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Eicosapentaenoic acid and docosahexaenoic acid moderate inflammation in porcine cartilage explants

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Abstract

Objective: To characterize the effects of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and arachidonic acid (ARA) in various combinations on indices of inflammation in recombinant porcine IL-1 beta stimulated porcine articular cartilage explants.

Methods: Cartilage was obtained from the humeral-ulnar joints of Yorkshire x Landrace market sized gilts. Explants were harvested from the humeral-ulnar joints within 8 h of slaughter. Explants were allocated to culture plates and cultured in 1 mL of Dulbecco's Modified Eagle serum free medium for 24 h with 10% fetal bovine serum. At 48 and 72 h, 1 mL of treatment media containing fatty acids and 15 ng/mL of recombinant porcine IL-1 was added to each well. At 48, 72, and 96 h after cartilage was allocated to wells, media were removed from each well and reserved for analysis. Media were analyzed for proteoglycan, nitric oxide (NO), interleukin-6 and prostaglandin E_2 concentrations.

Results: In general, when EPA and/or DHA are supplemented to explants in combination with linoleic acid (LA) NO and prostaglandin E_2 release is decreased. Explants treated with 25 µg/mL DHA released 53% less NO into the media than explants treated with the same level of EPA and 60% less than explants treated with LA alone.

Conclusions: These data demonstrate that EPA and DHA are capable of modulating the inflammatory response on porcine articular cartilage *in vitro*.

Keywords: Cartilage, omega-3, omega-6, fatty acids, swine

Introduction

Lameness is a common clinical condition in livestock usually resulting in culling of the affected animal. In livestock of all breeds this can be a cause of serious financial loss, including replacement cost, and is a source of major welfare concerns. Joint lesions have been identified as one of the main causes for culling sows in Danish herds, affecting 24% of sows [1], while in the U.S. 15.2% of sows are culled for locomotor problems [2]. Over a 6-month period, production sows culled for lameness were analyzed postmortem. It was found that 31 of the 45 sows were diagnosed with either osteochondrosis or athrosis [3].

The pathogenesis of osteoarthritis (OA) is strongly mediated by the pro-inflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor-α [4]. The inflammatory response in cartilage is regulated through cell signaling pathways that control gene expression of proteins responsible for the production of nitric oxide (NO), prostaglandin E_2(PGE_2), and interleukin-6 (IL-6). Nitric oxide production plays a significant role in the development of cartilage degradation through inhibition of proteoglycan and collagen synthesis [5]. When cartilage is stimulated with IL-1β and/or tumor necrosis factor-α, increased NO production occurs. Mastbergen et al., [6] demonstrated that increased proteoglycan release from human articular cartilage is positively correlated with an increase in both NO and PGE_2 production.

Omega-3 fatty acids have the potential to be potent modulators of osteoarthritic factors due to their ability to reduce prostaglandin and NO production. Eicosanoids formed from eicosapentaenoic acid (EPA) are 10- to 100-fold less potent than those produced from arachidonic acid (ARA) and therefore are associated with a decreased inflammatory response [7]. When mouse stromal cells were incubated with either 40 µM ARA or

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EPA, ARA increased PGE_2 production, while EPA treated cells did not differ from the control. Additionally, Razzak et al., [8] demonstrated that when murine macrophages were incubated with lipopolysaccharide and a cyclooxygenase-2 (COX-2) inhibitor, NO production increased; however, when the same cells were incubated with EPA or a COX-2 inhibitor and EPA, NO production was minimal.

Both EPA and docosahexaenoic acid (DHA) can reduce the inflammatory response and the production of NO and PGE_2 in other tissues. The objective of this study was to determine the concentration of EPA and DHA, both alone and in combination, necessary to reduce inflammatory mediators in porcine articular cartilage explants ex vivo relative to either linoleic acid (LA) or ARA.

