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Evidence for a Serotonin-Mediated Effect of Cocaine Causing Vasoconstriction and Herniated Umbilici in Chicken Embryos

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ZHANG, X., L. M. SCHROTT AND S. B. SPARBER. *Evidence for a serotonin-mediated effect of cocaine causing vasoconstriction and herniated umbilici in chicken embryos.* PHARMACOL BIOCHEM BEHAV **59**(3) 585–593, 1998.—Some of cocaine (COC)'s pathophysiological effects on exposed embryos likely result from its vasoconstrictive action, and serotonin$_2$ (5-HT$_2$) agonists such as dimethoxyiodophenylaminopropane (DOI) can mimic these effects. Infusions of COC (5 mg/kg/min) or DOI (0.5 mg/kg/min) for 15 min into chicken eggs with embryos on E15 caused a significant reduction in blood vessel diameters (14 and 30%, respectively). Pretreatment with the 5-HT$_2$ antagonist ritanserin (RIT, 0.9 mg/kg) 18–22 h earlier blocked the effect of COC and blocked or attenuated the effect of DOI. In separate groups of chicken embryos exposed to multiple injections of low doses of COC on E18, herniated umbilici were prominent in hatchlings. A single bolus injection of the same absolute amount of COC did not cause herniated umbilici. An additional experiment replicated the induction of herniated umbilici by multiple injections of COC and demonstrated the probable involvement of 5-HT$_2$ receptors because RIT blocked COC's ability to induce this anomaly. These data suggest that COC's vasoconstrictive effect, via 5-HT$_2$ receptors, may play a mechanistic role in some adverse outcomes in embryos exposed to COC. © 1998 Elsevier Science Inc.

5-HT$_2$ receptor DOI Cocaine multiple injections Vasoconstriction Herniated umbilici Chicken embryo

ADVERSE effects of COC abuse such as myocardial infarction and cerebrovascular accidents in adults have been well documented (25,34,48), and great effort is being made to determine the extent of risk to the embryonic and fetal human exposed to the direct and indirect effects of maternal COC abuse. These risks may include an increased incidence of placental abruption (18), structural malformation (19,26), spontaneous abortion, prematurity, low birth weight, as well as fetal death (17,27,40). Aside from its local anesthetic effects, COC has sympathomimetic and psychoactive properties that are primarily due to its ability to potently block the reuptake of dopamine (DA), norepinephrine (NE), and 5-HT. Hemodynamic changes associated with excessive vasoconstriction can result in ischemia in multiple organ systems and lead to an imbalance of oxygen demand and supply (12,22,24,30). This, together with decreased nutrient supply and metabolic waste removal by the circulation, can contribute to fetal growth disturbances (40).

Adrenergic activation has been proposed as a prime mechanism for these pathophysiological processes, and the effects of COC on systemic hemodynamics via indirect activation of the adrenoceptors have been studied in human and nonhuman species. A detailed discussion of this subject is beyond the scope of this article, and these findings have been reported or reviewed by Gillis et al. (15), Kenny et al. (20), Lange et al. (24), and Schindler et al. (42). However, an example of experiments to test the above adrenergic hypothesis was reported by Anderson-Brown and co-workers (1). COC was reported to transiently inhibit DNA synthesis in brains of rat pups; pretreatment with the α-adrenergic receptor antagonist phenoxybenzamine did not block the effect of COC on decreased incorporation of [^{3}H]thymidine into DNA. Nevertheless, phenoxybenzamine inhibited a similar effect caused by the α-receptor agonist methoxamine. It was concluded that the action of COC may be through a direct effect as a cellular toxin, rather than secondary to its vasoconstrictor action, even

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though vasoconstriction or other measures of hemodynamic homeostasis were not made. The possibility that vasoconstriction caused by excessive stimulation of 5-HT receptors might be responsible for the transient reduction in DNA synthesis was also not considered.

COC's effects on 5-HT and associated functional changes have been well documented. In fact, COC is thought to be more potent as a reuptake blocker of 5-HT than of the catecholamines (39,41). The vasoconstrictive action of COC and some of its pathophysiological consequences may be mediated, in great part, by 5-HT. 5-HT localization in platelets and the presence of 5-HT_2 receptors on platelet membranes that enhance aggregations, as well as 5-HT_2 receptors in vascular tissue suggest a strong role for 5-HT in vasoconstriction (10,11,50). In vitro and in vivo 5-HT agonists have mediated the contraction of umbilical blood vessels in pregnant sheep. The 5-HT_2 agonist, dimethoxymethylamphetamine (DOM), was the most potent among various 5-HT agonists used (57). DOM also caused a significant decrease in blood flow in uterine and umbilical arteries, and increased systemic blood pressure in the pregnant ewe and fetus in vivo. Thus, 5-HT and its agonists significantly increased the vascular resistance, and these responses were effectively inhibited by the 5-HT_2 antagonist ketanserin (58).

