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## Maternal reproductive health: Expression patterns of antioxidant enzyme selenoproteins of post-implantation embryos conceived by ethanol-treated murine mothers supplemented with $\alpha$ -tocopherol

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### ABSTRACT

**Objective:** To investigate if the protective effect of  $\alpha$ -tocopherol against the impact of ethanol on brain morphogenesis involved the activity of the selenoproteins phospholipid hydroperoxide glutathione peroxidase (PHGPx; *GPx4*) and selenoprotein P (SelPP) that have roles against oxidative stress. **Methods:** Forty female mice were randomly assigned into natural control (CON), positive control (ETOH), low-, medium-, and high-  $\alpha$ -tocopherol-supplemented-ethanol groups (LTOC, MTOC, HTOC, respectively). CON received drinking water without ethanol while ETOH, LTOC, MTOC and HTOC groups received 20% ethanol in drinking water. The supplemented groups were given respective dosages of  $\alpha$ -tocopherol, 0.410, 0.819, and 1.640 mg/g body weight, at day 14 before mating onwards to the day 9 of gestation. At 10.5 ED of gestation (1 100 h), the pregnant females were sacrificed by cervical dislocation and the embryos were harvested. Total RNA were extracted, cDNA synthesis and qRT-PCR analyses were carried out. **Results:** The level of expression of PHGPx in the positive control was significantly lower than that of the natural control. Among the three  $\alpha$ -tocopherol-supplemented groups, only the medium dose- group was significantly higher than the positive control. The level of expression of SelPP in the positive control was significantly lower than those of the natural control, the low- and medium- dose  $\alpha$ -tocopherol supplemented groups. In the high dose-  $\alpha$ -tocopherol supplemented group, the level of expression was not significantly different from the positive control but significantly lower than the natural control. **Conclusions:** The activity of the selenoproteins PHGPx and SelPP are involved in the internetwork of antioxidative enzymes with vitamin E when given up to a medium dose only and is one of the possible pathways of shielding embryonic development against the impact of ethanol on brain morphogenesis. This study strengthens the impact of dietary  $\alpha$ -tocopherol and Selenium supplement during the critical period of pregnancy.

## 1. Introduction

Copious studies that have accumulated through the years have made it certain that ethanol exposure during pregnancy poses damaging effects to the conceptus[1]. Fetal alcohol syndrome (FAS),

characterized by craniofacial deformations, growth and mental retardations among other various forms, is well-claimed as due to the impact of alcohol consumption during pregnancy. Based on a comprehensive review[2], FAS is evident in 4%–6% of infants of heavy drinking mothers, to minor effects, such as low birth weight,

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Intra Uterine Growth Retardation (IUGR), a slight reduction in intelligence quotient of the infants and increased rate of congenital anomalies.

An increased oxidative stress is believed to be one of the different mechanisms that may explain alcohol-induced effects to the developing embryo[3]. Ethanol can induce oxidative stress directly by formation of free radicals in the form of reactive oxygen species (ROS) which react with different cellular compounds, or indirectly by reducing intracellular antioxidant capacity, such as decreased glutathione peroxidase levels. Alcohol-induced oxidative stress was also found to increase lipid peroxidation and damage protein and DNA[1,4].

$\alpha$ -tocopherol (vitamin E) is a lipid-soluble vitamin which functions primarily as a chain-breaking antioxidant that prevents propagation of lipid peroxidation[5]. In our previous study[6],  $\alpha$ -tocopherol exhibited a protective effect on dysmorphogenesis in post-implantation embryos induced *in vivo* by treating dams with ethanol prior to and during pregnancy. This could strengthen a claim that of  $\alpha$ -tocopherol as a significant supplement for pregnancy that may be able to minimize the adverse effects of alcohol during the critical period of organogenesis. Hence, an attempt to find out if the protective capacity of  $\alpha$ -tocopherol against the impact of ethanol on brain morphogenesis in our previous study involved the activity of selenoproteins.

Selenoproteins are proteins containing selenium in the form of the 21st amino acid, selenocysteine. Members of this family of proteins have roles in a variety of cell processes and diseases[7] and are key defense against oxidative stress where the selenocysteine residues serve as catalytic sites as they neutralize reactive oxygen species (ROS)[8]. Selenoproteins that are involved in antioxidant defense include the phospholipid hydroperoxide glutathione peroxidase (PHGPx) and Selenoprotein P (SelPP). PHGPx, also known as *GPx4*, is an intracellular enzyme that reduces hydroperoxides in membranes and lipoproteins[9,10] while SelPP is involved in selenium transport[11]. In addition, evidences showed that SelPP has both antioxidant and selenium transport functions[12].

