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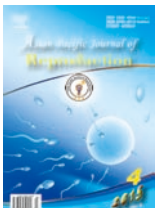

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Protective potential of α-tocopherol supplementation against ethanol-induced dysmorphogenesis in postimplantation murine embryos

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ABSTRACT

Objective: To assess the protective potential of α-tocopherol on ethanol-induced dysmorphogenesis in 10.5 embryonic day (ED) mouse embryos. **Methods:** Forty female mice were randomly assigned into control (CON), positive control (ETOH), low-, medium and high- -tocopherol-supplemented-Ethanol groups (LTOC, MTOC, HTOC respectively). CON received drinking water without ethanol, ETOH LTOC, MTOC and HTOC groups received 20% ethanol in drinking water. The supplemented groups were given respective dosages of α-tocopherol, 0.410, 0.819 and 1.640 mg/g body weight, at 14 days before mating until the 9th day of gestation. The 10.5 ED embryos were assessed for embryo weight, head- and crown-rump length, and morphological scoring of brain and sensory vesicles, flexion and somites. The embryo yield was assessed by counting the number of full-term developed embryos from the bulging implantation sites while resorption was assessed by counting the bulging implantation sites but without formed embryos. **Results:** The weight and head- and crown-rump length of the embryos from the α-tocopherol supplemented groups were comparable to the control. These were significantly higher than that of positive control (P<0.05). Overall morphological scores of the hindbrain and sensory vesicles were significantly higher in the supplemented and control groups than that of the positive control (P<0.05). The number of full-term developed embryos was neither affected by ethanol alone nor with supplementation with α-tocopherol. Resorption was significantly lower in the supplemented groups than that of positive control (P<0.05). **Conclusion:** The medium and high dosages of α-tocopherol exhibited a protective effect on ethanol-induced dysmorphogenesis.

1. Introduction

Alcohol, ethanol in a more specific form, is a substance widely used in pharmaceutical and fermentation industries. However, the misuse of ethanol, particularly its chronic consumption, can bring about adverse health conditions. Prior to and during gestation, ethanol consumption has been proven to bring about disturbed embryonic development in humans as well as in experimental animals. As ethanol crosses the placenta, it causes the continuous production of oxygen-containing free radicals, in the form of reactive oxygen species (ROS), while reducing the generation of antioxidants, which are capable of eliminating ROS[1]. Elevated levels of ROS lead to oxidative stress, which is believed to be the main mechanism of ethanol-induced damage. More often, the fetus is more vulnerable to ethanol than the mother[2]. Some manifestations of the ethanol-induced toxicity in the fetus are craniofacial dysmorphisms, skeletal and limb developmental anomalies, cardiovascular dysfunction as well as hormonal and protein insufficiency.

Oxidative stress may be prevented with the ingestion of exogenous antioxidants such as Vitamin E[3-5]. As the most biologically active form of vitamin E, -tocopherol was first discovered and recognized as a factor essential for reproduction[6]. It is known as a lipid-soluble antioxidant in cellular membranes capable of protecting free

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radicals such as ROS against peroxidative damage[7]. Studies have shown that after placental formation, a low -tocopherol environment results in necrosis of syncytiotrophoblast cells and fetal endothelium which can lead to placental failure and improper implantation of the embryo along with neural tube malformation and death of embryos[4,8]. Studies on ethanol-induced events in cell cultures and animals models have shown the protective properties of vitamin E in terms of fetal survival and normal development, as well as decreased lipid peroxide levels[2,9,10]. Therefore, the investigation on the indispensable effects of maternal α -tocopherol supplementation is important to strengthen its vital role in ensuring proper embryonic growth and development both at pre- and postimplantation stages, by counteracting the ethanol-induced embryonic damage. The study aims to assess the beneficial effects of maternal -tocopherol supplementation on protecting the postimplantation embryos from ethanol-induced dysmorphogenesis. Specifically, it examined the effect of three different dosages of α -tocopherol on E.D. (embryonic day) 10.5 embryos through the scoring of morphological characteristics such as brain vesicles (forebrain, midbrain and hindbrain), sensory vesicles (otic vesicles, optic vesicles, and olfactory vesicles), flexion, number of somites, and assessment of embryo yield and number of resorptions, embryo weight, head length and crown-rump length.

