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Inflammatory response following *in vitro* exposure to methylmercury with and without n-3 long chain polyunsaturated fatty acids in systemic lupus erythematosus patients compared to healthy controls.

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Running title: Methylmercury and inflammation

Abstract

The heavy metal methylmercury (MeHg) is proposed as an environmental stimulus in the pathogenesis of systemic lupus erythematosus (SLE). Humans are primarily exposed to MeHg through fish consumption. Fish are the main dietary source of n-3 long chain polyunsaturated fatty acids (n-3 LCPUFA), which have been shown to be anti-inflammatory and beneficial to health. This *ex-vivo* study investigated the inflammatory response of isolated peripheral blood mononuclear cells (PBMCs) from SLE patients and healthy controls when exposed to $MeHgCl_2$. The difference in inflammatory response between SLE patients and healthy controls following co-exposure of cells to $MeHgCl_2$ and n-3 LCPUFA was also investigated. Separately, in the SLE and in the control group, the effect of co-exposure to $MeHgCl_2$ and n-3 LCPUFA on cytokine concentrations compared with the effect of $MeHgCl_2$ alone was investigated. A total of 12 SLE patients and 12 age and sex matched controls were recruited. PBMCs were isolated and exposed to 200nM of $MeHgCl_2$ in the presence of lipopolysaccharide (LPS) for 24 hr with or without co-exposure of the n-3 LCPUFA eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) at a concentration of 100 μM. Supernatants were analysed for the inflammatory markers IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13 and TNF-α. Following exposure to $MeHgCl_2$ and LPS, the mean TNF-α concentrations in the supernatants from SLE patients (2226.01 ±348.98 pg/ml) was significantly higher than those from matched controls (701.40 ±680.65pg/ml) (P=0.008). Co-exposure of cells with $MeHgCl_2$, EPA and LPS resulted in a significantly higher concentration of IL-8 in supernatants from SLE patients (2137.83 ±1559.01 pg/ml) compared to that of healthy controls (879.26 ±979.49 pg/ml) (P=0.030). Cells from SLE patients co-exposed to $MeHgCl_2$, n-3 LCPUFA and LPS elicited significantly lower concentrations of IL-1β, IL-2, IL-8 and TNF-α compared with SLE cells exposed to $MeHgCl_2$ and LPS. Cells from healthy controls co-exposed to $MeHgCl_2$, n-3 LCPUFA and LPS elicited significantly lower concentrations of IL-1β, IL-2, IL-4, IL-6 and

TNF-α compared with healthy cells exposed to $MeHgCl_2$ and LPS. This study suggests that SLE patients are hyper-responsive to $MeHgCl_2$ exposure in the presence of LPS and supports a role for n-3 LCPUFA in ameliorating inflammation in SLE patients.

Keywords: methylmercury, EPA, DHA, lupus, inflammation

Introduction

Systemic lupus erythematosus (SLE) is a prototypic chronic autoimmune disease with a variable presentation characterised by a loss of tolerance to self-antigen, autoantibody production and a dysregulated inflammatory response[1]. A number of environmental factors are implicated in the pathogenesis of SLE[2] including that of mercury (Hg) exposure[3]. Humans are exposed to Hg in several forms including, elemental mercury (Hg^0) through dental amalgams, ethyl mercury (EtHg) through some vaccinations and methylmercury (MeHg) through contaminated fish consumption. MeHg is an organic compound found in aquatic environments following the methylation of inorganic Hg (iHg). MeHg accumulates in fish, with higher amounts being found in large predatory species such as shark and swordfish[4,5]. MeHg exposure from fish consumption is of public health concern because once consumed MeHg is distributed widely throughout the human body and can cross the placental barrier, and the blood brain barrier[6]. The developing brain is particularly vulnerable to neurotoxic effects of MeHg[7]. Conversely, fish is a valuable source of protein contributing to 17% of the world's animal protein intake[8]. Fish also contain n-3 long chain polyunsaturated fatty acids (n-3 LCPUFA) which have been shown to have numerous beneficial effects on health[9].