Materials and methods

Explant cultures

Yorkshire x Landrace cross gilts were slaughtered at market weight following the standard practices of the MSU Meat Laboratory. For experiments 1 and 2, front legs were collected from 8 gilts, and for experiments 3 and 4, front legs were collected from 6 gilts. Front legs were removed within 30 min of slaughter. Each experiment was conducted separately. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified.

The left and right humeral-ulnar joints were opened aseptically under sterile conditions and 50 cartilage disks were harvested with a 6 mm biopsy punch (Miltex, York, PA, USA) from the weight-bearing region of the articular surface of each gilt within 6 h of slaughter. Only visually normal cartilage was selected for biopsy.

Cartilage discs were washed twice in Dulbecco's modified Eagle's medium: nutrient mixture F-12 (Ham) (DMEM:F12; Invitrogen, Carlsbad, CA, USA) containing 100 units/mL penicillin-streptomycin (Life Technologies, Carlsbad, CA, USA). Explant discs were randomly placed into the wells of a 24-well culture plate until each well contained two discs. Explants were conditioned for 24 h in 1 mL of base media, containing DMEM:F12 supplemented with amino acids [9], 10% fetal bovine serum (FBS; Gibco, Invitrogen, Carlsbad, CA, USA), 50 µg/mL ascorbate, and 100 units/mL penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA), in a humidified incubator at 37°C with 7% CO_2.

After 24 h of conditioning media explants were washed twice with 1 mL of sterile phosphate buffered saline to remove FBS from the wells. Saline was completely removed from each well after each wash. Then, 1 mL of treatment media consisting of FBS free base media plus 1 µL/mL insulin-transferrin-sodium selenite supplement (Roche Applied Science, Mannheim, Germany), 0.02 µg/mL thyroxine, and long chain polyunsaturated fatty acids (LCPUFA; Camen Chemical, Ann Arbor, MI, USA) were added to each well (**Table 1**).

Each of the four experiments used a different combination of LCPUFA. Experiment 1 examined concentrations of EPA

Table 1. Detailed list of all experiments conducted, the concentration of IL-1β, the control fatty acids.

Experiment No.	Treatment	IL-1β, ng/mL	LA, µg/mL	EPA, µg/mL	DHA, µg/mL	AA, µg/mL
1	100 LA	15	100	0	--	--
	6.25 EPA	15	93.75	6.25	--	--
	12.5 EPA	15	87.5	12.5	--	--
	18.75 EPA	15	81.25	18.75	--	--
	25 EPA	15	75	25	--	--
2	100 LA	15	100	--	0	--
	6.25 DHA	15	93.75	--	6.25	--
	12.5 DHA	15	87.5	--	12.5	--
	18.75 DHA	15	81.25	--	18.75	--
	25 DHA	15	75	--	25	--
3	100 LA	15	100	0	0	--
	25 EPA	15	75	25	0	--
	18 EPA, 6 DHA	15	75	18.75	6.25	--
	12.5 EPA, 12.5 DHA	15	75	12.5	12.5	--
	6 EPA, 18 DHA	15	75	6.25	18.75	--
	25 DHA	15	75	0	25	--
4	100 AA	15	--	0	0	100
	12.5 EPA	15	--	12.5	0	87.5
	25 EPA	15	--	25	0	75
	12.5 DHA	15	--	0	12.5	87.5
	25 DHA	15	--	0	25	75

linoleic acid (LA) and arachidonic acid (AA) as well as the treatment fatty acids: eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) for each treatment.

