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Effect of dipentyl phthalate in 3-dimensional *in vitro* testis co-culture is attenuated by cyclooxygenase-2 inhibition

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Exposure to phthalate esters is associated with changes in steroidogenesis, leading to the hypothesis that this is a primary mechanism of phthalate reproductive toxicity. However, some phthalate-induced male reproductive toxicity has been demonstrated in the absence of changes to testosterone production, suggesting additional mechanisms of action. There is evidence that phthalate exposure increases expression of the inflammatory enzyme cyclooxygenase 2 (cox-2). Furthermore, inhibition of cox-2 enhances expression of the steroidogenic acute regulatory protein (StAR), which mediates the rate-limiting step in steroidogenesis. This study hypothesized that phthalate-induced toxicity and testosterone perturbation are mediated in part by cox-2. A 3D *in vitro* rat testis co-culture to explore the role of cox-2 in phthalate toxicity was employed. Cells were treated with 100 µM dipentyl phthalate (DPP) with and without pre-treatment with the specific cox-2 inhibitor NS-398. Effects were evaluated after 8, 24, and 72 h. DPP exposure significantly increased cox-2 expression at 8 and 24 h ($p<0.01$) and resulted in significant, dose-dependent cytotoxicity. Pre-treatment with NS-398 significantly reduced the cytotoxicity of DPP at 8 and 24 h ($p<0.01$). NS-398 also mitigated the effects of DPP on testosterone regulation. Total testosterone concentrations in cell culture media were significantly increased following 8 and 24 hr of DPP exposure ($p<0.001$) and NS-398 reduced this effect ($p<0.05$). Simultaneously, DPP significantly decreased StAR protein expression after 8 h ($p<0.01$) and this effect was significantly attenuated by the presence of NS-398 ($p<0.01$). These results suggest that the DPP-induced changes in testosterone regulation observed in this experiment are mediated in part by an inflammatory response that is cox-2 dependent.

Key words: dipentyl phthalate, testosterone, cyclooxygenase 2, in vitro toxicology

INTRODUCTION

Phthalates are a class of chemicals widely used as plastic softeners and stabilizers in a range of products including adhesives, lubricants and cosmetics. Because they are not bound covalently to these materials, phthalates are prone to leaching out of common consumer products, raising concern over ongoing human

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exposures. Monoester phthalate metabolites have been detected in maternal urine during pregnancy, amniotic fluid and breast milk, indicating that low levels of exposure can occur during critical periods of fetal development (Hauser and Calafat, 2005; Silva et al., 2004; Fromme et al., 2011).

A subset of structurally similar phthalate esters are widely recognized as male reproductive toxicants. Male rats exposed to developmentally toxic phthalates *in utero* exhibit reduced testes size, undescended testes, decreased (feminized) anogenital distance, and decreased sperm count (Mylchreest et al., 1998; Gray et al., 2000). Related effects have been documented in humans as well. Male infants whose mothers had high levels of phthalate metabolites (MEP, MBP, MBzP, and MiBP) in their urine during pregnancy had significantly shortened anogenital distance (Swan et al., 2005; Swan, 2008) and *in utero* exposures to diethylhexyl phthalate (DEHP) and dibutyl phthalate (DBP) metabolites were associated with decreased masculine play behavior in boys (Swan et al., 2010). In adult men, urinary concentrations of phthalate metabolites (MEHP, MINP, MBzP, MBP, and MEP) have been associated with a decline in sperm quality (Jurewicz et al., 2013). Dipentyl phthalate (DPP), a phthalate commonly used to soften PVC plastic, appears to have particularly potent effects on male reproductive development (Foster et al., 1980; Hannas et al., 2011).

Because fetal testosterone and insulin-like 3 peptide hormone are key regulators of male reproductive development, the decrease in these hormones observed in response to a range of phthalates (Howdeshell et al., 2008; David, 2006) is considered an important mechanism of male reproductive toxicity of phthalates. Indeed, exposure to DBP has been shown to alter expression of a range of key steroidogenic factors, including transcription factors (e.g. GATA4, C/EBPbeta), cholesterol transporters (e.g. the HDL receptor, steroidogenic acute regulatory protein) and enzymes involved in the conversion of cholesterol to testosterone (e.g. hydroxysteroid dehydrogenase) (David, 2006; Lehmann et al., 2004; Barlow et al., 2003). Altered expression of the steroidogenic acute regulatory protein (StAR) is of particular interest because StAR mediates the transfer of cholesterol across the inner membrane of the mitochondria, the rate-limiting process in steroidogeneis (David, 2006; Clark and Cochrum, 2007).

The mechanism of altered StAR expression and testosterone regulation in response to phthalate has not been fully elucidated. Characterization of upstream mechanisms that lead to reduced testosterone synthesis could offer a broader understanding of the full range of possible toxic responses to phthalate exposure. This is particularly important given that some aspects of the toxic responses to phthalate cannot be attributed to altered hormone levels. While the effects of phthalate on hormone level have been shown to be species-specific, phthalates have been shown to alter germ cell development independent of effects on testosterone in mice, rats and human tissue (Gaido et al., 2007; Alam et al., 2010; Lambrot et al., 2009; Heger et al., 2012; Johnson et al., 2012).

Several studies point to the inflammatory enzyme cyclooxygenase 2 (cox-2) as a negative regulator of StAR expression. The cyclooxygenases are a family of enzymes responsible for the conversion of fatty acids into prostaglandins and thromboxanes, mediators of inflammation and vasoconstriction. Cox inhibitors are a widely used class of anti-inflammatory drugs. In cultured mouse Leydig cells, specific inhibition of cox-2 has been shown to increase sensitivity to cAMP stimulation. This results in enhanced expression of both the StAR gene and StAR protein, promoting steroid production (Wang et al., 2003). Conversely, an age-related increase in cox-2 protein in Leydig cells has been associated with an inhibitory effect on StAR-mediated testosterone production (Wang et al., 2005). Furthermore, there is evidence that mono (2-ethylhexyl) phthalate exposure increases expression of cox-2 as well as mitochondrial expression of the redox protein peroxiredoxin 3 in spermatocytes (Onorato et al., 2008). However, the increase in cox-2 following phthalate exposure has not yet been directly linked to subsequent changes in StAR expression or testosterone synthesis. It was hypothesize that the change in StAR gene expression and testosterone production typically observed in response to phthalate exposure is mediated in part by cox-2.

