Organic Arsenical Exposure Stimulates Atherosclerosis through Oxidative Stress Increase and Adhesion Molecule Expression

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ARTICLE INFO

ABSTRACT

Approximately 100 million people are exposed to arsenic worldwide, majorly through drinking water and anthropogenic activities. Monosodium methylarsonate (MSMA) is a potent organoarsenical content of herbicides used in many Asian countries. Epidemiological studies have linked inorganic arsenic exposure with atherosclerosis, whereas organoarsenicals toxicological studies are scanty. Paraoxonase 1 (PON1) enzyme suppresses systemic Ox-LDL generation, thereby preventing atherosclerosis. We investigated effects of MSMA oral exposure on PON1, lipid peroxidation and atherosclerosis development. Five groups (n=11) of Sprague-Dawley rats received daily intubation of MSMA at 0 (control), 42.1, 63.2, 126.4 and 210.7 mg/kg BW respectively for 16 weeks. Serum samples were analysed for PON1 activities, Ox-LDL and MDA levels. Histomorphometric evaluation (H&E and VVG) and immunohistochemistry (VCAM-1 and ICAM-1) were done on aorta. High mortality rate led to discontinuation of 126.4 and 210.7 mg/kg BW treatment groups. Groups treated with 42.1 and 63.2 mg/kg B.W. MSMA had a significantly higher MDA (p=0.004,CI: 2.73-0.82) and Ox-LDL (p<0.0001,CI: 2425.07-955.45) levels but lower PON1:Ox-LDLratio (p<0.0001,CI: 0.49-1.07) compared to control. Microscopically, treatment groups showed early atherosclerotic intima thickening and positive VCAM-1 and ICAM-1 expressions. In conclusion, chronic MSMA exposure reduced PON1 ability to hydrolyse Ox-LDL and also induced inflammation by elevating oxidative stress that supports early atherosclerosis development.

INTRODUCTION

Health challenges associated with chronic and acute exposure to arsenic (As) have drawn attentions around the world (Hughes *et al.,* 2011; Bolt, 2012; Chen *et al.,* 2013). Agricultural practices have been identified to contribute immensely towards increasing arsenic exposure above normal healthy range in our environment (Garelick *et al.,* 2008; Bolt, 2013). MSMA is an organic form of arsenic used as active ingredient of some herbicides and pesticides for controlling weeds and insects in crops and non-crops areas worldwide particularly in Asian

countries (Arnold *et al.,* 2003; Albert *et al.,* 2008; Morrissey *et al.,* 2008; Hammid *et al.,* 2013). Humans are exposed to the toxicity of arsenic mainly via drinking water and anthropogenic activities. It is evident that various side effects of MSMA has been described in earlier studies which include hepatocellular damage, renal toxicity, neurological problems as well as skin problems (Hessl and Berman, 1982; De Capitani *et al.,* 2005; Yao *et al.,* 2013; Casale *et al.,* 2014). Atherosclerotic disorders have been linked to lipid peroxidation (Kei, 1978). On the same note, epidemiological studies have also established a link between high-chronic inorganic arsenic exposure with cardiovascular diseases such as stroke, coronary artery disease (CAD) and peripheral arterial disease (Stea *et al.,* 2014; Samsuddin *et al.,* 2015). Previous study also reported that inorganic arsenic induces atherosclerosis and endothelial dysfunction in experimental rats (Lee *et al.,* 2002).

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However, studies focusing on the adverse effects of organic arsenic are limited (Kato *et al.,* 2010; Hammid *et al.,* 2013; Ong *et al.,* 2013). PON1 is a lactonase cardio-protective enzyme that hydrolyses ox-LDL and prevents oxidation of LDL (Aviram *et al.,* 1998; Hao *et al.,* 2013). PON1 is also associated with HDL and plays a vital role in suppressing ox-LDL-generated inflammation on arterial endothelium that can initiate atherosclerosis (Eckerson *et al.,* 1983; Gan *et al.,* 1991; Durrington *et al.,* 2001; Efrat and Aviram, 2010). However, literature on organic arsenicals' effect on PON1 does not exist to the best of our knowledge. To bridge this literary deficiency, our study investigated popular agriculturally used organic arsenic (MSMA) oral exposure on PON1 activity, lipid peroxidation and atherosclerosis development in rat model.

MATERIAL AND METHODS

Animal housing and materials

Male Sprague-Dawley rats of approximately 250g, 3-4 months old, were purchased from Animal breeding centre, Universiti Putra Malaysia, Selangor. Animals were housed in polypropylene cages, two animals per cage, in Kulliyyah of Medicine, IIUM animal retention facility. They were exposed to 12 hours light and dark cycle at 25^0C throughout the study. The rats were allowed access to standard rat chow (Gold Coin, Malaysia) and reverse osmosis (RO) water (ELGA Prime system, USA) *ad libitum.*

Animals were allowed to acclimatize for one week in our animal retention area after which blood sample was taken through orbital sinus from all the animals, under inhalation anaesthesia, for the pre-treatment serum sample isolation. Then the animals were divided into five groups randomly. These groups include control group, treatment 1(T1), 2(T2), 3(T3) and 4(T4) groups. Research planning and protocols were approved by International Islamic University Malaysia (IIUM) Institutional Animal Care and Use Committee (IACUC) with approval number IIUM/IACUC Approval/2014/ (3)(11). Study was performed humanely in accordance to IACUC guidelines. MSMA was purchased from Ancom Corp (Malaysia) and all other chemicals used were analytical grade supplied by Mercks (Germany) or Sigma (Texas) chemicals except stated otherwise.

Dose Planning and Sample Collection

To the best of our knowledge after extensive search, no previous academic toxicology study of MSMA on murine exists. Hence, our dose planning was done using the toxicity information on the chemical data sheet (CDS) of the product published by the manufacturer, Ancom Corp; in addition to the reported inorganic arsenic contamination doses within the south Asian countries (Nordstrom, 2002). Ancom Corp reported 1264mg/Kg as the LD_{50} of MSMA for rats (Ancom, 2012). Previous study reported drinking water iAs contamination of up to 5000μg/L (5mg/L) for Thailand and up to 100,000μg/L (100mg/L) for USA and Canada (Nordstrom, 2002). Meanwhile, daily adult human water

consumption is approximately 4 litres (Sawka *et al.,* 2005). Therefore, our chosen doses were calculated to simulate a real life arsenic exposure as reported in previous studies (Nordstrom, 2002). Fifty five male Sprague-Dawley rats were divided into five groups (n=11) including a control group. Treatment groups 1, 2, 3 and 4 were given oral intubation of 42.13, 63.30, 126.40 and 210.67 mg/kg body weight of MSMA respectively daily for 16 weeks, which is equivalent to 1/30, 1/20, 1/10 and 5/30 LD_{50} of MSMA for rat reported by Ancom, (2012) and it also mimic the calculated daily drinking water arsenic exposure for some South Asian countries (Thailand) and some other countries (USA and Canada) around the world (Nordstrom, 2002, Ilyas*et al.,* 2009, Ancom, 2012). Pre and post treatment blood samples were collected into plain tubes through orbital sinus under inhalation anesthesia for serum isolation.