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varying from 0 to 25 µg/mL with LA added to total 100 µg/mL of total fatty acids in the media. Experiment 2 examined concentrations of DHA varying from 0 to 25 µg/mL with LA added to total 100 µg/mL of total fatty acids in the media. Experiment 3 examined EPA and DHA at the following concentrations: 25 µg/mL EPA, 18.75 µg/mL EPA with 6.25 µg/mL DHA, 12.5 µg/mL EPA with 12.5 µg/mL DHA, 6.25 µg/mL EPA with 18.75 µg/mL DHA, and 25 µg/mL DHA. The control treatment contained 100 µg/mL LA while all other treatments contained 75 µg/mL LA in addition to the EPA and DHA. Experiment 4 examined either EPA or DHA at the following concentrations: 12.5 µg/mL EPA, 25 µg/mL EPA, 12.5 µg/mL DHA, 25 µg/mL DHA. The control treatment contained 100 µg/mL ARA while all other treatments contained ARA at either 87.5 µg/mL or 75 µg/mL to total 100 µg/mL fatty acids in the media. Fatty acid concentrations used in these four experiments were based two factors. First, a preliminary study in which concentrations of 0, 25, 50, 75, and 100 µg/mL EPA were tested and it was determined that concentrations of EPA over 25 µg/mL provided no additional advantage. Second, LCPUFA concentrations less than 25 µg/mL are likely more biologically relevant.

At 48 and 72 h 15 ng/mL of recombinant porcine IL-1β (R & D Systems, Minneapolis, MN, USA) were added to the treatment media (**Figure 1**). Between 24 and 48 h the explants were not exposed to IL-1β to serve as the unstimulated control. At 48, 72, and 96 h media were removed from each well and separated into two tubes for analysis. The first tube contained 10 µg/mL indomethacin to prevent further metabolism of PGE_2 and was stored at -20°C until analysis. The second tube did not contain any additives and was stored at 4°C for NO and proteoglycan analysis. Media were analyzed for proteoglycans (PG), NO, interleukin-6 (IL-6), and PGE_2 concentrations.

Proteoglycan analysis

Proteoglycan release into media was measured using the dimethylmethylene blue assay [**10**]. Proteoglycan content was determined by measuring sulfated glycosaminoglycan content using a chondroitin sulfate standard and expressed as µg PG/well. Absorbance at 530 nm with a correction at 590 nm was determined using a Spectramax 300 plate reader (Molecular Devices, Sunnyvale, CA, USA).

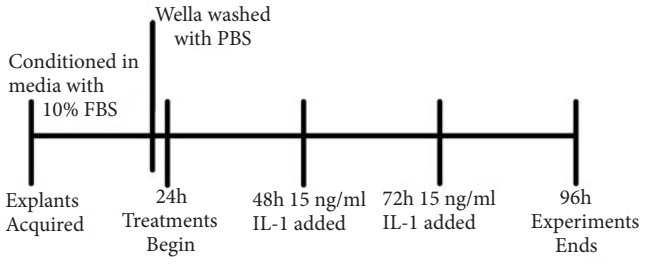

Figure 1. Diagram of explant culture process over 96 h.

Nitric oxide analysis

Nitric oxide was measured indirectly by quantifying nitrite, a stable end-product of nitric oxide metabolism, in the media by using the Greiss reaction and a sodium nitrite standard [**11**]. Absorbance at 540 nm was determined using a Spectramax 300 plate reader (Molecular Devices, Sunnyvale, CA, USA). Results are expressed as µM of NO/well.

Prostaglandin E_2 analysis

Prostaglandin E_2 was measured in the media using a commercially available ELISA kit(EHPGE2; Thermo-Fisher Scientific, Pittsburgh, PA, USA) following the manufacturer's instructions. Media samples were diluted as needed in the provided assay buffer and analyzed. Absorbance at 405 nm with a correction at 580 nm was determined using a Spectramax 300 plate reader (Molecular Devices, Sunnyvale, CA, USA).

Interleukin-6 analysis

Interleukin-6 was measured in the media using a porcine-specific, commercially available ELISA kit (Porcine IL-6 Duoset; DY686, R & D Systems, Minneapolis, MN, USA) following the manufacturer's instructions. Samples were diluted as needed in the reagent diluent and analyzed in duplicate. Absorbance at 450 nm with a correction at 570 nm was determined using a Spectramax 300 plate reader (Molecular Devices, Sunnyvale, CA, USA).