In this study, 3-dimensional *in vitro* testes-co-culture model was employed (Yu et al., 2005) to evaluate the role of cox-2 in mediating DPP-induced testicular toxicity. DPP was selected as a model phthalate due to its potency and selected treatment concentrations that had previously been demonstrated to alter gene expression in the absence of cytotoxicity (Yu et al., 2009). Specifically, the effects of DPP exposure on cox-2 protein expression, StAR protein expression and testosterone concentrations in the presence or absence of a specific cox-2 inhibitor was compared.

MATERIALS AND METHODS

Testis cell co-culture

Male Sprague-Dawley rat pups were obtained on postnatal day 4 (Harlan). On postnatal day 5, testis tissue was isolated and digested for cell culture as described previously (Wegner et al., 2013; Yu et al., 2005). Briefly, testes were removed and dissected to isolate seminiferous tubules, which were digested in a series of enzyme cocktails. Once dissociated, testis cells were suspended in serum-free Eagle's Minimal Essential Medium (Invitrogen) containing 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 3 mM sodium lactate, 1% ITS culture supplement (BD Biosciences) and plated in 35 mm culture dishes at a density of 1.6×10^6 cells/plate. Ice-cold Matrigel (BD Biosciences) extracellular matrix overlay (30 µl, for a final concentration of 200 µg/ml) was immediately added to the center of each dish to provide a 3-dimensional

scaffold.

Phthalate treatment

After plating (48 h), medium was removed and replaced with fresh medium containing 100 µM of the developmentally toxic phthalate DPP (Sigma Aldrich # 80154, 99% purity) or a DMSO (Sigma Aldrich) vehicle control. Pre-treatment with the cox-2 inhibitor NS-398 (EMD Chemicals Inc.) was done 30 min prior to treatment with DPP. Each treatment was repeated in a minimum of three plates for each experiment. Phthalate treatment concentration was selected based on a previous assessment in our co-culture that found less than a 15% reduction in cell viability measured by neutral red uptake assay (Yu et al., 2009).

Microscopy

Following 8, 24 or 72 h of treatment, each three-dimensional co-culture was visualized using an Olympus microscope with phase-contrast optics at 20X magnification. Images were captured and digitized with a Coolsnap camera (Roper Scientific, Inc.).

Lactate dehydrogenase cytotoxicity assay

Following 8, 24, or 72 h of phthalate exposure, 50 µl media was collected from each culture plate for a lactate dehydrogenase (LDH) cytotoxicity assay (Promega Corporation), performed according to the kit protocol. Briefly, prior to harvesting cell lysates, 50 µl of media from each plate was added to a 96-well plate in triplicate. Samples were incubated with 50 µl substrate mix in the dark. After 30 min, reactions were stopped with stop solution and absorbance at 490 nm was read with a plate reader. OD values were normalized to blanks and presented relative to LDH positive controls (media from control plates killed by a freeze/thaw cycle). Data reflect at least 3 independent experiments.

Western blotting

Following 8, 24, or 72 h of treatment, cells were harvested in lysis buffer (Cell Signaling Technology) and protein was isolated by a series of freeze-thaw steps followed by centrifugation. Protein concentration was determined using a commercially available protein assay kit (Protein Assay kit, Bio-Rad Laboratories) and protein samples were diluted with sample buffer, reducing agent and buffer so that all samples contained equal protein concentrations. Samples were loaded in 4 to 12% Bis-Tris NuPage precast minigels (Invitrogen) and separated by running at 200 V for approximately 45 min in running buffer containing 500 µl antioxidant (Invitrogen). Protein was then transferred to polyvinylidene difluoride nylon membranes (Bio-Rad Laboratories) for immunoblotting. Efficiency of transfer was confirmed by commassie stain of the gel after the transfer was complete. Membranes were rinsed in tris-buffered saline (TBS) pH 7.6 then blocked with 5% non-fat dry milk in TBS with 0.1% Tween 20 (TTBS) for 1 h. Membranes were rinsed with TTBS then incubated overnight with primary antibody and for 2 h with secondary antibody conjugated to horseradish peroxidase. Primary antibodies included StAR (a generous gift from Dr. Douglas M Stocco, Texas Tech University), cox-2 (Cayman Chemical), actin (Sigma), anti-rabbit secondary antibody (Cell Signaling Technology Inc.), and anti-mouse secondary antibody (BD Pharminogen). After antibody incubations, membranes were washed 5 times for 5 min with TTBS and incubated with enhanced chemiluminescence detection reagent (GE Lifescience) and exposed to X-ray films (GeneMate). Western blots were analyzed by densitometry using ImageJ (NIH). The intensity of each band of interest was normalized to corresponding actin loading controls. Data presented here reflect at least 3 independent experiments.

Testosterone assay

Media was collected from cells under each treatment condition at 8 and 24 h following treatment. Testosterone concentrations in each medium sample were determined by an ELISA assay for total testosterone according to kit protocol (Neogen Corporation #402510). Testosterone concentrations measured in media were normalized to the protein content of corresponding cell lysate (determined as described earlier). Data on testosterone production are presented in terms of fold change in testosterone/protein relative to control. Data presented here reflect at least 3 independent experiments.

Statistical analysis

Statistical significance was determined by ANOVA using a mixed effects model in R and results are presented as the mean and 95% confidence intervals of the mean. Random effects are the variations between digesting at least three different sets of rat pup testes.

RESULTS

Morphology

Phase-contrast images illustrate the three-dimensional complexity provided by Matrigel overlay in these testicular co-cultures (Figure 1). These images also provide initial qualitative evidence of the effects of phthalate treatment on the 3D co-cultures (indicated by the presence of condensed nuclei and some floating cells) and the prevention of these effects in cells pre-treated with NS-398, a specific cox-2 inhibitor.