Biochemical tests

Serum PON1 activities, paraoxonase and arylesterase were determined after hydrolysis of paraoxon and phenylacetate substrates respectively. Serum paraoxonase activity measured the rate of phenol production after hydrolysis by paraoxon as described by Eckerson *et al.* (1983). Molar extinction coefficient of $18290M^{-1}$ cm^{-1} (Eckerson *et al.*, 1983) was used to calculate activity. Arylesterse activity was carried out according to Gan *et al.* (1991) method. MDA was determined spectrophotometrically by TBARS assay as described by Kie, (1978) and Ox-LDL level was measured following Ox-LDL ELISA kit SEA527Ra (Cloud-Clone Corp, Houston, TX) manufacturer's instruction.

Aorta Histomorphometric Assessment

Formalin (10%) fixed aorta samples were processed and embedded in parafin. Aorta sections (4μm) were microscopically evaluated after staining with Haematoxylin and Eosin (H&E) using manual protocol. In order to precisely view the position of the internal elastic lamina (IEL) of the aorta, elactic fibre assessment was done by carrying out Verhorff Van Gieson (VVG) staining using connective tissue staining kit, ab150667 from Abcam (Cambridge, U.K). Immunohistochemistry (IHC) was performed to assess the expression of adhesion molecule and early inflammatory markers VCAM-1 and ICAM-1 using Dako autostainer and EnVision+System-HRP(DAB) kit with rabbit antibody (Carpinteria, CA, USA). VCAM-1 and ICAM-1 antibodies were diluted to 1:200 and 1: 150 respectively for IHC.

Statistical Analysis

Normality of data was determined by Kolmogrov-smirnov test. ANOVA was used to test parametric data of PON1 activities, oxLDL, PON1:Ox-LDL ratio and MDA levels between the study groups. Non-parametric data were tested using Kruskal-walis test. Paired t-test was also used to test between pre and post treatment measurements for normally distributed data while Friedman's test was used for non-normally distributed data. All analysis was performed using IBM SPSS version 21. P< 0.05 was chosen to be statistically significant at 95% confidence interval.

RESULTS

Clinical Signs and Mortality

Generally, the most common clinical signs observed at the beginning of the study in most of the treatment groups were diarrhea and weight fluctuation. These signs automatically alleviated or stopped, without any intervention, within few days in MSMA treated groups 1 (T1) and 2 (T2) that were given 42.13 and 63.20 mg/kg body weight MSMA respectively. However mortality was recorded in MSMA treated groups 3 and 4 that were administered with 126.4 and 252.8 mg/Kg BW. The mortality was associated with severe diarrhea and drastic weight decrease in both groups. Calculated mortality rate were 0.636 and 0.545 for groups 3 and 4 respectively. Therefore, treatment was discontinued for animals in groups 4 by fifth week and group 5 by second week because of the high mortality rate recorded in those groups. Control group animals were consistent in growth and did not show any sign of diarrhea.

Body and organ weight

The weight trend of each group is presented in Figure 1. Comparing the MSMA treated groups with Control group, there was a mean weight decrease of 10.36% in Treatment group 1 (42.13mg/Kg BW MSMA) while Treatment group 2 (63.20 mg/Kg BW MSMA) recorded reduction of 5% in its mean weight at week 16. There was no statistical significance in mean relative organ weight (defined as organ weight/body weight) for liver (p=0.761), kidney (p=0.684) and heart (p=0.379). A reduction of 17% and 8% was detected in actual mean weight of heart and kidney respectively in Treatment group 1 when compared to that of Control group. Tables 1 and 2 present actual organ weight and relative organ weights in detail.
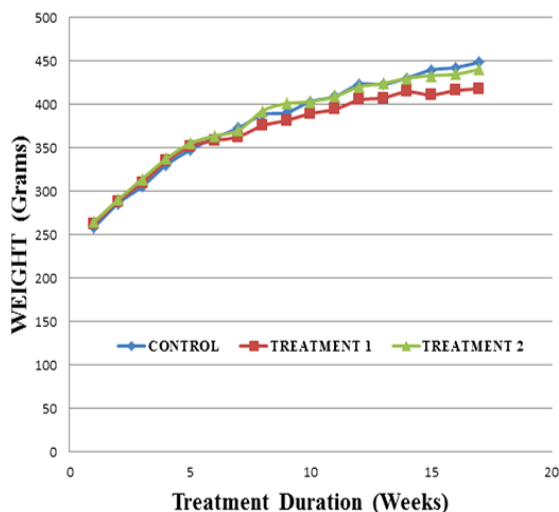

Fig. 1: Mean Weight Trend in Control and MSMA Treated Group.