Statistical analysis

Data were analyzed as a cumulative response following stimulation with IL-1, such that the response from media collected at 72 h was added to the response from media collected at 96 h. Data were analyzed using the mixed procedure of SAS (version 9.2; SAS Inst. Inc., Cary, NC, USA) with animal, treatment and time in the model. A random statement of animal nested in treatment was used. Differences between means were examined using the pdiff function. All data will be presented as lsmeans±SEM. P-values <0.05 will be discussed as significant while P-values <0.10 will be discussed as trends.

Results

Prior to IL-1β stimulation, release of proteoglycans, and production of nitric oxide and IL-6 did not exhibit a treatment effect for any of the 4 experiments. Additionally, experiments 1 and 3 did not exhibit a treatment effect for PGE_2 production prior to IL-1β stimulation. In experiment 2, explants treated with 12.5 and 25 µg/mL of DHA produced more PGE_2 prior to stimulation than those treated with 100 µg/mL of LNA, 6.25 µg/mL of DHA and 18.75 µg/mL of DHA ($P<0.01$; **Table 3**). In experiment 4, where ARA was used as the control fatty acid, prior to IL-1β stimulation the control produced 1.7 times more PGE_2 than the next highest treatment while the 25 µg/mL of DHA produced the least amount of PGE_2 ($P<0.01$; **Table 5**).

Cartilage explants treated with 0 to 25 µg/mL of EPA and stimulated with IL-1β exhibited a trend for an overall effect of

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treatment (P=0.09) in which the 6.25 and 12.5 EPA treatments released less proteoglycans than 100 LA or 18.75 EPA (**Table 2**; P<0.05, P=0.07). Proteoglycan release from cartilage explants treated with 0 to 25 µg/mL of DHA or 0 to 25 µg/mL of EPA and DHA and stimulated with IL-1β displayed no difference in cumulative release over 48 h (**Tables 3** and **4**). Post IL-1β stimulation, explants with ARA as the control fatty acid and treated with 12.5 or 25 µg/mL of EPA released more proteoglycans than those treated with 12.5 and 25 µg/mL of DHA (**Table 5**).

There was no difference in nitric oxide release from explants treated with varying concentrations of EPA and stimulated with IL-1β (P<0.05). Explants treated with DHA displayed an overall effect of treatment (P<0.01) and at 72 h the 12.5, 18.75 and 25 DHA treatments released less NO than the 100 LA or the 6.25 DHA (**Table 3**). In explants treated with EPA and DHA alone or in combination NO release exhibited an overall effect of treatment (P<0.01) and all fatty acid treatments released less NO than the 100 LA treatment (P<0.05; **Table 4** and **Figure 2**). Additionally, the 25 DHA treatment in experiment 3 released less NO than any other treatment (P<0.01). Explants with ARA as the control fatty acid exhibited a treatment effect for NO release with the 25 µg/mL of EPA treatment releasing the most NO (P<0.01; **Table 5**) while there was no difference between the control, 12.5 DHA and 25 DHA treatments.

Prostaglandin E_2 release was not affected by treatment with EPA and stimulation with IL-1β in experiment 1 (**Table 2**). In experiments 2 and 3, PGE_2 release exhibited an overall effect

Table 2. Cumulative proteoglycan(PG), nitric oxide (NO), prostaglandin E_2 (PGE_2), and interleukin-6 (IL-6) release from cartilage into media for experiment 1 when explants were treated with 0 to 25 µg/mL of eicosapentaenoic acid (EPA) (Table 1). Proteoglycan release exhibited trend for a treatment effect (P=0.087). Means with different letters differ (P<0.05) for a given analyte.

