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Preconception Omega-3 Fatty Acid Supplementation of Adult Male Mice with a History of Developmental TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) Exposure Prevents Preterm Birth in Unexposed Female Partners

Melinda E. McConaha[1], **Tianbing Ding**[1], **John A. Lucas**[2], **Joe A. Arosh**[3], **Kevin G. Osteen**[1], and **Kaylon L. Bruner-Tran**[1]

[1]Women's Reproductive Health Research Center, Vanderbilt University School of Medicine, Nashville, TN 37232

[2]Division of Reproductive Endocrinology and Infertility, Department of Obstetrics and Gynecology, Vanderbilt University School of Medicine, Nashville, TN 37232

[3]Department of Veterinary Integrative Biosciences, Texas A and M University College of Veterinary Medicine, College Station, TX 77843

Abstract

We recently reported that adult male C57BL/6 mice exposed in utero to the environmental toxicant TCDD confer an increased risk of preterm birth (PTB) to unexposed females. Risk of PTB was coincident with decreased placental progesterone receptor (PR) mRNA expression and increased toll-like receptor-4 (TLR-4) mRNA expression, suggesting toxicant exposure induced a heightened inflammatory response at the maternal-fetal interface. Since omega-3 fatty acids exhibit anti-inflammatory activity, herein, we provided TCDD-exposed males a fish oil-enriched diet prior to mating. Although PTB was common in control females mated to TCDD-exposed males on the standard diet, fish oil supplementation of TCDD-exposed males *eliminated* PTB in unexposed partners. We also determined the influence of preconception, paternal fish oil supplementation on the placental inflammatory response in late pregnancy (E18.5) by examining expression of PR and TLR-4 mRNA as well as expression of 15-hydroxy prostaglandin dehydrogenase (PGDH). PGDH catabolizes the inflammatory PGE2 to an inactive form; thus, reduced expression of this enzyme would promote tissue inflammation. Compared to control pregnancies, examination of E18.5 placentas arising from TCDD-exposed males on the standard diet revealed a significant increase in TLR-4 mRNA expression corresponding to a reduction in PR mRNA and PGDH protein expression. In contrast, fish oil supplementation of toxicant-exposed males led to normalization of placental expression of both PR and TLR-4 mRNA and a marked increase in PGDH expression. These studies suggest that a *paternal* preconception diet which includes omega-3 fatty acids prevents the toxicant-associated increase in the placental inflammatory response at late gestation, preventing PTB.

To whom correspondence and reprint requests should be addressed: Kaylon L. Bruner-Tran, PhD, Women' s Reproductive Health Research Center, Department of Obstetrics and Gynecology, Vanderbilt University School of Medicine, 1161 21st Ave S MCN B-1100, Nashville TN 37232, Phone: 615-322-4196, Fax: 615-343-7913, Kaylon.bruner-tran@vanderbilt.edu.

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Introduction

Despite significant medical advances within the reproductive sciences, preterm birth (PTB), occurring before 37 weeks gestation, remains the leading cause of perinatal mortality and morbidity in industrialized nations (Kramer *et al.* 2000). Although chorioamnionitis due to bacterial or viral infection is a well-recognized cause of early human parturition (Challis *et al.* 2009), our understanding of other causes of PTB remains limited and the incidence of this condition continues to increase. Nevertheless, regardless of the origin, inappropriate or uncontrolled inflammation is clearly a significant contributor to the initiation of early parturition. Importantly, normal nidation is also an inflammatory event; however, inflammation is well-controlled and localized to the site of embryo implantation by maternal progesterone which acts to inhibit a generalized expression of inflammatory cytokines at the maternal-fetal interface. Although the precise mechanisms by which progesterone limits inflammatory events associated with pregnancy are not fully known, Su *et al.* (2009) recently demonstrated that the immunosuppressive effects of this steroid were related to the suppression of toll-like receptor-4 (TLR-4), resulting in a reduced inflammatory response. During late human pregnancy, progesterone dominance gradually subsides, eventually permitting the onset of an inflammatory cascade and parturition (Challis *et al.* 2009). Similarly, numerous studies in mice demonstrate that parturition in this species is also an inflammatory event, preceded by a disruption in progesterone action at the placental-decidual interface (reviewed by Mendelson 2009). Therefore, in either women or mice, impaired progesterone action prior to the end of pregnancy has been associated with PTB (Mendelson 2009).