Studies on the reaction and sensitivity of human placental and umbilical vessels to various vasoactive substances after full-term vaginal delivery have demonstrated that 5-HT increased perfusion pressure and contractile responses in both fetal and placental segments of umbilical arteries and veins in vitro (2,38,49,56). 5-HT also caused contraction of chorionic arteries and veins that are near the point of insertion of the umbilical cord, and this effect could be blocked by the 5-HT_2 antagonist ketanserin (29). Additional support for a major role for 5-HT in mediating COC-induced vasoconstriction in umbilicoplacental vasculature comes from studies that found no adrenergic nerve fibers in the human placenta and umbilical cord (37,52). Furthermore, studies in human umbilical arteries have demonstrated that NE induced only a weak contractile response, even at 100-fold higher concentrations than that of 5-HT. DA and the β-receptor agonist isoproterenol failed to induce any vascular response (38,56), suggesting that α- and β-adrenoceptors, as well as dopaminergic receptors, appear to have little control of this circulation, which may be due to a low density or lack of these receptors in this vasculature, respectively. These studies suggest that 5-HT is the more potent vasoconstrictor in this particular vascular bed and plays an important role in umbilicoplacental circulation, mediated mainly by 5-HT_2 receptors.

Strong evidence has accumulated that 5-HT plays an important role in COC's effects on the developing chicken embryo, and that the 5-HT_2 receptor subtype is involved. Prior studies in our laboratory have demonstrated that COC exposure can alter embryonic behavior (21). The selective 5-HT_2 receptor agonist DOI can mimic COC's depressant effect on embryonic motility and hatchability, while the selective 5-HT_2 antagonist RIT can block these effects (5,44). In a prior study done by Sparber and co-workers (46), a single bolus injection of DOI into eggs with chicken embryos on days 3, 14, or 18 of embryogenesis (E3, E14, E18) led to the development of herniated umbilici in hatchlings. Severe herniated umbilici is a reflection of incomplete abdominal wall closure shortly before hatching and it was, at times, accompanied by abdominal organ protrusion. Although the mechanism(s) by which herniated umbilici are induced is unknown, 5-HT_2 mediated vascular insults just prior to hatching (at E20−) are likely involved, because RIT effectively antagonized this effect of DOI.

Preliminary studies had previously failed to induce clear evidence of herniated umbilici with a single injection of high doses of COC, even at acutely lethal doses (unpublished data). One possibility for our failure to observe herniated umbilici after injection of a single dose of COC is its relatively short half-life (54), and thus multiple injections may be necessary for their induction. This may be a more relevant clinical model because pregnant women abusing COC are more likely to self-administer the drug repeatedly than to take a single dose (13,14). Thus, the aims of the present study were to 1) compare and contrast the effects of COC and DOI upon extraembryonic blood vessel diameter as a measure of their ability to constrict blood vessels; and 2) determine if the same pattern of herniated umbilici induced by DOI could be reproduced by repeated administration of COC over a longer period of time, and if so, would RIT block these effects of DOI and/or COC.

METHOD

Subjects

Fertilized chicken eggs (from a cross between a White Leghorn female and a Rhode Island Red male) were obtained from the Poultry Nutrition Research Facility, University of Minnesota (St. Paul, MN). The eggs were refrigerated at 14–16°C for 24 to 48 h to synchronize embryogenesis, and were set in a rotating, forced-air incubator (Humidaire Hachette, New Madison, OH) at 37.5°C and relative humidity at 56–58%. The day the eggs were set was regarded as E0.

Eggs were candled on E14 for viability, and eggs with nonviable embryos were discarded. An injection site for drug administration was marked about 1.0 cm below the air cell, avoiding membrane-bound blood vessels that could be observed during the candling procedure. The shell surface at the injection site was sterilized with a small drop of 2% iodine tincture and wiped off with a gauze pad moistened with 70% ethanol. A 1.2-mm diameter dental burr and a small drill were used to drill the holes through the shell without puncturing the underlying membrane. Holes were then covered with a small piece of transparent plastic tape (3M, St Paul, MN). The eggs for Experiment 1 were kept in the incubator until the next day (E15). The eggs for Experiments 2 and 3 were transferred to the forced-air hatcher (37.5°C; 58–60% relative humidity) on E19, which was checked twice daily on E20–E22 for new hatchlings.

Treatment

COC HCl was generously provided by the National Institute on Drug Abuse (Rockville, MD). The selective 5-HT_2 agonist (±)DOI HCl and its antagonist RIT were purchased from Research Biochemicals International (Natick, MA). The solutions were made just prior to the experiments and kept chilled on ice.

Experiment 1. DOI and COC were dissolved in avian isotonic saline (SAL, 0.85% NaCl), and RIT was dissolved in 0.1 M tartaric acid (TA). A total of 54 eggs with chicken embryos were used. They were injected on E14 with 0.9 mg RIT/kg egg (n = 27) or 20 μl of 0.1M TA (n = 27), 18–22 h prior to the infusion of DOI (0.5 mg/kg egg/min), COC (5 mg/kg egg/min), or 5 μl SAL/min. Eggs were randomly assigned to six groups (n = 9/group): 1) TA + SAL, 2) TA + DOI, 3) TA + COC, 4) RIT + SAL, 5) RIT + DOI, and 6) RIT + COC. The average egg weight was 56 ± 0.3 g (mean ± SEM). The dosages of DOI and COC were chosen based upon previous experiments

in our laboratory (3–5,21,44,45), within the range that produces moderate to severe effects, including lethality in chick embryos. Prior studies have found no noteworthy effects of this dose of RIT or TA on baseline (BSL) values.