The outcome of this study may further enhance the awareness on the benefits of dietary antioxidants like vitamin E and selenium in improving maternal oxidative status during pregnancy.

## 2. Materials and methods

### 2.1. Test animals

Eight-week old ICR female mice, with an average weight of 30 g, and fifteen to twenty-week old ICR males were obtained from the Natural Products Research Laboratory of the Marine Science Institute, University of the Philippines, Diliman.

### 2.2. Chemicals and reagents

Vitamin E in its natural form d- $\alpha$ -tocopherol containing 400 IU, contained as 260 mg d- $\alpha$ -tocopherol in 0.50 mL gel capsule, was purchased from DynaDrug (Manila, Philippines). Ethanol and components of phosphate-buffered saline were purchased from Ajax Finechem Pty Ltd (Bay Road, Taren Point, Australia). RNA extraction kit was purchased from Invitrogen, USA and Taqman Universal PCR Master Mix Kit from Applied Biosystems, USA.

### 2.3. Diet regimen and mating

The mice were allowed free access to water and food pellet *ad libitum* while being acclimatized for 1 wk in their new cages. After 1 wk, the females were then randomly assigned to five test groups. Individual mouse per group were fed with determined diet regimen and supplement. A natural control group (CON) was provided with food pellet and plain drinking water, a positive control (ETOH) was supplied with food pellet and 20% ethanol-drinking water (v:v), as liquid source. The remaining three test groups were provided with 20% ethanol- drinking water (v:v) and fed with food pellets mixed with varying amounts of  $\alpha$ -tocopherol. The computation of the dosage was based on 0.819 mg/g body weight as normal dose[11]. Given that 400 IU contains 260 mg d- $\alpha$ -tocopherol in 0.50 mL of gel capsule, a 0.023 mL, 0.047 mL, and 0.094 mL would contain 12.285 mg, 24.570 mg and 49.140 mg respectively. These were administered per mouse and were designated as low dose (LTOC=0.410 mg/g body weight), medium dose (MTOC=0.819 mg/g body weight) and high dose (HTOC=1.640 mg/g body weight) respectively. The d- $\alpha$ -tocopherol was delivered via dietary route by coating onto food pellets. To make sure that the mice would consume the d- $\alpha$ -tocopherol, it was initially mixed into 0.5 g of food pellets and given at 800 h. After the initial food supply was consumed, the remaining 3.5 g food pellet of an ideal daily consumption was given. The drinking water-ethanol was supplemented with 50 g/L glucose to mask the ethanol taste, following previous method. Supplementation of the three groups was performed continuously for 14 d before mating. On the day 15, the females were joined with a male (2:1 ratio) in one cage at 1 800 h. The female mice were monitored the following day, between 600 h and 700 h, for the presence of vaginal copulation plug. The females that were identified positive for plugs were housed in separate cages. These were considered pregnant bearing embryos aged at 0.5 d post coitus (dpc). Their respective diets were continued up to the 9th d of gestation.

### 2.4. Collection of postimplantation embryos

On the 10th day of gestation (1 100 h), the pregnant females were

sacrificed by cervical dislocation and the embryos were harvested. The embryos were aged 10.5 dpc on this time.

### 2.5. RNA extraction, cDNA synthesis, and real-time PCR

Total RNA were extracted from post-implantation embryos using Trizol reagent kit (Invitrogen, USA). cDNA synthesis and real-time PCR was carried out in a 25 uL volume using the Taqman One-Step RT-PCR Master Mix Reagents Kit (Applied Biosystems, CA, USA). Reactions were performed using a Rotor-Gene Q 5Plex HRM.

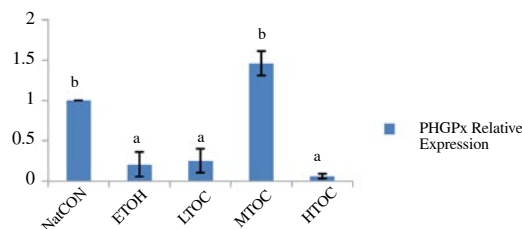
Taqman probe and primers to mouse PHGPx (applied biosystems), and SelPP (applied biosystems) were used. Mouse GAPDH mRNA was used as an internal standard (applied biosystems) and to normalize the levels of the target transcripts. The data represent three independent assays performed in triplicates, and were analyzed using the comparative Ct method, as previously described[13].

### 2.6. Data analysis

The differences among the groups were analyzed by oneway ANOVA and means were compared by Tukey's test using Statistical Package for the Social Sciences (SPSS) Version 17. The level of significance in all cases was  $P < 0.05$ .