2. Materials and methods

2.1. Test animals

Eight-week old ICR female mice, with average weight of 30 g, and twenty- week old ICR males were obtained from the University of the Philippines National Institutes of Health (UP-NIH), Manila. These were housed individually in 5 in. × 10 in. × 6 in. plastic cages with wire mesh and kept on a 14:10 h light-dark cycle at room temperature in specific pathogen-free animal laboratory of the University of the Philippines-National Institute of Health (NIH). The use of animals was in accordance with the ethical guidelines of the Institutional Animal Care and Use Committee of UP-NIH, Manila, Philippines.

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).

2.3. Diet regimen and mating

The mice were allowed free access to water and food pellet *ad libitum* while being acclimatized for one week in their new cages. After one week, the females were then randomly assigned to five test groups. Individual mouse per group were fed with determined diet regimen and supplement. The 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 -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- α -tocopherol was delivered *via* dietary route by coating onto food pellets. To make sure that the mice would consume the d- α -tocopherol, it was initially mixed into 0.5 g of food pellets and given at 0800H. 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 [12].

Supplementation of the three groups was performed continuously for fourteen[14] days before mating. On the 15th day, the females were joined with a male (2:1 ratio) in one cage at 1 800 hrs. The female mice were monitored the following day, between 0600 to 0700 hours, 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 day post coitus (dpc). Their respective diets were continued up to the 9th day of gestation.

2.4. Collection of postimplantation embryos and morphological assessment

On the 10th day of gestation (1100 hrs), the pregnant females were sacrificed by cervical dislocation and the embryos were harvested. The embryos were aged 10.5 dpc on this time.

The uterine horns were dissected and transferred to a petri dish with phosphate-buffered saline (PBS), pH 7.5, and examined using a dissecting microscope. The embryo yield was assessed by counting the number of full-term developed embryos and the number of resorptions was identified by the very evident number of bulging

sites but without formed embryos. Each embryo was weighed using an analytical top-loading balance (Sartorius Brand, Model #8 120S). The crown-rump length and head length were measured using the built-in toolbar software in an inverted microscope (Nikon Stereozoom SMZ800). Each embryo was evaluated based on craniofacial characteristics (forebrain, midbrain, hindbrain, otic vesicles, opic vesicles, olfactory vesicle), flexion and somites using the morphological scoring system[13,14]. Images of the embryos were digitized using Nikon Stereozoom SMZ800. To eliminate bias, blinding maneuvers were done during embryo collection, grading and scoring. A research assistant was tasked to make a classified code for the embryos from each treatment group. Researchers #1, #2 and #3 took turns in gathering their own measurements, grading and scoring. The final grade and score per embryo was based on the tally of 2 over 1 of the 3 researchers.

2.5. Statistical analysis

Results were expressed as Mean ± SD. Morphological scores were subjected to Kruskal-Wallis test and Mann-Whitney pairwise comparisons using Paleontological Statistics (PAST) Version 2.02. The data on embryo yield, number of resorptions, embryo weight, head length and crown-rump length were subjected to one-way analysis of variance (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

3.1. Embryo yield and number of resorptions

A total of 199 embryos were obtained from 35 pregnant females. The control group incurred the highest embryo yield but not significantly different ($P=0.27$) from the high dose alpha-tocopherol supplemented group. The positive control incurred the lowest yield. The mean number of resorptions in the control and high-supplemented groups were 25% less than those of the positive control, low-, and medium-supplemented groups (Table 1, Figure 1).

Table 1
Mean embryo yield and number of resorptions in the uterine horns of female mice.

Groups	*n*	Mean embryo yield	Mean number of resorptions
CON	7	7.43±2.23	0.43±0.53[a]
ETOH	8	3.88±3.23	3.50±3.25[b]
LTOC	7	4.86±4.10	4.57±3.74[b]
MTOC	6	6.33±3.88	3.67±3.14[b]
HTOC	7	6.29±2.43	0.86±1.21[a]

Values of same letters are not significantly different from each other, *n*=sample size of pregnant female.