Experiment 2. COC was dissolved in distilled water. Eggs were injected on E18, between 0900–1500 h, with either a single, large bolus dose (56.25 mg/kg egg) or divided into three or five smaller doses such that the total amount of COC was equivalent for all three groups. The control group received five injections of SAL. The total volume of each injection was 20 μl. Table 1 displays the treatment groups and injection parameters.

Experiment 3. Eggs containing viable embryos were randomly assigned to four groups (n = 26/group): 1) TA + SAL, 2) RIT + SAL, 3) TA + COC, 4) RIT + COC. In the late afternoon of E17, eggs containing viable embryos were injected with either 0.4 mg RIT/kg egg or the RIT vehicle 0.1 M TA. The RIT dosage was chosen because of its efficacy in attenuating or blocking herniated umbilici formation in DOI-treated chicken embryos in prior studies (46). The following morning, on E18, eggs in each of these groups received either five COC injections (11.25 mg COC/kg egg) or five SAL injections, as described in Experiment 2. Injection volume was 20 μl for all injections.

Experiment 1: Vascular Response to DOI or COC

Equipment. An Olympus endoscope (Model A5257), mounted on a Palmer metal stand, was connected to a Toshiba CCD color camera-Panasonic digital AV mixer-VCR system. The imaging system was connected to a TV monitor and a Macintosh computer equipped with a frame grabber card for capturing images. An Olympus CLK-4 halogen light source was connected to the endoscope and used for illumination of the vasculature. A circular hole of 3 cm in diameter was made above the aircell with a special puncturing device (Tri-R Instruments, Jamaica, NY). The partial egg shell was carefully removed with a taper-tipped forceps. Mineral oil (4–5 drops) was placed onto the exposed membrane to render it translucent. A membrane-bound blood vessel within the area that could be easily reached by the endoscope was chosen. A piece of mineral oil-soaked 3-0 black silk surgical suture (1 cm long, 250 μm in diameter) was placed near the desired vessel for reference. The egg was placed upright in an incubator maintained at 37°C on a sponge cradle under the endoscope with the injection hole facing toward the incubator door, allowing easy access for injection of drugs. The endoscope was carefully lowered until the blood vessel was in focus when viewed on the TV monitor.

Experimental protocol. The eggs were numbered and randomly assigned to treatment. The image was recorded throughout the experiment and the egg number (not treatment) was displayed on the monitor to avoid the experimenter bias when measuring the vessel diameter. After a 5-min acclimation, a 5-min BSL video tape recording was taken. A PE-20 polyethylene tubing was inserted into the injection hole in the egg shell extending 3.5 mm into the egg. The other end of the tubing was attached to a 3-ml syringe mounted on an infusion pump (Harvard Apparatus, Millis, MA) and infusion of drugs commenced. The image was recorded for 15 min during infusion and for 5 min postinfusion. The total volume for the 15-min infusion was 75 μl/egg.

Vessel diameter measurement. After recording the vessel on videotape, the vessel diameter was measured by transferring the images to the computer. Images were captured and "frozen" on the computer screen from the recording at the end of the first 5 min (BSL) and at 5, 10, and 15 min after the infusion was started and 5 min postinfusion. Thus, effects of cumulative doses of DOI (2.5, 5.0, and 7.5 mg/kg egg), COC (25, 50, and 75 mg/kg egg) or SAL (25, 50, and 75 μl/per egg) upon this parameter were determined. The vessel and suture widths were measured in pixels using NIH image software (Division of Computer Research and Technology, NIH, Bethesda, MD) and converted to μm by comparison with the suture as a reference. Pilot studies confirmed the linear relationship and reliability of this procedure for estimating apparent vascular diameters.

Experiments 2 and 3: COC-Induced Herniated Umbilici

Umbilici assessment. For Experiments 2 and 3, the number of chicks hatching and their body weight was recorded in each treatment group. The presence or absence of a herniated umbilicus was noted and if present, measured with a caliper to the nearest 0.5 mm. Umbilicus color and presence of inflammation, organ protrusion, and other notable features were recorded.

Statistical Analysis

Chi-square analysis or single-factor/multivariate analysis of variance (ANOVA), for repeated measures were used followed by Dunnett's or Fisher's PLSD test where appropriate. The criterion for significance was set at $p \leq 0.05$.

RESULTS

Experiment 1: Vascular Responses to DOI or COC Infusion

Examples of the video images of the blood vessels with various treatments are shown in Figs. 1 and 2 . The mean absolute value for BSL vessel diameter was 199 ± 7 μm (mean ± SEM), ranging in size from 126–396 μm (n = 54). Although there was no significant difference between BSL values for any of the six groups, $F(5, 48) = 0.75$, $p > 0.05$, the effects of infusion of SAL, DOI, or COC were analyzed as a percent of their individual BSL values because of the large variability between subjects within a group and are expressed as such.