Serum Paraoxonase 1 (PON 1) Activities

After 16 weeks of MSMA treatment, Treatment group 1 has 50.91% increase in mean PON1 activity as compared to that of Control group, while PON1 activity in Treatment group 2 was decreased by 4.378% compared to Control. There were no significant difference (p=0.097) in mean arylesterase activities between Control and MSMA treated groups (Table 3). Although insignificant (p>0.05), comparing PON1 activity between pre and post treatment in each group, paraoxonase activity in Control group increased by 19.32% which is higher increment as compared to 6.82% increase recorded in Treatment group 1. On the other hand, Treatment group 2 recorded a decrease in paraoxonase activity after MSMA treatment by 5.86% (Figure 2). Likewise, arylesterase was also insignificant (p>0.05) after comparing pre and post MSMA treatment in each group (figure 3). The arylesterase activity in Control group was reduced by 0.3% and arylesterase activity in MSMA treated groups 1 and 2 increased by 4.7% and 16.4% respectively.
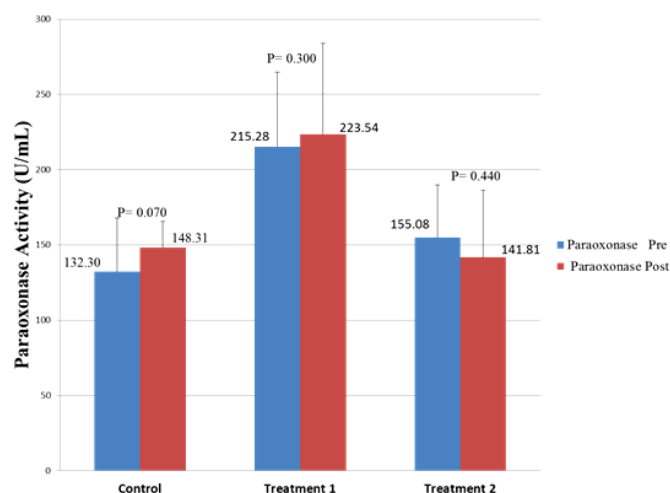

Fig. 2: Comparison of Serum Paraoxonase Activity during pre and post MSMA treatment. Results presented as mean (SD). Paired t-test. P< 0.05 is taken as statistical significant at 95% confidence interval.
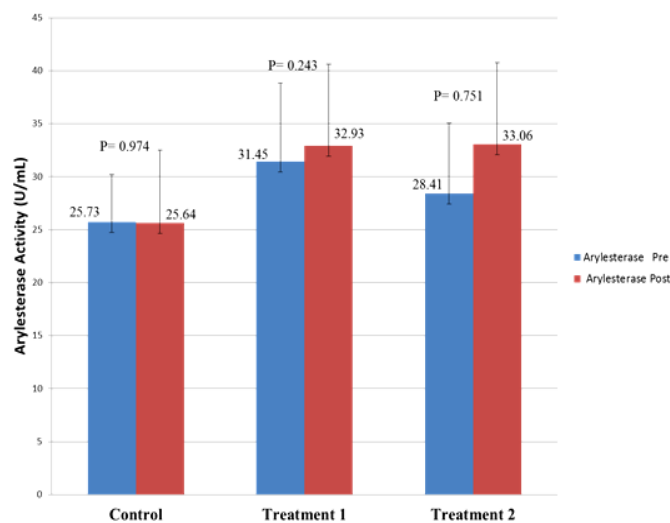

Fig. 3: Comparison of Serum Arylesterase Activity Between Pre and Post MSMA treatment. Results presented as mean (SD). Paired t-test. P< 0.05 was taken as statistically significant at 95% confidence interval.

Table 1: Mean Organ Weight in Control and MSMA Treated Groups.

Weight (grams)	Mean Organ Weight (grams)			
	Control n= 8	Treatment 1 n= 8	Treatment 2 n= 8	P-value
Liver	11.34 (1.56)	10.53 (1.41)	11.16 (2.12)	0.639
Kidney	2.92 (0.32)	2.68 (0.22)[b]	2.93 (0.55)	0.340
Heart	1.52 (0.24)	1.26 (0.19)[a]	1.39 (0.32)[b]	0.163

Values represent mean (SD). [a]=17%, [b]=8% organ weight reduction compared with control group. $P<0.05$ was taken to be statistically significant at 95% CI.

Table 2: Mean Relative Organ Weight in Control and MSMA Treated Groups.

Organ	Mean Relative Organ Weight			
	Control n= 8	Treatment 1 n= 8	Treatment 2 n= 8	P-Value
Liver	0.023(0.009)	0.024 (0.003)	0.024 (0.004)	0.379
Kidney	0.006 (0.001)	0.006 (0.000)	0.006 (0.001)	0.379
Heart	0.003 (0.001)	0.003 (0.001)	0.003 (0.001)	0.379

Values represent median (IQR). Kruskal-Walis test. $P<0.05$ was taken as statistically significant at 95% confidence interval. IQR= Interquartile range.

Table 3: Paraoxonase 1 (PON1) Activity in Control and MSMA treated groups.

Test Parameter	Treatment Group	n	Mean (SD)	F-Stat (df)	P-Value
Paraoxonase (U/ml)	Control	8	148.31(17.18)		
	Treatment 1	8	223.54(60.45)	8.326(2,21)	0.002*
	Treatment 2	8	141.81(44.72)		
Arylesterase (U/ml)	Control	8	25.64(6.888)		
	Treatment 1	8	32.93(7.666)	2.615(2,21)	0.097
	Treatment 2	8	33.06(7.696)		

Results presented as mean (standard deviation, SD). One way ANOVA and Tukey post hoc test. $P<0.05$ was taken as statistically significant at 95% confidence interval. *Significant difference ($p<0.05$) in mean paraoxonase activity between Control and Treatment group 1 with mean difference of 75.51U/ml (CI: 37.06 – 111.41) and between Treatment group 1 and Treatment group 2 with mean difference of 81.73U/ml (CI: 68.57 – 94.89). No significant difference in mean arylesterase activity between Control and MSMA treated groups ($p>0.05$).

Table 4: Lipid Peroxidation Parameters in the Control and MSMA treated groups.

Test Parameter	Control Mean (SD) n=8	Treatment 1 Mean (SD) n=8	Treatment 2 Mean (SD) n=8	F-Stat (df)	P-Value
MDA (nmol/ml)	4.70(0.93)	6.75(2.64)	5.96(0.87)	2.93(7)	0.1010
Ox-LDL (pmol/ml)	199.79(37.66)	1890.06(18.71)	1222.03(95.94)	17.05(7)	<0.0001*

Results presented as mean (SD). One way ANOVA and Tukey post hoc test. $P<0.05$ was taken as statistically significant at 95% confidence interval.
*Significant difference ($p<0.001$) in mean ox-LDL level between Control and Treatment group 1 with mean difference of 1690.26 pmol/ml (CI: 2425.07 – 955.45) and between Control and Treatment group 2 with mean difference of 1022.23 pmol/ml (CI: 1757.04 – 287.42).
Ox-LDL= oxidized low density lipoprotein, df= degree of freedom.

Lipid Peroxidation Assays: Malondialdehyde (MDA) concentration and ox-LDL Level

Table 4 presents mean (SD) of MDA concentration and ox-LDL level in Control and MSMA treated groups after 16 weeks treatment. The mean MDA concentrations were 43.60% and 26.73% higher in T1 and T2 respectively as compared to that of Control (Table 4) although there was no significant statistical difference ($p>0.05$).