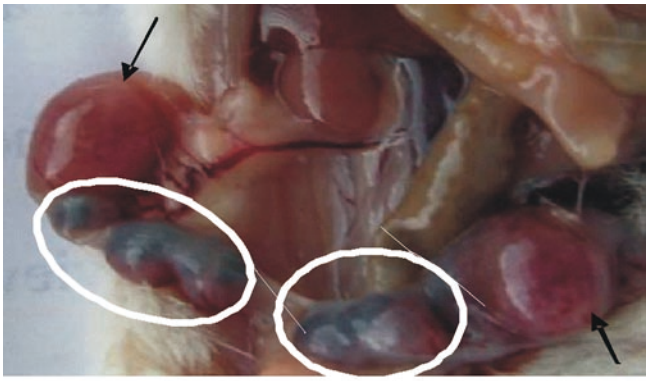

Figure 1. Mouse uterine horns from the positive control (ETOH) group showing two implantation sites with embryos (arrows) and five resorbed areas (circles).

3.2. Embryo weight, head length and crown-rump length

The embryo weights, and head- and crown-rump lengths of the three supplemented groups and the control group were significantly higher than that of the positive control group ($P=0.05$) (Table 2).

Table 2
Mean weight (g), head length (mm) and crown-rump length (mm) of 10.5 E.D mice embryos from all groups.

Groups	*n*	Mean embryo weight (g)	Mean head length (mm)	Mean crown-rump length (mm)
CON	52	0.021±0.007[a]	2.71±0.26[aa]	5.080±0.470[a]
ETOH	31	0.012±0.007[b]	2.18±0.49[b]	4.330±0.780[b]
LTOC	34	0.019±0.007[a]	2.56±0.34[a]	4.860±0.530[a]
MTOC	38	0.027±0.006[a]	3.11±0.43[a]	5.680±0.740[a]
HTOC	44	0.028±0.005[a]	2.72±0.42[a]	5.060±0.710[a]

Values of same letters are not significantly different from each other; *n*=total number of embryos.

3.3. Morphological scores

The mean scores of the three brain vesicles were lowest in the positive control. The α -tocopherol-supplemented groups were comparable to the control group. Based on post hoc analysis, forebrain scores were not significantly different among the five groups (Table 3). At least 80% of the embryos from each group obtained the highest score of 6 for the forebrain morphological scores (Figure 2A). Occurrences of neural tube defects, represented by scores of 0-2, however, were most evident in the positive control and low-supplemented groups at 13% and 2.9%, respectively.

The midbrain morphological scores were not significantly different among all groups. More than 90% of embryos from the three supplemented groups and the control group obtained the highest score of 5, in contrast with only 77% in the positive control (Figure 2B). The midbrain showed scores of 0 and 2 similar to the forebrain charcteristics of the positive control and low-supplemented groups (Figure 3).

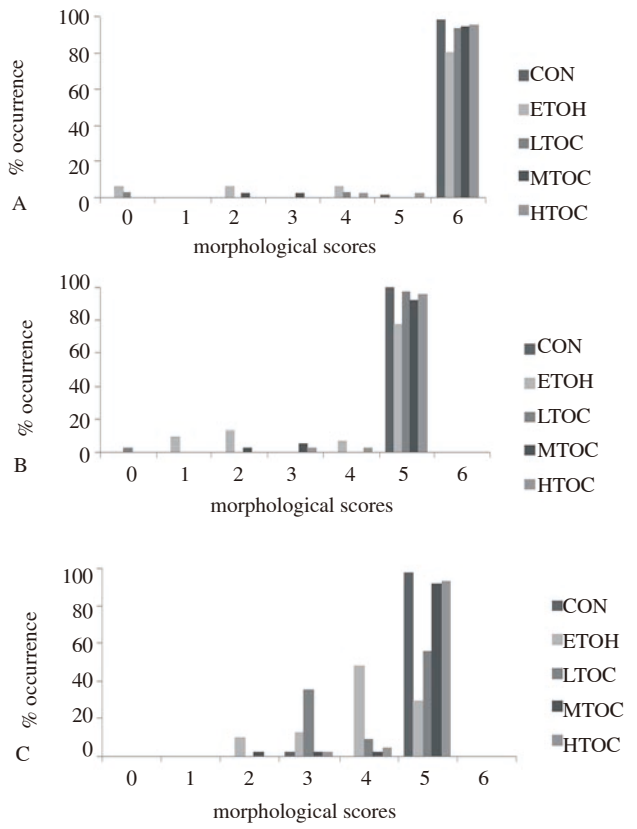

Figure 2. Percent occurrences of brain vesicles morphological scores of embryos from all experiment groups a) forebrain b) midbrain c) hindbrain.