Equally critical in regulating the timing of human and murine parturition is the synthesis and metabolism of the prostaglandins (PGs), inflammatory agents which stimulate uterine contractions and cervical ripening (Challis *et al*, 2002). Both PGE2 and PGF2α are produced by maternal and fetal tissues during late pregnancy, with their concentrations increasing prior to and during labor (Wang & Hirsch, 2003). Within the placenta and fetal membranes, biosynthesis of these PGs is governed by cyclooxygenases 1 and 2 (COX-1 and COX-2) and PGE/PGF synthases (Smith and Song, 2002). Importantly, these tissues also produce 15-hydroxyprostaglandin dehydrogenase (PGDH), the enzyme which catabolizes PGE2 and PGF2α to their inactive forms (Tai *et al*, 2002); thereby preventing active PGs from reaching the myometrium prior to the onset of labor. In contrast, levels of PGDH decline at the end of pregnancy; thereby permitting the transport of active PGs to the myometrium which promotes uterine contractions and cervical ripening (Myatt and Sun, 2010). Roizen *et al*, (2008) demonstrated the significance of this regulatory system using mice genetically altered to produce reduced expression of PGDH. As expected, PTB was common in pregnancies in which one or both parents were hypomorphic for PGDH expression.

Across many species, inflammation associated with infection can influence the timing of birth as a result of altered progesterone synthesis and action, resulting in inappropriate expression of PGs (Giannoulias *et al.* 2005; Challis *et al.* 2009; Bruner-Tran & Osteen, 2011); however, little is known regarding the role environmental toxicants may play in promoting non-microbial, intra-uterine inflammation which would also disrupt maintenance of pregnancy. Among the ubiquitous toxicants present within our environment is the known endocrine disruptor TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin or dioxin), which has been linked to reproductive failure in acutely exposed women (Sharara *et al.* 1998). Since recent studies suggest that early life toxicant exposure may alter adult reproductive function (Miller *et al.* 2004), we established a murine model of TCDD exposure during pregnancy in order to examine the potential developmental effects of this toxicant on reproductive function in first generation adult offspring (toxF1 mice). Our initial study revealed that female mice exposed

to TCDD in utero exhibited a dose-dependent reduction in uterine PR mRNA and protein expression as adults (Nayyar *et al.* 2007). Not surprisingly, toxF1 female mice frequently exhibited infertility and, among mice achieving pregnancy, PTB was common (Bruner-Tran & Osteen, 2011). Additionally, PTB in toxF1 female mice was associated with a heightened sensitivity to an inflammatory challenge, a likely consequence of reduced uterine responsiveness to progesterone (Bruner-Tran & Osteen 2011). These phenotypic changes persisted for three generations among female offspring in the absence of additional toxicant exposure. In a companion study, we found that in utero TCDD exposure of *male* mice conferred a similar risk of PTB following mating to an unexposed female partner (Ding *et al.* 2011). Thus, in utero TCDD exposure led to alterations in normal adult reproductive tract function which negatively influences the maintenance of pregnancy, regardless of which parent was toxicant-exposed. In summary, our previous developmental studies demonstrate that the toxicant-associated risk for inflammation-related PTB exists *prior to conception* and appear to be biologically linked to reduced placental responsiveness to progesterone. In humans and mice the placenta is largely a paternally-derived organ (Kajji & Ohama 1977; Barton *et al.*1984); therefore, a previously unrecognized therapeutic option for prevention of PTB may involve paternal intervention strategies.

In the current study, we utilized our murine model to examine the potential utility of prenatal *paternal* intervention using fish oil supplementation to prevent early parturition in female partners. Compared to females mated to TCDD-exposed males on the standard diet, we found that fish oil supplementation markedly increased pregnancy length in their unexposed partners, resulting in term delivery. Currently, women at imminent risk for PTB are most often treated with tocolytic agents; however these anti-contraction medications cannot inhibit the inflammatory mechanisms which have already triggered the induction of labor (Simhan & Caritis 2007). Our current and previous findings in mice indicate that targeting the inflammatory phenotype *before* establishment of the maternal-fetal interface is a highly effective therapeutic strategy. Since placental-decidual communication in women also affects the timing of parturition, our studies suggest that similar preconception preventive measures may be more effective than those taken after establishment of the maternal-fetal interface.