In vitro research has reported that MeHg exposure results in an increase in interleukin (IL)-1β concentrations from human peripheral blood mononuclear cells (PBMCs)[10] whilst MeHg exposure in animal studies has been shown to increase concentrations of interferon (IFN)-γ[11]. Furthermore, MeHg exposure resulted in the development of a systemic autoimmunity in murine models that are genetically predisposed to metal-induced autoimmunity[12-14]. Murine MeHg-induced autoimmunity appears to be less severe than murine iHg-induced autoimmunity in that no immune complexes (IC) are deposited in organs of MeHg exposed mice[15]. A limited number of human studies have investigated the effects of MeHg on

autoimmunity. Two observational studies have reported an association between MeHg exposure and the production of antinuclear autoantibodies, suggesting that long term low dose exposure to MeHg may increase the risk of autoimmunity in healthy individuals[16,17].

Fish contain several health promoting nutrients, most notably the n-3 LCPUFA eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) that have been shown to have potent anti-inflammatory effects.[18] Several *in vitro* studies have shown that EPA and DHA can decrease the production of the pro-inflammatory cytokines IL-1, IL-6 and tumour necrosis factor (TNF)-α from human (endothelial, monocyte) and murine (macrophage) cell lines.[19-24] Furthermore, fish oil supplementation in mice that are genetically susceptible to autoimmunity has been shown to attenuate the secretion of pro-inflammatory cytokines,[25,26] delay the onset of autoimmunity,[27,28] and in some cases ameliorate autoimmunity entirely.[29,30] The results from human studies concur with those reported from *in vitro* and animal research in that supplementation of autoimmune patients with EPA and DHA has been shown to decrease pro-inflammatory cytokines,[31,32] and improve health outcomes.[33-36]

Although fish are rich in EPA and DHA which have been shown to be beneficial to SLE patients, fish are also a source of MeHg which has been suggested to be an environmental stimulus in the pathogenesis of SLE. This *ex-vivo* study will investigate the inflammatory response of isolated PBMC from SLE patients and healthy controls when exposed to $MeHgCl_2$ and LPS. The difference in inflammatory response between SLE and healthy control PBMCs following *ex-vivo* co-exposure to $MeHgCl_2$, LPS and n-3 LCPUFA will be compared. Separately, in the SLE and in the control group, the effect of co-exposure to $MeHgCl_2$ and n-3 LCPUFA on cytokine concentrations compared with the effect of $MeHgCl_2$ alone will be investigated.

Materials and Methods

Study population

Ethical approval (15/NI/0062) was granted by the Office of Research Ethics Committees Northern Ireland (ORECNI). PBMCs were isolated from 12 female SLE patients and 12 age (+/-5 years) and sex matched healthy controls. Participants suffering from SLE, who previously participated in research and consented, were contacted and recruited to take part in the current study. Each SLE participant met at least 4 of the 11 American College of Rheumatology diagnostic criteria[37] and were previously verified by a consultant rheumatologist in 2011.[38] SLE patients were excluded if they self-reported that they were currently taking steroids, non-steroidal anti-inflammatory drugs or supplementing with fish oils. Healthy controls were excluded if they had a history of autoimmune disease, were currently suffering from an acute illness or were currently taking non-steroidal anti-inflammatory drugs, or supplementing with fish oils.

Materials

All chemicals were purchased from Sigma-Aldrich unless otherwise stated. Phosphate buffered saline (PBS) was obtained from Analab (Lisburn, UK). Roswell Park Memorial Institute medium (RPMI) 1640, penicillin streptomycin, L-glutamine and heat inactivated foetal bovine serum (hi-FBS) were purchased from Invitrogen (Paisley, UK). $MeHgCl_2$, the commonly used analog of MeHg, was reconstituted in PBS at a stock concentration of 3mM and sterile filtered before being diluted to a working concentration of 200 nM. EPA and DHA were each dissolved in ethanol at a concentration of 10 mM and stored at -80^{o}C. EPA and DHA were diluted in RPMI to a final concentration of 100 µM each with 1% hi-FBS, 2% w/v

bovine serum albumin and 1% ethanol. The EPA and DHA mixtures were vortexed for 10 minutes and incubated for 2 h at 37ºC prior to addition with cell culture. LPS was prepared in PBS at a stock concentration of 1g/ml and frozen at -80ºC. All experiments used LPS aliquots from the same batch. All lab ware was endotoxin free (less than 0.5 EU).

