FULL ARTICLE

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The *in vitro* effects of n-3 fatty acids on immune response regulation of bovine *ex vivo* endometrial explants

Os efeitos in vitro dos ácidos graxos n-3 na regulação da resposta imune de explantes endometriais bovinos ex vivo

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ABSTRACT

Fatty acids are considered metabolic intermediaries, although new facts indicate they also work as signaling molecules with different roles in the immune response. Based on that, in this study, we investigated the anti-inflammatory effects of n-3 polyunsaturated fatty acids (PUFAs) as eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and α-linolenic acid (LNA) in *ex vivo* bovine endometrial explants. For this, two groups were formed: (1) LPS-challenged and (2) control, both to evaluate the accumulation of proinflammatory cytokines as interleukin 1β (*IL1B*) and interleukin 6 (*IL6*). To develop the study, bovine female reproductive tracts from non-pregnant Angus heifers without evidence of reproductive diseases were selected. Endometrial explants were processed and treated for 24 h with EPA, DHA, and LNA in five different concentrations (0μM, 50μM, 100 μM, 200μM and 400 μM) and then, challenged with LPS for 24 h. Supernatants were collected to evaluate the concentration of *IL1B* and *IL6* by ELISA. Explants treated with EPA from control groups reduced the concentrations of *ILB* (200μM) and *IL6* (400 μM), and *IL6* (50 μM; 100 μM) from the LPS-challenged group. DHA decreased the accumulation of *IL*1B and IL6 at 200 μM on explants from the LPS-challenged group, and 200 μM reduced *IL6* from the control group. In contrast, explants treated with LNA only reduced the accumulation of *IL1B* to 400μM (from both groups). In conclusion, the EPA acid is the best anti-inflammatory option to decrease the concentration of both pro-inflammatory cytokines (*IL1B* and *IL6*) from LPS-challenged and control groups in bovine endometrial explants; while LNA evidence to be the last option to promote an anti-inflammatory response.

Keywords: Cytokines. Fatty acids. Reproduction. Uterus.

RESUMO

Os ácidos graxos são considerados intermediários metabólicos, embora novos fatos indiquem que eles também atuem como moléculas sinalizadoras com diferentes papéis na resposta imune. Dessa forma, este estudo investigou os efeitos anti-inflamatórios de ácidos graxos poliinsaturados n-3 (PUFAs) como ácido eicosapentaenóico (EPA), ácido docosahexaenóico (DHA) e ácido α-linolênico (LNA) em explantes endometriais *ex vivo* de bovinos. Para tal, o experimento foi divido em dois grupos: (1) Desafiado-LPS e (2) Controle, para que então pudesse avaliar o acúmulo de citocinas pró-inflamatórias como interleucina *1β (IL1B)* e interleucina *6 (IL6)*. Foram selecionados tratos reprodutivos de fêmeas bovinas de novilhas Angus não prenhes sem evidência de doenças reprodutivas. Explantes endometriais foram processados e tratados por 24h com EPA, DHA e LNA em cinco concentrações diferentes (0μM, 50μM, 100 μM, 200μM e 400 μM) e, em seguida, desafiados com LPS por mais 24h. Os sobrenadantes foram colhidos para avaliar a concentração de *IL1B* e *IL6* pelo teste de ELISA. Os explantes tratados com EPA dos grupos de controle reduziram as concentrações de *ILB* (200 μM) e *IL6* (400 μM) e no grupo desafiado com LPS houve redução das concentrações de *IL6* (50 μM; 100 μM). Nos explantes do grupo desafiado com LPS, o DHA diminuiu o acúmulo de *IL1B* e *IL6* nas concentrações de 200 μM, e no grupo controle reduziu IL6 nas concentrações de 200 μM, enquanto os explantes tratados com LNA reduziram apenas o acúmulo de *IL1B* a 400 μM (de ambos os grupos). Em conclusão, o ácido EPA provou ser a melhor opção anti-inflamatória para diminuir a concentração de ambas as citocinas pró-inflamatórias (*IL1B* e *IL6*) de grupos desafiados com LPS e controle em explantes endometriais bovinos; enquanto o LNA evidencia ser a opção menos viável para promover uma resposta anti-inflamatória.

