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Does Methadone Exposure During Avian Development Alter Sex Ratio of Survivors?

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Authors' contributions

This work was carried out in collaboration between all authors. Author SD designed the study, assisted with experiments and prepared the manuscript. Author MMO worked on literature searches, ran experiments, collected data and assisted with data analyses. Author ZG supervised the sexing protocols with the assistance of author KA. All authors read and approved the final manuscript.

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ABSTRACT

Aim: Methadone is commonly used to treat pregnant heroin users and presumed to be safe for developing offspring. An avian model, free of confounding maternal variables, was used to investigate sex differences from methadone exposure during development.
Place and Duration of Study: Studies were conducted at Marist College, Poughkeepsie, NY between June 2012 and May 2013.
Methodology: In the first experiment, methadone in phosphate buffered saline was administered to fertilized eggs at one of two doses (0.458 mg/kg or 1.75 mg/kg) for one of three durations of exposure (Late, Incubation Days 12 to 19; Mid to Late, Days 9 to 19; or Early to Late, Days 5 to 19) with six eggs in each dose x duration condition and six controls (N = 42 eggs). Feathers were taken from eggs with developed embryos from this study for DNA analysis. DNA analysis was not attempted on embryos that had died early in development and decomposed before eggs were opened on Day 20. In a second experiment, methadone (1.00 mg/kg) was administered to eggs from which embryos were sacrificed at four time points during development to investigate growth

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retardation in methadone exposed embryos as compared with controls. Feathers were taken from randomly selected eggs exposed from Day 8 to Day 19 for sexing (N = 10 exposed and N = 10 controls.

Results: Methadone exposure significantly affected embryo viability. The sex ratio of exposed specimens was 2:1 in favor of females, a departure from the normal 1:1 ratio. The results suggest that most of the embryos that died during incubation were male.

Conclusion: The skewed sex ratio observed in this study suggests a sex difference in mortality from methadone exposure during development.

Keywords: Methadone; development; chicks; toxicity; opioids; sex differences.

1. INTRODUCTION

Fodor, Timar and Zelena provide a comprehensive overview of the behavioral effects of perinatal opioid exposure, which include sex differences [1]. The developing brain is vulnerable during development because of the blood brain barrier permeability [2]. In rodent models, administering opiates during fetal development results in higher rates of intrauterine death [3] and smaller brains [4]. Exposure to opiates during development may restrict cortical cell proliferation and maturation in rats [5] and results in decreased dendritic branching in mice [6].

Effects from prenatal drug exposure are sexually dimorphic, so understanding the impact of prenatal exposure on adult brains will require longitudinal studies that take into account sex differences [7]. Sex differences in the pharmacology of opiates include analgesic properties, dependence proclivities, and pharmacokinetics [8]. In rodents, sex differences that have been observed after prenatal exposure to opioids including differences in anxiety and depression-like behaviors, dysregulation of the stress axis [9], and performance on tasks requiring learning and spatial memory [10]. In rodent models, prenatal exposure feminizes male sexual behavior in adulthood [11] and increases sensitivity to opiates in different ways depending on sex [12].

When female rats are administered opioids during puberty, the female offspring later display heightened anxiety-like behaviors during elevated maze tasks and in novel environments; and offspring of both sexes display enhanced sensitivity to morphine [13]. Results like these are evidence that some adverse effects of opioids are mediated through maternal behaviors. It is also known that exogenously administered opiates inhibit maternal behaviors in lactating rats, affecting pups [14].

Adverse effects of prenatal opioid exposure are of concern because methadone maintenance is standard treatment for managing pregnant opioid addicts [15,16]. Adverse outcomes for exposed children are often attributed to environmental factors, such as maternal lifestyles and lack of prenatal care [17]. However, attention problems, hyperactivity, impulsivity and aggressiveness persist in exposed children even those who are adopted [18]. It is notoriously difficult to investigate longitudinal outcomes for prenatally exposed children, and reviewers fail to find published studies that follow cohorts of children to grade school age [19,20]. Maternal factors impact child development, and having a mother who is using opioid drugs affects a child's environment; but there is also evidence that opioids such as methadone directly affect human brain development [21,22].

Examining newborns in the hospital for birth weight, head circumference, and neonatal abstinence syndrome is easier than tracking children exposed *in utero* and suitable case controls longitudinally. A search of PubMed for the term "neonatal abstinence syndrome" produced 980 references dating back to 1974. By contrast, only five case control studies suitable for inclusion in a meta-analysis of neurobehavioral consequences of prenatal opioid exposure were identified [23]. Few studies of developmental outcomes for prenatally exposed children report results by sex, but some find sex differences [24,25].

