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Review

TOPICAL DOSE DELIVERY IN THE REPTILIAN EGG TREATMENT MODEL

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Abstract—Developing assays to detect endocrine-mediated toxicity from in ovo or in utero exposure is a current challenge in regulatory toxicology. Some species of reptiles exhibiting a unique mode of sex determination, in which the incubation temperature during a critical period determines gonadal sex, have been explored as an in ovo model to screen environmental contaminants for endocrine effects. We critically review published egg-exposure studies and conclude that data regarding the pharmacokinetics of topically applied substances are insufficient to validate dose–response relationships for the effects of chemicals on in ovo endocrine function or gender determination in reptiles. The insufficiencies in these data largely result from methodological failures, including lack of measurement verification, failure to investigate and control extraneous factors affecting the measurements, and lack of independent replication of results. Considerable additional research will be necessary to alleviate these methodological inadequacies. Given the current status of the data, topical treatment of reptilian eggs cannot be considered to be a valid means of establishing causal relationships between chemical treatment and biological outcome.

Keywords—Temperature-dependent sex determination Reptile Egg In ovo exposure Method validation

INTRODUCTION

Background

Identifying endocrine-mediated toxicity that occurs from in ovo or in utero exposure is an unresolved challenge, and its importance for hazard identification has been debated for decades. In 1996, by the passage of the Food Quality Protection Act and amendments to the Safe Drinking Water Act, the U.S. Environmental Protection Agency (U.S. EPA) was mandated to develop a screening program to detect human estrogenic effects of pesticides and chemicals in drinking water. Later that same year, the U.S. EPA convened the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) as a federal advisory committee on how to develop such a program. In its final report, EDSTAC struggled with the issue of in ovo or in utero exposure as it devised a tiered approach to screen chemicals for their potential to act via estrogenic, androgenic, and thyroidogenic pathways and, subsequently, to test for adverse effects that might occur as a consequence of disrupting those pathways.

One of the difficulties faced by EDSTAC was that mechanistic assays, which evaluate a well-defined activity, may fail to identify all known modes of hormonal action. On the other hand, more apical assays that evaluate a broader range of activities at a higher biological order (i.e., organ function, reproduction, etc.) often are difficult to interpret and can respond irrespective of an endocrine mechanism. The EDSTAC report [1] expressed the concern that ''chemical substances or mixtures could produce effects from prenatal/prehatch exposure that would not be detected from pubertal or adult exposure.'' Specific concerns included how to expose test organisms before birth (in utero or in ovo) and whether the screening program would detect effects on early development, especially effects in species that may not be identified by mammalian screening assays. Therefore, EDSTAC recommended the development of a full life-cycle exposure screening assay that ''must involve prenatal or prehatch exposure and retention of offspring through puberty to adulthood and structural, functional, and reproductive assessment.''

Eggs from some species of reptiles have been explored as in ovo models to screen environmental contaminants for endocrine effects, in part because of their unique mode of sex determination. All crocodilians and several species of turtles exhibit temperature-dependent sex determination (TSD), in which the incubation temperature during a critical period determines gonadal sex. Temperature-dependent sex determination is dissimilar from mammalian genotypic sex determination in that no sex chromosome is involved. Temperature-dependent sex determination has been observed in the laboratory using a range of constant-temperature incubations [2–4] and in the field through the study of natural nests or nests constructed in the sun or shade to produce various nest temperatures [2,5].

The temperature-sensitive period (TSP), usually the middle third of the incubation period, is the time frame when gonadal sex determination occurs. Temperature-sensitive periods have been identified for many reptilians, including freshwater turtles (map turtle [*Graptemys ouachitensis*] [6], painted turtle [*Chrysemys picta*] [6], European pond turtle [*Emys orbicularis*] [7], red-eared slider turtle [*Trachemys scripta*] [8], and snapping turtle [*Chelydra serpentina*] [9]), marine turtles (green turtle [*Chelonia mydas*], loggerhead turtle [*Caretta caretta*], olive ridley turtle [*Lepidochelys olivacea*], Hawksbill turtle [*Eretmochelys imbricata*], and leatherback turtle [*Dermochelys coriacea*]; reviewed in Davenport [10]), and several crocodilians, including the American alligator (*Alligator mississippiensis*) [5,11]. In most turtles, females are produced at high temperatures and males at low temperatures. Temperature is