Palavras-chave: Citocinas. Ácidos graxos. Reprodução. Útero.

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Introduction

Polyunsaturated fatty acids (PUFAs) supplementation has become a common tactic to enhance the energy density of diets and decrease the effects of negative energy balance (Bionaz et al., 2020). In this scenario, the PUFA from omega 3 as α-linolenic acid (ALA), eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), and docosahexaenoic acid (DHA) and those from omega 6 as linoleic acid (LA) and arachidonic acid, receive special consideration due to their roles in reproductive and immune function (Moallem, 2018; Moallem et al., 2020). Lackey & Olefsky (2016) state that fatty acids are metabolic intermediaries. However, new facts indicate that they also work as signaling molecules with different roles in immune response, thus establishing a link between metabolism and immunity. More specific studies that explore which cell lines these fatty acids act to protect the bovine reproductive system are not yet elucidated (Dirandeh & Ghaffari, 2018).

When bovine *ex vivo* endometrium explants are the focus of scientific research, a potential link between animal and cellular studies are offered. Thus, research using intact endometrial explants as technical tools directly links *in vitro* and *in vivo* observational studies (Borges et al., 2012).

It has been widely discussed that immune mechanisms protect different types of pathogens. These mechanisms are based on a limited repertoire of germline-encoded receptors called pattern recognition receptors (PRRs) due to their capacity to identify conserved microbial components known as pathogen-associated molecular patterns (PAMPs) (Albiger et al., 2007). In this context, during cows postpartum, an increase of cytokines (inflammatory mediators) as nuclear factor-kappa b (*NFKB*), interleukin 8, 1, 6 (*IL8*, *IL1*, *IL6*), and acute phase proteins are characteristics of a local uterine response to activate the immune system feedback mediated by PRRs and their PAMPs (Chapwanya et al., 2009).

As part of the immune response, the increased or decreased cytokines regulate acute and chronic inflammation by activating neutrophils and macrophages and enhancing the endothelial expression from cell adhesion molecules (Collins et al., 1995; Koh et al., 2007). In this context, it was stated that the endometrium is a dynamic site of cytokine release and that the embryo, in its development process, can communicate with it using the same "cytokine receptor language" (Roberts et al., 2008).

The reason why PUFAs, such as EPA, DHA, and LNA, induce changes in *ex vivo* and *in vivo* cytokine production is not well established. What has been explored is that n-3 PUFAs have inflammatory resolution properties (Calder, 2006), including both pro and anti-inflammatory roles (Fritsche, 2008). The DHA, for example, alters gene transcription and translation via direct or indirect actions on intracellular signaling pathways (Gorjão et al., 2006; Lee et al., 2004), while EPA can compete with arachidonic acid as a substrate for cyclooxygenase (COX)-2 and 5-lipoxygenase (5-LO) enzymes and be transformed to less inflammatory eicosanoids (Thien et al., 1993).

In this study, we hypothesized that an *in vitro* inclusion of PUFAs in a cow's endometrium treated by lipopolysaccharide (LPS) can alter the inflammatory response by reducing the production of pro-inflammatory cytokines. For this, the objective of this study was to investigate the effects of three different n-3 PUFAs as DHA, EPA, and alpha-linolenic acid (LNA) against LPS – stimulated inflammatory response on bovine *ex vivo* endometrial explants by measuring the accumulation of pro-inflammatory cytokines as *IL6* and *IL1B* as they are directly implicated in the pathogenesis of cow's postpartum uterine diseases.