Results and Discussion

Omega-3 fatty acids, found in fish oil, are necessary for optimal cell function, but must be obtained through diet since mice and humans have only limited ability to synthesize these compounds (Fetterman & Zdanowincz 2009). In particular eicosapentaenoic acid (EPA) and decosahexaenoic acid (DHA), incorporate into the plasma membrane of all cell types, displacing omega-6 fatty acids such as arachidonic acid and reducing the availability of substrates needed for inflammatory prostaglandin synthesis (reviewed by Moreno 2009). Recent studies have examined the efficacy of providing fish oil supplementation to women at risk for PTB (reviewed by McGregor *et al.* 2001 and Jordan 2010); however, an increase in gestational length has not been consistently observed. Whether early life toxicant exposure affects human risk for PTB remains unclear; nevertheless, a nutritional strategy employed *prior to pregnancy* may be optimal for addressing developmental defects leading to reproductive failure. Additionally, recent studies in mice which demonstrate the male partner can influence the timing of parturition further argue in favor of preconception therapy (Roizen et al, 2008; Ding et al, 2011). Data in both women and mice suggest a placental-decidual dialogue controls levels of PR expression within the inflammatory microenvironment of late pregnancy, regulating the timing of parturition (Houbon *et al.* 2009; Mendelson 2010). Since placental development in both species is markedly influenced by paternally-derived genes (Kajji & Ohama 1977; Barton *et al.*1984); targeting the

preconception paternal diet would likely influence placental development and potentially reduce the inflammatory responses that affect timing of birth.

In the current study, we evaluated whether or not preconception fish oil supplementation of toxicant-exposed male mice ($toxF1_{fish}$) improves pregnancy outcomes in unexposed females via a reduction in the placental response to inflammation. To this end, young adult (6–8 weeks of age) C57BL/6 male mice with and without a history of developmental TCDD exposure were transferred to a low phytoestrogen diet with or without 5% Menhaden fish oil. Both $conF1_{fish}$ and $toxF1_{fish}$ males were maintained on the supplemented diet for 2–3 weeks prior to mating at which time they were transferred to the standard diet in order to avoid maternal fish oil supplementation. As shown in Table 1 and Figure 1, all control female mice mated to control male (conF1) mice achieved pregnancy and delivered at term, regardless of diet. In contrast, toxicant-exposed F1 male mice maintained on the standard diet (toxF1) were frequently infertile and gestational length was significantly reduced, averaging 18.8 days ($p<0.0001$, compared to conF1 males). Toxicant-exposed males maintained on the fish oil-enriched diet ($toxF1_{fish}$) exhibited a markedly improved fertility rate and gestation length in their female partners was significantly increased (19.8 days; $p<0.0001$, compared to toxF1 mice) which was statistically similar to control pregnancies ($p=0.1192$). Given the striking improvement in fertility among $toxF1_{fish}$ males, following mating, a subset of mice was euthanized and epididymal sperm collected. Compared to conF1 males, toxF1 mice exhibited a significant reduction in sperm density while sperm numbers were markedly improved in $toxF1_{fish}$ males (Supplementary Table 1). These results are not entirely unexpected, since infertility in men has been linked to a reduction in spermatocyte omega-3 fatty acid content while DHA/EPA supplementation of similalr patients was associated with improved semen quality and enhanced fertility (Safarinejad 2011).

Improved fertility and longer gestational length in female partners mated to $toxF1_{fish}$ males clearly indicate preconception paternal fish oil supplementation can positively influence pregnancy. Taken with our current understanding of inflammation-driven term and preterm parturition, we next examined whether the beneficial effects of this dietary intervention containing fish oil protected pregnancy via a reduction in the placental response to inflammation. Since several studies suggest a balance between PR and TLR-4 expression may be an important determinant in the timing of parturition (Ding *et al.* 2011 and references therein), we conducted quantitative RT-PCR analysis for these genes using placental tissues from mice euthanized in late pregnancy (E18.5). As we previously reported, placental tissues from control females mated to control males exhibited abundant PR mRNA and minimal TLR-4 mRNA expression (Figure 2). In contrast, placental samples from control females mated to toxF1 males exhibited a significantly higher level of TLR-4 mRNA expression ($p=0.0171$), which correlated with diminished PRB/PRAB mRNA expression ($p<0.025$ for both isoforms). However, PR expression was maintained in placental samples taken on E18.5 from control female mice mated to $toxF1_{fish}$ males ($p=0.4851$, PRAB; $p=0.2984$; PRB, compared to conF1 mice). Among these same mice, TLR-4 mRNA expression was also reduced compared to toxF1 mice on the standard diet and was similar to observations in control animals ($p=0.1230$) (Figure 2). Activation of TLRs has been linked to preterm parturition following bacterial infection as well as induction of normal term delivery via interaction with endogenous ligands (Patni *et al.* 2009). Additionally, in a mouse model, Wang & Hirsch (2003) demonstrated that TLR-4-mediated induction of term and PTB was coincident with a decrease in placental expression of PGDH. Consistent with these observations, we found that late pregnancy samples obtained from females mated to toxF1 males also exhibited a dramatic decrease in PGDH immunolocalization within the fetal membranes (Figure 3). In contrast, preconception fish oil supplementation of toxF1 male mice prevented the premature loss of PGDH (Figure 3),

likely contributing to the increase in length of gestation observed in their female partners. Taken together with our previously published findings (Bruner-Tran & Osteen 2011; Ding *et al*. 2011), our current studies suggest developmental TCDD exposure negatively impacts placental development in adulthood, resulting in alterations in inflammatory signaling at the maternal-fetal interface which compromises pregnancy maintenance.