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believed to have an all-or-nothing effect on sex determination; it is the sex ratio of a clutch that varies with temperature (intersexes rarely are seen) [2,4]. In alligators and snapping turtles, females are produced at both low and high temperatures, and males are produced at intermediate temperatures [5,9]. Like other turtles, the sex ratio of snapping turtle and alligator clutches varies with temperature; however, male gonadal characteristics can be found in some females produced at high temperatures [9,12].

Temperature-dependent sex determination is thought to involve temperature-induced changes in the steroid hormone environment during incubation. Thus, the presence of exogenous hormone or hormone agonist/antagonist in the egg could alter normal endocrine function and, ultimately, influence gender determination. Indeed, exposure-dependent increases in the proportion of females were observed when reptilian eggs were treated with 17β-estradiol during incubation at male-producing temperatures [8,11,13–30]. At incubation temperatures that typically produce a 1:1 sex ratio, treatment with nonaromatizable androgens increased the proportion of males [19,23,31,32]. Treatment with inhibitors of aromatase or reductase (the enzymes that convert testosterone to estradiol or dihydrotestosterone) increased the proportion of males or females, respectively, at incubation temperatures favoring the opposite sex [21,31,33–37].

Although the exact mechanism of TSD is unknown, these results have led some to conclude that temperature changes the in ovo hormonal environment by regulating the expression of steroid-metabolizing enzymes and hormone receptors. However, studies examining hormone levels and aromatase expression or activity in various tissues (gonad, brain, and adrenal-mesonephros-gonad) have produced mixed results for different species. These data are well reviewed by Murdock and Wibbels [38] and by Crews et al. [39]. In general, hormone levels do not consistently differ between the sexes, and although aromatase activity increases at female-producing temperatures, in most species tested it does so only after the TSP. Thus, the proposed relationship between temperature, steroid hormones, and sex ratios may be too simplistic to account for the data. Consequently, extragonadal tissues, such as the hypothalamus and adrenals, are being probed for their role in sex determination, and mechanistic research into TSD currently is focused on identifying and quantifying expression patterns of sex-related transcription factors and genes in relation to incubation temperature [38,40–50].

Methodological concerns

The impact of temperature on endogenous sex steroid hormone concentrations or receptor characteristics within the developing reptilian embryo has not been well characterized. The estrogen and progesterone receptors have been isolated from the American alligator [51]. However, these receptors have not been studied in other species, nor have receptors for other hormones potentially involved in sex determination (e.g., androgens) been investigated. Few studies have characterized receptor binding of endogenous steroid hormones, including estrogens, or compared the potencies of exogenously applied steroids and chemicals to endogenous hormone activity in the various reptilian models. It is difficult to interpret the effects of exogenously applied substances without the benefit of this information.

Similarly, few data exist concerning the relationship between egg treatment dose and internalized dose. The methods for experimental exposure of reptilian eggs to various hormones, pharmaceuticals, and environmental contaminants have included painting the egg surface with a solution containing the substance or injection of the solution into the yolk or albumin. Although injection directly into the egg likely would deliver a reliable dose, injection produces high embryonic mortality, presumably from subsequent bacterial infection [14,16,21]. Most published egg-exposure studies have employed topical treatment (surface painting) using various carriers (ethanol, acetone, or dimethyl sulfoxide) in differing volumes, all of which produced low mortality (Table S1, *Supplemental Data* [http://dx.doi.org/10.1897/06-290.S1]). It is uncommon to find reports of dosing solution analysis or mass-balance equations to determine dosing efficiency. Only a few researchers have recognized that transfer of topically applied substances into the egg is an unpredictable process, and they are among the first to emphasize that interpreting the effect of a chemical on sex ratio requires a measurement of internal dose [52,53].

