Reproductive Toxicology 78 (2018) 102–110

Contents lists available at ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox

Implantation loss induced by ethanolamine in the rat is ameliorated by a choline-supplemented diet

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ARTICLE INFO

Article history:
Received 8 September 2017
Received in revised form 3 April 2018
Accepted 6 April 2018
Available online 7 April 2018

Keywords:
Ethanolamine
Embryo
Choline
Implantation
Rat

ABSTRACT

Ethanolamine (EA) reduced implantation success in a two-generation reproduction toxicity study; the aim of this work was to explore the underlying basis for this response. When administered to pregnant rats during gestation days (GD) 1–3, 4–5, or 6–7, EA had no effect upon implantation success. In a second experiment, EA was administered either in the diet or by oral gavage from two weeks prior to mating through to GD 8. Parallel groups also received a diet supplemented with choline. In the absence of supplementary choline, EA induced early resorptions, statistically significant only when administered in the diet. A slight reduction in implantation success was ameliorated by supplementary choline. We conclude that implantation is affected by EA only when exposure starts before mating; that dietary administration is more effective than gavage dosing; and that interference with choline homeostasis may play a role in the aetiology of this lesion.

1. Introduction

Ethanolamine (EA; 2-aminoethanol; CAS RN 141-43-5) is a primary amine and primary alcohol that is used in gas treatment; metalworking fluids; as an acidity regulator; and as an intermediate in the synthesis of, *inter alia*, pharmaceuticals, detergents, and corrosion inhibitors. Biologically, it is derived from the decarboxylation of serine [1], and utilised in the synthesis of membrane phospholipids via the CDP-ethanolamine pathway [2]. Choline- and ethanolamine-derived glycerophospholipids are quantitatively the most important in mammalian cell membranes [3].

EA inhibits the sequestration of choline by cultured mammalian cells and isolated organs *in vitro* [4–12], including mouse preimplantation embryos [13], and from ligated rat jejunum *in situ* [14]. An analogue of EA, diethanolamine, inhibited the uptake of choline into Chinese hamster ovary cells *in vitro* [15], and depleted the levels of choline and its metabolites in the mouse liver *in vivo* [16]. Therefore, it is apparent that EA has the potential to inhibit the uptake of choline, both on a systemic level from the gastrointestinal tract, and on a tissue or cellular level from the blood or extracellular space.

A two-generation reproductive toxicity study was conducted in which EA, hydrochloride salt (EA.HCl; CAS RN 2002-24-6), was administered in the diet to Wistar rats. The concentration of EA.HCl in the feed was adjusted to maintain received daily doses of 100, 300, and 1000 mg/kg (1.03, 3.08, and 10.25 mmol/kg). There were statistically significant reductions in implantation sites and litter size as well as increased post-implantation loss in both generations exposed at the highest dietary inclusion level, compared to the concurrent control group [17].

The purpose of the studies reported herein was to investigate the potential role of choline antagonism in the aetiology of EA-induced implantation loss. The work was conducted in two phases: the first to determine if there was a critical period of sensitivity during the pre- and peri-implantation period; the second to evaluate the impact of choline co-administration on implantation success following treatment with EA. Due to the relatively low potency and efficacy of EA, a structural analogue, *N*-(isopropyl)ethanolamine (IPEA; 2-(isopropylamino)ethanol; CAS RN 109-56-8)—which is a known potent antifertility agent in rodents [18–22]—was used as a positive control in the first phase.

Abbreviations: AEA, *N*-arachidonylethanolamine; CBR, cannabinoid receptor; EA, ethanolamine; EA.HCl, ethanolamine, hydrochloride salt; GD, gestation day; IPEA, *N*-(isopropyl)ethanolamine; PAF, platelet activating factor.

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https://doi.org/10.1016/j.reprotox.2018.04.005
0890-6238/

2. Materials and methods

All procedures were conducted in an AAALAC-approved laboratory, in compliance with European Communities Council Directive 86/609/EEC. The experiments were conducted to the standards of Good Laboratory Practice.

2.1. Animal husbandry

Rats of the Wistar Crl:WI (Han) strain, 10–12 weeks of age, were from Charles River Laboratories, Research Models and Services, Germany GmbH. They were supplied either as time-mated pregnant females, with the day following mating being designated gestation day (GD) 0, or as males and nulliparous, non-pregnant females. After veterinary inspection by the receiving laboratory, animals were randomly assigned to treatment groups and housed singly in Makrolon type M III cages, with dust-free wood chip bedding and suitable environmental enrichment, at a temperature of 20–24 °C, 30–70% humidity, and 12-h light/dark cycle. Kliba mouse/rat maintenance diet (Provimi Kliba SA, Kaiseraugst, Switzerland), ground for females pelleted for males, and municipal drinking water were provided *ad libitum*.

2.2. Chemicals

EA.HCl and choline chloride (CAS RN 67-48-1) were from Sigma Aldrich (Saint Louis, MO) and had stated purities of 99.4–100.5% and 99.6% respectively; IPEA (Tokyo Chemical Industry Co. Ltd., Tokyo, Japan) had a stated purity of 99.7%. No confirmation of purity was undertaken.