Cytotoxicity

In order to evaluate the cytotoxic response to DPP in the presence or absence of NS-398 in the testicular co-culture, a lactate dehydrogenase (LDH) release assay was used. It was found that treatment with DPP resulted in significant cytotoxicity at all time points. Pre-treatment with NS-398 significantly decreased the cytotoxicity of DPP treatment at 8 and 24 h (Figure 2). By 72 h, cox-2 inhibition was less effective in preventing cytotoxicity (data not shown). In order to ensure that NS-398, which has a slightly yellow color, did not interfere with the calorimetric LDH assay reading, an additional set of LDH assays using medium from a single untreated plate was run. No difference was found in LDH reading with and without the addition of NS-398 (data not shown).

Cox-2 protein expression

To further demonstrate that cox-2 is a plausible mediator of DPP-induced toxicity, Western blotting was used to

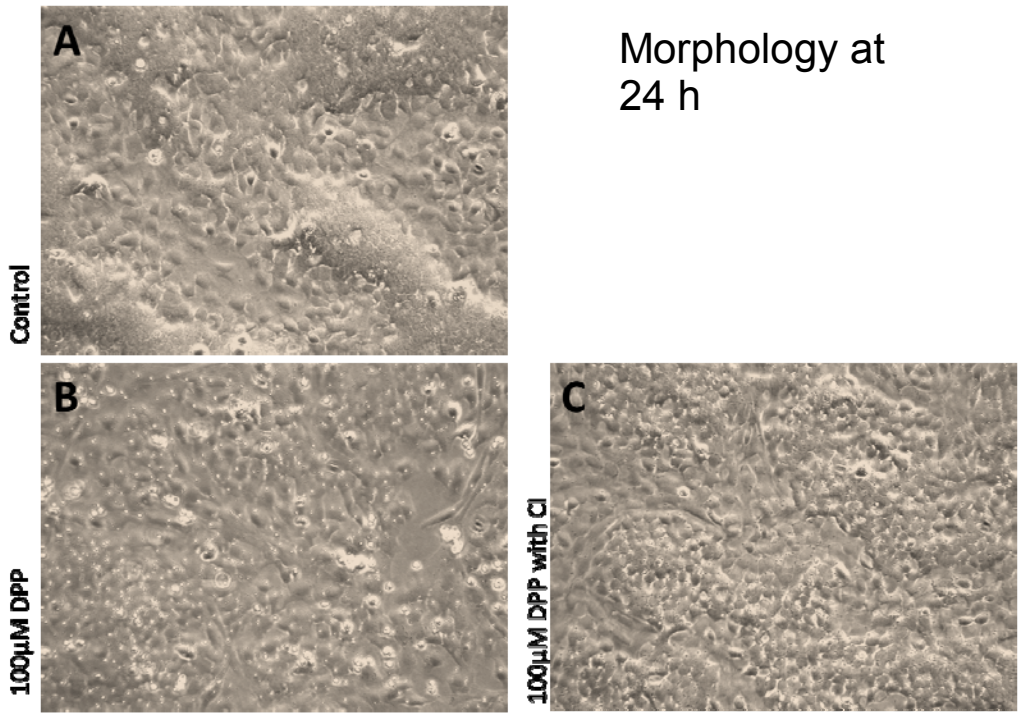

Figure 1. Morphological changes in response to DPP exposure with and without Cox2 inhibitor. (A) Representative morphology of control plates 24 h post treatment (72 h post plating). Cultures contain germ cells, Sertoli cells, Leydig cells in three-dimensional Matrigel matrix. (B) Representative morphology of 24 h 100 µM DPP exposed plates. Note the increase in dense nuclei, indicative of apoptotic cells. (C) Representative morphology of 24 h 100 µM DPP exposed plates with NS-398 cox2 inhibitor (CI) pre-treatment.

measure the effect of DPP exposure on cox-2 protein expression. Cox-2 protein was significantly increased in response to 100 µM DPP following 8 and 24 h of exposure (Figure 3). Interestingly, inhibition of cox-2 ultimately led to a dramatic increase in cox-2 expression by 72 h (data not shown), perhaps due to a feedback mechanism in response to the decrease in cox-2 activity.

Testosterone production

In order to determine the role of cox-2 in mediating DPP induced changes in testosterone concentration, an ELISA assay was used to measure total testosterone in the culture media relative to total protein content in the cell lysate. There was a significant, dose-dependent increase in cell culture media testosterone concentrations following 8 and 24 h of exposure to a cytotoxic concentration of DPP (100 µM). Pre-treatment with NS-398 significantly attenuated the DPP-induced increase in testosterone concentrations after 24 h ($p<0.05$) (Figure 4).

StAR protein expression

Western blotting was used to evaluate the effect of DPP exposure on expression of StAR protein, which mediates the rate limiting step in testosterone synthesis. In agreement with previous reports (Clark and Cochrum, 2007), it was found that exposure to 100 µM DPP significantly decreased expression of StAR protein relative to β-Actin loading control after 8 h of treatment (Figure 5). In order to evaluate the role of cox-2 in this observed decrease in StAR expression, the effect of cox-2 inhibition on the DPP-induced decrease in StAR expression was tested. Pretreatment of co-cultures with NS-398 prevented the DPP-induced decrease in StAR protein at 8 h (Figure 5). By 72 h of treatment, however, the preventive effect of NS-398 was done.

DISCUSSION

By attenuating the effects of DPP with cox-2 inhibitor pretreatment, cox-2 activity is involved in mediating DPP toxicity. Inhibition of cox-2 prevents DPP-induced cytotoxicity and reduces the effect of DPP on media testosterone concentrations and StAR protein expression.