Despite advances in prenatal care, premature birth in industrialized countries remains a significant health care problem, perhaps suggesting environmental exposures may contribute to this condition. We previously demonstrated that a single in utero exposure to TCDD led to reduced fertility and increased risk of PTB in three subsequent generations, strongly implicating the occurrence of epigenetic alterations within the germline of our murine model. Significantly, these data suggest that the risk of delivering preterm as an adult may be determined by the maternal or paternal fetal environment (Bruner-Tran & Osteen, 2011 and references therein). Although using animal models to unravel the potential epigenetic mechanisms of environmental toxicant action remains important, it is equally essential to translate this information into effective strategies which may reduce the negative impact of toxicants in humans. In this regard, it is currently known that the omega-3 fatty acids play an important role in regulation of inflammation via multiple mechanisms, including displacement of omega-6 fatty acids and competition for cyclooxygenases (reviewed by Calder 2003). A human recent human study demonstrated incorporation of omega-3 fatty acids into leukocyte phospholipids and plasma within one week of nutritional supplementation with fish oil (Faber et al, 2011). These studies further revealed fish oil supplementation led to displacement of omega-6 from the cell membranes, which likely contributed to the observed modulation of in vitro immune responses. These human studies clearly support a role of dietary fish oil supplementation in rapidly modulating cell membrane composition and potentially influencing local tissue inflammation.

Although fish oil supplementation for prevention of PTB in high-risk women is beginning to be examined, intervention is typically initiated during mid-pregnancy, well after establishment of the maternal-fetal interface. Since the placenta is a key contributor in determining the timing of parturition (Ding *et al*, 2011; Houbon *et al.* 2009), modulating the preconception paternal diet provides a novel and as yet unexplored strategy for the treatment of this condition in women. In contrast to most current fish oil studies, our findings suggest that dietary supplementation initiated *before* pregnancy and including the male partner would be more effective in preventing inflammation-related PTB.

Material and Methods

Animals

C57BL/6 mice were purchased from Harlan Spraque-Dawley Laboratories (Indianapolis, IN) and housed in the Animal Care Facility according to National Institutes of Health and institutional guidelines for laboratory animals. Animal rooms were maintained at a temperature of 22–24°C and a relative humidity of 40–50% on a 12-hour light:dark schedule. Experiments described herein were approved by Vanderbilt University Institutional Animal Care and Use Committee.

Chemicals

TCDD (99%) in nonane solution was obtained from Cambridge Isotope Laboratories (Andover, MA). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Rodent Diets

Purina Mills (TestDiet division) provided the 5% fish oil diet, which also contained 1.5% corn oil to prevent depletion of omega-6 fatty acids. Menhaden fish oil, generously donated by OmegaProtein (Houston, TX), has an established fatty acid profile (~40% omega-3 fatty acids) and was processed to remove dioxins/PCBs. The fish oil diet is a modification of Purina's low phytoestrogen rodent chow, V502, which was used as the control (standard) diet. Both diets are matched for protein, total fat and energy content. The fish oil diet is maintained in vacuum-sealed bags at −20°C until use and once provided to mice, replaced every 3 days.

In utero TCDD Exposure

Virgin C57BL/6 females (N=20), aged 10–12 weeks, were mated with intact males of similar age and examined each morning for the presence of a vaginal semen plug, denoting copulation had occured. The majority of normal mice will become pregnant after successful mating; thus males are removed at this time. The morning a vaginal plug is identified is considered embryonic day 0.5 (E0.5). Females were weighed prior to mating and again on E15.5 to assist with confirmation of pregnancy. Pregnant mice (F0) were exposed to TCDD (10μg/kg) or vehicle corn oil by gavage at 1100 hours CST on E15.5. Since the half-life of TCDD in C57bl/6 mice is 11 days (Vogel *et al*, 2003), exposure on E15.5 results in *in utero* plus lactational exposure. Although this is considered a high dose of TCDD, our goal was to determine the ability of a fish oil diet to reduce the adverse affects of this compound on fertility and pregnancy; examination of the toxicologic profile of TCDD was beyond the scope of this study. Importantly, the dose of TCDD used in these studies reflects the more rapid clearance of this toxicant in mice compared to humans and is well below the LD_{50} for adult mice of this strain (230μg/kg) (Vogel *et al.* 2003). TCDD given at this time and dose during pregnancy is not overtly teratogenic and gestation length was not affected in the F0 animals; pups (F1 mice) were born on E20±12 hrs.