Repeated-measures ANOVA revealed a significant main effect of treatment, $F(5, 48) = 8.30$, $p < 0.001$, and a repeated measures (i.e., cumulative dose) effect, $F(3, 144) = 13.34$, $p < 0.001$. A marginal 10% decrease and a siginificant 27% decrease in vessel diameter were found 5 and 10 min into the infusion with DOI (cumulative acute dose = 2.5 and 5 mg/kg egg, respectively). Pretreatment with RIT blocked the effect of DOI at this time. The effect of COC at these times (25 and 50 mg/kg egg) was not noteworthy (Fig. 3A and B). After 15 min of infusion, both COC and DOI decreased vessel diameter significantly: COC by 10% and DOI by 26%. Both effects

TABLE 1

TREATMENT GROUPS AND INJECTION PARAMETERS FOR EXPERIMENT 2

Treatment	n	Each Injection Dose	Total Dose	Injection Interval
1 COC inject.	34	56.25 mg/kg	56.25 mg/kg	—
3 COC inject.	34	18.75 mg/kg	56.25 mg/kg	3 h apart
5 COC inject.	34	11.25 mg/kg	56.25 mg/kg	1.5 h apart
5 SAL inject.	29	—	—	1.5 h apart

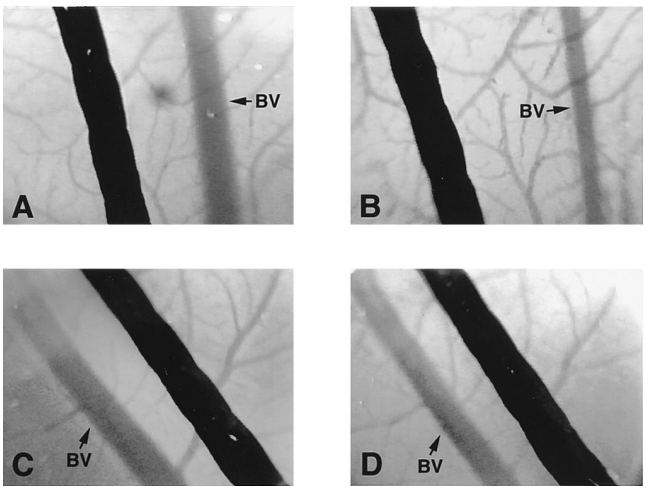

FIG. 1. A representative example of the effects of DOI and RIT on blood vessel diameter. This image was captured from video tape to the computer via Adobe Photoshop software. (A) Depicts the baseline blood vessel diameter; (B) displays the vasoconstriction after DOI infusion. (C,D) These panels display the effect of RIT pretreatment on blood vessel diameter. Note that C shows the baseline blood vessel diameter with RIT pretreatment before DOI infusion, while D displays RIT's ability to attenuate the vasoconstrictive effects of DOI. Arrow indicates the blood vessel (BV); the darker line is the black silk suture used as a reference.

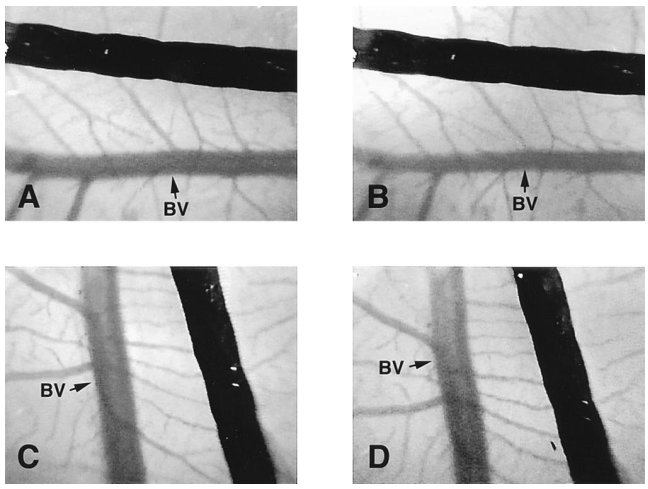

FIG. 2. A representative example of the effects of COC and RIT on blood vessel diameter. The image was processed as in Fig. 1. (A) Depicts the baseline blood vessel diameter; (B) displays the vasoconstriction after COC infusion. (C,D) These panels display the effect of RIT pretreatment on blood vessel diameter. Note that C shows the baseline blood vessel diameter with RIT pretreatment before COC infusion, while D displays RIT's ability to block the vasoconstrictive effects of COC. Arrow indicates the blood vessel (BV); the darker line is the black silk suture used as a reference.

were blocked or significantly attenuated by pretreatment with RIT (Fig. 3C). Thus, DOI caused a significant reduction in extraembryonic blood vessel diameter within 5 min, while COC's vasoconstrictive effects became significant 10 min later. Blood vessel diameters were still significantly reduced 5 min after infusions were stopped, at which time the experiment was terminated. In fact, the maximal effects of COC or DOI were observed 5 min after the 15 min infusion, with vessels exposed to the COC showing a 14% reduction in apparent diameter and DOI causing a 30% reduction. Again, these effects were significantly attenuated by pretreatment with RIT, which itself had no apparent effect upon this variable (Fig. 3D).

Experiment 2: COC-Induced Herniated Umbilici

Hatching measures. Hatching was decreased 15–18% in the five COC injection group compared to the other groups (Table 2). However, χ^2 analysis failed to find a signficant overall treatment effect or a difference between the five COC- and five SAL-injected groups. Body weight at hatching was also unaffected by treatment (Table 2), as analyzed by a one-way ANOVA.