Peripheral blood mononuclear cell isolation

A fasting blood sample was obtained from participants by venepuncture from the median antebrachial vein into 3 x 9ml sodium heparin tubes for isolation of PBMCs. Blood samples were diluted 2:1 (v/v) with RPMI 1640 media and added to a LeucoSep™ tube (Aquilant Scientific, Belfast, UK) and centrifuged at 1000*g* for 10 minutes. The buffy layer containing PBMCs was isolated and subsequently washed using RPMI 1640.

Cell culture and exposure

PBMCs were maintained in culture at a density of 1 x 10^6 cells/ml in RPMI 1640 with 25mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPEs) supplemented with % l-glutamine, 5% penicillin streptomycin, and 10% hi-FBS along with 100µM EPA, 100µM DHA or vehicle control at 37ºC and 5% CO_2 for 24 hours. Cells were then exposed to 200nM $MeHgCl_2$ and 50ng/ml LPS or vehicle control for a further period of 24 hours at 37ºC (Figure 1). Following centrifugation, supernatants were immediately stored at -80ºC until analysis.

Cytotoxicity assay

Previous studies have reported that 200nM of $MeHgCl_2$ is not cytotoxic to PBMCs[10,39]. In order to confirm that the change in inflammatory markers were not owing to non-specific cytotoxicity a 200µl aliquot of cells from each participant post exposure was seeded in a 96 well plate. A 15µl aliquot of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (500 µg/ml) was added to each well and incubated for 4 hours at 37ºC followed by the addition of 100µl solubilizing solution (10% triton X in acidic isopropanol-2) and a further incubation period of 2 hours at 37ºC. Absorbance values were measured on a VersaMax plate reader (Molecular devices, Sunnyvale CA) at a wave length of 570nm and 630nm and expressed as optical density (OD). This assay is based on the principle that viable cells metabolise tetrazolium salt to a purple formazan as previously described.[40]

Cytokine analysis

Cytokine secretion by PBMCs was measured by the electro chemiluminescence-based Meso Scale Discovery (MSD) immunoassay (Meso Scale Discovery, Gaithersburg, MD, USA). MSD plates were analyzed on the MS2400 imager MSD. IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13 and TNF-α concentrations were calculated using the manufacturer's software and results are presented as mean (± SD) in pg/ml. The lower limits of detection for each cytokine were: IL-1β, IL-8 and TNF-α; 0.4 pg/ml, IL-2; 0.09 pg/ml, IL-4; 0.02, IL-6; 0.06 pg/ml, IL-10; 0.03 pg/ml, IL-12p70; 0.11 pg/ml, IL-13; 0.24 pg/ml. The CVs for each cytokine were IL-1β = 4.4%, IL-2 = 7.1%, IL-4 =18%, IL-6 =7.2%, IL-8 = 5.8%, IL-10 = 8.2%, IL-12p70 = 11.7%, IL-13 = 7.8% and TNF-α = 5.2%.

Statistical analysis

Statistical comparisons were completed using IBM® SPSS® Statistics v22. Skewed data were transformed for analysis using the Shapiro-Wilk test. Results are displayed using the original mean (± SD). Mean OD values post MTT were converted to percentage change relative to cells exposed to media alone. The mean cell viability from all 24 participants following each condition was compared with the mean cell viability from media exposed cells using analysis of variance (ANOVA). The differences in cytokine concentrations between SLE participants and healthy controls exposed to $MeHgCl_2$ and LPS was assessed using analysis of covariance (ANCOVA), with cytokine concentrations from cells exposed to LPS alone as a covariate. The difference in cytokine concentrations between SLE participants and healthy controls exposed to $MeHgCl_2$ and EPA or DHA was assessed using ANCOVA with cytokine concentrations from cells exposed to LPS and $MeHgCl_2$ as a covariate. Further analysis compared the within group (SLE and healthy controls separately) effect of co-exposure to $MeHgCl_2$, EPA and DHA on cytokine concentrations compared with the effect of $MeHgCl_2$ alone using an independent T test.

Results

Study population

All SLE patients recruited to this study were female, the mean age (± SD) of the SLE cohort was 42.08 (± 13.34) years. The mean age (± SD) of the matched controls was 41.89 (±14.12) years.