Materials and Methods

Preparation of bovine endometrial explants and bovine endometrial explants samples

The study was performed at the Large Animals Health Laboratory (LASGRAN) from the Federal University of Uberlândia - FAMEV/UFU. Bovine female reproductive tracts of non-pregnant Angus heifers were provided from a local abattoir (Uberlândia, Minas Gerais, Brazil) and were selected based on their healthy and normal appearance. Only uteri with no gross evidence of reproductive disease were chosen for the experiment.

The uteri were kept on ice (~2-8 °C) for a maximum of 2 h until processing in the laboratory and sprayed its surface with

ethanol 70% (EtOH, Sigma –E7023) to avoid contamination during the next steps. After samples arrived, the carcass number from each tract was recorded. The estrous cycle stage was determined by examining ovarian morphology and vasculature, as Ireland et al. (1979) described

The tissue culture technique (endometrial explants) was performed as described in a work developed by Borges et al. (2012), who standardized and validated the use of explants. The ipsilateral horn of the uterus with an active *corpus luteum* (CL) was opened longitudinally with sterile scissors. The endometrium was exposed and washed with HBSS media (Sigma-Aldrich, Dorset, UK) supplemented with 50 IU/mL penicillin, 50 µg/mL streptomycin, and 2.5 µg/mL amphotericin B (all Sigma-Aldrich).

In the intercaruncular area of the endometrium, sterile biopsy punches (KRUUSE, Saint-Priest, France) were used to select tissues around 8mm in diameter. A total of 26 explants per animal (n=5) were collected and transferred to an HBSS media (Sigma-Aldrich, Dorset, UK) supplemented with 50 IU/mL penicillin, 50 µg/mL streptomycin and 2.5 µg/mL amphotericin B (all Sigma-Aldrich). Explants were washed three times in a safety cabinet and then transferred into a 24-well DNAse, RNAse, and pyrogen-free plates for 24 h in 1 mL of Complete Media (500 mL of RPMI 1640 medium with Glutamax (Gibco 61870-044), with 10% of FBS, heat-inactivated (Biosera S1810), with 50 IU/mL penicillin, 50 µg/mL streptomycin and 2.5 µg/mL amphotericin B (all Sigma-Aldrich)).

Bovine ex vivo endometrial explants treatment

Bovine *ex vivo* endometrial explants were treated with three different n-3 PUFAs, two of them were from a fish oil source: EPA (90110, Cayman Chemical, USA) and DHA (90310, Cayman Chemical, USA), and one was derived from a vegetable oil source, LNA (90210, Cayman Chemical, USA). Stock solutions of these fatty acids were diluted in ethanol (50 mg/mL), aliquoted, and stored at -80 °C.

The explants were pre-treated for 24h (for sensitization) with DHA, EPA, and LNA in five concentrations: 0µM, 50µM, 100µM, 200µM and 400 µM (control group; adjusted from (Mattos et al., 2003). After that, they were treated with DHA, EPA, and LNA and challenged with 1 µg / mL of LPS (L2880, *E. coli* 055: B5 lipopolysaccharides, Sigma-Aldrich, LPS-challenged group) for another 24 h in those same concentrations. For that, explants were treated for 48 h (24 h with PUFAs and 24 h with PUFAs + LPS).

The explants remained in a humidified atmosphere with 5% CO2 at 37 °C, that is, for 48 h. They were only removed after 24 h to change the culture medium to challenge LPS. However, this process of switching media was done quickly. Supernatants were collected (stored at -20 °C), and explants were weighed for subsequent correlation of interleukin production as a function of tissue mass of each sample in picogram per milligram (pg/mg).

Cytokines accumulation from bovine endometrial explants supernatants

The concentrations of *IL6* (ESS0029, Bovine IL6 Reagent Kit, Thermo Scientific, Rockford, USA.; ESS0027) and I*L1B* (Bovine *IL-1β* ELISA Kit, Invitrogen, Vienna, Austria) were measured by ELISA according to the manufacturer's instructions.