Ox-LDL level in T1 was more than 8 times that of Control group while ox-LDL level in T2 was more than 5 times that of control group content. There was a significant difference ($p<0.001$) in ox-LDL level between Control and MSMA treated groups (Table 4). Comparing pre and post MSMA treatment in each group, although insignificant ($p>0.05$), Control and T1 recorded a decrease in mean MDA concentration of 4.374% and 0.604% respectively.

On the other hand, a significant increase in MDA concentration of 42.49% was recorded in Treatment group 2. Figure 4 shows the details. The levels of ox-LDL in T1 and T2 groups were significantly increased ($p<0.0001$) by more than 4 times and 3 times respectively after MSMA treatment as compared to before treatment. Figure 5 presents this pairwise comparison.
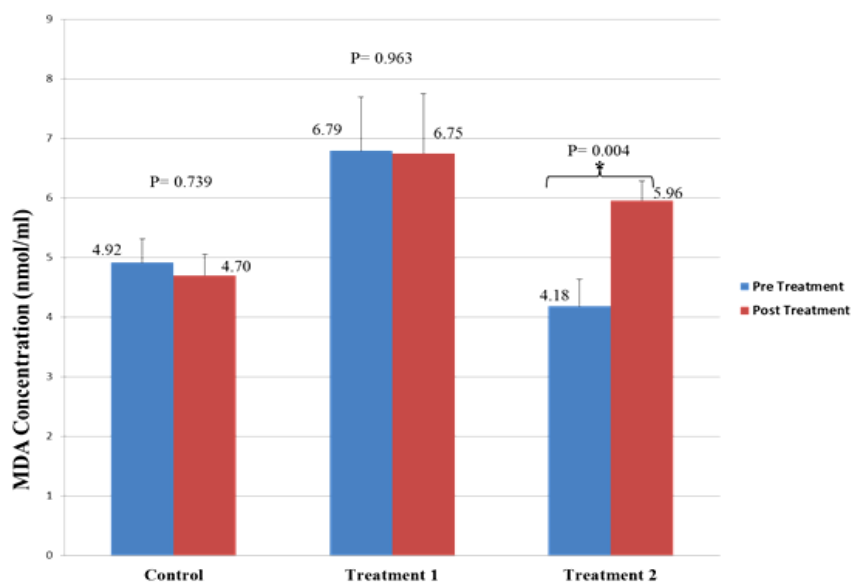
Fig. 4: Comparison of MDA Concentration Between Pre and Post MSMA Treatments. Data presented as mean (SD). Paired t-test. P<0.05 was taken as statistical significant at 95% confidence interval. *Significant difference in MDA concentration between pre and post MSMA treatment in Treatment group 1 with mean difference of 1.78 nmol/ml (CI: 2.73 – 0.82).
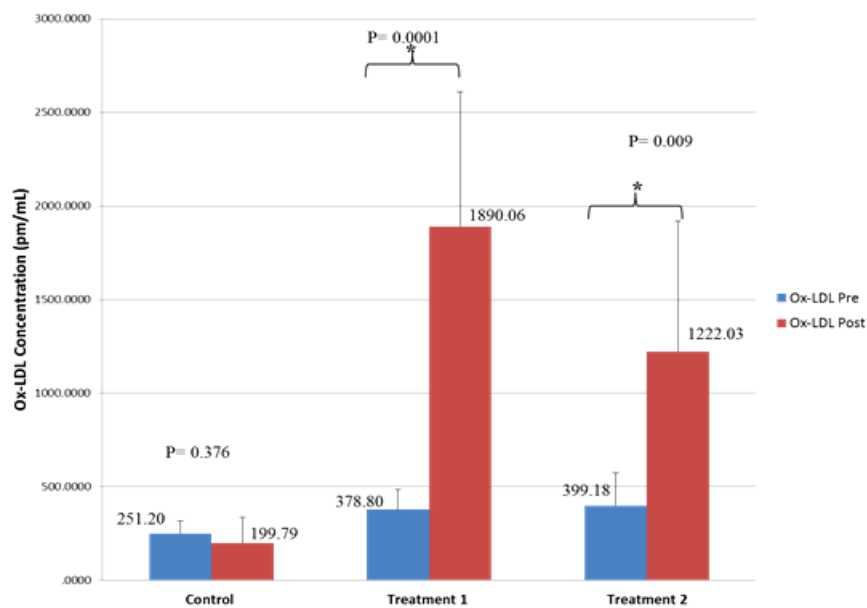
Fig. 5: Comparison of Serum Ox-LDL Level During Pre and Post MSMA Treatment. Data presented mean (SD). Paired t-test. P<0.05 was taken as statistically significant at 95% confidence interval. * Significant difference (p<0.05) between pre and post ox-LDL level in Treatment group 1 with mean difference of 1511.25 (CI: 2064.93 to 957.56) and in Treatment group 2 with mean difference of 822.843 (CI: 1366.68 to 279.01). Ox-LDL= oxidized low density lipoprotein.

PON1 activities: Ox-LDL Ratios

In order to investigate the effectiveness of PON1 ability to hydrolyse Ox-LDL in all the research groups, paraoxonase:Ox-LDL and arylesterase:Ox-LDL ratios were calculated for all the groups. After 16 weeks treatment, paraoxonase:ox-LDL was significantly reduced (p<0.0001) in both MSMA treated groups 1 and 2 as compared to that of Control group. MSMA Treatment groups 1 and 2 had 84.54% and 81.90% reduction in PON1 effectiveness in hydrolyzing ox-LDL respectively as compared to that of Control group (Table 5). Likewise, arylesterase:oxLDL were also significantly lower (p<0.0001) in both MSMA treated groups 1 and 2 as compared to that of Control group. MSMA treatment groups 1 and 2 had 87.07% and 77.03% reduction in

PON1 effectiveness in hydrolyzing ox-LDL respectively as compared to that of Control group (Table 5). Comparing the pre and post paraoxonase:ox-LDL and arylesterase:ox-LDL ratios, MSMA treatment groups 1 and 2 have significant differences (p<0.05) in their readings.

