



Effect of dietary n-3 polyunsaturated rich fish oil supplementation on ovarian function and interferon stimulated genes in the repeat breeding cow



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ARTICLE INFO

Keywords:

ISG
n-3 PUFA
Repeat breeding

ABSTRACT

Dietary n-3 polyunsaturated fatty acids (n-3 PUFA) improve utero-ovarian functions and embryonic survival in postpartum dairy cows. Because early embryonic mortality is the major cause of repeat breeding (RB) in cows, there was investigation of the effect of dietary supplementation of n-3 PUFA [eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)] rich fish oil (FO) from -2 to +2 weeks of artificial insemination on the size of preovulatory follicle (POF), serum progesterone (P₄) and relative abundance of the mRNA of interferon stimulated genes (ISG) that encode for these proteins in the peripheral blood leukocytes (PBL) in the RB cow ($n = 12$). The diet of control group was supplemented with palm oil (PO). The results indicated serum concentrations of EPA and DHA were greater by 4.6- and 3.5-fold, respectively at the end of feeding study in the RB cows of the FO group. The diameter of POF was larger by 2.2 mm in FO group; however, serum P₄ did not vary from day 14–20 post-artificial insemination. Greater abundance of ISG mRNA transcripts such as *ISG15*, *RTP4*, *Mx2* and *OAS1* in the PBL of pregnant cows of FO group indicates day 20 conceptuses produced more IFN- τ . It is concluded that supplementation of FO during the breeding period increased the size of POF and enhanced the abundance of ISG mRNA transcripts in RB cows that became pregnant.

1. Introduction

Genetic selection for high milk production has been associated with a decrease in reproductive efficiency in lactating dairy cows (Butler, 2003). At present, repeat breeding (RB) is the second most prevalent reproductive problem after postpartum anestrus in the high milk production dairy cow. The repeat breeding results in economic loss for the dairy producer due to more inseminations, an

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<https://doi.org/10.1016/j.anireprosci.2019.106230>

Received 2 May 2019; Received in revised form 24 August 2019; Accepted 6 November 2019

Available online 07 November 2019

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extended calving interval and increased cow culling rates from the herd (Bartlett et al., 1986; Lafi et al., 1992). The etiology of RB is broadly classified into fertilization failure or early embryonic mortality (EEM). There is consensus that in cows with normal fertility, the incidence of fertilization failure is about 10% whereas EEM within 3 weeks following fertilization accounts for about 30% of the fertility problems (Sreenan et al., 1986). Effects of embryonic mortality contribute to a lesser pregnancy rate after breeding and reduced litter size in polytocous species. The maintenance of the corpus luteum and its production of progesterone (P_4) depend on the temporal production of interferon-tau (IFN- τ) from the trophoectoderm of the elongating conceptus in the ruminants. Compromised anti-luteolytic effects of IFN- τ are a major cause of reproductive wastage in cattle (Robinson et al., 2008).

In addition to increasing energy stores, fatty acids (FA) affects PG production, steroidogenesis, maintenance of cell membrane properties and cholesterol metabolism of animals (Abayasekara and Wathes, 1999; Jump and Clarke, 1999; Cheng, 2004; Leroy et al., 2005). Results from isocaloric dietary studies indicate dietary supplementation of n-3 polyunsaturated fatty acids (PUFA) has anti-luteolytic effect by inhibiting the pulsatile release of $PGF_{2\alpha}$ from the endometrium in the food-producing animals (Gulliver et al., 2012) and improves embryonic survival (Diskin and Morris, 2008). Feeding alpha linolenic acid rich flax seed for 28 days before breeding led to an increase in the diameter of preovulatory follicle (POF) and reduction in pregnancy losses in Holstein cows (Ambrose et al., 2006). Feeding n-3 or n-6 PUFA rich diets altered the FA composition of granulosa cells and oocytes in ewes (Wonnacott et al., 2010). A relatively larger proportion of saturated FA in the oocyte is one of the reasons for a relatively lesser pregnancy rate in cows during the summer as compared with other times of the year (Zeron et al., 2001). Imposing management regimens that result in altering of the membrane composition of the oocyte, therefore, is a potential way to reduce the embryo mortality in cows (Hansen, 2002). In contrast, feeding rumen protected n-3 PUFA did not improve the embryo quality following superovulation in beef heifers (Childs et al., 2008a). This may be due to dose dependent effect of DHA on oocyte quality and steroid metabolism of cumulus cells (Osekria et al., 2016).