A final concern is that results from laboratory exposures at constant incubation temperatures may have little relevance to wild nesting conditions. In wild nests, the sex ratio is influenced by fluctuations in daily temperature, positional differences in temperature within the nest [2,5], and variation in the maternal contribution to the in ovo steroid environment, all of which can change significantly over the course of the reproductive season [54,55].

Objectives

The objective of the present report is to critically review the body of literature employing reptilian egg exposure. We first present a brief literature summary (Table S1, *Supplemental Data* [http://dx.doi.org/10.1897/06-290.S1]). We then offer our analysis of the studies in aggregate and make recommendations for interpretation and future research.

LITERATURE SEARCH STRATEGY

A search of the published literature was conducted in Medline and BIOSIS databases using the search strategy ''((reptile OR reptilian OR turtle OR alligator) AND egg AND (exposure OR treatment OR treated))''. From this search, literature was selected that described experimental exposure of eggs to any substance. Reference lists from the collected articles were examined, and additional literature fulfilling the inclusion criteria was identified and collected. The methods of each experiment were reviewed to determine if a measurement of internal dose was made. All literature collected was then summarized in tabular form.

EXPOSURE–DOSE CHARACTERISTICS

Of 45 published studies employing topical exposure or egg injection, five quantified the amount of chemical transferred into the egg (Table S1, *Supplemental Data* [http://dx.doi.org/10.1897/06-290.S1]) [16,52,53,56,57], and only three discussed the importance of quantifying the internalized and target dose [52,53,56]. Crews et al. [16] painted radiolabeled 17β-estradiol or estradiol benzoate, dissolved in 95% ethanol, onto the surface of red-eared slider turtle eggs just before the onset of the TSP (stage 17). Radioactivity in the dissected embryo indicated that 0.2 to 0.8% of the initial label was inside the embryo anywhere from 2 to 216 h after application; yolk and albumin measurements were not taken. Portelli et al. [52] topically applied dichlorodiphenyldichloroethylene (DDE) in an

ethanol carrier to snapping turtle eggs. Seventy-two hours after treatment, internal DDE concentrations were an order of magnitude less than the concentration applied, and percentage transfer ranged from 1.6 to 20%, depending on the dose applied. The results of that study should be viewed with caution: No estimation of internal dose variability was available because only one egg per treatment was analyzed for each DDE concentration. Gale et al. [53] reported that 16 d after topical treatment, 4 and 10% of tetrachlorodibenzo-*p*-dioxin (TCDD) and polychlorinated biphenyl (PCB) 126, respectively, crossed from an acetone carrier into the contents of red-eared slider turtle eggs, of which 0.8 and 1.7%, respectively, was in the developing embryo. Muller et al. [56] reported that 14 d after topical treatment, percentage transfer of topically applied DDE, dieldrin, and chlordane from a dimethyl sulfoxide carrier into the contents (albumin, yolk, and embryo) of American alligator eggs was very low (0.0–5.5%), highly variable, and did not correlate with applied dose. Podreka et al. [57] treated green sea turtle eggs with various concentrations of DDE dissolved in 5, 10, or 20 μl of 95% ethanol and found that approximately 34% of the applied dose (regardless of concentration or volume) transferred into the total contents of the egg, with 8% found inside the embryo. These authors incubated eggs for 34 d, more than twice the incubation time used by Gale et al. [53] or Muller et al. [56], which may have allowed for greater chemical transfer over time.

In the remaining 40 studies, a total of 64 substances were applied to eggs, none of which was quantified internally. Only five of these studies discussed the uncertainties regarding applied versus internal doses [8,18,22,58,59]. One publication presented internalized dose data from a study that employed a single treatment level (2.5 μg of estradiol) alongside results from a multilevel (0.1–20 μg of estradiol) treatment study for which exposure–response was mislabeled as dose–response [16]; those authors neglected to document that absorption was similar at the various treatment levels or to discuss potential differences in absorption that may occur at different exposure levels.