First, the ELISA plates were sensitized with capture antibody diluted in Phosphate Buffered Saline (PBS) and kept at room temperature overnight. Next, the plates were washed three times with wash buffer (0.05 Tween™-20 Detergent in D-PBS, pH 7.4). Then, blocking was performed by adding the diluent reagent to each well and keeping the plates at room temperature for one hour. After this interval, the plates were rewashed three times with wash buffer.

The samples or standard solution were added to their respective wells, and then the plates remained at room temperature for 2 h. Subsequently, the plates were washed three times with a wash buffer. The detection antibody was diluted in the reagent diluent and was added to each well, keeping the plates at room temperature for another hour. After that, the plates were washed three times with wash buffer. Diluted StreptadirectP solution was added to each well, and the plates were left at room temperature for 30 min, protected from direct light incidence.

Afterward, the plates were washed three times with a wash buffer. Substrate solution was added to each well, and the plates were kept at room temperature for 20 min, protected from direct light. Then, stop solution (sulfuric acid) was added to each well, and the plates were carefully shaken. The plates were quickly transported, protected from light, to the SpectraMax® M2/M2e device (Molecular Devices Corporation, San Jose, CA, USA).

To consider differences between explants weights, cytokines concentrations were reported as pg/mg of tissue. Optical density was measured on the 450 and 550 nm filters on a SpectraMax M2/M2^e apparatus (Molecular Devices Corporation, San Jose, CA, USA) using the SoftMax Pro Software program (Molecular Devices Corporation). Once this was done, the values were transferred to Excel and corrected by subtracting the absorbance found in the 450 nm filter from the 550 nm filter and then analyzed by the MyAssays Analysis Software Solutions program

(https://www.myassays.com /home.aspx) to obtain the values referring to the production of interleukins in the analyzed supernatant (pg/mL).

The standard curve was generated for each ELISA plate, and samples were made in duplicate for *IL1B* and *IL6,* starting at 2,000 pg/mL and 6,000 pg/mL, respectively. The inter- and intraassay coefficients of variation were all < 10% and the detection limits were 12.5 pg/mL for *IL1B* and 75 pg/mL for I*L6*.

Statistical analysis

Data and graphics were performed by GraphPad Prism 9 statistical software (GraphPad Software, San Diego, CA, USA). Cows were considered an experimental unit. Data were tabulated initially in Excel spreadsheets, and descriptive statistics were presented as the mean and standard error of the mean (SEM).

Quantitative variables were subjected to the Kolmogorov-Smirnov test to verify whether or not they had a parametric distribution. Variables with parametric distribution were subjected to analysis of variance (parametric ANOVA) and Tukey's Multiple Comparison post-test. Variables with non-parametric distribution were analyzed using the Kruskall-Wallis test (non-parametric ANOVA) and Dunn's Multiple Comparison post-test. Statistical significance was established as $P \leq 0.05$.

Results and Discussion

A lack of studies is observed exploring the effects of n-3 PUFAs on bovine endometrial cells related to the immune system. Based on the large number of epidemiological studies that provide the effects in animals that consume diets rich in n-3 PUFAs, we decided to study the effects of n-3 PUFA *in vitro* by measuring the accumulation of cytokines *IL6* and *IL1B* as they have been directly implicated in the pathogenesis of uterine diseases in cows. In this study, we mainly explore n-3 long-chain PUFAs as they have been proposed to enhance innate immune function in dairy cows (Olmo et al., 2019).

An anti-inflammatory response was observed in this study when bovine *ex vivo* explants were treated with n-3 PUFAS and challenged or not with LPS. What made this study interesting was that even in non-stimulated bovine endometrial explants (not treated with LPS, control group), an anti-inflammatory effect of these acids was observed, which reaffirms the beneficial function of these acids even without an inflammatory process.