Fish oil (FO) supplementation from day 30–160 postpartum reduced the pregnancy loss at first artificial insemination and improved the overall pregnancy rate (Silvestre et al., 2011). Recently, Sinedino et al. (2017) reported that feeding DHA rich marine algae from 27 to 147 days postpartum upregulated IFN stimulated genes (ISG) in the peripheral blood leukocytes (PBL), and increased the pregnancy rate in primiparous and multiparous cows, which was attributed to enhanced development of conceptus with increased production of IFN- τ . Further, the conceptus of cattle utilizes FA for growth and genes related to FA metabolism are upregulated during early gestation (Ribeiro et al., 2016a, 2016b). It, therefore, was hypothesized that dietary supplementation of EPA and DHA rich fish oil would modulate the ovarian function and the expression of ISG in the PBL in RB cows.

2. Materials and methods

2.1. Experimental animals

The study was conducted with crossbred (Haryana \times Holstein Friesian/Brown Swiss/Jersey) RB cows maintained at Livestock Production and Management Section, Indian Veterinary Research Institute, Izatnagar, which is located at an altitude of 564 feet above the mean sea level, at latitude of 28 °N and a longitude of 79 °E. Agro-ecologically, the experimental site is located in north-western plains of Uttar Pradesh having hot humid sub-tropical climate. The animal experimentation was conducted from September 2017 to April 2018 during which period, the mean temperature was 26.3 °C (Range 13 to 30 °C) and mean relative humidity was 69.4 % (Range 45–80%). The experimental RB cows ($n = 24$) were 3–8 years of age; 400 ± 50 kg mean (\pm SEM) body weight and were in parity one to four. At the time of enrollment in the study, the milk yield of the RB cows ranged from 9.5 to 12.0 L/day. During the experiment, there was imposing of an intensive production management regimen; concentrate feed was offered twice a day with access to wheat straw, green fodder and water *ad libitum*. Milking was conducted twice a day at around 4 am/pm. Assessment of the reproductive data sheet resulted in the determination that RB animals had a median artificial insemination number of four (least and greatest quartile ranges were three and five) at the time of assignment to groups in the present feeding study. Reproductive ultrasonography indicated the RB cows did not have any confounding conditions such as ovarian cysts or ovaro-bursal adhesions. Results from assessing samples collected using the endometrial cytology cytobrush technique indicated the animals did not have subclinical endometritis. The experiment was approved by the Institute Animal Ethics Committee, IVRI, Izatnagar.

2.2. Diagnosis of subclinical endometritis

Endometrial samples for cytologic examination were collected using the cytobrush technique with minor modifications. At least 200 cells were counted (Supplementary Fig.1) and the threshold value for diagnosing subclinical endometritis was fixed at 10 % polymorphonuclear leukocytes using procedures that were previously described (Kasimanickam et al., 2005).

2.3. Experimental diets and feeding regimen

The RB cows were randomly assigned to a FO supplemented treatment group ($n = 12$) and palm oil (PO) supplemented control group ($n = 12$). The cows of FO group were supplemented with FO (Avestia Pharma, Mumbai, India) at 0.6 ml/kg body weight to provide 156 mg EPA and DHA per kg body weight. The dose of FO was extrapolated from the results of a dose-response study in goats (Verma et al., 2018). The FO was extracted from deep sea sardine fish (*Sardinella longiceps*) and had EPA and DHA 16.21 % and 10.15 %, respectively. The control group was supplemented with PO (Raag®, Adani Wilmar limited, Ahmedabad, India) at the same dose (0.6 ml/kg body weight) so that the diets were isocaloric. The FO and PO was from the same batch used in a previous study (Mahla