	100 LA	6.25 EPA	12.5 EPA	18.75 EPA	25 EPA	SEM	P-value
PG							
Pre IL-1	101.9	101.0	122.8	96.8	103.9	18.4	0.872
Post IL-1	540.1^{a}	423.2^{b}	452.8^{ab}	526.2^{a}	478.7^{ab}	32.8	0.087
NO							
Pre IL-1	15.7	15.2	14.9	15.5	14.8	0.58	0.795
Post IL-1	86.7	77.6	74.7	80.9	76.1	3.9	0.228
PGE_2							
Pre IL-1	84.0	92.4	71.06	103.7	112.8	19.7	0.584
Post IL-1	917.5	903.3	486.3	865.1	640.4	177	0.346
IL-6							
Pre Il-1	63.2	55.9	34.39	36.0	44.8	11.5	0.323
Post IL-1	6390.3	6059.0	6475.7	6194.7	6419.5	724	0.993

Table 3. Cumulative proteoglycan (PG), nitric oxide (NO), prostaglandin E_2 (PGE_2), and interleukin-6 (IL-6) release from cartilage into media for experiment 2 when explants were treated with 0 to 25 µg/mL of docosahexaenoic acid (DHA) (Table 1). NO and PGE_2 release exhibited a treatment effect (P<0.01). Means with different letters differ (P<0.05) for a given analyte.

	100 LA	6.25 DHA	12.5 DHA	18.75 DHA	25 DHA	SEM	P-value
PG							
Pre IL-1	70.2	71.1	66.6	72.2	75.8	5.3	0.81
Post IL-1	380.7	347.9	282.8	298.0	312.3	34.7	0.285
NO							
Pre IL-1	14.3	14.5	13.9	13.0	12.3	0.8	0.270
Post IL-1	74.7^{a}	66.12^{a}	52.05^{b}	52.75^{b}	46.58^{b}	4.8	0.001
PGE_2							
Pre IL-1	22.3^{a}	39.3^{ab}	153.0^{c}	56.3^{a}	104.7^{bc}	24.3	0.008
Post IL-1	1060.1^{a}	431.2^{b}	523.2^{b}	382.3^{b}	474.9^{b}	102	<0.001
IL-6							
Pre IL-1	42.7	43.3	19.0	13.4	7.1	21.6	0.66
Post IL-1	3923.3	4380.1	3446.5	3844.1	3642.7	938	0.967

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Table 4. Cumulative proteoglycan (PG), nitric oxide (NO), prostaglandin E_2 (PGE_2), and interleukin-6 (IL-6) release from cartilage into media for experiment 3 when explants were treated with 0 to 25 μg/mL of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)alone or in combination (Table 1). NO and PGE_2 release exhibited a treatment effect (P<0.01) post interleukin-1 (IL-1) stimulation. Means with different letters differ (P<0.05) for a given analyte.

	100 LA	25 EPA	18.75 EPA, 6.25 DHA	12.5 EPA, 12.5 DHA	6.25 EPA, 18.75 DHA	25 DHA	SEM	P-value
PG								
Pre IL-1	66.1	62.8	68.2	69.6	65.5	65.1	4.92	0.941
Post IL-1	473.7	427.7	383.4	478.5	386.1	352.5	40.7	0.189
NO								
Pre IL-1	13.5	12.6	12.6	12.0	11.8	12.1	1.4	0.963
Post IL-1	72.7^{a}	59.1^{b}	52.54^{b}	54.7^{bc}	47.72^{c}	28.5^{d}	3.6	<0.001
PGE_2								
Pre IL-1	91.0	222.0	292.7	235.3	218.5	376.8	87.44	0.302
Post IL-1	1379.8^{a}	578.2^{b}	471.7^{b}	521.8^{b}	357.1^{b}	641.9^{b}	149.8	0.001
IL-6								
Pre IL-1	25.5	120.5	28.4	145.4	114.0	38.4	57.95	0.529
Post IL-1	6830.8	9870.4	10172	12468	7255.2	6852.9	1768	0.163

Table 5. Cumulative proteoglycan (PG), nitric oxide (NO), prostaglandin E_2 (PGE_2), and interleukin-6 (IL-6) release from cartilage into media for experiment 4 when explants were treated with 12.5 or 25 μg/mL of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Table 1). All variables exhibited a treatment effect post interleukin-1 (IL-1) stimulation (P<0.01). Means with different letters differ (P<0.05) for a given analyte.