Mating Scheme of F1 mice and Monitoring of Pregnancy

A single female was placed with a single male and observed each morning for a vaginal plug. Males which produced 3 positive vaginal plugs, but no observable pregnancy, were considered infertile. Following identification of vaginal plug (E0.5), males were removed and parturition expected on E20. Preterm parturition in mice has been defined as spontaneous labor ≥24 hrs prior to term (Roizen *et al.* 2008; Ding *et al.* 2011). After E17, all mice were monitored twice daily for timing of delivery of the first pup.

Euthanasia of Pregnant Females and Collection of Tissues

Spontaneous PTB in toxicant-exposed animals typically occurs on or before E18.5 (Ding et al. 2011; Bruner-Tran & Osteen 2011); therefore, in the current study, a subset of pregnant females from all groups were euthanized at 1400–1500 hours CST on E18.5 and tissues collected as previously described (Ding *et al.* 2011). Briefly, pregnant females were weighed immediately prior to euthanasia by cervical dislocation under anesthesia. The uterus and cervix were removed *in toto* and placentas dissected from the decidua. Half of the placenta were flash frozen for RNA extraction and stored at −80°C, the remainder was formalin fixed. All cervices were formalin-fixed. Since only a subset of females mated to toxF1 males can be expected to deliver preterm, cervices were histologically examined for evidence of cervical ripening (mucin via PAS staining and collagen degradation via Masson's Trichrome staining, data not shown) to aid in selection of appropriate tissues for RT-PCR analysis. No pregnancies resulting from matings with conF1 or $toxF1_{fish}$ males exhibited extensive cervical ripening.

Histochemistry/Immunohistochemistry

Fixed samples were processed, paraffin-embedded and sectioned at 5 um by the Vanderbilt Histology Core Laboratory. Cervices were subjected to hematoxylin and eosin (H & E), PAS and Masson's trichrome staining by standard methodology.

Immunohistochemical localization of PGDH protein in placenta and fetal tissues was performed using a Vectastain Elite ABC kit (Vector Laboratories Inc, Burlingame, CA) according to the manufacturer's protocol. Endogenous peroxidase activity was removed (0.3% hydrogen peroxide/methanol) and sections blocked in 10% goat serum for 1 h at room temperature. Slides were incubated with the anti-human rabbit polyclonal PGDH antibody (1:500, Cayman Chemicals, Ann Arbor, MI) overnight at 4°C followed by goat anti-rabbit IgG biotinylated secondary antibody for 45 min at room temperature. Rabbit serum was used as the negative control (1:500). Slides were viewed with an Olympus BX51 microscope system and images captured using an Olympus DP71 digital camera.

Quantitative RT-PCR analysis

Total RNA was isolated from frozen placental tissues with Trizol (Invitrogen, Carlsbad, CA) and purified using the RNeasy Mini Kit (Qiagen, Valencia, CA). cDNA from 1μg of total RNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad) and random decamer primers. Reactions were performed in duplicate in a Bio-Rad CFX96 Real-time thermocycler system. The ribosomal protein, large, P0 (RPLP0) gene was used as an endogenous control. Results were evaluated using the delta-delta Ct method, where delta was calculated as (Target Ct)-(RPLP0 Ct), and the relative quantity of target gene expression was calculated by the delta-delta Ct as $2^{-[(\text{experiment sample delta Ct})-(\text{control sample delta Ct})]}$. Primers (forward and reserve) and the thermal cycling protocol using the CFX96 Real-time System have been previously described (Ding *et al.* 2011).