There were decrease in paraoxonase:ox-LDL ratio from pre to post Treatment in MSMA Treatment groups 1 and 2 by 77.06% and 62.00% respectively. Conversely, control group reading increased by 61.82%. Arylesterase:ox-LDL in both treatments 1 and 2 were significantly decreased (p<0.05) by 76.45% and 54.18% respectively (Figure 6 and 7). In contrast, control group increased by 44.61% and was not significant (p>0.05).

Table 5: Paraoxonase:Ox-LDL and Arylesterase:Ox-LDL Ratios in Control and MSMA Treated Groups After 16 Weeks of Treatment.

Test Parameter	Control Mean (SD) n = 8	Treatment 1 Mean (SD) n = 8	Treatment 2 Mean (SD) n = 8	F-stat (df)	P-Value
Paraoxonase:OxLDLRatio	0.93(0.39)	0.14(0.09)	0.17(0.13)	27.408 (7)	<0.0001**
Arylesterase:OxLDL Ratio	0.16(0.08)	0.02(0.01)	0.03(0.02)	22.685 (7)	<0.0001**

Results Presented as Mean (SD). One way ANOVA. P<0.05 was taken as statistically significant at 95% confidence interval. **Significant difference (p<0.0001) in paraoxonase: ox-LDL ratio between Control and Treatment group 1 with mean difference of 0.79 (CI: 0.49 – 1.10) and between Control and Treatment group 2 with mean difference of 0.77 (CI: 0.46 – 1.07). Likewise, *significant difference (p<0.0001) in arylesterase:ox-LDL ratio between Control and Ttreatment group 1 with mean difference of 0.14 (CI: 0.08 – 0.20) and between control and treatment 2 group with mean difference of 0.13 (CI: 0.07 – 0.18). Paraoxonase:Ox-LDL = paraoxonaseto oxidised low density lipoprotein ratio. Arylesterase:Ox-LDL= arylesterase to oxidised low density lipoprotein ratio.
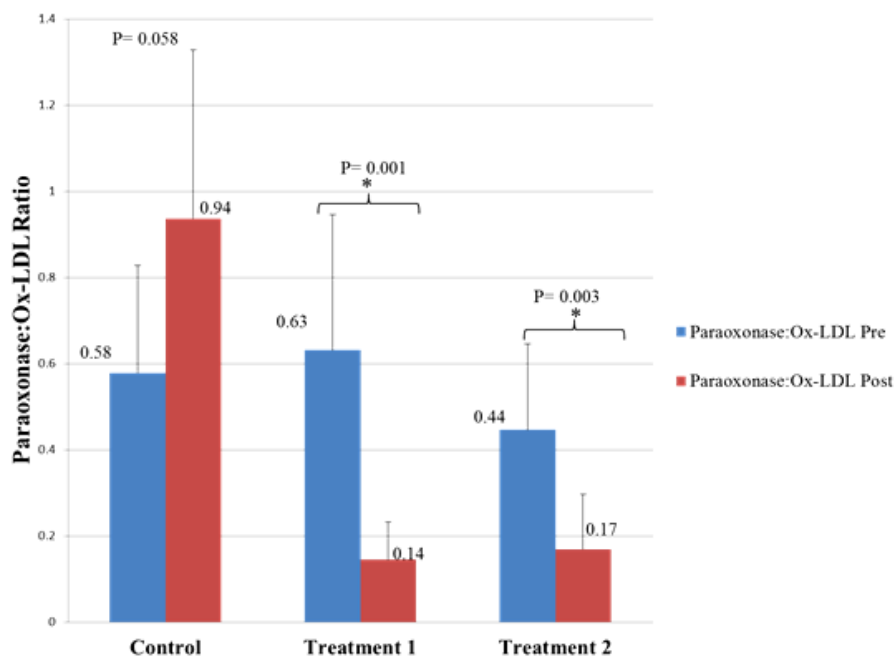
Fig. 6: Comparison of Paraoxonase:OxLDL During Pre and Post MSMA Treatment. Data presented as are mean (SD). Paired t-test. P<0.05 was taken as statistically significant at 95% confidence interval. *significant difference (p<0.05) in paraoxonase: Ox-LDL ratio between pre and post MSMA treatment in Treatment group 1 with mean difference of 0.49 (CI: 0.28 – 0.70) and in Treatment group 2 with mean difference of 0.28 (CI: 0.13 – 0.43). Paraoxonase: Ox-LDL = paraoxonase oxidized low density lipoprotein ratio.
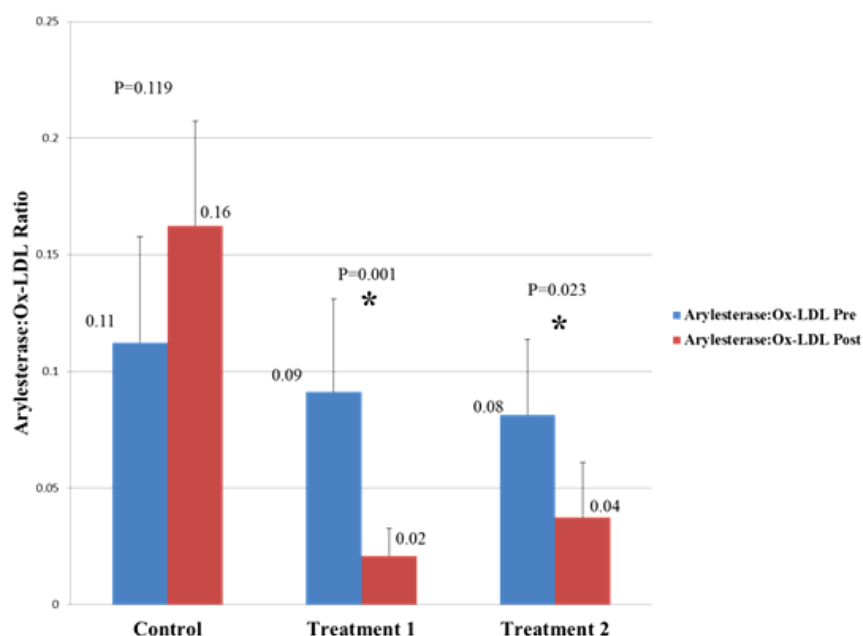
Fig. 7: Comparison of Arylesterase: OxLDL During Pre and Post MSMA Treatment. Data presented as are mean (SD). Paired t-test. P<0.05 was taken as statistically significant at 95% confidence interval. *significant difference (p<0.05) in Arylesterase:Ox-LDL ratio between pre and post MSMA treatment in Treatment group 1 with mean difference of 0.07 (CI: 0.04 – 0.10) and in Treatment group 2 with mean difference of 0.04 (CI: 0.01 – 0.08). Arylesterase:Ox-LDL = Arylesterase oxidized low density lipoprotein ratio.