	100 AA	12.5 EPA	25 EPA	12.5 DHA	25 DHA	SEM	P-value
PG							
Pre IL-1	60.9	64.5	78.8	57.9	61.4	6.0	0.125
Post IL-1	126.6^{ac}	208.3^{b}	230.8^{b}	152.2^{a}	96.6^{c}	16.6	<0.001
NO							
Pre IL-1	6.9	7.0	4.0	7.0	4.1	1.7	0.471
Post IL-1	9.2^{a}	17.4^{b}	21.8^{c}	10.0^{ad}	8.9^{ad}	1.3	<0.001
PGE_2							
Pre IL-1	34695^{a}	20240^{b}	11180^{bc}	19880^{b}	8680^{c}	3304	<0.001
Post IL-1	122110^{a}	76420^{b}	30290^{c}	80135^{b}	81670^{b}	7080	<0.001
IL-6							
Pre IL-1	1.8	3.7	1.7	2.3	2.4	1.5	0.884
Post IL-1	585.8^{a}	2287.6^{b}	4136.4^{c}	2309.5^{b}	238.7^{a}	601.1	<0.001

of treatment (P<0.01) in which all EPA and/or DHA treatments decreased release when compared to the control (P<0.05; **Table 3** and **Figure 3**) with no difference between the fatty acid treatments. Cumulative release of PGE_2 in experiment 4 was also affected by treatment (P<0.01) with the 25 EPA treatment producing the least amount of PGE_2 and the ARA control treatment producing the most (P<0.01; **Table 5**).

Experiments 1, 2, and 3 did not have any effect on IL-6 release into the media regardless of fatty acid or concentration. However, experiment 4 demonstrated that cumulative release of IL-6 was affected by treatment (P<0.01) such that the 25 μg/mL of EPA treatment produced 50% more than the 12.5 EPA and DHA treatments and 86% more than the 100 ARA or 25 DHA treatments (P<0.01; **Table 5**).

Discussion and conclusion

This work investigated the anti-inflammatory effects of EPA and DHA by measuring inflammatory mediators and tissue degradation in a porcine explant model of inflammatory joint disease. The *in vitro* system of cartilage explants does mimic physiological conditions. The experiments were designed to determine the potential for omega-3 fatty acids to influence inflammatory molecules in porcine cartilage. Supplementation of EPA and/or DHA into porcine explant cultures consistently caused a reduction in NO and PGE_2 concentrations in the media; however, only EPA alone was able to alter PG release. In previous studies, Il-1β has been successfully used to induce cartilage degradation [**12-14**]. DHA was successful at altering chondrocyte metabolism to inhibit NO and PGE_2 production.

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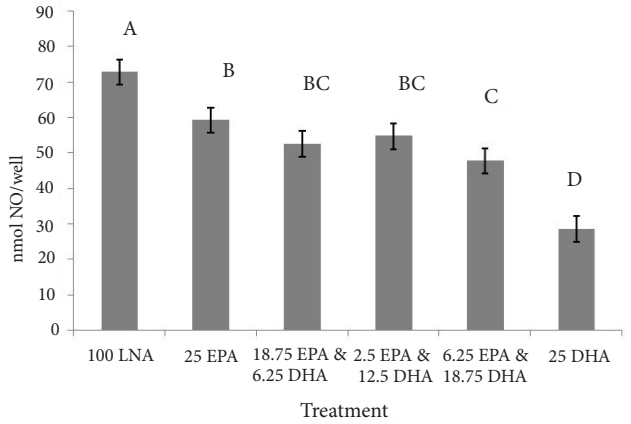

Figure 2. Cumulative nitric oxide (NO) release from cartilage into media for Experiment 3 when explants were treated with 0 to 25 μg/mL of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)alone or in combination (**Table 1**). NO exhibited a treatment effect (P<0.01). Bars with different letters differ at P<0.05.