Cell viability

The average absorbance of the negative control was 0.2 OD. The MTT assay confirmed that cells exposed to LPS, $MeHgCl_2$, $MeHgCl_2$ and LPS, $MeHgCl_2$ LPS and EPA, $MeHgCl_2$ LPS and DHA did not cause a significant decrease in cell viability in comparison to cells exposed

to media alone. The mean change in cell viability relative to the media control was shown to be LPS = -11%, $MeHgCl_2$= -5%, $MeHgCl_2$ and LPS = -8%, $MeHgCl_2$ LPS and EPA = +7%, $MeHgCl_2$ LPS and DHA = +3%.

$MeHgCl_2$ exposure: comparing inflammatory response between SLE and controls

Similar to Gardner *et al*. (2010),[39] no differences were observed in cytokine concentrations between cells exposed to $MeHgCl_2$ alone compared with cells treated with media (data not shown). Exposure of SLE patients' PBMCs to $MeHgCl_2$ and LPS resulted in a significantly higher concentration of TNF-α (P=0.008) when compared to cytokine concentrations from healthy control PBMCs also exposed to $MeHgCl_2$ and LPS (Table 1).

$MeHgCl_2$ and LCPUFA exposure: comparing inflammatory response between SLE and controls

PBMCs from SLE patients secreted significantly higher concentrations of IL-8 following exposure to $MeHgCl_2$, LPS and EPA compared to PBMCs from healthy controls under the same conditions (P=0.030) (Table 2).

There was no difference in cytokine concentration between SLE patients and healthy controls following exposure to $MeHgCl_2$, LPS and DHA (Table 3).

Inflammatory response of SLE patients following $MeHgCl_2$ and LCPUFA exposure

PBMCs from SLE patients exposed to $MeHgCl_2$, LPS and EPA had significantly lower mean concentrations of IL-1β (P=0.001), IL-8 (P=0.007) and TNF-α (P=0.048) compared with PBMCS from SLE patients exposed to $MeHgCl_2$ and LPS in the absence of EPA. PBMCs from SLE patients exposed to $MeHgCl_2$, LPS and DHA had significantly lower mean

concentrations of IL-1β (P=0.003), IL-2 (P=0.003), IL-8 (P=0.010) and TNF-α (P=0.009) compared with PBMCs from SLE patients' cells exposed solely to $MeHgCl_2$ and LPS.

Inflammatory response of controls following $MeHgCl_2$ and LCPUFA exposure

PBMCs from healthy controls exposed to $MeHgCl_2$, LPS and EPA had significantly lower mean concentrations of IL-1β (P=0.002), IL-2 (P=0.015), IL-4 (P=<0.001), IL-6 (P=<0.001), and TNF-α (P=0.002) compared with PBMCs from healthy controls exposed to $MeHgCl_2$ and LPS in the absence of EPA. PBMCs from healthy controls co-exposed to $MeHgCl_2$, LPS and DHA had significantly lower mean concentrations of IL-1β (P=0.001), IL-2 (P=0.015), IL-4 (P=<0.001), IL-6 (P=<0.001), and TNF-α (P=0.002) compared with PBMCS from healthy controls exposed solely to $MeHgCl_2$ and LPS.

Discussion

SLE patients appear to be more responsive to the inflammatory inducing effects of *ex vivo* MeHg exposure, as PBMCs from SLE patients elicited higher concentrations of TNF-α compared to those of the control group. Co-exposure to MeHg and n-3 PUFA resulted in a lower inflammatory response albeit PBMCs from SLE patients secreted higher concentrations of IL-8 following $MeHgCl_2$, LPS and EPA exposure when compared with healthy controls under the same conditions. Overall, both EPA and DHA appear to mitigate the $MeHgCl_2$-induced inflammatory response, albeit the effect was weaker in SLE patients than in controls suggesting SLE patients may require higher concentrations of n-3 LCPUFA.