The increase in testosterone concentrations and simultaneous decrease in StAR protein expression observed following DPP treatment in our co-culture suggests a temporarily dynamic response. While exposure to a range

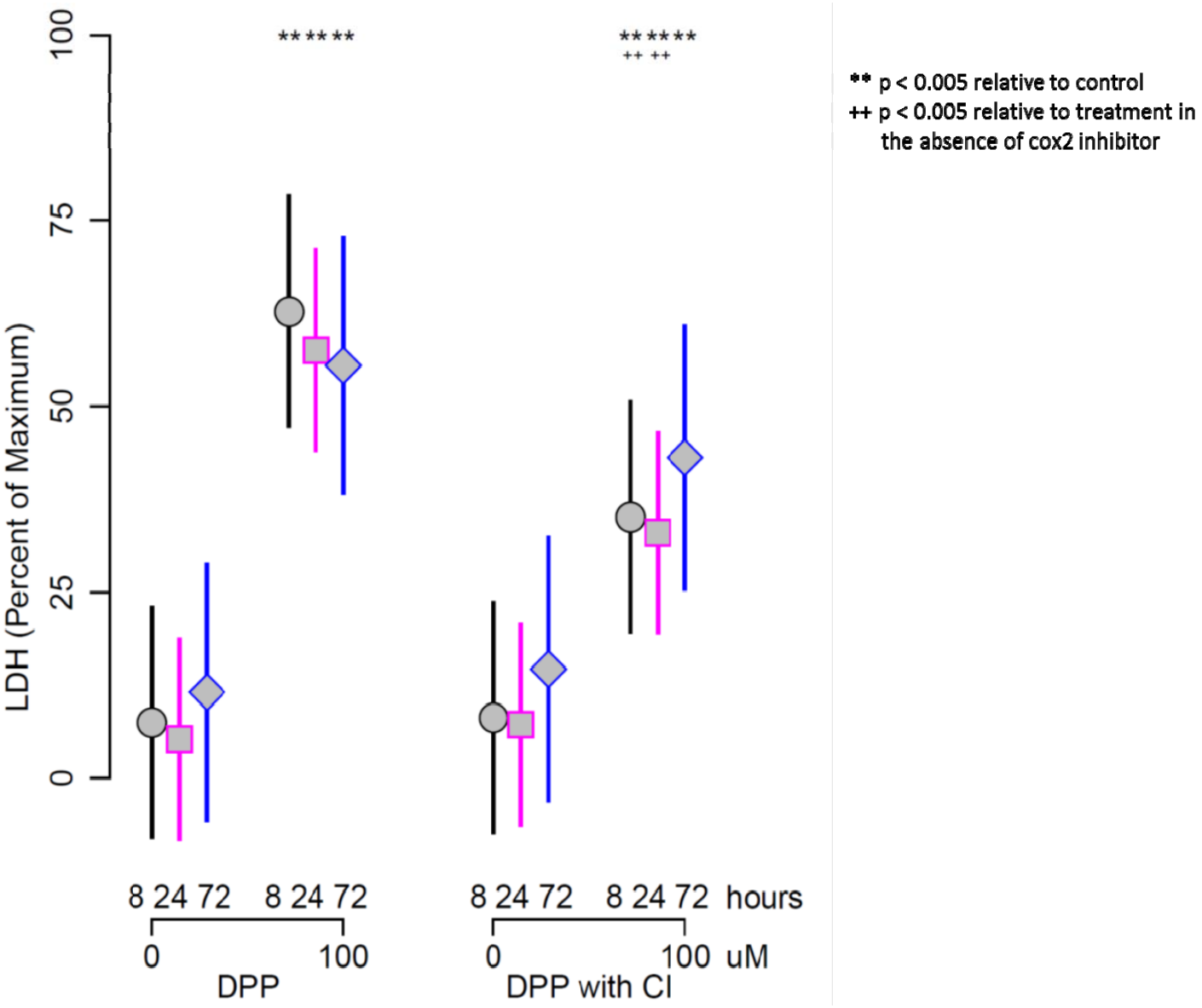

Figure 2. Effect of Cox2 inhibition on DPP Phthalate-Induced Cytotoxicity. After 8 or 24 h of treatment with 100 µM DPP with or without Cox2 inhibitor, medium was harvested and assayed for lactate dehydrogenase (LDH) as an indicator of cytotoxicity. 100 µM DPP dramatically increases cytotoxicity relative to controls. After 8 and 24 h of exposure, pre-treatment with NS-398 cox inhibitor (CI) significantly reduces cytotoxicity. Each data point represents results from at least 3 independent experiments. Error bars indicate 95% confidence intervals determined by a mixed effect ANOVA.

of reproductively toxic phthalates is typically associated with a decrease in testosterone (David, 2006), an initial increase in testosterone concentrations in response to DPP at 8 and 24 h was seen. The simultaneous down regulation of StAR protein at these time points may foreshadow an eventual decrease in testosterone concentrations. Our previous research has demonstrated a dramatic increase in StAR gene expression following 24 h of exposure to several different reproductively toxic phthalates, including DPP (Yu et al., 2009). These observations are consistent with previous reports that the direction and magnitude of StAR gene expression responds to reproductively toxic phthalates in a time and dose-dependent manner (Lahousse et al., 2006). It still remains to be seen how cox-2 influences StAR protein expression and testosterone concentrations in seemingly contradictory ways. Our current studies do not reveal whether DPP acts on StAR directly, or indirectly via effects on other aspects of testosterone production and metabolism.

Upregulation of cox-2 protein by DPP may be due to feedback in response to direct inhibition by phthalate.