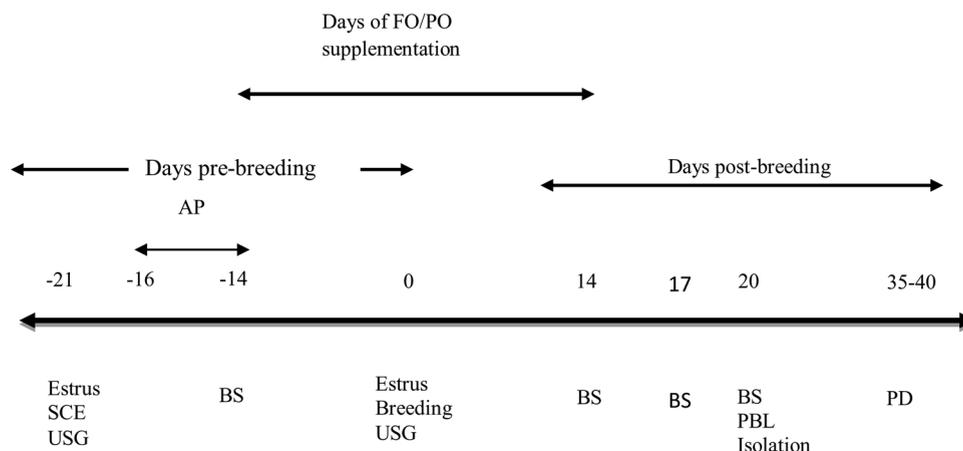


Fig. 1. Depiction of feeding regimen, collection of samples, ultrasonography and pregnancy diagnosis: PBL = Peripheral blood leukocytes; AI = Artificial Insemination; SCE = Subclinical endometritis; USG = ultrasonography; BS = Blood sampling; FO = Fish oil; PO = Palm oil; AP = Acclimatization period; PD = Pregnancy Diagnosis.

et al., 2017; Verma et al., 2018) and the composition is provided in Supplementary Table 1. Before the commencement of actual feeding regimen, there was an acclimatization period of 2 days during which respective oils were supplemented to allow for animals to adjust to the experimental diet. The diet was supplemented with n-3 PUFA rich FO or PO for about 4 weeks (2 weeks before expected date of breeding and 2-weeks post-artificial insemination) by direct drenching to prevent feed refusal. The schematic presentation of the feeding regimen and sampling schedule is depicted in Fig. 1. The RB cows were evaluated for symptoms of estrus twice a day (0600 and 1800 h) using a vasectomized “teaser” bull accompanied by a trained stockman for 30 min. Cows in estrus were bred twice at 12 h interval by artificial insemination using semen of a bull with proven fertility.

2.4. Ultrasonographic examination

A real time ultrasonic device with a linear array transducer of 7.5 MHz frequency connected to B-Mode high resolution real-time echo camera (Exago, France) was used for the trans-rectal ultrasonography examination of ovary and uterus. The ultrasonography assessments were conducted on the day of breeding to determine the follicle with largest size, which was considered to be the POF. Confirmatory diagnosis of pregnancy was conducted on day 35–40 post-breeding using trans-rectal ultrasonography of the uterus (Supplementary Fig. 2 and 3).

2.5. Blood sampling

Blood samples were collected for the assay of serum concentrations of EPA, DHA, and P_4 on day -14 (commencement of feeding) followed by day 14, 17 and 20 post-breeding by jugular venipuncture in vacutainers (SSTTM II Advance, Becton Dickinson Vacutainer Systems, Plymouth, UK) and centrifuged at 800 g for 15 min. Serum was collected and stored at -20°C until assay. For estimating the concentration of EPA and DHA, the sera collected on day -14 and day 14 post-breeding (end of feeding trial) were used. The sera samples, within a group, were pooled such that the sample size was three per group. Concentration of EPA and DHA was estimated using gas chromatography. Briefly, fatty acid methyl esters were prepared from lipids present in supplemented oils and serum samples. Oil samples were methylated by direct trans-esterification using the method of O’Fallon et al. (2007). The fatty acid methyl esters were separated using gas chromatography (450-GC; Bruker, Billerica, MA, USA) equipped with SGE Forte GC capillary column ($60\text{ m} \times 0.25\text{ mm} \times 70\text{ }\mu\text{m}$ -BPX70). Serum P_4 was quantified on day 14, 17 and 20 post-breeding using a radioimmunoassay (Immunotech, France). Analytical sensitivity for the detection of P_4 was $< 0.03\text{ ng/mL}$. Coefficients of intra and inter assay variation were $< 8.15\%$ and $< 8.66\%$, respectively.