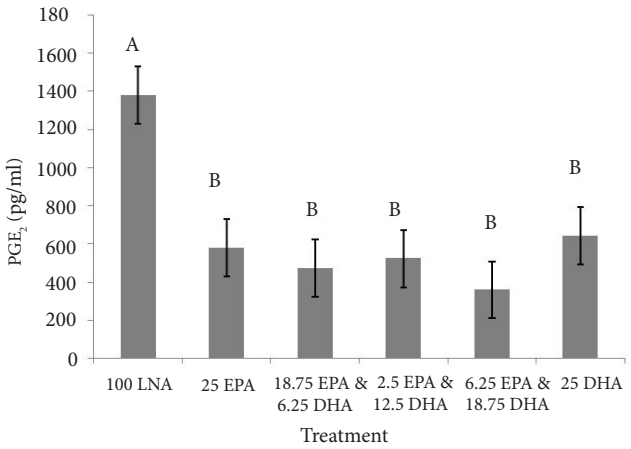

Figure 3. Cumulative PGE_2 release from cartilage into media for Experiment 3 when explants were treated with 0 to 25 μg/mL of EPA and DHA alone or in combination (**Table 1**). PGE_2 exhibited a treatment effect (P<0.01). Bars with different letters differ at P<0.05.

The reduction in NO is likely due to a concurrent reduction in PGE_2.

Omega-3 fatty acids are known for their anti-inflammatory properties with a reduction in PGE_2 being a consistent finding regardless of tissue. Arachidonic acid is an omega-6 fatty acid, which generally initiates a pro-inflammatory response via its metabolism to PGE_2 in the cell. Arachidonic acid is the preferred substrate for COX-2; however, EPA can also be used. *In vitro* COX-2 has higher specificity for ARA than for EPA and preferentially oxygenates ARA when both fatty acids are present even if ARA is at a low substrate concentration [**15**]. In the current experiments, when only EPA or DHA and LA were present in the media, once all ARA was released from the cellular membrane any additional reactions would utilize EPA as a substrate for COX-2, resulting in the production of PGE_3. Since the ELISA used in the current study to measure the concentration of PGE_2 in the media has minimal cross reactivity with PGE_3, the lower concentration of PGE_2 in the EPA-treated media may be due to the production of PGE_3 and not alterations in COX-2 expression. In experiment 4 when ARA was present in the media as the control fatty acid instead of LA the PGE_2 levels were 122 times higher after IL-1β stimulation than in the LA treatment of the other experiments. Addition of EPA or DHA at any level was sufficient to reduce PGE_2 production even with an unlimited supply of ARA; however the 25 EPA treatment was able to create the most substantial reduction of approximately 75%. In the experiments using LA as the control, the presence of either EPA or DHA at any concentration or combination reduced PGE_2 concentrations similarly. The data from experiment 4 suggests that EPA may be utilizing competitive inhibition with ARA while DHA may beregulating COX-2 at a molecular level to reduce the production of PGE_2 post-stimulation.

In addition to a reduction in PGE_2, supplementation of EPA and/or DHA resulted in the reduction of NO production. Nitric oxide production plays a significant role in the development of cartilage degradation through inhibition of proteoglycan and collagen synthesis [**5**]. Mastbergen et al., [**6**] demonstrated that increased proteoglycan release from human articular cartilage is positively correlated with an increase in both NO and PGE_2 production. Cyclooxygenase-2, inducible nitric oxide synthase (iNOS), and fatty acids have a very complicated interrelationship that has been explored by many researchers in various cell types [**8**,**16**-**18**]. For example, when ARA was added to osteoblast cell cultures an increase in iNOS gene expression occurred; however, when EPA was added to ARA-treated cells, EPA prevented an increase in iNOS expression [**16**]. This suggests that EPA may be a more potent regulator of iNOS expression than ARA. However, when ARA was used as the control fatty acid NO production increased when EPA was added to the wells. Little research has been conducted using chondrocytes and it is possible that other cell types respond differently under these conditions.