There are sex differences in the pharmacokinetics of opioids [26] and common mammalian sex differences in behavior may be mediated by the opioid system, as high mu-opioid availability is associated with a temperament that seeks to avoid harm, typical of females, and low mu-opioid receptor availability may be associated with a more impulsive temperament, typical of males [27]. The brain's central reward pathway can be altered in sex specific ways by modifying the expression of

components of its opioid and dopaminergic signaling pathways [28]. A recent study found that individuals who are genetically predisposed to impulsivity and sensation seeking seek similar peers. In male children of alcoholics, peer selection is found to be genetically influenced when the children were homozygous for the A allele of a gene that codes for mu-opioid receptors. The allele is associated with elevated risk of alcohol abuse, and males carrying the A allele are more likely than others to affiliate with alcohol-promoting peers [29]. Evidence that genetic variations may predispose bearers to addictions is profoundly troubling in light of evidence that exposure to opioids such as methadone may modify brain development in the womb.

We are engaged in a research project comparing direct effects of exposure to methadone and heroin during development. In mammals, epigenetic events in the womb are mediated maternally; and even cross fostering does not resolve this issue. As the mu-opioid system is conserved across vertebrate species, chicken embryos were an ideal model organism for eliminating maternal variables. Outcomes for prenatally exposed children are frequently attributed to maternal factors such as lack of prenatal care, poor nutrition, poverty, and poly-substance abuse [30,31]. A number of adverse outcomes have been reported from prenatal exposure to methadone and other opioids [32,33,34].

The chick embryo (*Gallus gallus domesticus*) is a well-accepted research model in developmental biology, genetics, immunology, embryology, pharmacology, and toxicology. The goal of our research is twofold: first, to identify direct drug effects from exposure to methadone during development; and second, to compare developmental consequences of methadone to heroin. Because early studies reported sex differences for prenatally exposed children [35], we intended to analyze avian data for sex differences. Male and female chick embryos are indistinguishable morphologically before about 8 days of development, but earlier identification is possible using other techniques, such as a polymerase chain reaction (PCR) based sexing protocol [36]. The chicken genome was sequenced in 2004, and investigators identified genes which are only present on the W chromosome of female birds [37] allowing sex to be genetically determined using PCR and conventional agarose gel electrophoresis [38]. We assumed that about half of the embryos in our samples would be male and half female, as this is the normal ratio for domestic chickens [39,40].

2. MATERIALS AND METHODS

2.1 Experimental Model

Specific pathogen-free fertilized eggs (44-60 g) from white leghorns were obtained from Charles River Laboratories in North Franklin, Connecticut. Eggs were placed in a forced-air incubation chamber at 38±1ºC with constant humidity in the range 50-55%. The incubator's automatic turner changed egg position every four hours. Eggs were incubated for 20 days. Eggs were randomly assigned to experimental conditions and distributed throughout the incubator. Methadone, at the dose specified for the condition was administered in a vehicle of 10 µl phosphate buffered saline, or if eggs were controls a 10 µl drop of vehicle, using ½ cc insulin syringes to place the drop over the airsac through a 1 mm hole drilled in the shell. By this method, drugs diffuse across membranes into the embryo's circulatory system. N = 43 eggs were used in study one (37 exposed and 6 controls, an additional egg was incubated in one condition). N = 36 eggs were incubated in each condition in the second study (N = 18 exposed and N = 18 controls). From the long exposure (Day 8 to Day 19) middle dose (1.00 mg/kg) condition in this second study, we randomly selected N = 10 exposed and N = 10 controls for sexing. Because of the high death rate from early exposure in study one, the second study protocol was altered to begin injections on Day 8 rather than Day 5. Drug doses for these studies were calculated based on an average weight of 20g embryo on Incubation Day 18. Eggs were opened and chicks sacrificed by cervical decapitation on Incubation Day 20.

2.2 Treatment Groups

DNA samples were collected from feathers of specimens in two studies on effects of methadone exposure during development. The first study investigated potential interactions between dose and exposure duration. Eggs in this study were administered either a high (1.75 mg/kg) or low (0.458 mg/kg) dose of methadone for one of three exposure duration conditions (Incubation Days 12 to 19, Days 9 to 19, or Days 5 to 19) with six eggs in each dose by duration condition (with one additional egg is in a high

dose condition) and six controls (N = 43 eggs). Doses were selected to approximate the exposure of developing humans born to women in methadone treatment programs, either during the early years of this practice (40 mg a day dose for a woman) or the current trend of using up to a four-fold higher dose. Duration of exposure conditions were selected to simulate treatment beginning at different trimesters of pregnancy. Feathers were collected from all embryos that had not died days earlier in development.