Umbilical measures. There was a marginal treatment effect, $\chi^2(3) = 7.60$, $p = 0.055$, for the incidence of herniated umbilici. Individual χ^2 analysis revealed that the five COC- and three COC-treated groups had more affected individuals (50 and 42%, respectively) than the five SAL group (13.6%; Fig. 4, upper panel). There was no increased incidence in the group receiving a single COC injection (28%). The umbilical size was also affected by treatment. The umbilici of chicks receiving five COC injections was 3.75 ± 0.99 mm (mean ± SEM), and for those receiving three injections was 2.67 ± 0.66 mm compared to 1.92 ± 0.65 mm for the bolus COC and 0.77 ± 0.43 mm for the SAL-treated group (Fig. 4, lower panel).

Experiment 3: Can RIT Protect Against COC-Induced Herniated Umbilici?

Hatching measures. The data were analyzed as in Experiment 2. Treatment did not affect the rate of hatching (<10% difference between TA + SAL and other groups) or body weight (TA + SAL = 46.00 g ± 1.00; RIT + SAL = 45.32 g ± 1.04; TA + COC = 46.00 g ± 0.77; RIT + COC = 45.88 g ± 1.29).

Umbilical measures. There was no difference between the TA + SAL and the RIT + SAL groups on the incidence (TA + SAL = 15% and RIT + SAL = 21%) or the size (TA + SAL = 0.90 ± 0.53 mm and RIT + SAL = 0.87 ± 0.42 mm) of herniated umbilici, so these two groups were collapsed to form a single control group to which the TA + COC and RIT + COC subjects were compared. There was an overall treatment effect, $\chi^2(2) = 7.45$, $p < 0.03$, for the incidence of herniated umbilici, with COC increasing the incidence and with RIT attenuating the effect of COC administration. Individual χ^2 analysis revealed that the TA + COC group (53%) differed from the control group (18%), while the RIT + COC group (35%) did not (Fig. 5, upper panel). Umbilical size was also affected by treatment, $F(2, 72) = 3.92$, $p < 0.03$. The umbilici of chicks receiving TA followed by COC (2.84 ± 0.74 mm) were significantly larger than controls (0.88 ± 0.33 mm), while those receiving RIT followed by COC (1.76 ± 0.62 mm) were not (Fig. 5, lower panel). Thus, the findings in Experiment 2 of induction of herniated umbilici following multiple injections of COC were replicated in Experiment 3, and similar to the results of Sparber et al. (46) with DOI, RIT attenuated this induction.