2.6. RNA extraction and cDNA synthesis

Blood was collected by jugular venipuncture in EDTA coated vacutainers on day 20 post-breeding and PBL were isolated using density gradient technique (Histopaque-1077, Sigma life sciences). Total RNA was isolated from PBL pellet using Trizol reagent (Qiagen, Germany) as per the manufacturer’s instructions. First strand cDNA was synthesized using the reverse transcription kit according to manufacturer’s instructions (Qiagen, Germany). Total RNA ($1\text{ }\mu\text{g}$) from each sample was reverse transcribed into cDNA using random hexamers. The cDNA was quantified by an absorbance of 260 nm and stored at -80°C for subsequent analysis. Based on the results of pregnancy diagnosis on day 35 post-breeding, cDNA samples from 12 pregnant cows ($n = 6/\text{group}$) were used for real-time quantitation of ISG.

Table 1

Oligonucleotide primers used to amplify the interferon stimulated genes in the peripheral blood leukocytes of experimental repeat breeding cows.

Gene	Sequence 5'-3'	Amplicon length	Annealing Temp (°C)	Accession No.
<i>ISG 15</i>	Forward: GAAGATCAATGTGCCTGCITTC Reverse: CAGGATGGAGATGCAGTTCT	145	60	NM_174366.1
<i>OAS1</i>	Forward: GTTGCTGGTAAAGACGCAAATAG Reverse: GGAGACCCATCCCATTTCTTAAA	95	60	AY243505
<i>RTP4</i>	Forward: CAAGCCTCAGAGCAAGAGAATA Reverse: CATCCATCTTCAGGGTCCATC	110	60	NM-001075961
<i>Mx2</i>	Forward: GTCATGCAGAACCTCACCTATC Reverse: TTCITTCCTGGTTGCCTCTG	111	58	NM-173941.2
<i>ACTB</i>	Forward: CTGGACTTCGAGCAGGAGAT Reverse: GATGTCGACGTACACTTC	207	59	AY141970.1

2.7. qPCR for IFN stimulated genes (ISG)

Primers were designed for ISG such as *ISG15*, *Mx2*, *RTP4* and *OAS1* of cattle (Table 1) using the integrated DNA technology (IDT, <https://eu.idtdna.com/Primerquest/> Home/Index) and synthesized commercially (Eurofin Genomics, Bangalore, India). Annealing temperatures of the different primers were optimized using gradient thermal cycler PCR (Agilent Technologies, USA). The cDNA of target genes was amplified in triplicate using Quantifast™ SYBR® Green PCR Master Mix (Qiagen, Germany) in a Real-time qPCR instrument (BIORAD -CFX96 TOUCH™). Briefly, PCR templates containing 10 ng (1 µL) reverse transcribed total RNA (cDNA) were added to 0.2 µL forward and reverse primers (10 pmol each), and 5 µL 2X SYBR Green Master Mix to a final volume of 10.0 µL. The specificity of the primers was confirmed by amplification plot and dissociation curve assessments. The β -actin (*ACTB*) served as endogenous reference gene to calculate Δ Ct for each target gene and six samples from non-pregnant cows (three each from FO and PO) were used as calibrator to determine the relative fold change in abundance of mRNA transcripts (Livak and Schmittgen, 2001). The mean Ct value of *ACTB* in non-pregnant and pregnant cows of FO or PO did not vary ($P = 0.159$) and the intra-assay CV% of the target genes were less than 3.5 % (Supplementary Table 2 and 3).

2.8. Statistical analysis

Concentration of serum EPA, DHA and P₄ was analyzed using a mixed model ANOVA utilizing Proc Mixed procedure of SAS software with terms for group, time and the interactions included in the model. An independent *t*-test with an unbalanced/unequal sample size was used to compare the mean difference of POF size between the groups on the day of estrus. Relative fold change of ISG mRNA transcripts in the PBL of non-pregnant and pregnant (FO and PO) cows was analyzed using a one-way ANOVA with Bonferroni *post-hoc* test. Conception rate was analyzed using the Fisher's exact chi-square test. Results were expressed as mean \pm SE for the metric variables. Significance was set at 95 %. Line and bar charts were prepared using GraphPad Prism 5.0.