2.3. Experimental procedures

In the first experiment, groups of twelve time-mated pregnant female rats were administered either EA.HCl (1000 mg/kg b.w./day; 10.25 mmol/kg b.w./day) or IPEA (100 mg/kg b.w./day; 0.97 mmol/kg b.w./day) in distilled water, once daily by gastric intubation on GD 1–3, GD 4–5, or GD 6–7. Doses levels were selected based on those previously shown to reduce implantation success [17,20]. A control group was administered distilled water throughout the period GD 1–7. The IPEA solution was adjusted to pH 7.0 by the addition of hydrochloric acid prior to administration. Blood was collected by retroorbital venous puncture under isoflurane anaesthesia for standard haematological and clinical chemistry analyses. Food consumption and body weight were recorded throughout gestation, and daily cage-side checks were made for clinical signs of toxicity or morbidity. On GD 20, all animals were anaesthetised with isoflurane and sacrificed by cervical dislocation. After gross pathological examination, the ovaries were removed and the corpora lutea were counted, and the uterus was removed and weighed. The uterus was then opened and evaluated for the number of implantations which were differentiated according to live and dead foetuses, and early or late resorptions. Early resorptions in animals that did not appear to be pregnant, or which had single-horn pregnancy, were confirmed by ammonium sulphide staining [23]. Foetuses were sacrificed by subcutaneous injection of sodium pentobarbital (Narcoren®, 100 μL/foetus).

In the second experiment, after a seven-day acclimatisation period, groups of twelve non-pregnant female rats were given standard diet or diet supplemented with choline chloride to achieve choline intakes of approximately 1.2–1.5 and 6.2–7.5 mmol/kg b.w./day respectively (Table 1). Subsets of these groups were administered EA.HCl (1511 mg/kg b.w./day; 15.49 mmol/kg b.w./day) in distilled water adjusted to pH 7.0 with aqueous sodium hydroxide, once daily by gastric intubation; EA.HCl in the diet at an inclusion level to achieve an equivalent delivered daily dose; or distilled water by oral gastric intubation. A higher dose of EA was selected compared to that used in Experiment 1, based on the OECD limit dose of 1000 mg/kg/day as EA free base. Therefore, there were six experimental groups in a 2×3 block design: untreated control (group 0); choline-supplemented diet (group 1); EA administered by gastric intubation (group 2); EA administered by gastric intubation with choline-supplemented diet (group 3); EA-supplemented diet (group 4); and diet supplemented with both EA and choline (group 5). After fourteen days of treatment each female was paired overnight with an untreated male in the male's home cage. In cases where females were treated via the diet, the males' diet was replaced by that of the females. The pairing procedure continued until evidence of copulation, either vaginal plug or sperm in the vaginal smear. This day was denoted GD 0, after which the females remained in their own cages and were treated as before until GD 8. Blood was collected approximately 1–1½ hours after the final dose administration, by retroorbital venous puncture under isoflurane anaesthesia, for standard haematological and clinical chemistry analyses as well as the measurement of EA concentration in the plasma. All animals were then given standard untreated diet from GD 9 onwards and gavage treatment was curtailed. Food consumption and body weight were recorded throughout gestation, and daily cage-side checks were made for clinical signs of toxicity or morbidity. The experiment was terminated on GD 20 in the same manner as the first.

2.4. Determination of EA and choline in diet and EA in plasma

The concentrations of EA.HCl and choline chloride in the diet were determined by HPLC/MS, monitoring the protonated $[M+H]^+$ ions formed by heated electrospray ionisation. A sample of the treated diet was accurately weighed and suspended in 0.1% aqueous formic acid by shaking for one hour. An aliquot was then diluted further with 0.1% aqueous formic acid and filtered through a Millex-GV 0.45 μm filter prior to direct injection (10 μL) onto the column (Primesep 200, 250×3.2 mm, 5 μm). Separation was by means of gradient elution between water and acetonitrile, each containing 0.1% formic acid, at a constant flow rate of 0.5 mL/min.

The concentration of EA (as free base) in rat serum was also determined by HPLC/MS. An aliquot (20 μL) of serum was mixed with methanol (980 μL), centrifuged (13000 rpm, 5 min), and an aliquot (10 μL) of the supernatant was applied directly to the column. The mobile phase consisted of 80% acetonitrile and 20% aqueous ammonium formate (50 mM, pH 3.7) at a flow rate of 0.5 mL/min. Again, the protonated $[M+H]^+$ ions were monitored following heated electrospray ionisation in positive mode. The working limit of quantitation was approximately 50 ng/mL; in comparison, the concentration of EA in serum from an untreated rat was 445 ng/mL.

2.5. Statistical analyses

Statistical analyses of growth, food consumption, and reproductive parameters were restricted to those animals with confirmed pregnancy.

If Bartlett's test for homoscedasticity was not significant, following transformation if necessary, continuous data were analysed by unpaired *t*-test or by ANOVA followed by either Dunnett's test (experiment 1) or the Tukey-Kramer test (experiment 2); the central tendency is presented as the mean, with dispersion as standard error. Proportional data were analysed by χ^2 and Fisher's exact tests. Count data, and continuous data for which parametric analysis was not appropriate, were analysed by unpaired *U* test or by Kruskal-Wallis ANOVA followed by either Steel's test (experiment 1) or the Dwass-Steel-Critchlow-Fligner test (exper-