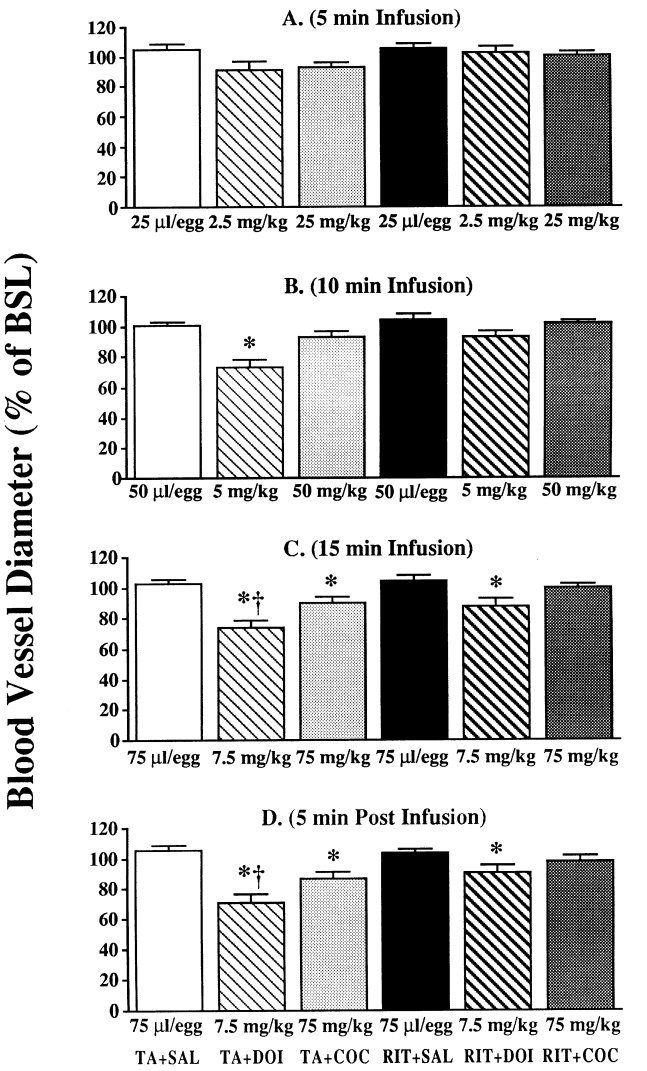

FIG. 3. Blood vessel diameters during and after drug infusion. (A) Displays the effects at the end of 5 min, (B) at the end of 10 min, and C at the end of the 15 min of infusion, and D 5 min after the termination of the infusion. A displays a marginal ($p = 0.053$) 10% reduction in blood vessel diameter by DOI. A more dramatic effect was found at the end of 10-min infusion where DOI reduced vessel diameters by 27%. Pretreatment with RIT blocked this reduction (B). (C) Shows continued vasoconstriction by DOI (26%), as well as COC induced vasoconstriction (10%) at the end of the 15-min infusion. Maximal effects of DOI or COC are depicted in D, where there was a 30% and 14% reduction of vessel diameters, respectively, 5 min after the termination of the infusion. Pretreatment with RIT significantly attenuated DOI's effect and blocked COC's effect on this parameter. In A–D (Fisher's PLSD): *$p < 0.05$ vs. SAL; †$p < 0.05$ vs. RIT + DOI. $n = 9$/group.

TABLE 2
HATCHING DATA

Treatment	Number Hatched	% Hatched	Hatch Weight (g) Mean ± SE
1 COC inject.	25/34	73.5	44.88 ± 0.75
3 COC inject.	26/34	76.5	45.15 ± 1.07
5 COC inject.	20/34	58.8	45.05 ± 1.26
5 SAL inject.	22/29	75.8	44.32 ± 1.01

DISCUSSION

The present study has demonstrated similar acute effects of COC and the 5-HT_2 agonist DOI on extraembryonic vascular diameters and effects of COC on development of herniated umbilici following repeated COC injections into eggs with chicken embryos. Pretreatment with RIT did not alter the vessel BSL diameters, nor did infusion of 75 µl SAL over the 15-min period and 5 min after terminating the infusion. Infusion of COC at 5 mg/kg/min or DOI at 0.5 mg/kg/min caused significant extraembryonic vasoconstriction. The vascular response to DOI appeared sooner, and was greater than that of COC, which may be due to its greater potency as a vasoconstrictor and/or its greater resistance to metabolic degradation, as it is not hydrolyzed by esterases, as is COC. The vasoconstrictive effects of both drugs were effectively antagonized by the 5-HT_2 receptor antagonist RIT. RIT did not completely abolish the effect of DOI at the higher cumulative dose (7.5 mg/kg). This may be due to an inappropriate ratio of the antagonist relative to the rather higher dose of DOI. For example, Bollweg and Sparber (5) demonstrated that 0.3 and 0.9 mg RIT/kg egg, administered 1 h after the injection of 1 mg DOI/kg egg, could block DOI-induced embryonic motility suppression on E15 and the alteration of detour learning on posthatch days 6–9 caused by the DOI.

Multiple injections of a low dose of COC, unlike a bolus injection, were indeed capable of inducing more and larger herniated umbilici, even though the total dose was identical for all COC groups. RIT was able to block this effect of COC. The dose of COC used in Experiments 2 and 3 (total dose 56.25 mg/kg egg) was not lethal to the developing embryos, because the rates of hatching and body weight did not differ between the embryos treated with COC or vehicle. This is in contrast to the prior study (46), where herniated umbilici were induced by DOI treatment, and there was also a dramatic decrease in hatching. Thus, in the present study, we have demonstrated a minor developmental structural malformation following multiple injections of a low dose of COC, which did not affect hatching. The incidence of herniation was approximately 50% in the two experiments and RIT effectively blocked the herniation. Future studies will determine if RIT can block the formation of more severe herniated umbilici with higher doses of COC, doses that may also be lethal to the embryo.

COC is a potent vasoconstrictor, and thus, its absorption and distribution away from the injection site may be self-limiting. This, combined with its relatively short half-life, could be responsible for the lack of herniated umbilici after a single COC injection. The multiple dosing procedures have been used to demonstrate the behavioral effects of COC in mature rodents (43,47), as well as the teratogenic, toxic, and/or behavioral effects on their offspring of COC-exposed dams [i.e., (7–9,51,53)]. For example, a possible cumulative effect of COC following repeated injection was demonstrated by Webster and Brown-Woodman (53). Rat fetuses were exposed to COC on day 16 of gestation resulting in hemorrhage and sometimes edema in limbs, examined 2 days later. A "margin-