The incubation for 48 h with EPA from the control group ($P < 0.05$; Figure 1A) reduced the concentration of

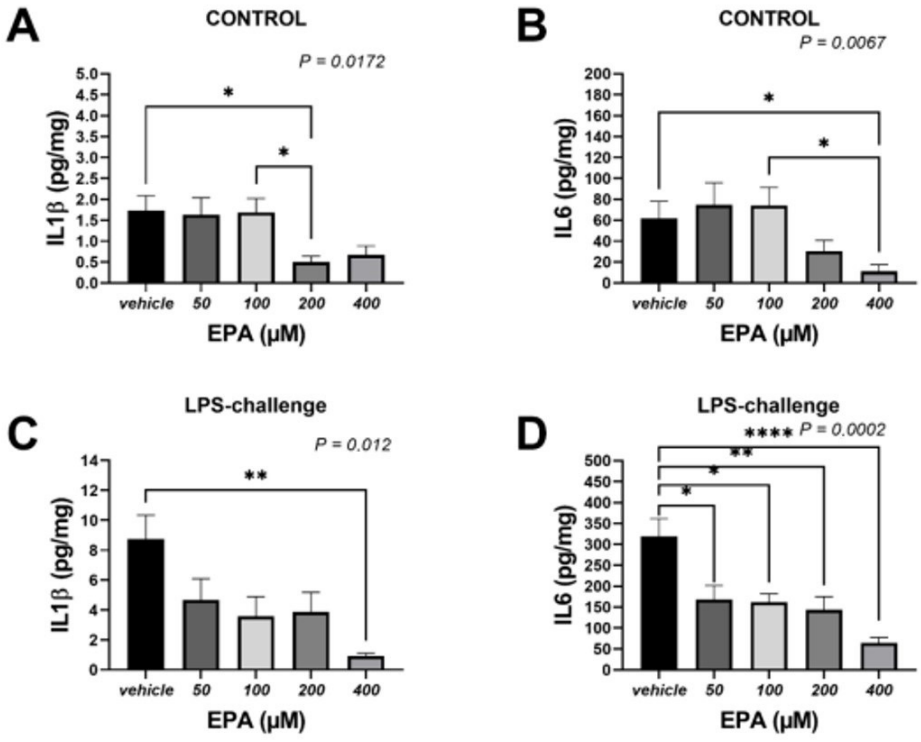

Figure 1 - Accumulation of IL-1β (A) and IL-6 (B) in cows *ex vivo* endometrial explants after being treated with eicosapentaenoic acid (EPA) in concentrations at 0 μM, 50 μM, 100 μM, 200 μM, and 400 μM challenge (LPS-challenge) or not (control) with heat-killed Gram-negative bacteria lipopolysaccharide (LPS at 1 μg/mL). Test for normal distribution (Kolmogorov-Smirnov test) and One-way ANOVA with Tukey's multiple comparisons tests (A, B, C, D). *P<0.05; **P<0.001;

IL1B in 200 μM and *IL6* in 400 μM (P < 0.05; Figure 1B). On the other hand, in the LPS-challenged group, bovine endometrial explants reduced the concentration only for *IL6* at 50 and 100 μM of EPA (P < 0.05; Figure 1D). In a study conducted by Penrod et al. (2013), they evaluated the treatment with 100 μM of EPA on equine endometrial explants in the release of PGF2α and PGE2, and they observed no effect of this acid on these cells. On the other hand, Yan et al. (2013) noticed that EPA suppressed the inflammation process by inhibiting NLRP3 inflammasome from reducing proinflammatory cytokines.

When immune mediators were explored in goat *in vitro* neutrophils, Pisani et al. (2009) found that treating these cells with EPA up-regulated the phagocytosis activity on these immune cells. Even though the phagocytic action was not explored in this experiment, it is essential to emphasize that using *in vitro* n-3 PUFAs for anti-inflammatory properties can act in other lines of animal immune cells' defense.