3. Results

The concentration of serum EPA and DHA was relatively greater ($P < 0.05$) in the FO- than that of PO- supplemented RB cows at the end of the feeding study. The increase in the serum EPA and DHA was by 4.6- and 3.6-fold, respectively, in the FO group on day 14 post breeding as compared with pre-feeding and PO group values (Fig. 2). The size of POF was larger by 2.2 mm in the FO than that of PO group (Fig. 3; $P = 0.007$). The mean concentration of serum P₄ did not differ between the two groups across the days of

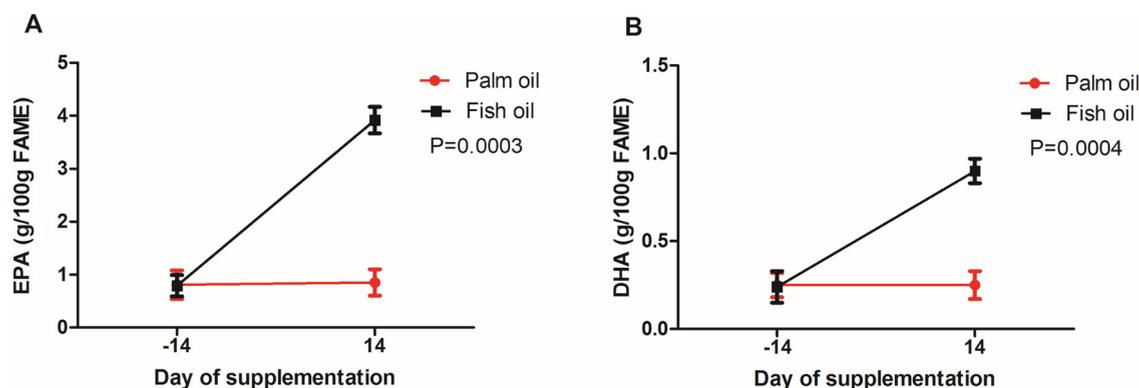


Fig. 2. Effect of dietary FO or PO supplementation on serum EPA (a) and DHA (b) pre and post- feeding in the repeat breeder cow (g/100 g fatty acid); Sera in each group was pooled such that the sample size was $n =$ three/treatment for fatty acid profile by gas chromatography.

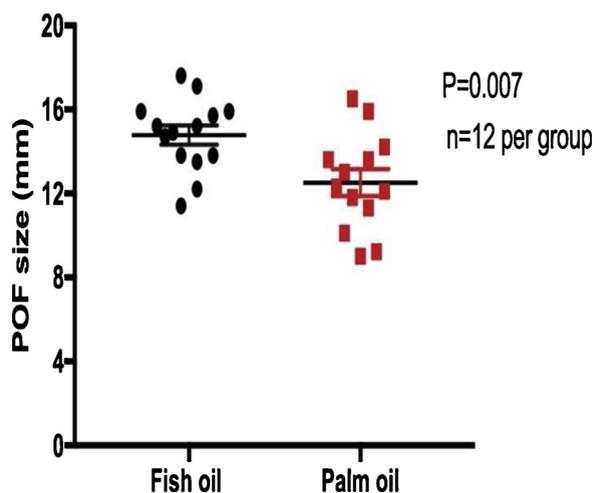


Fig. 3. Effect of dietary supplementation of n-3 PUFA rich fish oil on the diameter of POF in the repeat breeding cow ($n = 12$) in each group; $P = 0.007$.

sampling ($P = 0.25$). There was no effect of day ($P = 0.14$) and interaction on mean concentration of serum P_4 in the RB cows that became pregnant (Fig. 4a). As expected, there was a lesser ($P < 0.001$) serum P_4 concentration on day 20 post-breeding than those of other days in RB cows that failed to conceive (Fig. 4b). The conception rate (%) was 75 (9/12) in the FO group, while it was 58.3 (7/12) in the PO group; however, the difference was non-significant which is likely due to the small sample size.