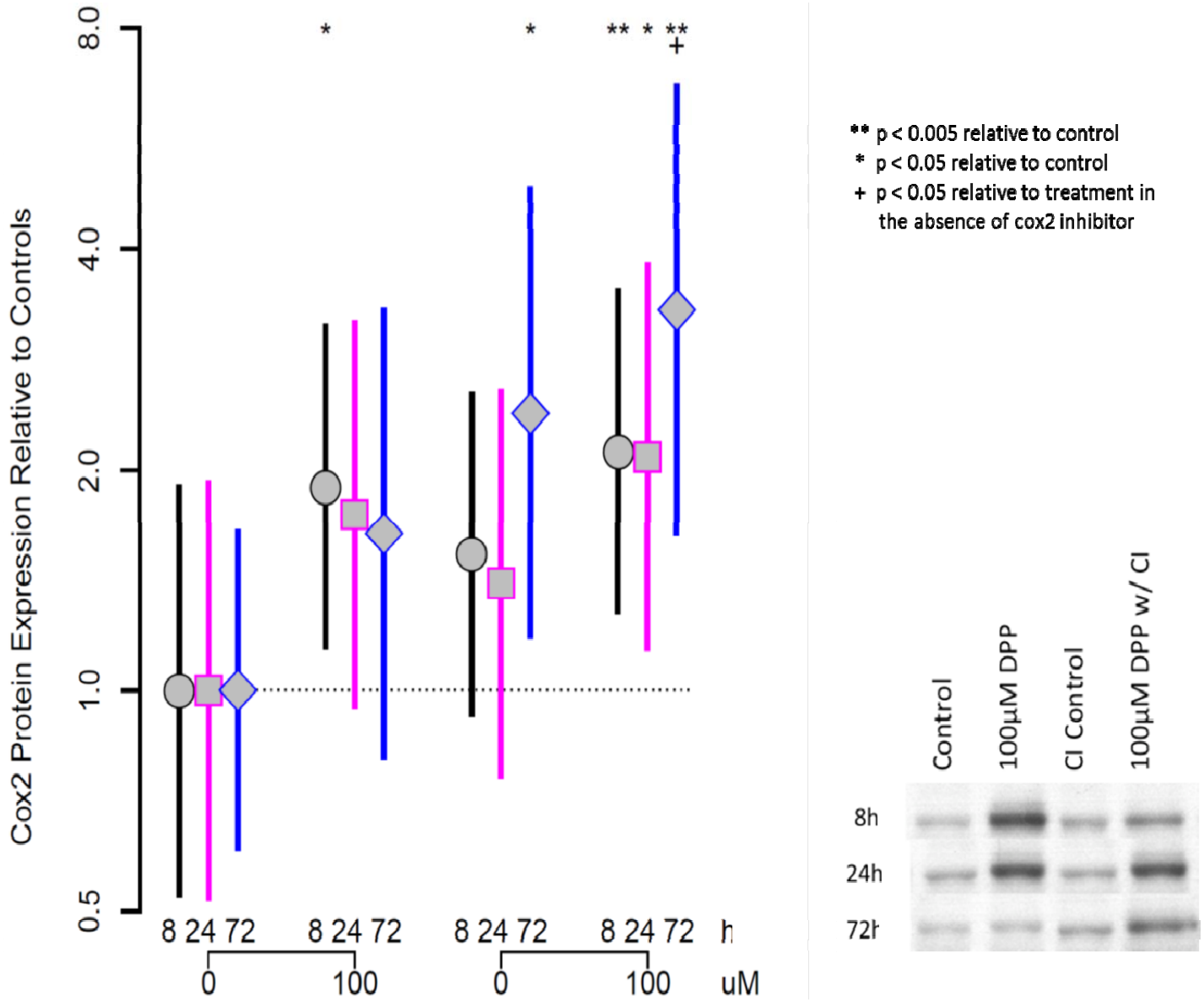

Figure 3. Effect of COX2 inhibition on DPP-induced changes in Cox2 protein expression. After 8 or 24 h of treatment with 100 µM DPP with or without Cox2 inhibitor, protein was harvested, run on western blot, and probed with an antibody to Cox2 protein. Cox2 protein expression was significantly increased in response to DPP phthalate after 8 and 24 h. Cox inhibition significantly increases Cox2 protein expression by 72 h. Each datapoint represents Western results from at least 3 independent experiments. Error bars indicate 95% confidence intervals determined by a mixed effects ANOVA.

Indeed, several reproductively toxic phthalates have been shown to reduce prostaglandin synthesis following 24 h exposures *in vitro*, suggesting a decrease in cyclooxygenase activity. Computer modeling simulations indicate that several phthalates can directly inhibit cyclooxygenase enzymes by direct binding (Kristensen et al., 2011). Because cox-2 is known to suppress testosterone, such inhibition of cox-2 by phthalate could explain the higher levels of testosterone measured in the cell culture media following DPP exposure. DPP induced inhibition of cox-2 may underlie the ultimate upregulation of cox-2 observed in this study. Inflammatory signals like cox-2 can also be induced by cell death (Rock and Kono, 2008). Therefore, it was difficult to distinguish in our studies whether effects were also being seen on cox2 via cell death.

The effect of DPP on cox-2 expression has interesting implications. In addition to its effect on StAR expression, cox-2 contributes to prostaglandin and thromboxane synthesis and induces expression of other cytokines including IL1a and IL6 (Ishikawa et al., 2005). A phthalate-induced increase in cox-2 may therefore increase