Cells from SLE patients secreted higher concentrations of TNF-α than healthy controls following *ex vivo* $MeHgCl_2$ exposure in the presence of the adjuvant LPS. TNF-α is upregulated in lupus nephritis[41] and is positively correlated with disease activity.[42] Infliximab

is a medication directed against TNF-α and its use has been shown to have a beneficial effect on joint tenderness and proteinuria in SLE patients with arthritis and nephritis.[43,44] Therefore, the $MeHgCl_2$-induced rise in TNF-α concentrations observed in this study could potentially be detrimental to health. However, we observed that co-exposure with both EPA and DHA ameliorated this significant increase in TNF-α and as MeHg is typically encountered in the presence of n-3 LCPUFA this suggests that n-3 LCPUFA outweigh any detrimental inflammatory effects of $MeHgCl_2$. Similar findings have been reported when assessing the effects of fish consumption on child development.[45-48]

This study demonstrated that the anti-inflammatory effects of n-3 LCPUFA are more potent in PBMCs from healthy controls compared to SLE patients. SLE patients had significantly higher concentrations of IL-8 compared with healthy controls following $MeHgCl_2$, LPS and EPA exposure. The role of IL-8 is critical to leukocyte infiltration which could lead to neutrophil-induced tissue damage in patients with SLE through enhanced secretion of neutrophil elastase, cathepsins and myeloperoxidase.[49] Neutrophil elastase and cathepsins can breakdown renal tissue through the proteolysis of both collagen-IV and elastin[49,50] whereas myeloperoxidase elicits deleterious effects through oxidative damage.[51] Urinary concentrations of IL-8 are higher in SLE patients with glomerular diseases compared with healthy controls.[52] It is plausible that SLE patients require a higher intake of n-3 PUFAs to achieve the same anti-inflammatory effects as in healthy controls.

EPA mitigated the MeHg-induced rise in IL-1β, IL-8 and TNF-α in SLE patients and the MeHg-induced rise in IL-1β, IL-2, IL-4, IL-6, and TNF-α in healthy controls. DHA mitigated the MeHg-induced rise in IL-1β, IL-2, IL-8, and TNF-α in SLE patients and the MeHg-induced rise in IL-1β, IL-2, IL-4, IL-6, and TNF-α in healthy controls.

Both EPA and DHA mitigate the $MeHgCl_2$-induced rise in IL-2, IL-4, and IL-6 in healthy controls and only DHA mitigated the $MeHgCl_2$-induced rise in IL-2 in SLE patients. These cytokines are involved in B cell differentiation and subsequently humoral immunity, which is dysregulated in SLE.[53] The raised concentrations of IL-2, IL-4 and IL-6 in SLE could result in an enhanced production of Th2 cells, which could further perpetuate the production of autoantibodies.[54] EPA and DHA significantly decreased IL-8 in SLE patients but not in healthy controls. IL-8 is secreted from macrophages, which are part of the innate immune system. It is possible that n-3 LCPUFA are unable to decrease cytokines regulated by humoral immune cells due to the dysregulation of humoral immunity in SLE. The mechanism responsible for the difference in response to EPA and DHA between SLE patients and healthy controls needs to be investigated.

The n-3 and n-6 LCPUFA are precursors to leukotrienes, prostaglandins and thromboxanes. Arachidonic acid, a potent n-6 LCPUFA is a substrate in the production of leukotrienes LTC4 and LTD4 through lipoxygenase (LOX) or prostaglandins (PGD2, PGE2, PGF2) and thromboxane (TXA2) through cyclooxygenase (COX). These leukotrienes, prostaglandins and thromboxanes lead to an increase in pro-inflammatory cytokines.[55] EPA and DHA are also substrates for eicosanoids producing less pro-inflammatory derivatives such as PG3, LTB3 and LTC5. Therefore, when n-3 LCPUFA are available as substrates there is an inhibition of n-6 LCPUFA produced eicosanoids in favour of n-3 derived eicosanoids and a less pronounced pro-inflammatory effect results.[56] A further mechanism that could be acting in conjunction with the above is the activation of nuclear transcription factors peroxisome proliferator-activated receptors (PPARs). EPA and DHA are agonists for the PPAR-γ/RXR complex which upon activation will subsequently translocate to the nucleus to alter gene expression. Activation of the PPAR-γ/RXR complex has been shown to prevent the

translocation of NFκB to the nucleus which results in decreased transcription of its pro-inflammatory targets including TNF-α, IL-1, IL-6 and IL-8.[57] The decrease in production of pro-inflammatory cytokine elicited by EPA and DHA has been suggested to contribute to the clinical benefits noted following n-3 LCPUFA supplementation. Several human intervention studies have reported a decrease in disease activity in SLE patients following n-3 LCPUFA supplementation,[33-36] albeit the molecular mechanism responsible for this effect in human studies remains to be elucidated.