Treatment affected ($P < 0.05$) the relative abundance of all ISG mRNA transcripts, which was greater in PBL of pregnant cows fed FO than those of PO group. There was a greater relative abundance of the mRNA transcripts of the *ISG15* gene by 5.6-fold in the FO- than that of PO-pregnant cows ($P < 0.05$; Fig. 5a). The relative abundance of the *Mx2* mRNA transcripts was nearly 8.81-fold greater ($P < 0.05$) in the cows of FO than PO group (2.9-fold change, Fig. 5b). The fold change of mRNA transcripts of the *RTP4* and *OAS1* genes in the pregnant cows fed FO was 7.92 and 6.91 as compared to 2.68 and 2.33 in the pregnant cows fed PO, respectively (Fig. 5c-d).

4. Discussion

Studies indicate that dietary supplementation of either saturated or unsaturated fat enhance milk production and fertility, which is likely due to minimizing the adverse effect of negative energy balance. Results from studies where there were isocaloric diets fed clearly indicate that apart from energy mediated positive effects, n-3 and n-6 PUFAs have specific effects on many reproductive processes (Staples et al., 1998; Abayasekara and Wathes, 1999; Mattos et al., 2004). Because the feeding of dietary n-3 PUFAs is reported to improve fertility as a result of enhanced embryonic survival (Ambrose et al., 2006; Wathes et al., 2007; Santos et al., 2008), there was investigation of the effect of n-3 PUFA rich FO supplementation in the RB cow in the present study. The EPA and DHA rich FO were selected because of greater bioavailability than that of short chain alpha linolenic acid (Ashes et al., 1992; Deckelbaum and Torrejon, 2012). To ensure the specific effects of long chain n-3 PUFA, an isocaloric experimental design was used. To the best of our knowledge, this is the first investigation on the effect of dietary FO on the ovarian function and ISG gene expression, as indicated by relative abundance of mRNA transcript, in the PBL of the RB cow.

The feeding of FO resulted in an increase in the serum EPA and DHA by 4.6- and 3.6-fold, respectively, on day 14 of the

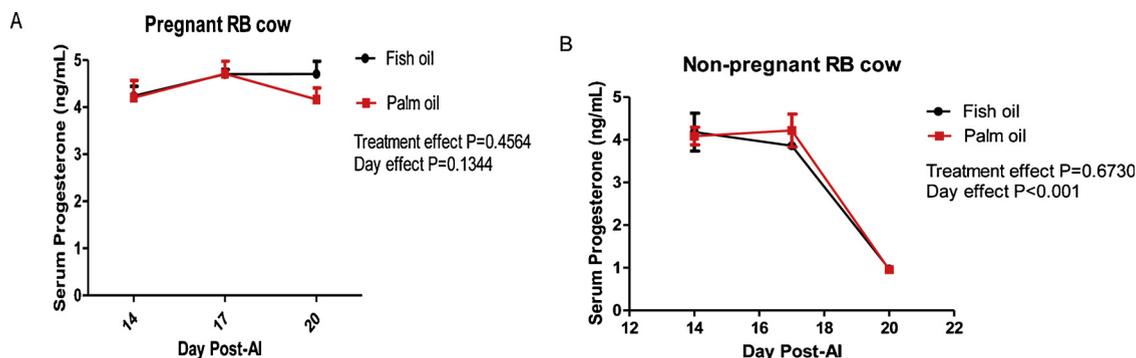


Fig. 4. Effect of dietary n-3 PUFA rich fish oil supplementation on serum progesterone concentration (ng/mL) in the pregnant ($n = 6$) (A) and non-pregnant ($n = 3$) (B) repeat breeding cow.