Euthanasia of Males and Analysis of Sperm Density

Following a minimum of 72 hrs after mating, adult (12–16 weeks) males were euthanized by cervical dislocation under anesthesia; sperm was collected from epididymal cauda and density determined by standard methodology (Wang 2002). Two counts were taken for each sample and density was calculated using the following equation: Sperm density = (mean count × dilution factor)/cauda weight (mg).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism. All data are expressed as mean value ± SD. Statistical comparisons between two experimental groups were determined using the parametric Student's *t*-test (for normally distributed populations). A p value of less than 0.05 was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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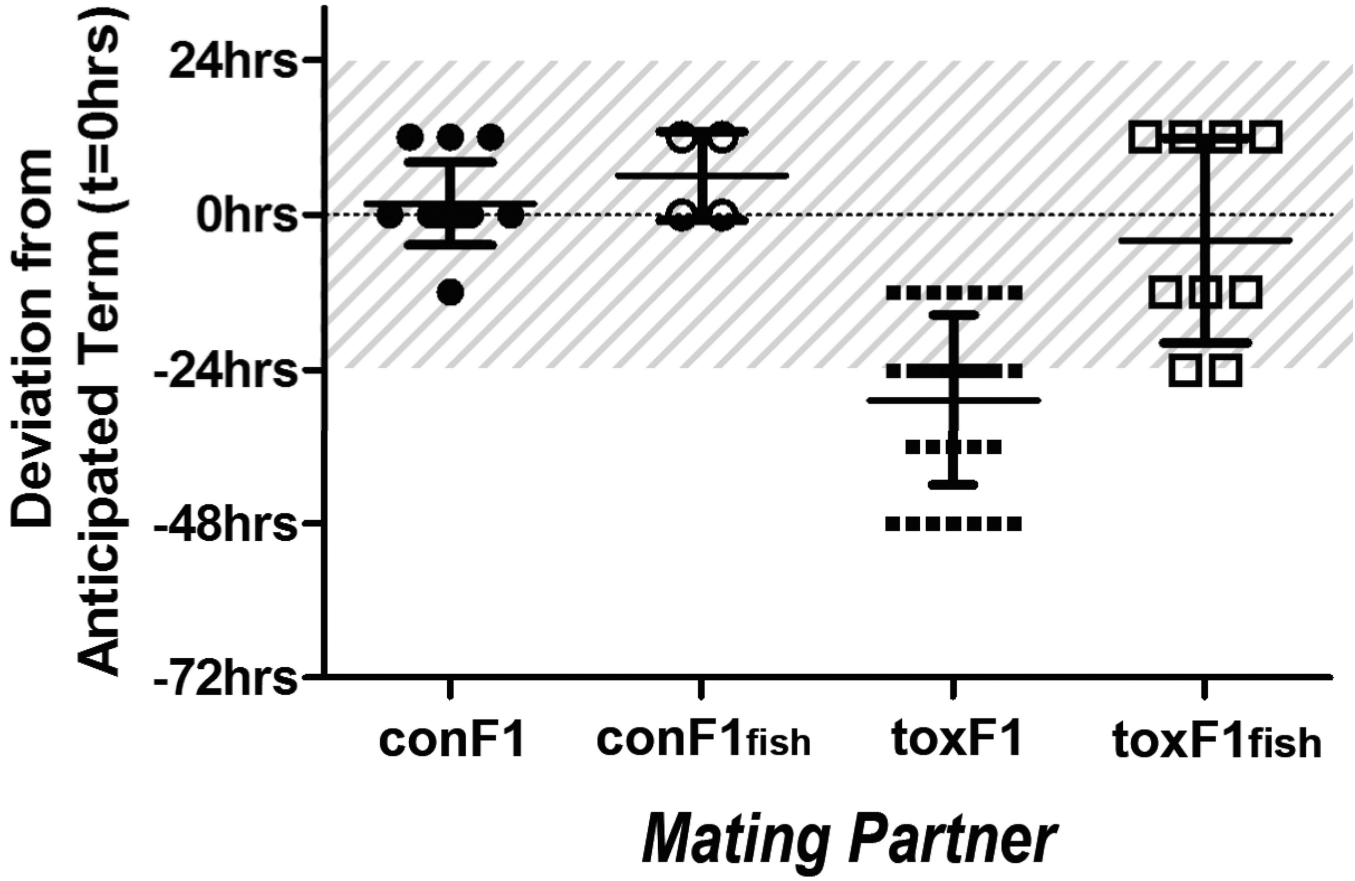

Figure 1.

Effect of a fish oil-enriched diet on gestation length in control females mated to males with and without a history of *in utero* TCDD exposure and undergoing spontaneous labor and delivery. Anticipated time of delivery (E20) was denoted t=0 and actual delivery times were plotted as hours deviating from that time. Each geometric symbol represents one mouse. The inner horizontal line indicates the average hours from t=0 for each group of mice while the SD is indicated by the outer lines.

ConF1 female mated to conF1 male (●; N=15); ConF1 female mated to $conF1_{fish}$ male (○; N=4); ConF1 female mated to toxF1 male (■; N=25); ConF1 female mated to $toxF1_{fish}$ male (□; N=9). Shaded area represents the acceptable variation in normal delivery time (±24 hrs) and which is also considered term.

All males within a litter were placed on the same diet (fish oil enriched or standard diet) and multiple litters were used per group (conF1, N=4; $conF1_{fish}$, N=2; toxF1, N=12; $toxF1_{fish}$, N=3). $ConF1_{fish}$ and $toxF1_{fish}$ males were provided fish oil supplemented diet for 2–3 weeks prior to mating.