Histomorphometric Assessments of Aorta

The histological examination of the aorta sections stained by H&E showed early atherosclerosis changes observed in MSMA treated groups (Figure 8). There were some areas that showed intracellular lipid deposition between the internal elastic lamina (IEL) and endothelial layer indicating a thickened tunica intima. The endothelial cells seem sparse and distorted as compared to regular lining in Control group (Figure 8). Special staining of the aorta's elastic fibre with VVG stain precisely showed the position of the IEL in relation to the endothelium thus delineate better the tunica intima and the tunica media (Figure 9). More precisely, Immunohistochemistry (IHC) of the aorta showed positive adhesion molecules VCAM-1 and ICAM-1 (Figure 10) expression in aorta of both Treatment 1 and 2 groups (Figure 11).
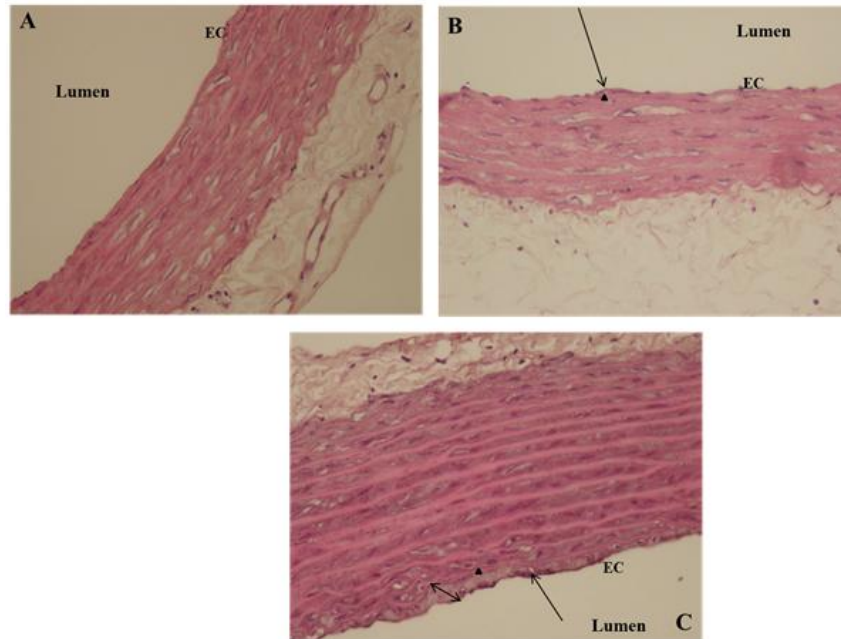
Fig. 8: Microscopic View of Aorta Stained with H&E in Control and MSMA treated groups at x40 magnification. A: Control, B: Treatment 1, C: Treatment 2. Arrows point to the areas of endothelial malformation in MSMA treated groups (B and C). Black triangle shows the position of the internal elastic lamina (IEL) and EC is the endothelial cell. Black double head arrow shows the distance between the endothelium and the IEL, indicative of thickened intima (C) due to the presence of early deposition of fatty deposits in the tunica intima (B and C).
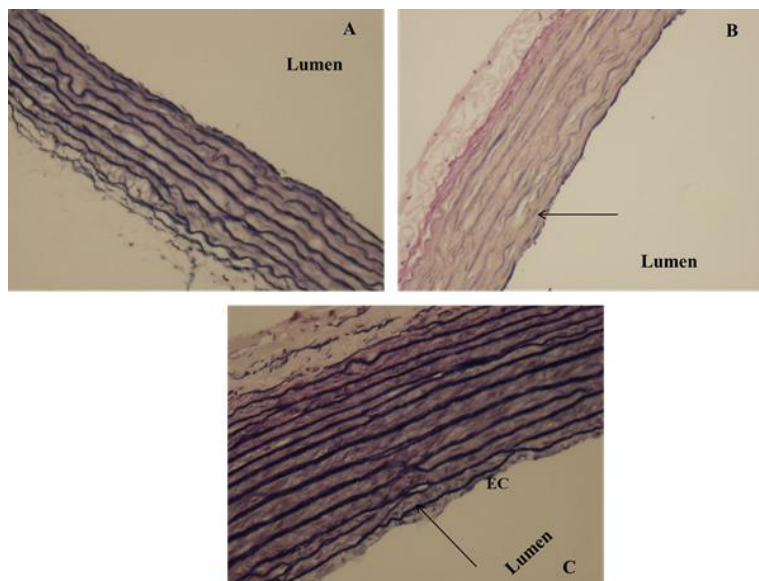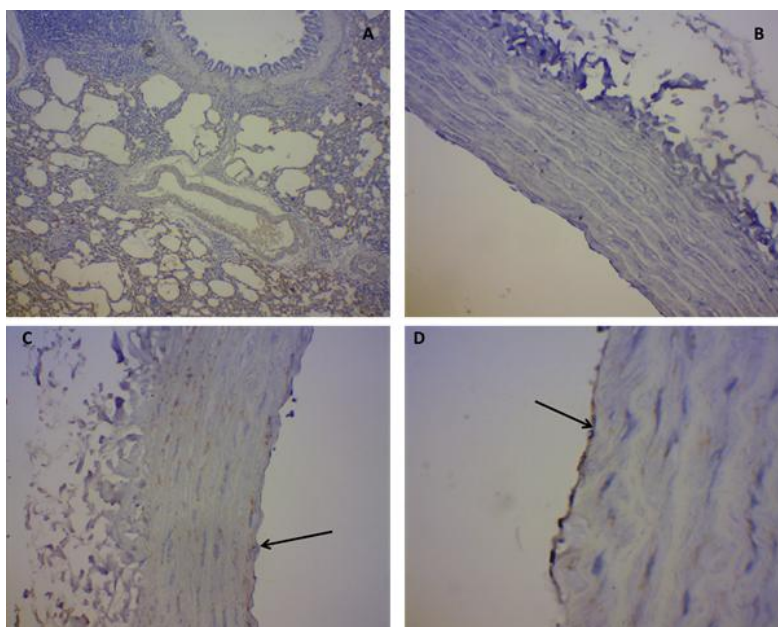