## 3. Results

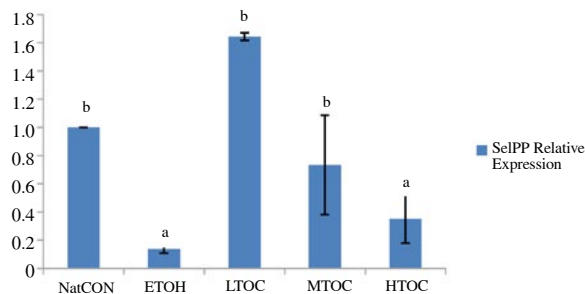
The level of expression of PHGPx (Figure 1) in the positive control (ETOH) was significantly lower than that of the CON. When the level of expression in the positive control was compared with the three  $\alpha$ -tocopherol-supplemented groups (LTOC, MTOC, HTOC), it is the MTOC group only that incurred significantly higher level. MTOC incurred about 1.2 and 1.3 fold differences from the ETOH and the LTOC groups respectively. As compared with that of the CON, the level of expression in MTOC group incurred a slim fold difference of 0.5. This difference was found not significant. The HTOC group exhibited the lowest level of expression that is significantly lower than the CON.



**Figure 1.** Relative expression of PHGPx of 10.5 ED mouse embryos from all the experimental groups.

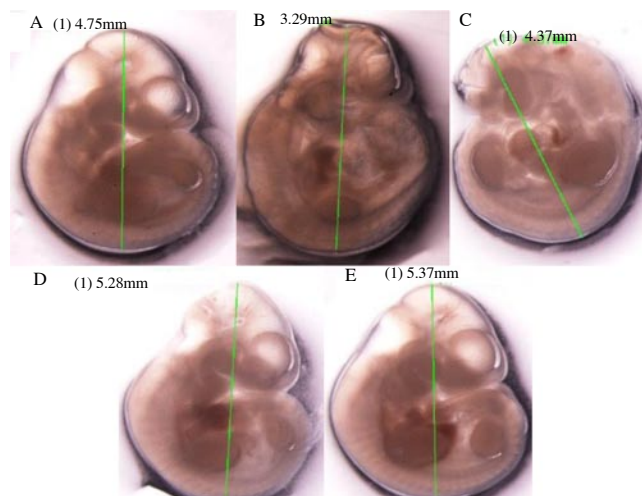
Values with different letters are significantly different from each other,  $P < 0.05$ . Data (mean $\pm$ SD) represent three independent assays performed in triplicates.

SelPP exhibited significantly lower level of expression in the positive control (ETOH) than those of the CON, the low- (LTOC) and medium- (MTOC) dose  $\alpha$ -tocopherol supplemented groups. The level of SelPP expression in the high- (HTOC)  $\alpha$ -tocopherol supplemented group appeared higher by 0.2 fold than that of ETOH group (Figure 2) but was not significant. HTOC was significantly lower than CON by 0.6 fold (Figure 3).



**Figure 2.** Relative expression of SelPP of 10.5 ED mouse embryos from all experimental groups.

Values with different letters are significantly different from each other,  $P < 0.05$ . Data (mean $\pm$ SD) represent three independent assays performed in triplicates.



**Figure 3.** Effects of ethanol and/or  $\alpha$ -tocopherol on 10.5 ED mouse embryo. (A) CON, (B) ETOH (C) LTOC, (D) MTOC, (E) HTOC. Values indicate crown-rump length (mm)[6].

## 4. Discussion

The significantly lower level of expression in the positive control (ETOH) than that of the CON is indeed indicative of the impaired effect of ethanol on the antioxidant activity of PHGPx of the embryo. This effect coincides with the low morphological scores of the primary brain vesicles of the ethanol-treated-mice-without- $\alpha$ -tocopherol supplement in our previous study[6].

PHGPx, also known as *GPx4*, exhibits dynamic distribution in the brain during rat embryogenesis and that the three isoforms,

mitochondrial (*mGPx4*), cytosolic (*cGPx4*) and nuclear (*nGPx4*) are highly expressed in the forebrain, midbrain and hindbrain regions during embryogenesis[8]. Hence, the presence of ethanol during the critical period of embryogenesis may most likely impact the expression of this selenoprotein enzyme in the developing brain.

Earlier reports on the physiological role of PHGPx in mammals[14] and its various roles in embryonic development[15,16] described that in a siRNA-mediated knockdown of mitochondrial PHGPx resulted to minor microcephaly and abnormal hindbrain development caused by increased apoptosis[14]. Increased apoptosis of the early embryo is associated with oxidative stress. Oxidative stress during early embryonic development comes as a result in the imbalance of reductive and oxidative homeostasis[17]. When there is imbalance, a significant amount of reactive oxygen species (ROS) is generated. ROS are involved in modifying biological molecules.