The n-6 LCPUFA were not present in any of the experimental conditions in this study and therefore the n-3 LCPUFA effect may have been heightened and it is important to consider this when interpreting these results. A strength of this study is that the dosage used of EPA and DHA translates to a blood concentration of 0.1mmol/l, which is achievable in the general population.[58] Nevertheless, the true effect of n-3 LCPUFAs on mitigating the inflammatory effect of MeHg exposure should be tested in individuals, with and without SLE, who regularly consume fish. Furthermore, the inclusion of patients who are suffering from a disease flare could further aid in the understanding of how environmental factors preclude and perpetuate disease severity. This study did not statistically control for medication intake such as hydroxychloroquine and methotrexate which have been shown to effect cytokine secretion from PBMC.[59,60] The use of $MeHgCl_2$ rather than MeHg in this study means it is not truly presentative of MeHg consumed through fish. MeHg when ingested is conjugated with L-cysteine and forms a molecule similar to L-methionine. Cells uptake $MeHgCl_2$ through diffusion whilst MeHg-cysteine is taken up via a carrier.[61]

Conclusion

This research investigated the difference in inflammatory response of SLE patients to that of healthy controls following *ex vivo* $MeHgCl_2$ and LPS exposure with or without pre-treatment with n-3 LCPUFA. The *ex vivo* exposure to $MeHgCl_2$ and LPS promotes inflammation in SLE patients and healthy controls; however, SLE patients have a stronger pro-inflammatory effect. Both EPA and DHA decreased MeHg Cl_2 induced inflammation albeit EPA and DHA appear to be less effective in SLE patients compared with healthy controls. Taken together the results from this study suggests that inflammation promoting effects from low dose $MeHgCl_2$ exposure can be mitigated by n-3 LCPUFA. Therefore, the benefit of consuming a diet of fish rich in n-3 LCPUFA for SLE patients appears to outweigh any potential inflammatory effect of MeHg. Further research is required to investigate the effect of MeHg exposure in individuals with SLE to investigate the risk/benefits of fish consumption in SLE.

Conflict of interest

The authors declare that there are not conflicts of interest

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Table 1: Mean (SD) cytokine concentrations from PBMCs of SLE patients (N=12) and healthy controls (N=12) following MeHg & LPS or LPS exposure alone

	SLE		Healthy		
Cytokine	LPS	LPS & MeHg	LPS	LPS & MeHg	P =
IL-1β	92.39 (79.65)	167.13 (98.07)	37.07 (38.12)	65.68 (68.09)	0.487
IL-2	59.62 (93.33)	84.99 (69.98)	34.97 (29.88)	46.26 (59.42)	0.554
IL-4	2.84 (6.91)	19.27 (45.63)	0.69 (1.40)	4.77 (3.32)	0.424
IL-6	1892.28 (3229.30)	3498.54 (7008.70)	510.46 (657.54)	861.45 (814.58)	0.597
IL-8	25209.33 (22986.28)	27587.60 (26469.74)	14419.11 (22084.62)	15316.33 (16876.38)	0.688
IL-10	152.23 (111.59)	93.30 (125.32)	180.71 (125.11)	96.63 (123.96)	0.933
IL-12p70	13.42 (11.60)	23.69 (53.88)	187.45 (9.43)	172.21 (25.22)	0.315
IL-13	219.58 (138.28)	190.92 (110.81)	187.45 (107.21)	172.21 (236.43)	0.992
TNF-α	979.30 (1656.39)	**2226.01 (348.98)**	390.93 (2752.68)	**701.40 (680.65)**	**0.008***

Analysis was conducted using ANCOVA, comparing cytokine concentrations of SLE patients with healthy controls following MeHg and LPS with cytokine concentrations from cells exposed to LPS alone as a covariate. Cytokine concentrations expressed in pg/mL. P values below 0.05 are considered significant.