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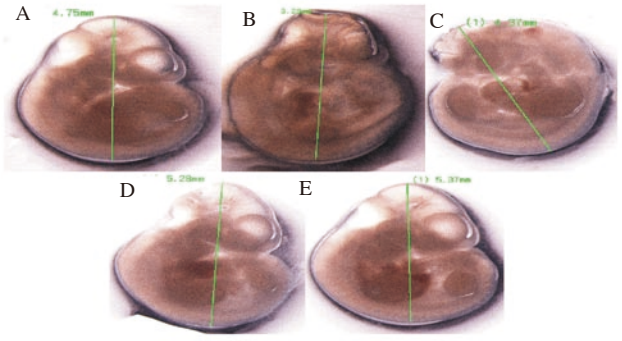

Figure 3. Effects of ethanol and/or α -tocopherol on E.D. 10.5 mouse embryo. (A) control, CON, group, (B) positive control, ETOH, group (C) low dose α -tocopherol supplemented, LTOC, group, (D) medium dose α -tocopherol supplemented, MTOC, group (E) high dose α -tocopherol supplemented, HTOC.

Values indicate crown-rump length (mm).

The hindbrain morphological scores were significantly higher in the control, medium- and high-supplemented groups than those of the positive control and low-supplemented groups (Table 3). There were 48.4% of embryos that scored 4.0, indicating the closure of anterior neuropore but the transparent roof of the 4th ventricle was not evident. The positive control and low supplemented groups incurred scores of 2 and 3 ranging from 20%-35%. Delayed fusion of neural tube were observed (Figure 4).

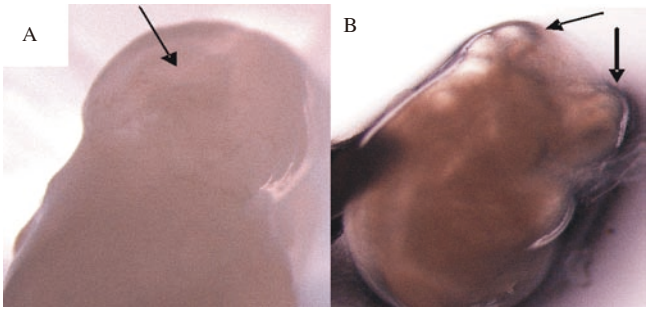

Figure 4. Embryos from positive control, ETOH, group showing anencephaly, wherein neural folds (arrows) were not fused(A-B).

The least score for the three sensory vesicles was incurred by the positive control group (Table 3) where 20% of the embryos obtained score 3 for the otic vesicles (Figure 5). This is significantly different from the control, medium- and high-supplemented groups.

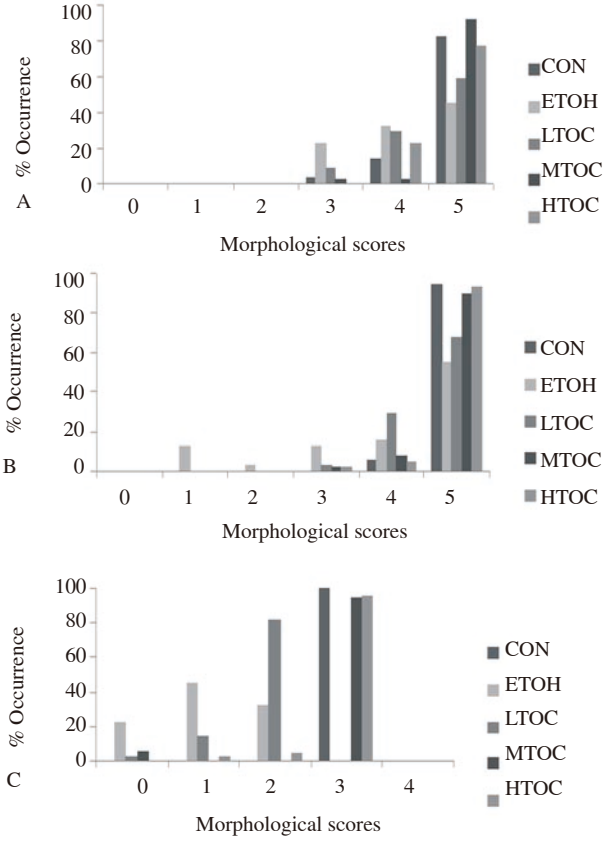

Figure 5. Percent occurrences of sensory vesicle morphological scores of embryos from all experiment groups a) otic b) optic c) olfactory.