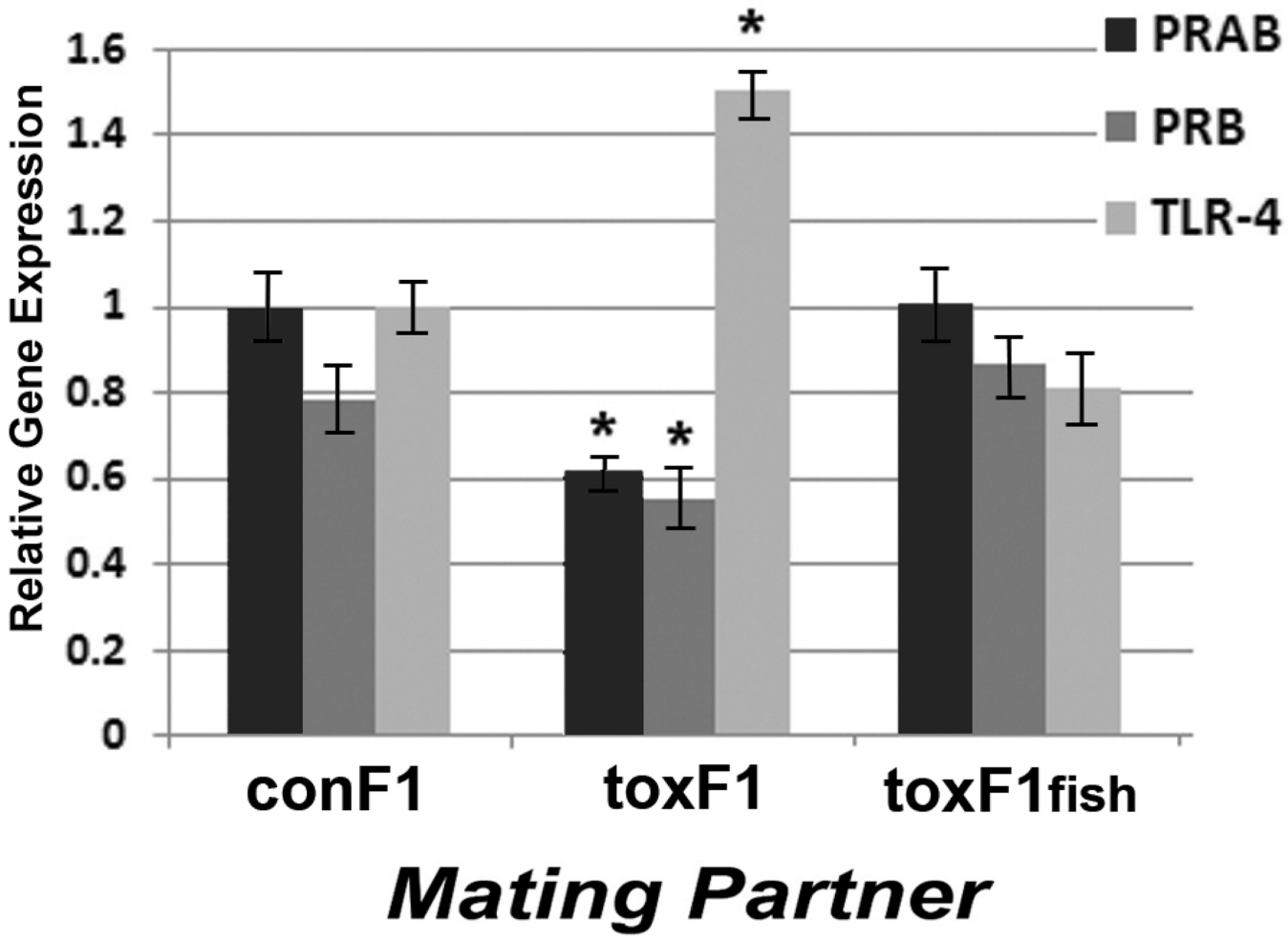

Figure 2.

Expression of PR-A/B, PR-B and TLR-4 mRNA in late gestation (E18.5) placental tissues. The mRNA level of target genes were analyzed by qRT-PCR and normalized to the expression level of the housekeeping gene RPLP0. mRNA is shown as fold changes compared with E18.5 control. Each bar represents the mean ±SD of mRNA for multiple samples from at least 3 different mice per group. Statistically significant changes ($p<0.05$) are denoted by *.