LPS: lipopolysaccharide, MeHg: methylmercury, SD: standard deviation, SLE: systemic lupus erythematosus, PBMC: peripheral blood mononuclear cells

Table 2: Mean (SD) cytokine concentrations from PBMCs of SLE patients (N=12) and healthy controls (N=12) following MeHg & LPS and EPA, MeHg & LPS exposure

	SLE		Healthy		
Cytokine	MeHg & LPS	EPA, MeHg & LPS	MeHg & LPS	EPA, MeHg & LPS	P =
IL-1β	167.13 (98.07)	28.8 (45.41)	65.68 (68.09)	2.97 (2.71)	0.270
IL-2	84.99 (69.98)	23.09 (51.23)	46.26 (59.42)	4.4 (3.60)	0.187
IL-4	19.27 (45.63)	2.42 (3.66)	4.77 (3.32)	0.08 (0.095)	0.062
IL-6	3498.54 (7008.70)	379.64 (617.78)	861.45 (814.58)	20.11 (24.02)	0.152
IL-8	27587.60 (26469.74)	**2137.83 (1559.01)**	15316.33 (16876.38)	**879.26 (979.49)**	**0.030**
IL-10	93.30 (125.32)	69.59 (189.23)	96.63 (123.96)	4.15 (4.95)	0.242
IL-12p70	23.69 (53.88)	0.89 (2.46)	172.21 (25.22)	0.13 (0.41)	0.513
IL-13	190.92 (110.81)	82.18 (104.96)	172.21 (236.43)	22.9 (25.30)	0.076
TNF-α	2226.01 (348.98)	234.42 (312.03)	701.40 (680.65)	37.96 (44.36)	0.132

Analysis was conducted using ANCOVA, comparing cytokine concentrations of SLE patients with healthy controls following exposure to MeHg, LPS and EPA with cytokine concentrations from cells exposed to MeHg & LPS alone as a covariate. Cytokine concentrations expressed in pg/mL. P values below 0.05 are considered significant

EPA: eicosapentaenoic acid, LPS: lipopolysaccharide, MeHg: methylmercury, SD: standard deviation, SLE: systemic lupus erythematosus, PBMC: peripheral blood mononuclear cells