The family of selenium-dependent *glutathione peroxidases* (*GPxs*) constitutes one of the protective systems against excessive ROS generation[16]. An impaired expression of PHGPx, otherwise known as *GPx4*, in developing embryos is reported to increase DNA fragmentation which is an indicator of an increased apoptotic cell death[18].

The impaired effect on the level of expression of PHGPx appeared to have been eased up with the  $\alpha$ -tocopherol supplementation. This attenuation, however, was significantly evident only when the supplement was given at a medium dose (MTOC). In our previous finding[6], both the medium and high doses of  $\alpha$ -tocopherol supplement exhibited protective effect on ethanol-induced dysmorphogenesis. These were evident by the high percent occurrences of high morphological scores in the primary brain vesicles of the  $\alpha$ -tocopherol supplemented groups relative to that of the ethanol-treated-without- $\alpha$ -tocopherol supplement. Hence, it can be postulated that the protective effect of vitamin E on ethanol-induced dysmorphogenesis may operate by its concerted antioxidant activity with that of PHGPx activity to offset the insult of ethanol on morphogenesis.

The result obtained for HTOC in this present study was unexpected. It appears that a supranutritional dosage of vitamin E might not always necessarily result to a higher magnitude of enhanced antioxidant activity. Although it is assumed[19] that vitamin E requirements increase during pregnancy, this has not been proven. The consumption of high doses of vitamin E during the first trimester of pregnancy does not appear to be associated with an increased risk for major malformations, but may be associated with decrease in birth weight[20] and when vitamin E, and also Selenium were administered at higher doses than recommended, their functions extend beyond the classical antioxidant properties to

immunomodulation[21].

Further assumption for the incurred result could be that at high dose, vitamin E might have worked with members of the selenoprotein family other than the PHGPx.

Similar with that of PHGPx, the significantly lower level of expression of SelPP in the positive control (ETOH) than that of the CON indicates impaired effect of ethanol on its activity. The impaired effect of ethanol was attenuated by  $\alpha$ -tocopherol supplement which was markedly significant even at a low dose (LTOC). At a high dose of  $\alpha$ -tocopherol, however, the alleviation on the effect of ethanol on the expression of SelPP was not as significant as those of the low and medium doses. Nonetheless, these higher levels of expression of SelPP in all the three  $\alpha$ -tocopherol supplemented groups than that of ethanol-treated-without- $\alpha$ -tocopherol supplement coincide with our previous results[6] where there were higher percent occurrences of high morphological scores of primary brain vesicles in the  $\alpha$ -tocopherol supplemented groups than that of ethanol-without- $\alpha$ -tocopherol supplement. These results can provide support to our assumption that the activity of selenoproteins like SelPP and PHGPx are involved in the internetwork of antioxidative enzymes with vitamin E as one of the possible pathways of shielding embryonic development from the adverse effects of ethanol.

SelPP together with another glutathione peroxidase (*GSHPx3*), is a known plasma selenoprotein that is synthesized in the liver and secreted in the plasma[12,22]. Within the brain, SelPP is synthesized and secreted primarily by astrocytes and its receptor ApoER2 mediates SelPP uptake into neurons. The selenium present is then utilized in selenoprotein synthesis[8].

Evidence showed that SelPP transported selenium to the brain[23] and it is claimed to be the main supplier of selenium to the brain[8] based on the report[24] that SelPP mRNA was homogeneously expressed in the mouse brain. Thus, SelPP is involved in maintaining a selenium pool in the brain that may prevent neurodegeneration. Based on knockout mice studies that were reviewed[8], neurological dysfunction was largely prevented when SelP<sup>-/-</sup> mice were fed a diet containing Se-levels at or above 0.25 mg/kg. Based on histological studies and reports, SelP<sup>-/-</sup> mice fed with Se-deficient diet upon weaning exhibited neurodegeneration[8,25]. These studies support the roles of Selenium in neurogenesis. According to previous studies on the regional analysis of brain tissue of rats that were fed with Se-adequate diet; the concentration of selenium is highest in the cerebellum, intermediate concentration in the forebrain, hindbrain and hippocampus and lowest in the brainstem.

In conclusion, the protective effect of  $\alpha$ -tocopherol on ethanol-induced brain dysmorphogenesis involves the activity of the selenoproteins PHGPx and SelPP antioxidative enzymes and this

internetwork of activity is one of the possible pathways of shielding embryonic development from the adverse effects of ethanol.

### Conflict of interest statement

The authors declare that they have no conflict of interest.

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