastic drugs like cisplatin (Sheng et al., 2016), or to decrease the detrimental side effects shown for mitosis inhibitors like docetaxel (Shekari et al., 2019) in gastric cancer cell lines.

Similar to previously reported data, a selective proapoptotic activity was found for DHA by our results obtained in Annexin V-PI assay, where DHA did not induced apoptosis in non-tumoral gastric cells (GES-1) at 75 µM concentration, but caused apoptosis in AGS cells. To further demonstrate that DHA induces apoptosis, we examined if pro-apoptotic and anti-apoptotic mediators were involved in this process, as many studies have shown that caspases play a critical role in apoptosis, identifying that caspase 3 activation can be driven out by either dependent or independent of mitochondrial cytochrome C release and caspase-9 function (intrinsic or extrinsic pathway, respectively). Consistent with our previous findings in the Annexin V-PI assay, DHA treatment on AGS cells induces a reduction of procaspase 3 protein levels, decrease in mRNA levels of anti-apoptotic mediators like Bcl-2 and Bcl-2, and an increase of the same levels of pro-apoptotic mediators like Bax and Smac/DIABLO. This proapoptotic situation on AGS cells is consistent with the increase in executioner caspases 3 and 7 activities, whose functionality is decreased both by DHA and cisplatin at similar extents, differing by their selectivity, where cisplatin portrayed proapoptotic effects on AGS and GES-1 cells. For GES-1 cells, we did not observed such aforementioned changes in pro and antiapoptotic mediators, except in the cisplatin-treatment, reflecting again the poor selectivity of this compound. Apoptosis induction via procaspase-3 protein levels decrease has also been described for DHA at 50–200 µM dosage on MDA-MB-231 human breast cancer cell line (Pizato et al., 2018). The absence of proapoptotic effects in GES-1 cells treated with DHA can be associated with the anti-inflammatory effects of this PUFA reported on other tissues (Kubo et al., 2020; Mason et al., 2020).

Finally, we tested DHA effects using *in vivo* models. For that purpose, we used BALB/c NOD/SCID mice xenografted with AGS cells in order to