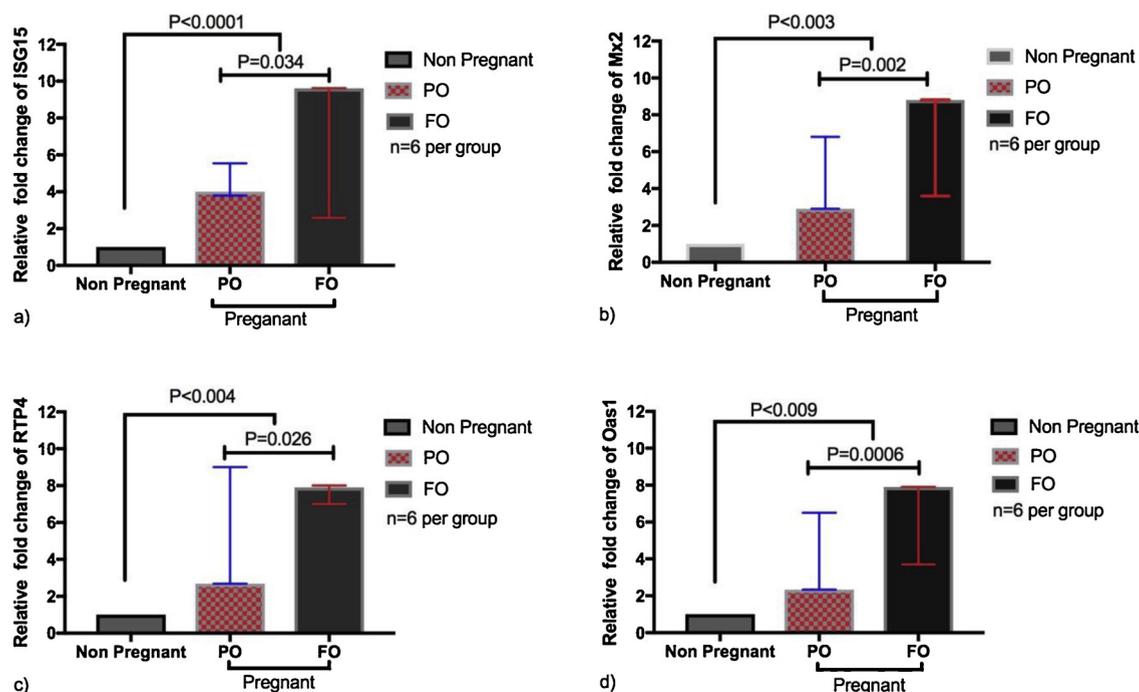


Fig. 5. Effect of dietary FO or PO supplementation on the relative fold change of abundance of interferon stimulated mRNA transcripts (ISG) in peripheral blood leukocytes of pregnant repeat breeding cows on day 20 post-breeding; a) *ISG15*, b) *Mx2*, c) *RTP4* and, d) *OAS1*; Error bars represent the confidence interval (CI) of normalized relative abundances generated from the least and greatest 95 % CI obtained for delta CT least-squares means differences; Mean difference were considered to have occurred $P < 0.05$.

experiment compared with pre-feeding and PO group values. There was an increase in the concentration of serum EPA and DHA in the present study that is consistent with results from previous studies in cows fed either FO (Mattos et al., 2004; Childs et al., 2008b) or fish meal (Burns et al., 2003). Long chain n-3 PUFAs such as EPA and DHA undergo minimum biohydrogenation (Ashes et al., 1992). Feeding protected FO increased plasma EPA (C20:5 n-3) and DHA (C22:6 n-3) in Holstein cows (Elis et al., 2016). The relative fold increase of EPA and DHA in the serum was less in the present study, which could in part, be attributed to the short duration of feeding as compared to what occurred in other studies. Feeding calcium salts of long chain PUFA increase the bioavailability (Silvestre et al., 2011). Collectively, the results of the present study support the thought that feeding protected or unprotected long chain n-3 PUFA can increase the serum EPA and DHA and alter the fatty acid profile in different tissues. A greater increase in the diameter of the POF in the FO group than that of PO group in the present study is consistent with the results from previous studies in dairy cattle supplemented with n-3 PUFA rich diets (Ponter et al., 2006; Ambrose et al., 2006; Dirandeh et al., 2013). Dietary supplementation of protected FO resulted in an increase in the number, as well as size, of large follicles in dairy cows (Elis et al., 2016). In contrast, there was no effect of n-3 PUFA on POF size following supplementation of DHA rich algae (Sinedino et al., 2017) or FO (Moallem, 2018) in cows or linseed in ewe diets (Wonnacott et al., 2010). Inconsistency among the findings on follicular growth may be attributable to differences between studies in the amounts of supplements, stage of lactation, metabolic status and sample size.