EXPOSURE–RESPONSE CHARACTERISTICS

Sixteen of the 45 studies employing topical exposure or egg injection did not perform statistical analysis on control versus treatment sex ratios (Table S1, *Supplemental Data* [http://dx.doi.org/10.1897/06-290.S1]) [8,11,15,16,18,19,21, 22,25,26,28,29,34,37,60,61]. Eleven of the 45 studies presented exposure–response data mislabeled or misinterpreted as dose–response data [8,17,18,23,24,26,27,29,30,32,62]. Of those that discussed dose–response, five provided statistical analysis on each treatment versus control [23,24,27,32,62], six calculated regression curves [18,23,26,27,32,61], and three did both [23,27,32].

Reported responses to in ovo exposures can be summarized based on the class of the applied compound. In general, the percentage of female hatchlings have been reported to increase following exposure during the TSP to various endogenous androgens [13,15,17,22,23,31,33], endogenous and synthetic estrogens [8,11,13–18,20–31,37,52,53,58,60,63,64], and endogenous and synthetic progestogens [22,23] at temperatures typically resulting in male-only, male-biased, 1:1, and female-biased sex ratios. Nonaromatizable androgens have been reported to increase the percentage of males when applied to eggs incubated at female-biased and 1:1 sex ratio–producing temperatures [19,23,31,32]. Of the 30 studies reporting changes in sex ratio caused by treatment with a hormone or hormone agonists, 16 statistically analyzed treatment versus control results [13,14,23,24,27–33,52,53,58,63,64], and two measured the internalized dose [52,53].

Application of antiandrogens at male- or female-producing temperatures have not resulted in effects on sex ratio [21,22,25], whereas the antiestrogen/partial estrogen agonist tamoxifen has been reported to increase the percentage of females at male-only and 1:1 sex ratio–producing incubation temperatures [21–23,25,60]. Reductase inhibitors have been reported to increase the percentage of female hatchlings at male-biased and 1:1 sex ratio–producing temperatures [33], whereas aromatase inhibitors have been reported to increase the percentage of male hatchlings at female-biased and 1:1 sex ratio–producing temperatures [31,33–35,37]. Of the 10 studies reporting sex ratios after treatment with synthetic hormone antagonists and enzyme inhibitors, four statistically analyzed treatment versus control results [23,31,33,35], whereas none measured the internalized dose.

No effect on resulting sex ratio has been reported after in ovo exposure to dieldrin, toxaphene, 2,4-dichlorophenoxyacetic acid, or atrazine [25,28,59,64]. Studies investigating in ovo chlordane exposure have consistently reported a significant increase in the percentage of female hatchlings from incubation at male-biased temperatures [62,64–66]. After exposure to either DDE, TCDD, or various PCBs, some studies report an increase in the percentage of female hatchlings from male-producing and 1:1 sex ratio–producing temperatures [30,58,62–66]; however, the three studies that measured the absorbed dose reported no difference in sex ratio between DDE- or PCB-treated and control eggs [52,53,57].

DISCUSSION AND CONCLUSIONS

Our review of published in ovo reptilian exposure studies reveals that fewer than 12% (5 of 45) measured the dose of chemical delivered into the egg. Given that our search terms were broad and all studies identified that involved hormone or chemical treatment were included, it is unlikely that our search was biased toward identifying studies that failed to verify a received dose. The majority of studies simply assumed that the applied substance was internalized and causally linked to observed changes in sex ratio, gonad structure, aromatase activity, and/or hormone levels. Of those studies reporting changes in sex ratio, 42% (16 of 38) did not use statistical analysis to verify the purported difference between treated and control eggs. With the exception of estradiol, no effects on hatchling health or sex ratio were noted in studies that measured internal concentrations (Table S1, *Supplementary Data* [http://dx.doi.org/10.1897/06-290.S1]).

Without verification of internalized dose and subsequent dose–response characterization, interpretation of toxicity data is dependent on the assumption of proportionality between the applied dose and internalized dose. Such assumptions seem unwarranted for TSD experiments in reptilian eggs given the highly variable but low rates of chemical transfer measured in the few studies that sought to verify internalized dose. The ambiguous relationship between applied and internalized dose thwarts a clear mechanistic interpretation of chemical-induced differences in sex ratio reported in this body of literature, because several alternative explanations cannot be discounted. Application of a substance to the eggshell surface may modulate critical processes within the eggshell independent of internal hormonal pathways. For example, obstruction of the

pores of the shell and shell membrane could alter oxygen, carbon dioxide, and water exchange that may secondarily affect energy metabolism and, perhaps, thereby alter internal temperatures. Regardless of the biological end point measured, the potential interpretational challenges created by ambiguous dose data also are critical for other experimental systems in which chemicals have been applied to eggs, such as topical application of herbicides or pesticides to avian eggs to simulate exposures arising from agricultural spraying practices [67–83].