Fig. 9: Microscopic View of Aorta Stained with VVG in Control and MSMA treated groups. A: Control, B: Treatment 1, C: Treatment 2. Black arrow shows displaced IEL as a result of thickened tunica intima (black arrow) in B and C (x40).
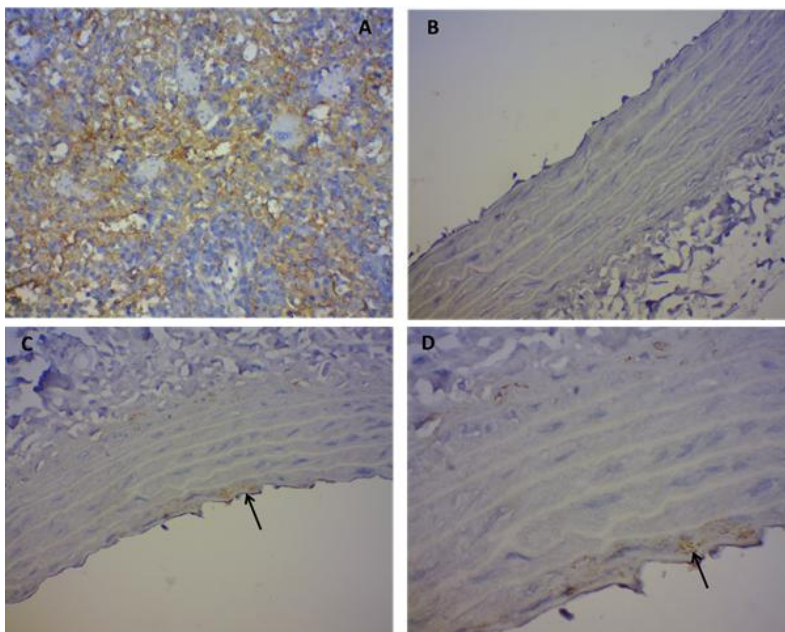
Fig. 10: Microscopic View of Aorta Stained with IHC VCAM-1 in Control and MSMA Treated Groups (x40 magnification). A: Positive control (rat spleen), B: Control group, C: Treatment 1 group, D: Treatment 2 group. Positive VCAM-1 expression in C and D (black arrow).

Fig. 11: Microscopic View of Aorta Stained with IHC ICAM-1 in Control and MSMA treated groups (x40 magnification). A: Positive control (rat lung), B: Control group, C: Treatment 1 group, D: Treatment 2 group. Positive ICAM-1 expression in C and D (black arrow).

DISCUSSION

Toxic effect of MSMA on body and organ weight of experimental subjects has been reported in a few studies (Arnold *et al.,* 2003, Albert *et al.,* 2007) involving different animals. For example, acute exposure to low and high doses (8, 24, 72 µg/g body weight) of MMA$^{(V)}$ was detected to significantly retard the growth and body weight of nestling Zebra Finches (Albert *et al.,* 2008). Our result is in agreement with a previous study on low dose (4 µg/g body weight) of MMA$^{(v)}$ which also reported non-significant weight decrease in their 2 weeks study (Arnold *et al.,* 2003). Slight weight reduction in MSMA exposed group could be due to stimulation of diarrhea as a result of MSMA ingestion (Arnold *et al.,* 2003).

Several investigations have identified the role of PON1 in cardiovascular diseases (Eckerson *et al.,* 1983, Aviram *et al.,* 1998, Durrington *et al.,* 2001, Mackness *et al.,* 2001, Li *et al.,* 2009, Mackness and Mackness, 2015). Human experimental study carried out on carotid atherosclerotic patients of arsenic endemic area in Taiwan showed that increased arsenic exposure in mg/L-year correlates with low PON1 activity (Li *et al.,* 2009). Apart from arsenic, various other metals such as lead (Pb), cadmium (Cd), Zinc (Zn), selenium (Se), mercury (Hg) and manganese (Mn) have also been discovered to also modulate PON1 activities (Hernández *et al.,* 2009, Ekinci and Beydemir, 2010, Ginsberg *et al.,* 2014, Laird *et al.,* 2015). Modulatory effects of these heavy metals were attributed to the binding of these metals to the free sulfydryl group on cysteine amino acid at position 283 of PON1 active site (Aviram *et al.,* 1998; Costa *et al.,* 2005; Afolabi *et al.,* 2014). Experimental studies investigating organic arsenic exposure effect on PON1 activities and antioxidant property are very limited. Reduced PON1 paraoxonase and arylesterase activities were reported in oral inorganic arsenic (sodium arsenite) exposed rats (Afolabi *et al.,* 2013; Afolabi *et al.,* 2014). However, in the present study, PON1 activities were not significantly different before and after oral MSMA exposure.

MDA is the peroxidative product of polyunsaturated fatty acids (PUFA) attacked by ROS and is used as a biomarker for oxidative stress (Mateos *et al.,* 2005). Oxidative stress has been implicated in the pathology of many diseases including inflammation, cancer, cellular aging, atherosclerosis and genotoxicity (Mateos *et al.,* 2005; Bhadauria and Flora, 2007). Arsenic toxicity has been postulated to occur through ROS generated oxidative stress deteriorated PUFAs (Bhadauria and Flora, 2007). This is supported by studies which discovered that iAs induces lipid peroxidation in a dose dependent manner in experimental animals (Odunola *et al.,* 2013). Likewise, epidemiological evidence of the cardiovascular toxicity of inorganic arsenic is also mounting. Arsenic toxicity has been identified to correlate with increased incidence of atherosclerotic cardiovascular diseases in Taiwan, Bangladesh and West Bengal, India (Chen *et al.,* 1996; Rabbani and Saha, 2000; Chen *et al.,* 2007; Chen *et al.,* 2009). In this current study, although insignificant, the MDA concentrations were found to be higher among MSMA exposed groups as compared to that of control group. Further, when compared between pre and post MSMA exposure, T2 group was found to have a significantly higher (p<0.05) MDA concentration after MSMA exposure. The present results agrees with previous animal experimental study investigating 12 months oral exposure to arsenic-containing well water which also discovered significant serum MDA increase in the exposed group (Santra *et al.,* 2000). Another study by Kaur *et al.* (2010) also reported a significant elevation in serum MDA in sodium arsenite exposed group as compared to that of control group (Kaur *et al.,* 2010). Likewise, arsenic trioxide also invoked significant increase in blood TBARS level after treatment for a week (Rabbani *et al.,* 2003). Conversely, a two days sodium arsenite (iAs) exposure animal study failed to discover any

significant increase in rat's liver MDA content (Schinella *et al.,* 1996). This disparity might be attributed to the very short exposure period and difference in samples assessed. Our result showed that organic arsenicals, MSMA, used as potent active ingredient of herbicides is capable of generating oxidative stress and lipid peroxidation *in vivo* similar to its inorganic counterpart. Hence, possessing potentials to contribute towards pathology of oxidative stress induced diseases such as atherosclerosis.