There was no difference in the serum P_4 concentration between the two groups among the days of sampling in the present study. This finding is consistent with those from previous studies which indicate there was no change in luteal P_4 concentrations following supplementation of n-3 PUFA rich flaxseed (Ponter et al., 2006), fish meal (Mattos et al., 2004; Wamsley et al., 2005) and FO (Childs et al., 2008b). In contrast, the greater concentration of P_4 in cows supplemented with flaxseed (Dirandeh et al., 2013) might be due to reduced clearance of P_4 from plasma rather than increased uptake of cholesterol and synthesis by luteal cells in cows fed Ca-LCFA (Hawkins et al., 1995). Greater plasma concentrations of EPA have been associated with increased peroxisome proliferator-activated receptors (MacLaren et al., 2006), that may result in reduced P_4 clearance (Galbreath et al., 2008).

During gestation day 13–15, IFN- τ stimulates the expression, as indicated by an increased abundance of mRNA transcripts, of a large number of ISGs in the endometrium of cattle (Gray et al., 2006; Chen et al., 2007). It has been hypothesized that the proteins encoded by these genes regulate the uterine receptivity for fetal attachment and conceptus development; however, the functional importance of expression of these genes in PBL is not clear (Ott and Gifford, 2010). Yankey and Kodali (2001) was the first to report results that during pregnancy there was an enhanced ISG expression, as indicated by an increased abundance of mRNA transcript, in the PBL. The relative abundance of *Mx1* mRNA transcripts in the PBL increased as early as day 13 and remained at these greater abundances to day 30 of pregnancy in sheep. Similarly, it was reported that pregnancy could be identified on day 18–20 in dairy cows by determining the abundance of mRNA transcripts for *ISG15*, *Mx1*, and *Mx2* in PBL (Gifford et al., 2007; Stevenson et al., 2007). Results of other studies also indicate that the *Mx2*, *ISG15* and *OAS1* genes were up-regulated in pregnant compared with estrous

cyclic heifers and cows (Stevenson et al., 2007; Green et al., 2010). In the present study, there was a greater abundance of *ISG15*, *Mx2*, *RTP4* and *OAS1* mRNA transcript, indicating there was an upregulation of these genes, in the FO group on day 20 of pregnancy indicating a positive correlation between IFN- τ production by the trophoblast and ISG in the PBL (Matsuyama et al., 2012) and a more advanced development of the conceptus (Ribeiro et al., 2014). The increase in relative abundance of ISG mRNA in the leukocytes of FO cows indicates that the day 20 conceptus was in a more advanced developmental stage and produced greater amounts of IFN- τ . The positive effect of n-3 PUFA on conception rate is likely due to a direct effect of n-3 PUFA on embryo development and an inhibitory effect on endometrial PGF_{2 α} production (Moallem, 2018). Sinedino et al. (2017) supplemented cows with an algae product containing 10 % DHA. The feeding started at 27 \pm 5 days postpartum and lasted 120 days. The authors reported that there was an upregulation of *RTP4* in cows supplemented with DHA, which is consistent with the findings in the present study. In contrast, *ISG15* expression, as indicated by abundance of mRNA, was not affected by n-3 PUFA supplementation in the DHA-supplemented cows (Sinedino et al., 2017). To the best of our knowledge, the present study is the first on the modulation of ISG expression, as indicated by abundance of mRNA transcript, by n-3 PUFA rich FO in the RB cow. Even though there was an apparent upregulation of the ISG, the conception rate did not differ between the groups, which could be explained by the small sample size with a consequent lesser-than-desirable statistical power.

In summary, results of the present study indicate that feeding n-3 rich PUFA rich diet around the time of breeding resulted in an increased size of POF and upregulated ISG expression, as indicated by abundance of mRNA transcript, in the RB cows that became pregnant. The effect on conception rate warrants further investigation.

Declaration of Competing Interest

The authors have no conflict of interest to declare.

Acknowledgements

The authors thank the Director, ICAR-IVRI for facilitating the study. We would like to thank Dr. Shahid Hussain, Dr. Mohd. Mashooq and Dr. Waseem Akram for help with qPCR. The work was supported by an intramural grant (Project code: IXX13792) to Dr. Harendra Kumar and ICAR-Junior Research Fellowship to Dr. Aamir S Teeli.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.anireprosci.2019.106230>.

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