The interpretational challenges presented by ambiguous dose assumptions are not unique to in ovo exposures or endocrine mechanisms; however, the challenges may be exacerbated disproportionately in complex experimental system and for complex biological pathways, such as those mediated by the endocrine system. Because of dose ambiguities, studies reporting effects of exogenously applied chemicals to reptilian eggs cannot address whether observations in the wild reflect endocrine responses to direct environmental exposure of the egg as opposed to maternal deposition of chemicals into the egg. For example, *p,p′*-DDE concentrations in yolks of American alligator eggs from Lake Apopka, Florida, USA, are two- to fourfold the concentrations measured in soil [84] and closely correlate with maternal adipose levels (~1:1 [85]), but *p,p′*-DDE absorption following topical application in the laboratory was less than 0.5% [56]. Given the low rates of transfer reported in the few studies that measured internal dose, the only inference that can be drawn is that in ovo chemical concentrations in the wild are more likely to reflect maternal deposition rather than absorption of contaminants from the environment. Thus, putative chemical effects in wild reptiles also are more likely to result from maternal factors than from chemical concentrations to which eggs may be exposed environmentally, and postulated endocrine mechanisms underlying such effects would need to account for maternal processes as well as potential chemical effects.

The summary of the current literature demonstrates that data regarding the pharmacokinetics of topically applied substances are insufficient to validate dose–response relationships for the effects of chemicals on in ovo endocrine function or gender determination in reptiles. The insufficiencies in these data are caused largely by methodological failures, which could be avoided in future studies by careful attention to three fundamental tenets for conducting reliable scientific investigations [86–88]. First, there must be verification that the measurement techniques measure what is purported without arbitrary assumptions and that the statistical error in the measurements is known. In general, the body of literature purporting to measure dose–response relationships for chemical treatment of reptilian eggs fails this tenet, because there has been no validation that topical treatment produces a quantifiable internal dose within specific statistical bounds. Indeed, the experimental data from those few reports quantifying internal doses cast doubt on whether a reproducible internal dose can be achieved using topical treatment. Second, there must be verification that extraneous factors affecting the measurements have been eliminated or controlled. It is nearly impossible to satisfy this tenet without satisfaction of the first, so the subject literature fails this tenet a priori based on the first. Few (if any) researchers have sought to determine the influence of extraneous factors on chemical transfer across reptilian eggs, including the effect of temperature on transfer, a parameter intentionally manipulated during the experiment. Third, independent researchers must replicate an experimental observation for it to gain general scientific acceptance. The few existing reports that characterized internal dose certainly indicate that percentage transfer is low; however, as a whole, the results were widely variable and certainly do not indicate replication of findings. Furthermore, it is impossible to satisfy this third tenet without meeting the first two; hence, the body of literature under consideration fails all three fundamental tenets of scientific investigation.

Until and unless these methodological deficiencies can be overcome, topical treatment of reptilian eggs cannot be considered a valid means of establishing causal relationships between chemical treatment and biological outcome. As discussed herein and in a companion paper in this issue [56], reasonable attempts to overcome dose-delivery problems have failed in this system. Consequently, the only reasonable recommendation at this time is to abandon this assay for consideration in regulatory screening programs. Instead of attempting to establish a reliable dose-delivery procedure for reptilian eggs, it would seem more productive to focus resources on understanding the basic biology of TSD in reptiles, as currently is underway in several laboratories [40–50], as well as on understanding the basic physical determinants of material transport and diffusion across reptilian eggs. A better understanding of these processes may increase the utility of this interesting system for studying chemical effects in the future.

SUPPORTING INFORMATION

Table S1. Results of reptilian in ovo experimentation listed by year.

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