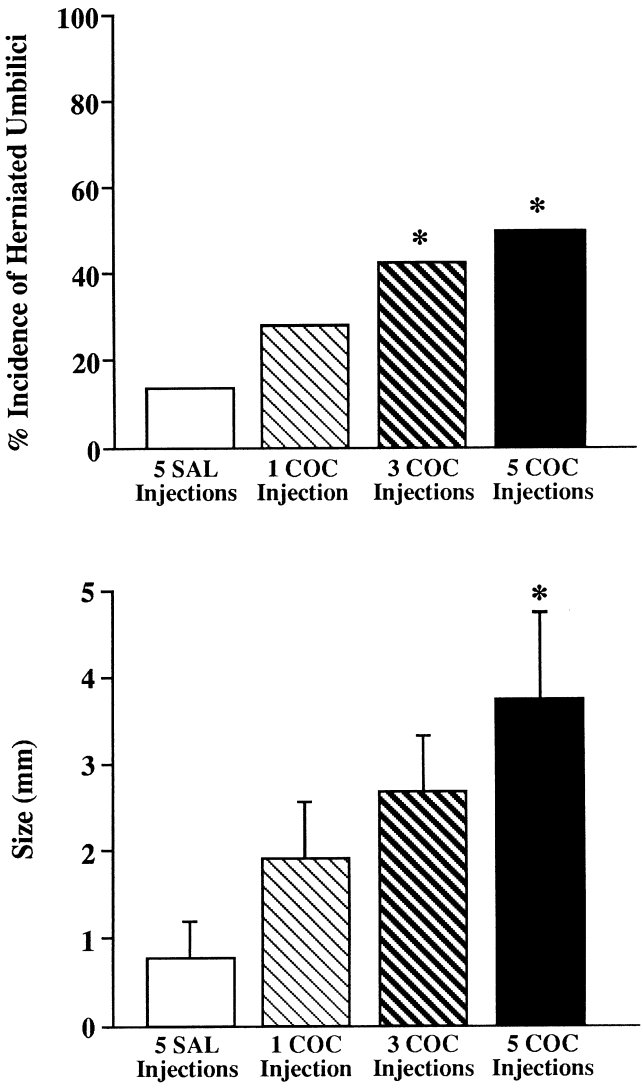

FIG. 4. Incidence and sizes of herniated umbilici following single or multiple injections of COC or SAL. Upper panel shows the incidence of herniated umbilici after treatment. $*p < 0.05$ vs. 5 SAL (χ^2 analysis). Lower panel dispicts the size of herniated umbilici after treatment. A marginal effect for three COC injections and a significant effect for five COC injections was found. $*p < 0.05$ vs. 5 SAL (Dunnett's test).

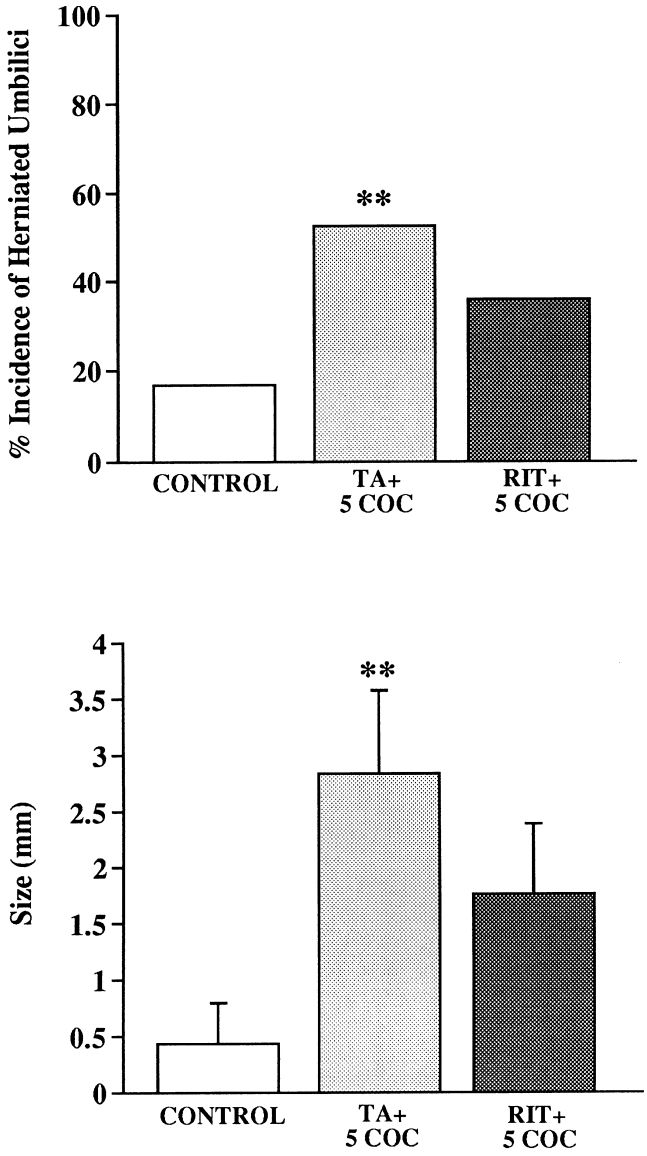

FIG. 5. Upper panel: incidence of herniated umbilici following pretreatment with RIT or TA, and treatment with multiple injections of COC or SAL. $**p < 0.01$ vs. Control (χ^2 analysis). Lower panel: herniated umbilical size following multiple injections of COC or SAL after pretreatment with RIT or TA. $**p < 0.01$ vs. Control (Dunnett's test).

ally teratogenic" dose of 50 mg COC/kg was given intraperitoneally to the mother, either as a single bolus injection or as two doses of 50 mg/kg, with intervals of 1, 2, 3, or 4 h between injections. The number of affected fetuses was significantly greater in the groups that received two doses with 1- or 2-h intervals compared with the control group (two injections of distilled water with a 1-h interval) and the single bolus group. In the Webster and Brown-Woodman's study, as well as the other studies using multiple dosing procedures, it was difficult to determine whether the teratogenic and behavioral effects were the result of the increased total dose or the lengthening of the duration of action by the multiple-dose procedure. Unlike the above-referenced studies, we used the identical total dose to compare the single-bolus with the multiple-dose injections. Constant low-dose infusion or repeated injections may make it possible for the drug to be more evenly distributed and to maintain sufficiently high concentrations for the time required to cause herniated umbilici. The multiple dosing/infusion paradigm may also be important for discerning the teratogenic cardiovascular and behavioral consequences of COC. From the clinical aspect, people abusing COC are more likely to self-administer the drug repeatedly than to take a single dose (13,14). These data suggest that in future COC studies, this regimen should be considered to more closely mimic the "real life" situation.