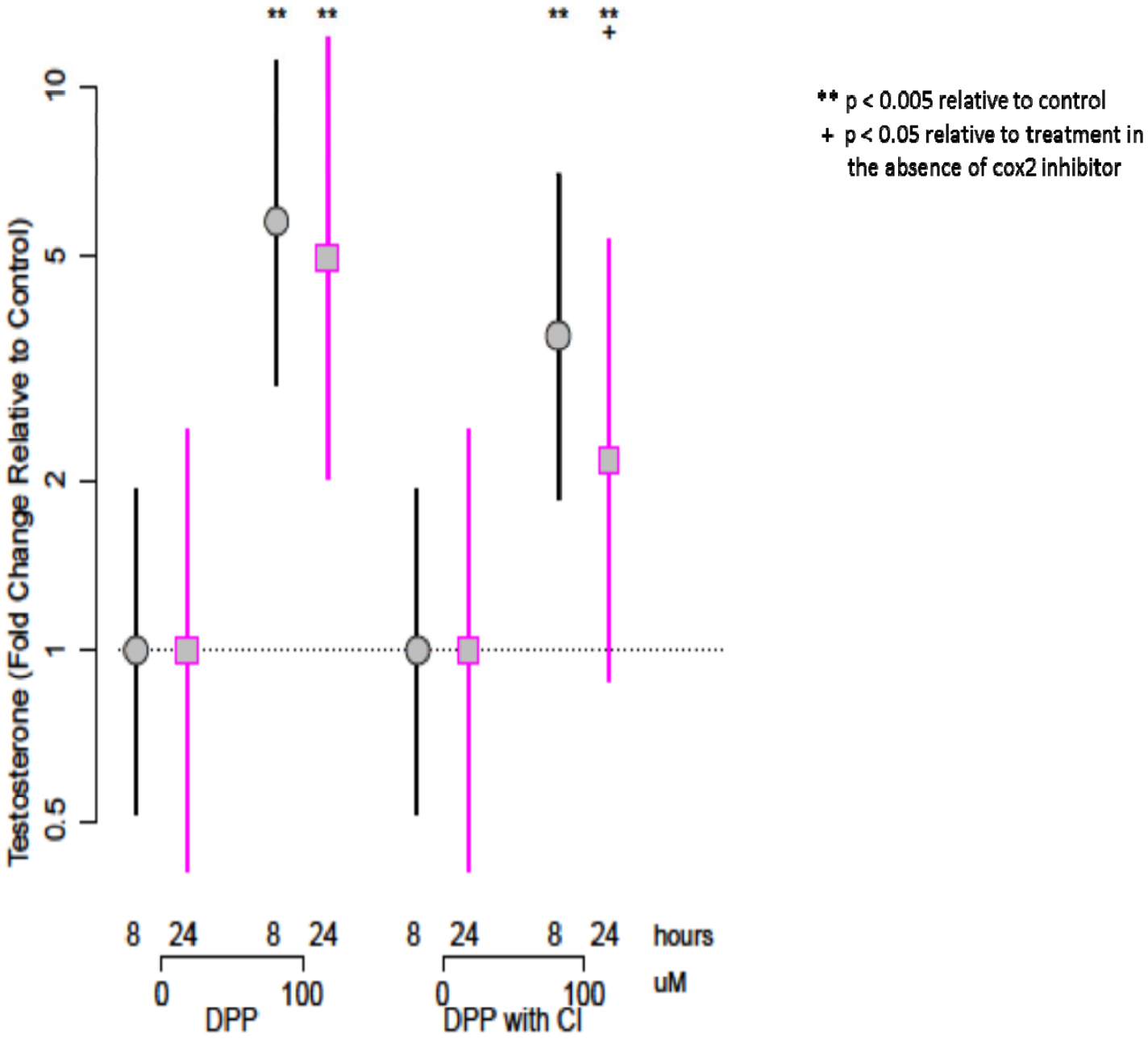

Figure 4. Effect of DPP on testosterone production. After 8 or 24 h of treatments with 100 μM DPP with or without Cox2 inhibitor, media was collected and assayed for testosterone concentration by ELISA assay. Testosterone concentrations were normalized to protein content and are presented here in terms of fold change relative to the control. Each datapoint represents Western results from at least 3 independent experiments. Error bars indicate 95% confidence intervals determined by mixed effects ANOVA.

inflammatory secretions by Sertoli cells. Such paracrine signaling by Sertoli cells may be an important secondary mechanism of phthalate toxicity.

Further exploration of the upstream events that lead to altered StAR expression and subsequently altered testosterone concentrations may reveal common upstream mechanisms for other important pathways of phthalate toxicity. In particular, the role of inflammatory responses in mediating phthalate toxicity warrants further investigation. There is a growing body of evidence to suggest that inflammation plays an important role in phthalate toxicity. For example, DPP has been linked to inflammatory response in adult male rat testes (Granholm et al., 1992) and MEHP exposure in immature rats has been shown to lead to an increase in recruitment of immune cells into the testis, followed by an increase in germ cell apoptosis