The score for optic vesicles was highest in the control and high-

supplemented groups and lowest in the positive control (Table 3). Ideal score of 5, showing indented lens plates, was observed in more than 90% of the embryos from the control, medium- and high-supplemented groups (Figure 5). A score of 4, characterized by primary optic vesicles with open optic stalk, was observed among 30% of embryos from the low-supplemented group (Figures 5 & 6). Low scores of 1 and 2, characterized by presence of sulcus opticus or optic primordium only, were observed among 15% of the embryos from the positive control (Table 3, Figures 5 & 7).

Distinct olfactory ridges, the characteristic score 3, was observed in more than 90% of the embryos from the control, medium- and high-supplemented groups, the average scores of which were significantly higher than those of the positive control and low-supplemented groups (Table 3). The low-supplemented group had 80% of embryos with score 2 characterized by olfactory plate with rims while the positive control showed 45% of embryos with score 1, showing olfactory plate (Figures 5 & 7). None of the embryos from positive control and low-supplemented groups obtained the high score of 3.

The mean somite scores in the control and supplemented groups were significantly higher than that of the positive control (Table 3). More than 70% of embryos from these four groups obtained the ideal score of 5, characterized by 26 to 30 somites in contrast with the 40% of embryos from the positive control group with scores of 3 and 4 (Figure 8). There were no significant differences among all groups for the flexion scores (Table 3).

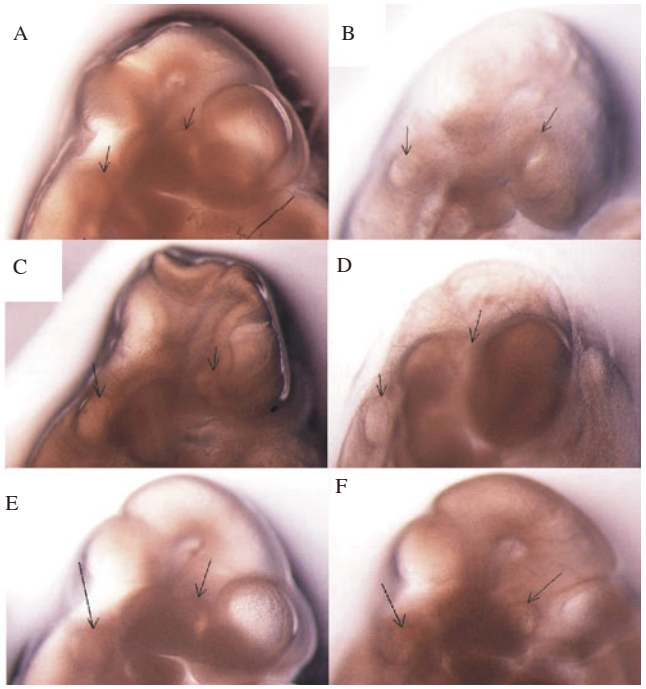

Figure 6. Effects of ethanol and/or α -tocopherol on E.D. 10.5 mouse embryo. (A) control, CON, group, (B-C) positive control, ETOH, group (D) low dose α -tocopherol supplemented, LTOC, group (E) medium dose α -tocopherol supplemented, MTOC, group (F) high dose α -tocopherol supplemented, HTOC, group. Arrows represent otic (left) and optic (right) vesicles.