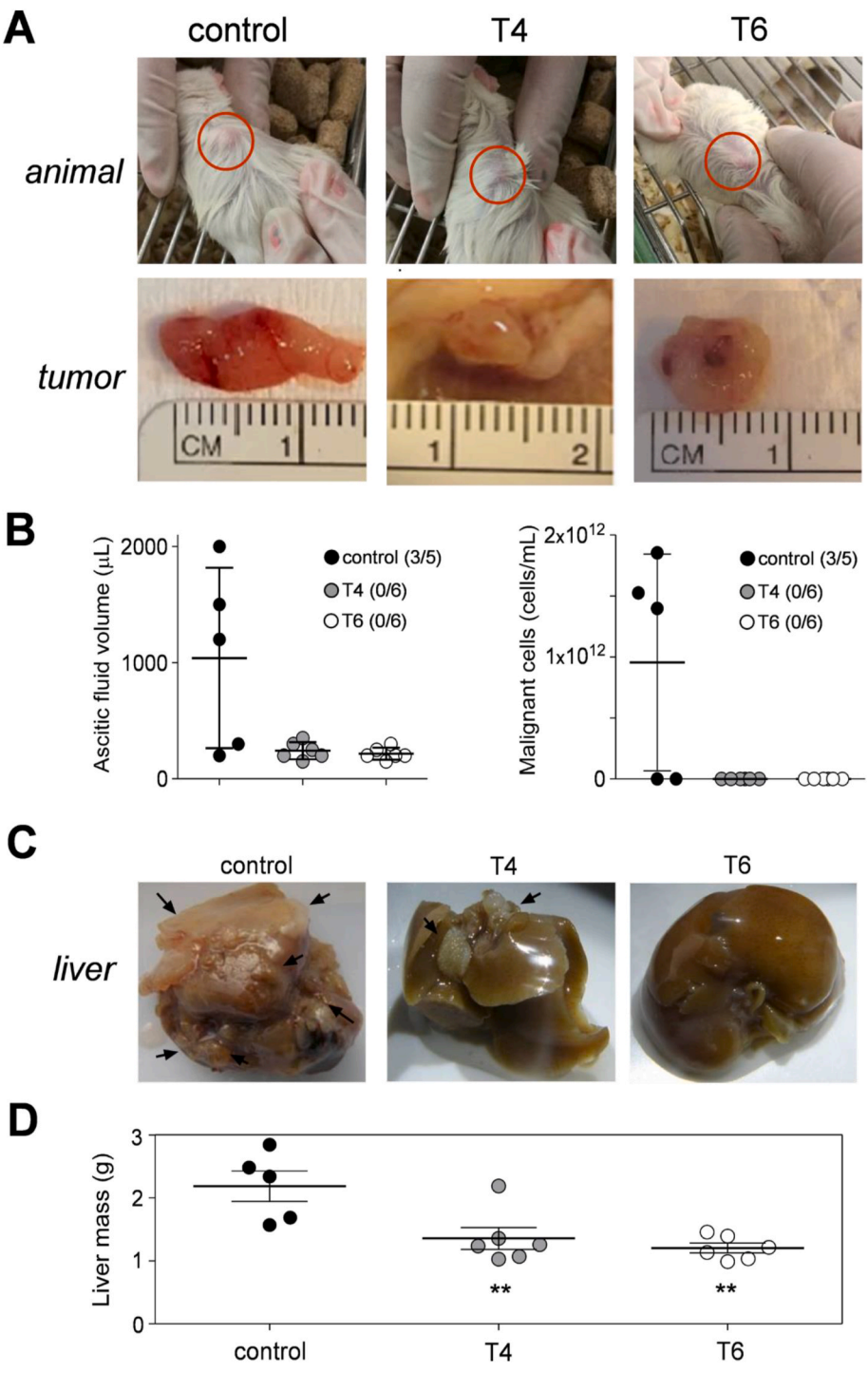

Fig. 7. DHA treatment in xenografted mice reduce spontaneous metastasis. A. Representative pictures of mice from control, T4 and T6 groups showing tumors induced in the abdominal cavity (upper pictures), and size of the extracted subcutaneous tumors after 52 days (lower pictures). **B.** Individual (circles) and average (horizontal lines) values of ascitic fluid volume (left graph) and number of malignant cells (right graph) are presented for control (black), T4 (grey) and T6 (white) groups. **C.** Representative pictures of the liver of animals from the 3 groups. Note the absence of metastasis-type processes in the T6 group liver. Arrows show metastasis examples. **D.** Liver mass quantification in control (black circles), T4 (grey circles), and T6 (white circles) groups. **P < 0.01, Dunnett's Multiple Comparison Test. n = 5–6.

induce tumors. After that induction, DHA treatment consistently reduced tumor volume and decreased signs of liver metastasis. These results suggest that DHA has potential as an anticancer drug, or as a chemosensitizer that could be administered together with other anti-GC treatments. Moreover, DHA did not show important side effects on treated animals, as could be noticed after monitoring the animal weights and other parameters during these experiments, finding no significant differences between non-treated and DHA-treated mice. Additionally, we did not find significant alterations on hematological and biochemical parameters, supporting the idea that DHA treatment is safe, with mild side effects. Generally, beneficial effects and no risks in humans has been associated to Ω-3 fatty acids consumption at doses up to 5 g/day (Bozzatello et al., 2016). This work is the first report showing *in vivo* effects for DHA on GC, although previous works reported *in vivo* effects of DHA on breast and pancreatic cancer models (Kang et al., 2010; Song et al., 2011), supporting the idea of using DHA as an attractive alternative for future treatments. Future experiments should consider the testing DHA in spheroid formation or clonogenic assays, techniques that have provided useful information in other types of cancer. In one study, it was observed that spheres treatment with DHA and EPA alone or combined for 72 h led to apoptosis, progressive loss of functionality, and DNA fragmentation in colorectal cancer stem-like cells (Yang et al., 2013). In prostate cancer models, the therapeutic efficacy of the novel omega-3 fatty acid conjugated taxoid prodrug DHA-SBT-1214 was

tested, finding that this nanoemulsion induced toxicity in 3D cultures of floating spheroids, and viable cells that survived DHA-SBT-1214 treatment from explanted *in vivo* xenograft were no longer able to induce floating spheroids and holoclone formation (Ahmad et al., 2017).

In summary, we reported a potent and selective antiproliferative and proapoptotic effect for DHA in both *in vitro* and *in vivo* models for GC. These effects are not observed for other Ω-3 PUFAs like EPA, nor precursor molecules like AA and ALA, highlighting a specific antiproliferative role for DHA. Our results support the idea of using DHA as a potential therapeutic agent, alone or associated with other chemotherapeutic drugs, or as preventive agent for gastric cancer as nutraceutical.

CRediT authorship contribution statement

Lorena Ortega: Investigation, Writing - review & editing, Visualization. **Lorena Lobos-González:** Investigation, Formal analysis, Writing - review & editing, Visualization. **Mauricio Reyna-Jeldes:** Conceptualization, Investigation, Writing - review & editing, Visualization. **Daniela Cerda:** Investigation, Formal analysis, Writing - review & editing, Visualization. **Erwin De la Fuente-Ortega:** Investigation, Formal analysis, Writing - review & editing. **Patricio Castro:** Writing - review & editing. **Giuliano Bernal:** Conceptualization, Writing - review & editing. **Claudio Coddou:** Conceptualization, Formal analysis, Writing - original draft, Writing - review & editing, Visualization, Supervision.

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