Omega-3 fatty acids, especially DHA, are likely to treat several diseases. Numerous animal and clinical studies indicated anti-inflammatory properties for DHA (Tortosa-Caparrós et al., 2017; Yates et al., 2014). In this study, the accumulation of *IL1β* was similar between all doses when DHA from the control group was measured (P >0.05; Figure 2A); in contrast, the collection of *IL6* decreased at 200 and 400 μM of DHA (P < 0.05; Figure 2B), on those endometrial explants incubated for 48 h with DHA (control group). Still, it was observed that 200μM on explants from the LPS-challenged group generated an an-inflammatory response for both cytokines (*IL1B* and *IL6*) (P < 0.05; Figures 2C and 2D).

In a study conducted by Parshyna et al. (2017), they evaluated DHA on stem cells and observed that this acid had anti-inflammatory properties by increasing *IL8* and decreasing *IL6* secretion. Also, in murine macrophages, DHA reduced tumor necrosis factor-alpha (TNFA), *IL6* monocyte chemoattractant protein-1, and *IL1B* production after an inflammasome activation (Oh et al., 2010; Williams-Bey et al., 2014).

According to He et al. (2017), DHA attenuated inflammatory response in primary bovine mammary epithelial cells; they found that DHA decreased RNA levels of TNFA, *IL6,* and *IL1B* in a dose-dependent manner when LPS-stimulated primary bovine mammary epithelial cells were explored.

Plewes et al. (2017) demonstrated that EPA and DHA have the same effects as fish oil, so a similar effect of both acids was expected in this experiment. However, it could be stated that EPA was more efficient in producing an anti-

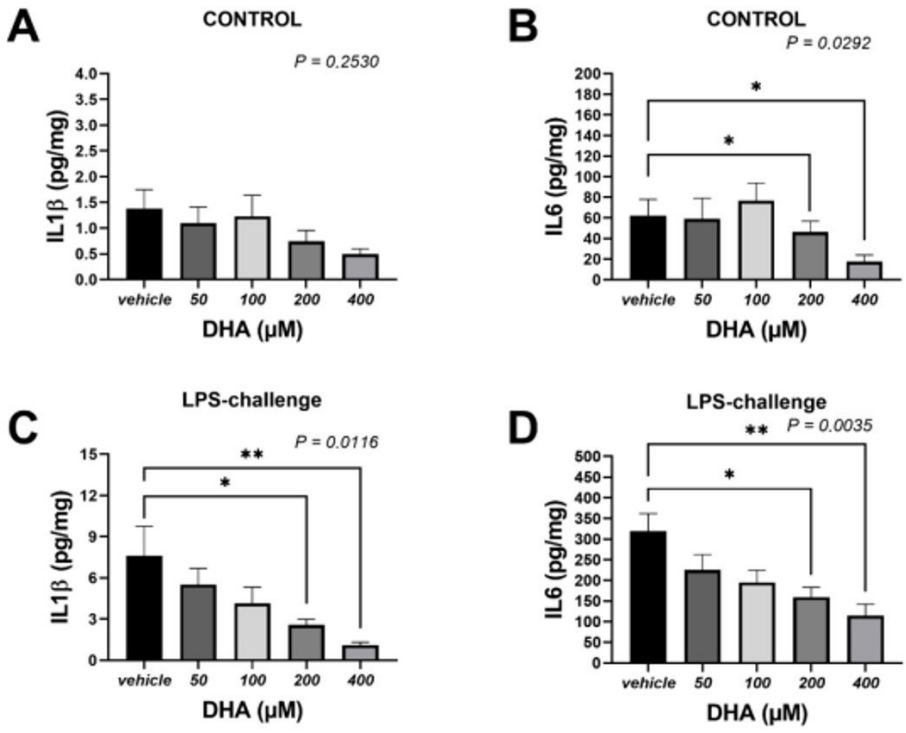

Figure 2 - Accumulation of IL-1β (A) and IL-6 (B) in cows *ex vivo* endometrial explants after being treated with docosahexaenoic acid (DHA) in concentrations at 0 μM, 50 μM, 100 μM, 200 μM, and 400 μM challenge (LPS-challenge) or not (control) with heat-killed Gram-negative bacteria lipopolysaccharide (LPS at 1 μg/mL).Test for normal distribution (Kolmogorov-Smirnov test) and One-way ANOVA with Tukey's multiple comparisons tests (A, B, C, D). *P<0.05; **P<0.001.