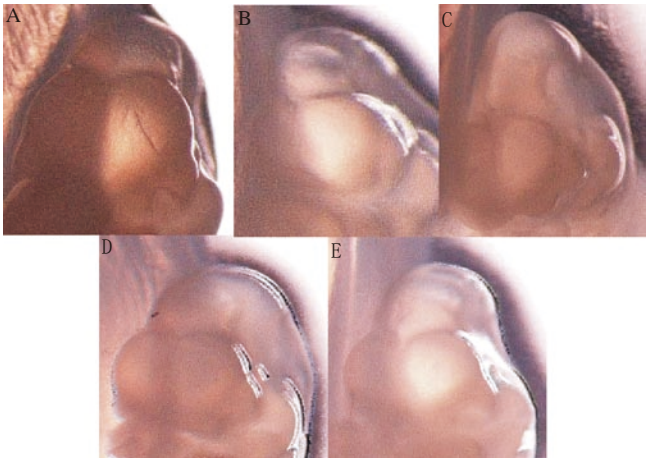

Figure 7. Effects of ethanol and/or α -tocopherol on olfactory vesicles of E.D. 10.5 mouse embryo. (A) control, CON, group (B) positive control, ETOH, group (C) low dose α -tocopherol supplemented, LTOC, group (D) medium dose α -tocopherol supplemented, MTOC, group (E) high dose α -tocopherol supplemented, HTOC, group. Arrows represent olfactory vesicles.

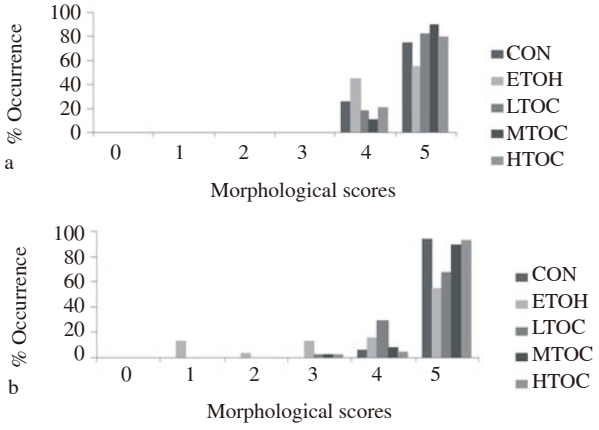

Figure 8. Percent occurrences of somite and flexion morphological scores of embryos from all groups a) somite b) flexion.

4. Discussion

4.1. Number of full-term developed embryos and number of visible resorption sites

The comparable mean number of resorptions of the control and the high-supplemented groups indicated the protective capacity of the highest dosage of α -tocopherol employed in the study. The dosage might have been able to prevent apoptosis in the trophoblast cells and of the blood vessels that could have led to improved placentation and vascularization thereby reducing the incidence of resorptions. These results on embryo yield and resorptions are parallel with a previous study[15]. A study[11] have shown that high incidence of resorption occurs due to excess reactive oxygen species (ROS) levels from ethanol consumption causing apoptosis in trophoblast

Table 3

Morphological scores of brain and sensory vesicles, somite and flexion of 10.5 E.D. mice embryos from all groups.

Groups	Forebrain	Midbrain	Hindbrain	Otic	Optic	Olfactory	Flexion	Somites	Total Score
CON(*n*=52)	5.98± 0.14	5.00± 0.00	4.96± 0.23[a]	4.79±0.50[a]	4.94±0.24[a]	3.00±0.00[a]	4.75±0.44	4.79±0.61[a]	38.27± 1.22
ETOH(*n*=31)	5.23 ± 1.77	4.42± 1.21	3.97± 0.91[b]	4.23±0.81[b]	3.97±1.43[b]	1.10±0.75[b]	4.55±0.51	4.39±0.80[b]	31.84± 0.80[b]
LTOC(*n*=34)	5.76 ± 1.08	4.85± 0.87	4.21± 0.95[b]	4.38±1.02[a]	4.56±0.93[b]	1.79±0.48[b]	4.82±0.39	4.79±0.59[a]	35.18± 4.71[b]
MTOC(*n*=38)	5.82± 0.80	4.82± 0.65	4.84 ±0.59[a]	4.79±0.88[a]	4.87±0.41[a]	2.84±0.68[a]	4.89±0.31	4.89±0.45[a]	37.76± 4.48[a]
HTOC(*n*=44)	5.93± 0.33	4.93± 0.33	4.91± 0.36[a]	4.77±0.42[a]	4.91±0.36[a]	2.91±0.36[a]	4.80±0.41	4.57±0.73[a]	37.73± 2.04[a]

Values of same letters are not significantly different from each other; *n*=total number of embryos.

cells leading to severely impaired placentae, making implantation unsuccessful. Excess ROS levels also lead to oxidative stress in both embryonic and maternal blood vessels causing insufficient placental vascularization, the results of which could be associated with early embryonic mortality and resorptions[16].