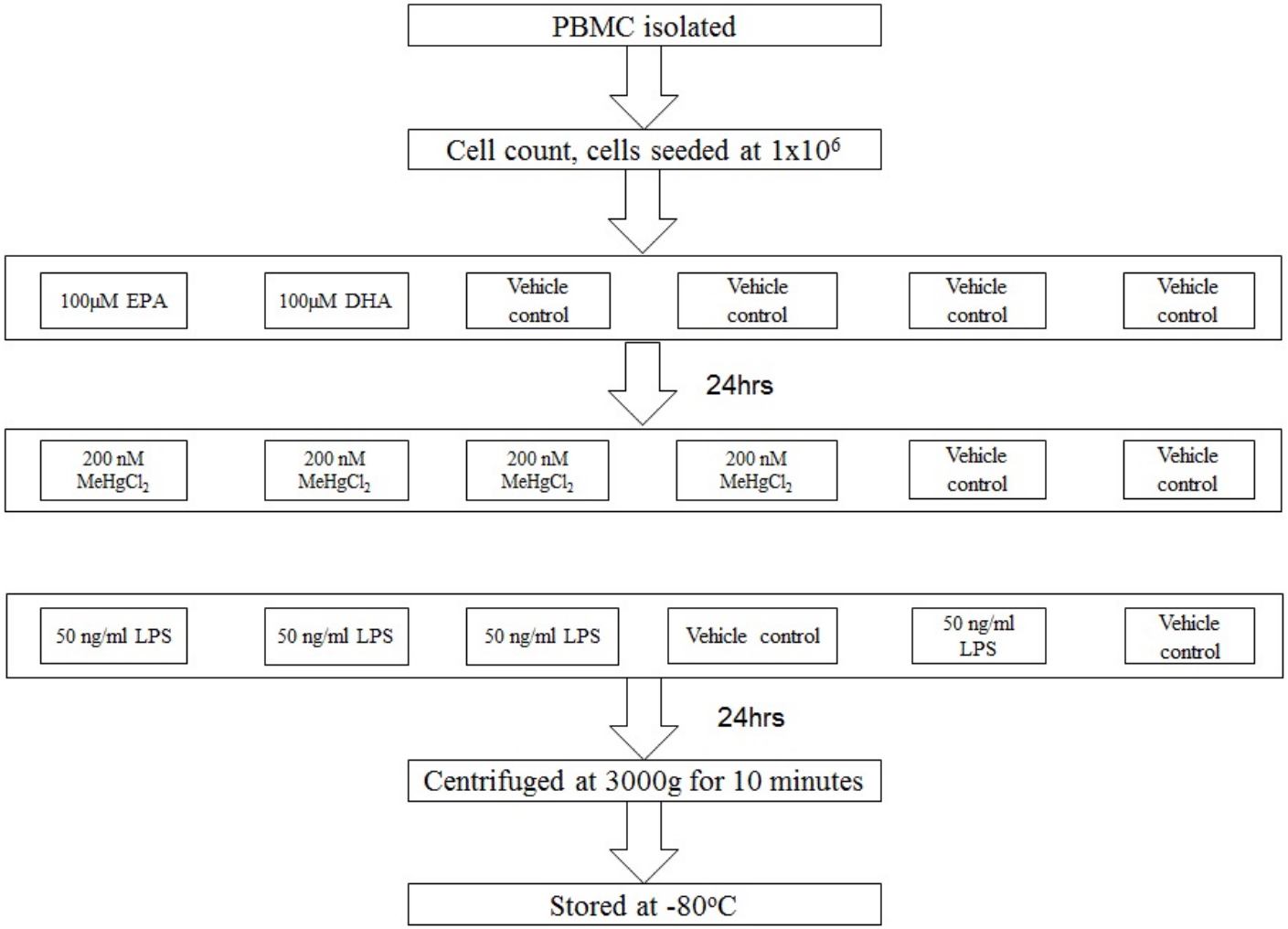
Table 3: Mean (SD) cytokine concentrations from PBMCs of SLE patients (N=12) and healthy controls (N=12) following MeHg & LPS and DHA, MeHg & LPS exposure

	SLE		Healthy		
Cytokine	MeHg & LPS	DHA, MeHg & LPS	MeHg & LPS	DHA, MeHg & LPS	P =
IL-1β	167.13 (98.07)	24.11 (48.99)	65.68 (68.09)	2.89 (2.24)	0.257
IL-2	84.99 (69.98)	3.71 (4.84)	46.26 (59.42)	7.6 (12.80)	0.254
IL-4	19.27 (45.63)	1.6 (2.93)	4.77 (3.32)	0.07 (0.16)	0.185
IL-6	3498.54 (7008.70)	683.25 (1961.84)	861.45 (814.58)	23.32 (29.59)	0.159
IL-8	27587.60 (26469.74)	2962.86 (4213.09)	15316.33 (16876.38)	1121.18 (1099.89)	0.165
IL-10	93.30 (125.32)	26.45 (60.10)	96.63 (123.96)	9.96 (10.87)	0.355
IL-12p70	23.69 (53.88)	4.01 (8.50)	172.21 (25.22)	0.13 (0.17)	0.154
IL-13	190.92 (110.81)	67.88 (91.15)	172.21 (236.43)	9.94 (11.45)	0.517
TNF-α	2226.01 (348.98)	515.07 (1276.77)	701.40 (680.65)	65.84 (55.88)	0.516

Analysis was conducted using ANCOVA, comparing cytokine concentrations of SLE patients with healthy controls following MeHg, LPS and DHA with cytokine concentrations from cells exposed to MeHg & LPS alone as a covariate. Cytokine concentrations expressed in pg/mL.

DHA: docosahexaenoic acid, LPS: lipopolysaccharide, MeHg: methylmercury, SD: standard deviation, SLE: systemic lupus erythematosus, PBMC: peripheral blood mononuclear cells

Figure 1: Flow diagram of experimental protocol.



Abbreviations; EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, LPS lipopolysaccharide, $MeHgCl_2$: methylmercury chloride. SLE: systemic lupus erythematosus, PBMC: peripheral blood mononuclear cells