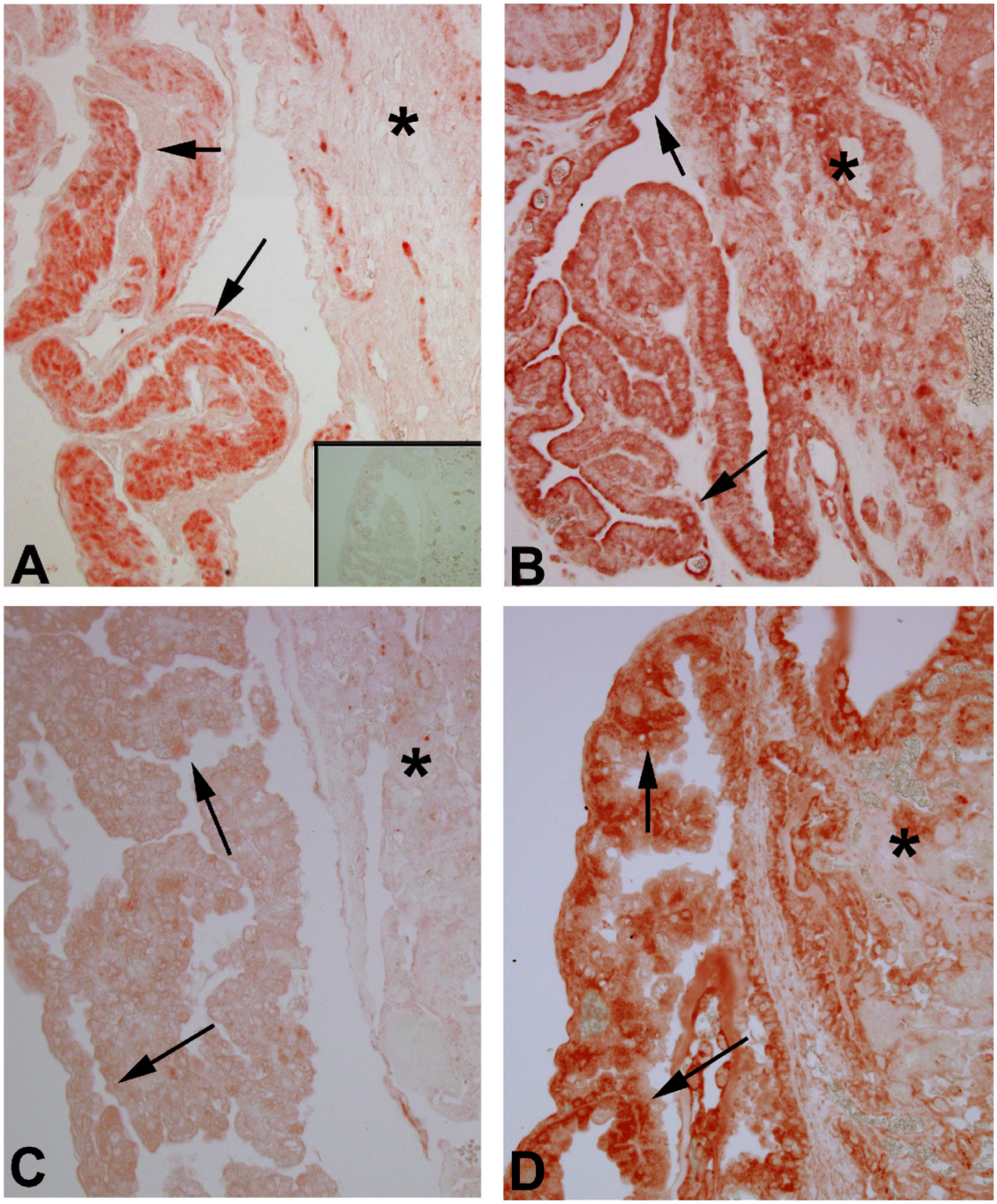

Figure 3.
Immunohistochemical localization of prostaglandin dehydrogenase (PGDH) in placental samples obtained on E18.5 from pregnant control females mated to (A) conF1 male, (B) conF1$_{fish}$ male, (C) toxF1 male or (D) toxF1$_{fish}$ male. PGDH, appearing as red staining, largely localizes to the fetal membranes (arrows) and is abundant in both control samples and in toxF1$_{fish}$ samples. PGDH expression is minimal in samples obtained from toxF1 mice maintained on the standard diet. Original magnification, 200×. IgG control is shown in inset in A. *denotes placenta. Results are representative of at least 4 samples per group.

Table 1

Impact of Paternal Fish Oil Supplementation on TCDD-Associated Male Infertility and Preterm Birth in Unexposed Female Partners

Mouse History	N	Pregnancy	Pregnancy Outcome	
			Full-Term	*Preterm*
conF1	15	15/15 (100%)	100%	0%
$conF1_{fish}$	4	4/4 (100%)	100%	0%
toxF1	53	25/53 (47%)	61%	39%
$toxF1_{fish}$	11	9/11 (81%)	100%	0%