Mouse macrophages treated with 60 μM of LA, LNA, ARA, EPA, or DHA and stimulated with lipopolysaccharide and interferon-γ to simulate a bacterial endotoxin demonstrated that both EPA and DHA inhibited NO production, while only DHA inhibited iNOS protein and mRNA expression [**17**]. This suggests that *in vitro* DHA is a more potent regulator of NO and PGE_2 release than EPA, which agrees with our results. This is likely due to the difference in the mechanisms by which each of these fatty acids regulate the inflammatory mediators at a molecular level. Razzak et al., [**8**] demonstrated that when murine macrophages were incubated with lipopolysaccharide (LPS) and a COX-2 inhibitor, NO production increased. However, when the same cells were incubated with EPA or a COX-2

inhibitor and EPA, NO production was minimal. The authors attributed this decrease in NO production to a series of omega-3 fatty acid related, multi-factorial events involving iNOS and COX-2.

When DHA was added in conjunction with LA similar results were found as when EPA was used. When either EPA or DHA was added in conjunction with ARA only the 25 DHA treatment was able to reduce NO production; all other treatments produced NO concentrations higher than the control. This suggests that EPA and DHA are not working through the same pathways. EPA, DHA, and LNA are potent inhibitors of COX-2 catalyzed prostaglandin biosynthesis in *in vitro* studies [18]. In activated macrophages the transcription factor NF-κB must be activated for iNOS gene expression. When macrophages were supplemented with DHA the amount of NF-κB binding decreased, thus inhibiting both iNOS expression and NO production [17]. In vascular endothelial cell cultures, when cells were exposed to 25 μmol/L DHA for 48 h before exposure to 10 ng/mL IL-1α, inhibition of COX-2 increased greater than 50% [19]. Following a series of experiments in which endothelial cells were incubated with 25 μmol/L DHA for 48 h the researchers concluded that DHA inhibits COX-2 expression through two mechanisms. First, DHA reducesprotein kinase C-εactivation thereby inhibiting COX-2 and iNOS gene expression. Secondly, DHA scavenges reactive oxygen species preventing the production of H_2O_2, which is necessary for nuclear factor kappa-light-chain-enhancer of B cells activation [19]. DHA should interface with IL-1 signaling pathways in a similar manner regardless of tissue type, therefore reduction in NF-κB activation can explain the decrease in both NO and PGE_2 production in DHA treated cell cultures since both iNOS and COX-2 require NF-κB activation.

The ability of EPA and DHA to alter inflammatory mediators depends highly on the incorporation of these fatty acids into the cell membrane. Dietary omega-3 fatty acids are preferentially incorporated into certain tissues. DHA content in plasma, liver, brain, and other organs is highly correlated to erythrocyte DHA levels [20]. A suitable biomarker that correlates with EPA and DHA status in articular tissue has not been identified. At the *in vivo* level the concern would be whether or not sufficient levels of EPA and/or DHA could be obtained in the cartilage to elicit the reduction in inflammatory mediators. Dietary supplementation of 1% protected fish oil increases the DHA concentration in the cartilage of sows 2.4 fold when compared to a control diet when fed for an average of 2 years [21]. Additionally, synovial fluid concentrations of EPA and DHA were higher in sows fed protected fish oil as 1% of their diet [21]. Although the aforementioned study measured increased concentrations of DHA in the cartilage following fish oil supplementation the changes may not have been substantial enough to be biologically significant.

These data provide evidence that both EPA and DHA are able to alter the production of NO and PGE_2 in porcine articular cartilage explants. Further research is necessary to more precisely explain the mechanisms by which EPA is altering PGE_2 production and DHA is altering the production of NO. Additionally, it has yet to be determined if EPA and DHA can be added to a porcine diet at a concentration that will modulate articular cartilage metabolism without adversely impacting other physiological processes in the animal.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Authors' contributions	CIR	MWO
Research concept and design	✓	✓
Collection and/or assembly of data	✓	--
Data analysis and interpretation	✓	✓
Writing the article	✓	--
Critical revision of the article	--	✓
Final approval of article	✓	✓
Statistical analysis	✓	--

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