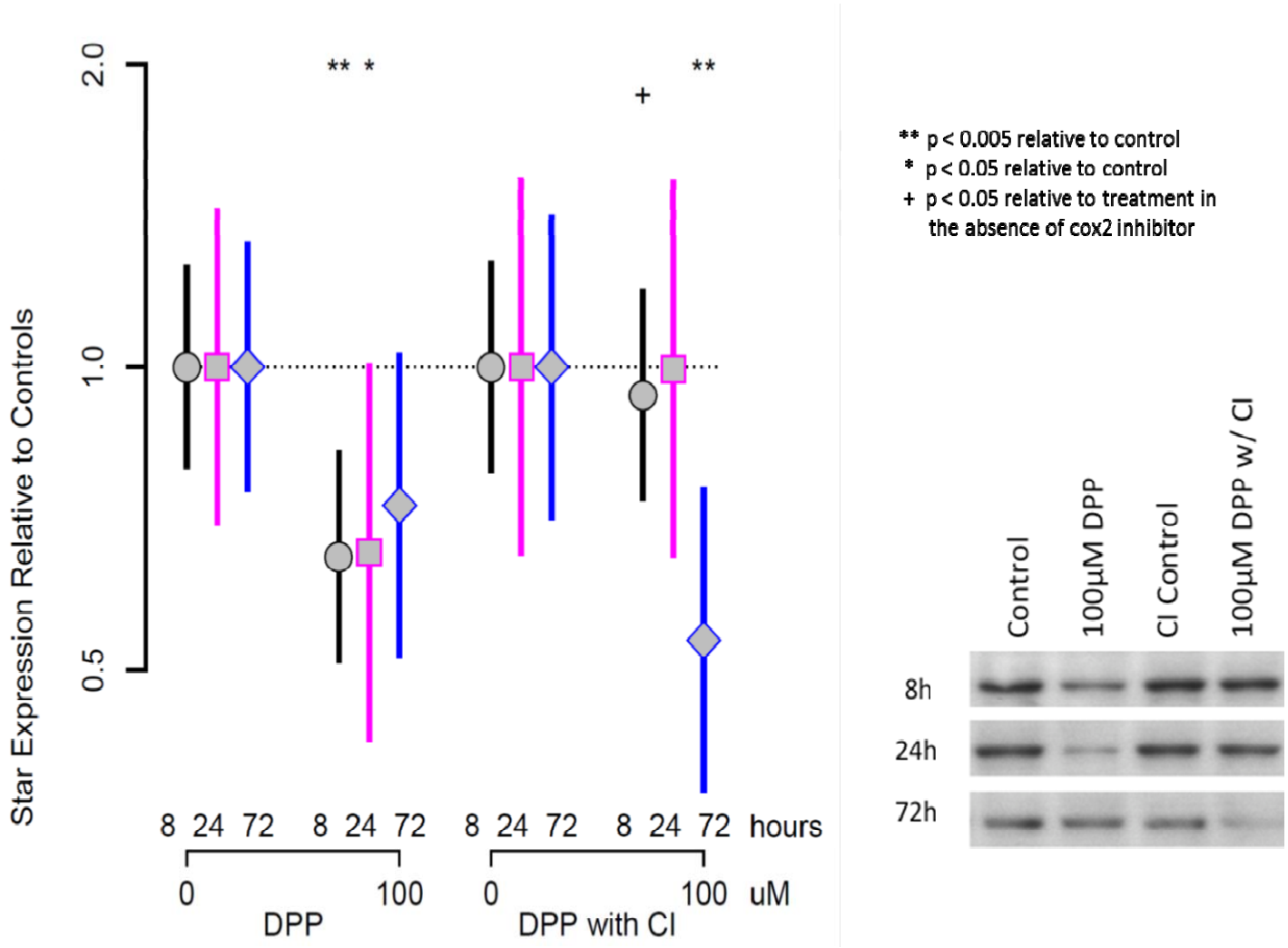

Figure 5. Effect of COX2 inhibition on DPP-induced changes in StAR protein expression. After 8 or 24 h of treatment with 100µM DPP with or without Cox2 inhibitor, protein was harvested, run on western blot, and probed with an antibody to StAR protein. At 8 h, StAR protein expression was significantly decreased in response to DPP phthalate (p=0.0025). Pre-treatment with Cox2 inhibitor (CI) prevented this effect (p=0.019). Each datapoint represents western results from at least 3 independent experiments. Error bars indicate 95% confidence intervals determined by a mixed effects ANOVA.

(Murphy et al., 2014). Furthermore, NHANES data shows a correlation between diethylhexyl phthalate and benzylbutyl phthalate metabolites and markers of inflammation and oxidative stress in humans (Ferguson et al., 2011. Epidemiological evidence suggests that exposure to a range of specific phthalates increases the risk of asthma and allergies (Bornehag and Nanberg, 2010; Kimber and Dearman, 2010). A clearer understanding of the underlying mechanism of the inflammation mediated effects of phthalates will inform risk assessment and facilitate construction of adverse outcome pathways for male reproductive toxicity.

Future studies should address the mechanism of phthalate influence on cox-2 activity. Cox-2 in Sertoli cells has previously been shown to be induced by the cytokine interleukin-1beta (IL1B) via JNK signaling (Ishikawa et al., 2005). This evidence warrants further exploration of these regulatory pathways in response to phthalate exposure. Further research should also explore the cell-type specific mechanisms of phthalate toxicity. These results also need to be replicated with other phthalates to determine whether this inflammatory mechanism is consistent across reproductively toxic phthalates or unique to DPP. Better understanding of the mechanisms of phthalate toxicity will contribute to more informed risk assessment of this ubiquitously used class of chemicals.

Conflict of Interest

Authors have not declared any conflict of interest.

REFERENCES

Alam MS, Ohsako S, Matsuwaki T, Zhu XB, Tsunekawa N, Kanai Y, Sone H, Tohyama C, Kurohmaru M (2010). Induction of spermatogenic cell apoptosis in prepubertal rat testes irrespective of testicular steroidogenesis: a possible estrogenic effect of di(n-butyl) phthalate. Reproduction 139 (2):427-37.

Barlow NJ, Phillips SL, Wallace DG, Sar M, Gaido KW, Foster PM (2003). Quantitative changes in gene expression in fetal rat testes following exposure to di(n-butyl) phthalate.Toxicol.Sci.73(2):431-41.

Bornehag CG, Nanberg E (2010). Phthalate exposure and asthma in children. Int. J. Androl. 33(2):333-45.

Clark BJ, Cochrum RK (2007). The steroidogenic acute regulatory protein as a target of endocrine disruption in male reproduction. Drug Metab. Rev. 39 (2-3):353-70.

David RM (2006). Proposed mode of action for in utero effects of some phthalate esters on the developing male reproductive tract. Toxicol. Pathol. 34(3):209-19.

Ferguson KK, Loch-Caruso R, Meeker JD (2011). Urinary phthalate metabolites in relation to biomarkers of inflammation and oxidative stress: NHANES 1999-2006. Environ. Res. 111(5):718-26.

Foster PM, Thomas LV, Cook MW, Gangolli SD (1980). Study of the testicular effects and changes in zinc excretion produced by some n-alkyl phthalates in the rat. Toxicol. Appl. Pharmacol. 54(3):392-8. Fromme H, Gruber L, Seckin E, Raab U, Zimmermann S, Kiranoglu M, Schlummer M, Schwegler U, Smolic S, Volkel W (2011). Phthalates and their metabolites in breast milk--results from the Bavarian Monitoring of Breast Milk (BAMBI). Environ. Int. 37(4):715-22.

Gaido KW, Hensley JB, Liu D, Wallace DG, Borghoff S, Johnson KJ, S Hall SJ, Boekelheide K (2007). Fetal mouse phthalate exposure shows that Gonocyte multinucleation is not associated with decreased testicular testosterone. Toxicol. Sci. 97 (2):491-503.

Granholm T, Creasy DM, Pollanen P, Soder O (1992). Di-n-pentyl phthalate-induced inflammatory changes in the rat testis are accompanied by local production of a novel lymphocyte activating factor. J Reprod. Immunol. 21(1):1-14. Gray LE Jr, Ostby J, FurrJ, Price M, Veeramachaneni DN, Parks L (2000). Perinatal exposure to the phthalates DEHP, BBP, and DINP, but not DEP, DMP, or DOTP, alters sexual differentiation of the male rat. Toxicol. Sci. 58(2):350-65.

Hannas BR, Furr J, Lambright CS, Wilson VS, Foster PM, Gray LE Jr (2011). Dipentyl phthalate dosing during sexual differentiation disrupts fetal testis function and postnatal development of the male Sprague-Dawley rat with greater relative potency than other phthalates. Toxicol. Sci. 120(1):184-93.