inflammatory effect. It could reduce the accumulation of both *IL6* and *IL1B* in explants from LPS-challenged or control groups, showing the beneficial effects of n-3 PUFA not only in inflammatory cells. Similarly, Mickleborough et al. (2009) evaluated the effects of EPA and DHA on eicosanoid and cytokine production (*IL1B* and TNFA) from LPS-stimulated human asthmatic alveolar macrophages. They indicated that EPA is a more potent inhibitor than DHA.

Quantities of DHA and EPA are found in marine products (fish oil) (Haag, 2003). However, alternatives to this oil have been studied to minimize fish oil dependence in aquaculture (Xie et al., 2017). A vegetable oil source is being explored as a substitute for fish oil. In contrast, vegetable oil sources lack long-chain PUFA but are rich in PUFA with 18 carbon atoms, known as LNA and linoleic acid (LA).

In this study, we tested LNA as an alternative and less expensive source of n-3 PUFA. An anti-inflammatory response was observed by LNA when bovine endometrial explants were challenged and not challenged with LPS, decreasing the accumulation of *IL1B* only at 400 µM. Interestingly, treatment of bovine endometrial explants for 48 h with LNA (control) and with LPS-challenged resulted in a lower concentration of *IL1B* at 400 µM ($P < 0.05$, Figures 3A and 3C) for both groups. Although, when *IL6* was evaluated, no difference was observed in its concentrations from those explants from LPS-challenged or control groups ($P > 0.05$, Figures 3B and 3D). We suggested that this may be because LNA is derived from a vegetable oil source, different from EPA and DHA, which are derived from fish oil sources. LNA may require higher concentrations to induce the anti-inflammatory effect of specific cytokines. The 400 µM of LNA was probably insufficient to stimulate the proinflammatory cytokines. In a study conducted by Chen et al. (2018), based on the intake of LNA by fish, they demonstrated that most parts of the LNA were bio-converted, being β-oxidized, which could be a possible explanation for the lower action of the LNA.

Mattos et al. (2003) evaluated the inhibitory effects of PUFAs (EPA, DHA, and LNA) on PGF2α secretion from bovine endometrial cells induced by stimulating protein kinase C with phorbol 12,13 dibutyrate. They observed that LNA was less inhibitory than DHA and EPA. These authors believed that as LNA is the precursor for the synthesis of DHA and EPA and can be converted to them in a process that depends on the activities of desaturase and elongase enzymes, it could be possible that some conversion of LNA to EPA and DHA during the period of incubation will result in a reduction of PGF2α secretion.

Presumably, pro-inflammatory cytokines secretion is regulated by a combination of different concentrations of