The significant difference of the three supplemented groups from the positive control group in terms of embryo weight, head length and crown-rump length are indications that the three dosages of α -tocopherol were able to exert preventive effect against ethanol-induced damage during embryonic development.

4.2. Morphological scores

The consistently lower scores for all the brain vesicles in the positive control in contrast with those of the control, medium- and high-supplemented groups may indicate that α -tocopherol-supplementation have protected brain development from ethanol-induced damage. Forebrain and midbrain scores may not be statistically significant from the other groups but marked indications for the protective potential of α -tocopherol persist such as 1) the high percent occurrence (80%) of score 6, (highest score for forebrain) from the medium- and high-supplemented groups in contrast with those of the positive control and low-supplemented groups 2) the evident score 0 and 2 with 13% and 2.9 % occurrences for positive control and low-supplemented groups, respectively and 3) the 90% occurrence of highest score 5 for midbrain in the control, medium- and high-supplemented groups in contrast with only 77% in the positive control group. Low percent occurrences of high scores in the positive control group could be due to reactive ROS generation. The anterior neural ridge (ANR), as the organizer for the prosencephalon, is documented to be one of the cell populations most susceptible to oxidative damage, hence the reason behind cranial dysmorphisms[17].

Comparing the brain vesicles, hindbrain development was most affected by ethanol exposure but was significantly improved by α -tocopherol supplementation at medium and high dosages. The presence of ethanol not only affect cell populations such as the ANR and neural crest cells but it also affects the activities of cell adhesion molecules (CAMs). These molecules are mainly involved in cell migration, cell-to-cell communication and connections and as ethanol impedes the activities of CAMs, this could delay fusion or incomplete fusion of neural folds as observed in anencephalic embryos in ethanol-exposed groups ([3, 17–19].

The results stress that α -tocopherol, at medium and high dosages, were able to protect development of sensory vesicles from ethanol-induced irregularities. The result that otic vesicle alone, at low dosage, was found significantly different from positive control, is an indication that amongst all three vesicles, otic development seems to be the one least sensitive to ethanol exposure and/or most responsive to -tocopherol-supplementation, that even at the lowest dosage, otic-associated malformations might have been prevented.

For the optic vesicles, indication of the protective potential of -tocopherol is manifested by the very closely similar scores of the control and the medium and high α -tocopherol-supplemented groups in contrat with those of the positive control and low-supplemented groups. The low scores obtained for the latter two groups may correspond to common ocular defects including anopthalmia, corneal and lenticular anomalies which are commonly seen in ED 18-19 embryos[20, 21]. As for the olfactory vesicle, a trend similar to the optic vesicles was observed which further support the beneficial effect of -tocopherol supplementation.

The observations on the optic and olfactory development of ethanol-exposed embryos were consistent with the morphological abnormalities observed in forebrain development, as these two sensory vesicles are associated with this brain region. In optic development, it could be possible that aside from ethanol mainly affecting cell migration activities of CAMs, the transcription of the Pax-6 gene, essential for lens formation[18], may have also been disturbed thus leading to delayed development as substantiated by lower optic vesicle scores. For the development of olfactory ridges, the susceptibility of ANR to ethanol, the fate of which is olfactory epithelium and medial nasal prominences, could be the reason behind the low morphological scores in embryos exposed to ethanol alone. The morphological scores of the sensory vesicles of the five groups have shown that the administration of both the medium and high dosages of α -tocopherol was able to yield marked improvement in both optic and olfactory development as indicated by the significantly higher morphological scores in the medium- and high- supplemented groups than that of the positive control group.

Flexion, more specifically along the cephalocaudal region, is of utmost significance in embryonic development because it aids in the proper positioning of the buccopharyngeal membrane and cardiogenic area as they adjust with the rapid growth of the forebrain. Flexion also repositions the transverse septum, which

eventually partitions the thoracic from the abdominal cavity[18].

Overall analysis on flexion scores revealed no significant differences among the five groups, suggesting that this aspect of embryonic development was not considerably affected by ethanol consumption. The three dosages of α -tocopherol were able to significantly exert their protective effect on somite formation.

The medium and high dosages of α -tocopherol, supplementations were able to protect ethanol-induced damage during embryonic development.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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