Hauser R, Calafat AM (2005). Phthalates and human health. Occup. Environ. Med. 62(11):806-18.

Heger NE, Hall SJ, Sandrof MA, McDonnell EV, Hensley JB, McDowell EN, Martin KA, Gaido KW, Johnson KJ Boekelheide K (2012). Human fetal testis xenografts are resistant to phthalate-induced endocrine disruption. Environ. Health Perspect. 120(8):1137-43.

Howdeshell KL, Rider CV, Wilson VS, Gray LE Jr (2008). Mechanisms of action of phthalate esters, individually and in combination, to induce abnormal reproductive development in male laboratory rats. Environ. Res. 108(2):168-76.

Ishikawa T, Hwang K, Lazzarino D, Morris PL (2005). Sertoli cell expression of steroidogenic acute regulatory protein-related lipid transfer 1 and 5 domain-containing proteins and sterol regulatory element binding protein-1 are interleukin-1beta regulated by activation of c-Jun N-terminal kinase and cyclooxygenase-2 and cytokine induction. Endocrinology 146(12):5100-11.

Johnson KJ, Heger NE, Boekelheide. K (2012). Of mice and men (and rats): phthalate-induced fetal testis endocrine disruption is species-dependent. Toxicol. Sci. 129(2):235-48.

Jurewicz J, Radwan M, Sobala W, Ligocka D, Radwan P, Bochenek M, Hawula W, Jakubowski L, Hanke W (2013). Human urinary phthalate metabolites level and main semen parameters, sperm chromatin structure, sperm aneuploidy and reproductive hormones. Reprod. Toxicol. 42:232-41.

Kimber I, Dearman RJ (2010). An assessment of the ability of phthalates to influence immune and allergic responses. Toxicology 271(3):73-82.

Kristensen DM, Skalkam ML, Audouze K, Lesne L, Desdoits-Lethimonier C, Frederiksen H, Brunak S, Skakkebaek NE, Jegou B, Hansen JB, Junker S, Leffers H (2011). Many putative endocrine disruptors inhibit prostaglandin synthesis. Environ. Health Perspect. 119(4):534-41.

Lahousse SA, Wallace DG, Liu D, Gaido KW, Johnson KJ (2006). Testicular gene expression profiling following prepubertal rat mono-(2-ethylhexyl) phthalate exposure suggests a common initial genetic response at fetal and prepubertal ages. Toxicol. Sci. 93(2):369-81.

Lambrot R, Muczynski V, Lecureuil C, Angenard G, Coffigny H, Pairault C, Moison D, Frydman R, Habert R, Rouiller-Fabre V (2009). Phthalates impair germ cell development in the human fetal testis in vitro without change in testosterone production. Environ. Health Perspect. 117 (1):32-7.

Lehmann KP, Phillips S, Sar M, Foster PM, Gaido KW (2004). Dose-dependent alterations in gene expression and testosterone synthesis in the fetal testes of male rats exposed to di (n-butyl) phthalate. Toxicol. Sci. 81(1):60-8.

Murphy CJ, Stermer AR, Richburg.JH (2014). Age- and Species-Dependent Infiltration of Macrophages into the Testis of Rats and Mice Exposed to Mono-(2-Ethylhexyl) Phthalate (MEHP). Biol. Reprod. biolreprod-113.

Mylchreest E, Cattley RC, Foster PM (1998). Male reproductive tract malformations in rats following gestational and lactational exposure to Di(n-butyl) phthalate: an antiandrogenic mechanism? Toxicol. Sci. 43(1):47-60.

Onorato TM, Brown PW, Morris PL (2008). Mono-(2-ethylhexyl) phthalate increases spermatocyte mitochondrial peroxiredoxin 3 and cyclooxygenase 2. J. Androl. 29(3):293-303.

Rock KL, Kono H (2008). The inflammatory response to cell death. Annu. Rev. Pathol. 3:99-126.

Silva MJ, Reidy JA, Herbert AR, Preau JL Jr, Needham LL, Calafat AM (2004). Detection of phthalate metabolites in human amniotic fluid. Bull. Environ. Contam. Toxicol. 72(6):1226-31.

Swan SH (2008). Environmental phthalate exposure in relation to reproductive outcomes and other health endpoints in humans. Environ. Res. 108(2):177-84.

Swan SH, Liu F, Hines M, Kruse RL, Wang C, Redmon JB, Sparks A, Weiss B (2010). Prenatal phthalate exposure and reduced masculine play in boys. Int. J. Androl. 33(2):259-69.

Swan SH, Main KM, Liu F, Stewart SL, Kruse RL, Calafat AM, Mao CS, Redmon JB, Ternand CL, Sullivan S. Teague. JL (2005). Decrease in anogenital distance among male infants with prenatal phthalate exposure. Environ. Health Perspect. 113(8):1056-61.

Wang X, Dyson MT, Jo Y, Stocco DM (2003). Inhibition of cyclooxygenase-2 activity enhances steroidogenesis and steroidogenic acute regulatory gene expression in MA-10 mouse Leydig cells. Endocrinol. 144(8):3368-75.

Wang X, Shen CL, Dyson MT, Eimerl S, J. Orly J, Hutson JC, D. M. Stocco DM (2005). Cyclooxygenase-2 regulation of the age-related decline in testosterone biosynthesis. Endocrinology 146(10):4202-8.

Wegner S, Hong S, Yu X, Faustman EM (2013). Preparation of rodent testis co-cultures. Curr. Protoc. Toxicol. 16-10.

Yu X, Hong S, Moreira EG, Faustman EM (2009). Improving in vitro Sertoli cell/gonocyte co-culture model for assessing male reproductive toxicity: Lessons learned from comparisons of cytotoxicity versus genomic responses to phthalates. Toxicol. Appl. Pharmacol. 239(3):325-36.

Yu X, Sidhu JS, Hong S, Faustman. EM (2005). Essential role of extracellular matrix (ECM) overlay in establishing the functional integrity of primary neonatal rat Sertoli cell/gonocyte co-cultures: an improved in vitro model for assessment of male reproductive toxicity. Toxicol. Sci. 84(2):378-93.