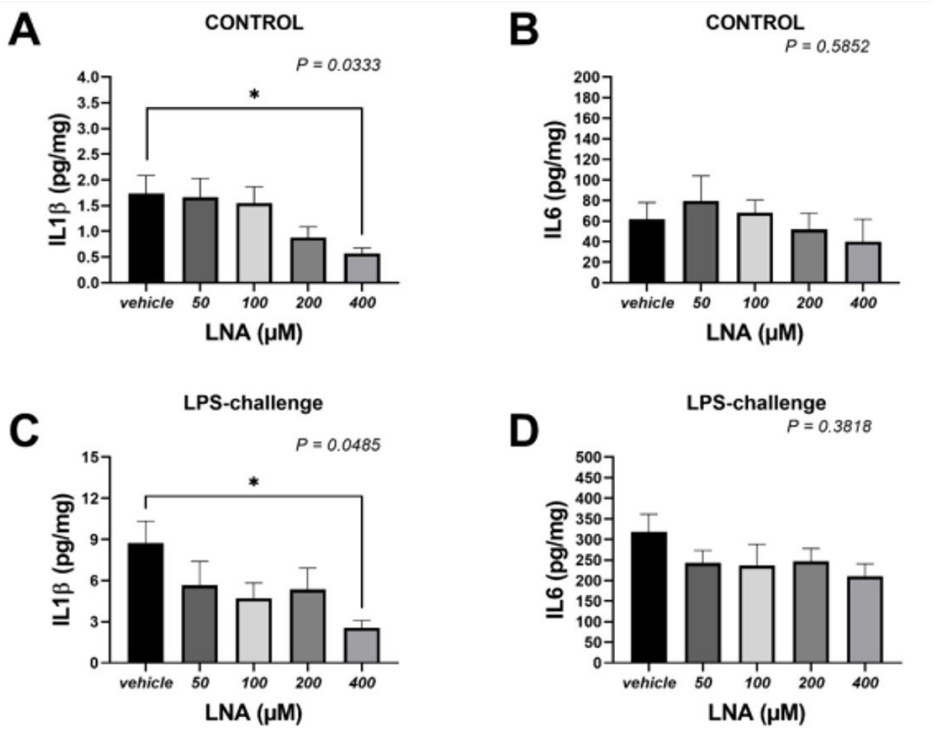

Figure 3 - Accumulation of IL-1β (A) and IL-6 (B) in cows' *ex vivo* endometrial explants after being treated with linolenic acid (LNA) in concentrations at 0 µM, 50 µM, 100 µM, 200 µM, and 400 µM challenge (LPS-challenge) or not (control) with heat-killed Gram-negative bacteria lipopolysaccharide (LPS at 1 µg/mL). Test for normal distribution (Kolmogorov-Smirnov test) and One-way ANOVA with Tukey's multiple comparisons tests (A, C, D) and Kruskal-Wallis test (B). *P<0.05.

EPA and DHA than by LNA as both EPA (Caughey et al., 1996) and DHA (Kelley et al., 1999) can efficiently decrease pro-inflammatory cytokine production. The few numbers of studies exploring a link between PUFAs and the immune system require further investigation. New studies are immediately needed to investigate the basic biology of n-3 PUFAs until their application in clinical and preventive fields to improve animal production systems worldwide.

Conclusion

Based on this experiment, we accepted our hypothesis that an *in vitro* inclusion of PUFAs in a cow's endometrium treated by lipopolysaccharide (LPS) can alter an anti-inflammatory response. Moreover, we also conclude that among the three different PUFAs evaluated, the EPA acid proves to be the best anti-inflammatory option to decrease the concentration of both pro-inflammatory cytokines (*IL1B* and *IL6*) from LPS-challenged and control groups in bovine endometrial explants; while LNA evidence to be the last option to promote an anti-inflammatory response, as it was capable only to reduce the accumulation of *IL1B* in higher concentrations (400 μM).

In vitro studies with PUFAs sources are essential for producing new supplements, especially with n-3, to control the inflammatory process in the postpartum period by activating immune mechanisms, which could impact uterine health.

Conflict of Interest

The authors declare no competing interests.

Ethics Statement

Not applicable.

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ERRATUM: The in vitro effects of n-3 fatty acids on immune response regulation of bovine ex vivo endometrial explants

Due to author's honest mistake the article "The in vitro effects of n-3 fatty acids on immune response regulation of bovine ex vivo endometrial explants" (DOI https://doi.org/10.11606/issn.1678-4456.bjvras.2023.202442), published in Brazilian Journal of Veterinary Research and Animal Science, 60, e202442, was published with errors.

On page 1, where the text reads:

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It should read:

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