Ox-LDL also contributes immensely toward endothelial injury that initiates atherosclerosis which leads to CVD (Steinberg, 1997, Steinberg and Witztum, 2010, Shah, 2013). Meanwhile, atherosclerosis and other CVD have been reported to be prevalent in arsenic contamination endemic regions of Taiwan and India (Tseng, 2008; Srivastava *et al.,* 2009). In this present study, a significant increase (p<0.0001) were noted in serum ox-LDL levels in MSMA treated groups 1 and 2 as compared to that of control group (Table 6). The present result agrees with previous human experimental study which also reported higher plasma oxLDL in Bangladeshi arsenic exposed people (Karim *et al.,* 2013). In the meantime, another study reported a significantly increased plasma ox-LDL in CAD patients (Holvoet *et al.,* 1998). Thus, current study draws our attention to the LDL oxidation capacity of MSMA and its imminent endothelial injury which can induce atherosclerosis and CAD.

PON1 ability to hydrolyse ox-LDL was assessed by calculating paraoxonase:oxLDL and arylesterase:ox-LDL ratios. In the present study, the paraoxonase:ox-LDL and arylesterase:ox-LDL ratios were found to be significantly lower (p<0.0001) in MSMA treated groups as compared to that of Control group. Similarly, when the paraoxonase:ox-LDL and arylesterase:ox-LDL ratios were compared between pre and post MSMA exposure, both Treatment groups 1 and 2 were found to have a significantly reduced (p<0.0001) PON1 ability to hydrolyse ox-LDL. Our results corroborate previous studies' postulation that PON1 activities and prevention of LDL oxidation are mutually exclusive (Aviram *et al.,* 1998; Durrington *et al.,* 2001; Li *et al.,* 2009; Efrat and Aviram, 2010). Previous study reported that arsenic binds to free cysteine 283 free sulfydryl group on PON1 and this sulfydryl group is needed for successful prevention of LDL oxidation by PON1 (Aviram *et al.,* 1998). Current study's findings suggested that PON1 cysteine 283 free sulfydryl group might be affected by MSMA exposure since paraoxonase:ox-LDL and arylesterase:ox-LDL ratios were lower in MSMA treatment groups as compared to control. Therefore, our study showed that PON1 antioxidant ability was also significantly reduced with MSMA exposure. Hence, MSMA exposure will encourage atherosclerosis-induced CVD development.

The histopathologic role of inflammation in atherosclerosis induced CAD had been established over the decades (Paeng, 2013; Shah, 2013). In the current study, H&E stained aortas of Control and MSMA treated rats showed early deposition of atherogenic plaques in the tunica intima in both Treatment group 1 and 2 administered with 42.1 and 63.2mg/kg body weight MSMA (Figure 11B and C) while aorta of rats in

control group showed a perfectly normal and consistent endothelium (Figure 11A). Present study's result also affirms previous study's view of inflammatory potential of MSMA toxicity (Jaghabir *et al.,* 1989). Similarly, more recent study reported that oral exposure to arsenic trioxide induced plaque deposition in aorta of rats (Cheng *et al.,* 2011). In order to provide a better assessment, Verhoeff Van Geison staining was used to stain the aorta elastic fibres as black and collagen as pink. Present study's result shows that the IEL of aorta in MSMA treated groups were displaced by atheromatus plague deposit from the endothelium (Figure 12). This indicates that there is thickening of the tunica intima of the MSMA treated groups which is an evidence of early atherosclerotic development. Current result is synonymous to that of a previous study which also reported displaced IEL in human early degenerative aortic stenosis: a condition that shared active inflammatory process with atherosclerosis (Otto *et al.,* 1994).

Pathologically, the onset of atherosclerosis is marked by transmigration of inflammation-stimulated leukocytes across the endothelium into the vessel wall. It requires the recruitment of various adhesion molecules such as vascular adhesion molecule (VCAM-1), intracellular adhesion molecule (ICAM-1), P-selectins and E-selectins (Roy *et al.,* 2009). In arsenic pollution endemic areas, epidemiological studies have found a significant relationship between plasma soluble vascular adhesion molecule (sVCAM-1) and soluble intracellular adhesion molecule (sICAM-1) (Chen *et al.,* 2007). Immunohistochemical analysis of atherosclerotic hypercholesterolemic rabbit's aorta showed considerable expression of both ICAM-1 and VCAM-1 (Koga *et al.,* 2004). Epidemiologic studies have established the involvement of ICAM-1 and VCAM-1 in arsenic pollution-induced CVD. Current study found out that organic arsenic, MSMA, exposed rats aorta showed positive VCAM-1 and ICAM-1 expression (Figure 13 and 14).

Our results agree with previous study which reported increased expression of ICAM-1 and VCAM-1 in aorta of atherosclerotic hypercholesterolemic rabbits using IHC as well (Koga *et al.,* 2004). It also conforms with epidemiological results previously reported (Chen *et al.,* 2007; Karim *et al.,* 2013). Therefore, our study is presenting for the first time that organic arsenic (MSMA) exposure is atherogenic and can contribute to the burden of CVD experienced worldwide.

CONCLUSION

In conclusion, this study showed that chronic MSMA exposure is capable of lowering PON1 antioxidant ability to hydrolyse ox-LDL. To the same effect, lipid peroxidation level, as measured by serum MDA and ox-LDL contents, is exacerbated by chronic MSMA exposure. It also initiates early histomorphological alteration of the aorta towards atherosclerotic changes and positively expressed inflammatory markers of VCAM-1 and ICAM-1. Therefore, these point to the fact that chronic MSMA exposure induces early atherosclerosis changes in rats,

which could be explained by elevated oxidative stress, inflammation and reduced ability of PON1 to hydrolyse oxidised LDL.

Conflict of Interests: There are no conflicts of interest.

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