Previous studies demonstrated that DOI administration induced herniated umbilici, suppressed motility, and interfered with hatching (46). Herniated umbilici were seen in all groups that were exposed to DOI injected on E3, E14, or E18. In a few instances, unresorbed yolk with partially externalized intestines were observed in the chicks hatched from eggs that received DOI. RIT significantly attenuated or blocked these effects of DOI, reducing the size of DOI-induced herniated umbilici and the number of chicks affected. Even though a single injection of DOI was given on E3, E14, or E18, at different stages of development, herniated umbilici were observed in all groups upon hatching. It was concluded that DOI (or its active metabolites) may remain in the egg or embryo for a long period of time, but that 5-HT_2-mediated effects on this organ are not manifest, and/or its associated vasculature is not sensitive to DOI until a later stage of development (E18+). In the present study, administration of COC via infusion or multiple injections has mimicked DOI-induced vasoconstriction and herniated umbilici, and both effects were blocked or attenuated by RIT. Thus, Experiment 3 demonstrated the involvement of 5-HT_2 receptors in mediating the COC-induced herniations, and supports the idea that vasoconstriction may be part of the mechanism.

Although COC can indirectly affect various receptors, studies showing blockade of 5-HT uptake in human placental brush border membrane vesicles and cultured placental choriocarcinoma cells by COC (36), provide evidence of COC's inhibitory effect on the 5-HT transporter in human placenta. This suggests that COC can directly act on this organ, which may contribute to complications resulting from maternal COC abuse. It is also known that human umbilicoplacental vessels generally lack adrenergic innervation. In addition, the concentration of 5-HT (together with bradykinin and thromboxane A_2) in the maternal blood present in umbilical vessels was found to be elevated from late pregnancy until delivery (28). Blood and placental tissue levels of 5-HT increased with gestation and reached a peak during and immediately after delivery (23,35). These studies, taken together, strongly support the idea that 5-HT is one of the few endogenous vasoconstrictor substances affecting umbilicoplacental vasculature in humans.

Vascular constriction or disruption, among other things (e.g., spasm, thrombi) has been strongly suspected to be directly or indirectly responsible for fetal structural malformations by interrupting blood flow. Hemorrhage and edema are seen in many organs, and are evidence of hypertension or impaired blood vessel wall integrity or both after developmental insults to chicken embryos causing ischemia and/or hypoxia (16), including injection of COC into eggs with embryos (unpublished observations), in rodents (7,53) and in humans exposed to this agent (19). A 14–30% decrease in blood vessel diameters induced by COC or DOI can theoretically result in a much greater increase in resistance, according to the Poiseuille equation ($R = 8\eta L/\pi r^4$). Despite other factors (e.g., tube length—L, fluid viscosity—η), the internal radius (r) has the greatest influence on the resistance (R) to flow in a cylindrical vessel (32). Because the greatest vascular effects were observed 5 min after the infusions were stopped in the present study, the peak of the vasoconstriction may be reached even later. Thus, DOI- or COC-induced changes in vessel diameter most likely cause a relatively great increase in pressure within mammalian umbilicoplacental vasculature, as well as decreases in fetal blood supply, resulting in hypoperfusion and hypoxia (33,55). Brace (6) demonstrated in a sheep model that fetal blood volume responses to acute hypoxia were very sensitive. A small (5%), but significant, reduction in fetal blood volume was observed after 10 min of mild hypoxia, when oxygen tension decreased by only 2–5 mmHg.

The nature of defects associated with vascular compromise following prenatal COC exposure may be a matter of timing and the location of vasoconstriction, so that the state of development of particular vascular beds can be crucial. Umbilical vessels may not develop the capacity to undergo severe spasm until late development, which may be a form of physiological self-protection and contribute to the closure of umbilical blood vessels at birth (28,49). If the 5-HT concentration appears higher in the umbilicoplacental circulation and causes vasoconstriction earlier than it needs for the physiological processes associated with hatching or parturition, it may lead to alterations of developing structures, interrupting the umbilical closure, and in the present study, inducing herniations and/or incomplete closure of the abdominal wall, at the extreme.

The effect of COC on herniated umbilici may be a secondary consequence of umbilical ischemia from 5-HT_2-induced vascular insults. Although there is no placenta in chicks, the chorioallantoic membrane is considered to be the "avian homologue of the mammalian placenta" (31). Chicken embryos were used in our experiments to study the direct embryonic effects by eliminating maternal factors. It may be a useful model for understanding some of the clinical complications, including cardiovascular and cerebrovascular insults, resulting from human maternal COC abuse. The blockade of 5-HT_2 receptors in relation to COC's effects on these parameters needs to be further studied to determine potential preventive and therapeutic benefits, as well as risks.

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