A second study was undertaken to investigate whether exposed embryos differed from controls at any of several time points during incubation. Beginning on incubation Day 8, eggs were dosed daily with methadone (1.00 mg/kg per day). On Days 11, 14, 17, and 20 eggs were opened (N = 18 exposed and N = 18 controls) and embryos sacrificed, weighed and measured for head circumference. Ten exposed specimens and ten controls were randomly selected for sexing from those opened on Day 20.

2.3 Sexing Samples

The DNA extraction and amplification protocol was tested prior to sexing embryos using a feather from an adult hen and an adult rooster. Fruit fly DNA was used as a negative control as insects lack the ZW chromosome present in avian species.

In this study feathers, with a small amount of attached epidermal tissue, were collected using flame sterilized tweezers on day 20. Feathers were stored in a vial containing 100% ethanol until DNA extraction. Genomic DNA samples were obtained from each specimen following standard methods for DNA purification. Total DNA was extracted using Qiagen's DNeasy® Tissue Kit (Venlo, Netherlands) protocol by digesting one feather from each specimen in 180 µL of lysis buffer with 20 µL proteinase K. Samples were incubated overnight for 15 hours at 56ºC until completely lysed. Primers were designed to amplify a 415 bp product of the XhoI repeat sequence from the W chromosome found only in female birds. Markers were amplified as a single fragment using the following primer pairs from Integrated DNA Technologies Inc. (Coralville, IA.):

XholA 5'CCCAAATATAACCACGCTTCACT 3'
and
XholB 5'CCCAAATATAACCACGCTTCACT 3'

Amplification was carried out in a 50 µL of buffer using a Quiagen Multiplex PCR kit. The PCR program consisted of an initial step at 95ºC for 15 minutes, followed by 35 amplification cycles (95ºC for 15 sec, 56ºC for 15 sec, 72ºC for 15 sec), and a final step at 72ºC for 6 minutes.

Polymerase Chain Reaction (PCR) products were analyzed by agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV light. If DNA of interest was present in a specimen, it indicated the sample came from a female embryo. Samples lacking amplified DNA were male. To verify our methods, tissue from samples were sent to Avian Biotech International, Tallahassee, FL for independent analysis to confirm results.

2.4 Statistical Analysis

Statistical analyses were conducted for the study using the Statistical Package for the Social and Behavioral Sciences. Chi square was used to determine whether there were significant differences in survival rates by condition.

3. RESULTS

In the dose by duration experiment, which employed a 2 x 3 design, Study One, methadone exposure had a significantly adverse effect on embryo viability. Twenty percent of controls died, a percentage within the range which the egg supplier, Charles River Laboratories, considers normal.

3.1 Early to Late Duration of Exposure (Days 5 to 19)

In the high dose long exposure condition (Incubation Days 5 to 19), significantly more exposed embryos died than did controls, 47 percent vs. 20 percent, Yates $X^2(1, N=15)$ = 43.89, $P<.001$. In the low dose long exposure condition, significantly more exposed embryos died than controls, 35 percent vs. 20 percent, Yates X^2 $(1, N=15)$= 13.14, $P<.001$. Methadone exposure beginning early in development through late development killed a significant number of embryos at both the high and low dose.

3.2 Middle to Late Duration of Exposure (Days 9 to 19)

In the high dose middle duration exposure condition (Incubation Days 9 to 19), significantly

more exposed embryos died than did controls, 36 percent vs. 20 percent, Yates $X^2(1, N=15)$= 51.02, P<.001. In the low dose middle duration condition, significantly more exposed embryos died than did controls, 43 percent vs. 20 percent Yates $X^2(1, N=15)$= 31.64, P<.001. Methadone exposure from middle to late in development killed a significant number of embryos at both the high and low dose.

3.3 Late Duration of Exposure (Days 12 to 19)

In the high dose late duration exposure condition (Incubation Days 12 to 19), there was not a significant difference in the number of dead embryos between exposed and control conditions at either the high dose Yates $X^2(1, N=15)$= 1.89, P = .17) or at the low dose Yates $X^2(1, N=15)$= 0.016, P = .90. Methadone exposure late in development did not kill a significant number of embryos. Dead embryos by condition in the first study are shown in Table 1.

Methadone exposure had a significant effect on embryo viability at both high (1.75 mg/kg) and low (.485 mg/kg) doses. Teratogens typically have more adverse impact early during development, which is consistent with these results.

3.4 Embryo Sex

In the dose by duration study, the sex ratio was normal in the low dose/late exposure condition (0.456 mg/kg/Days 12 to 19). Overall in this study N = 20 embryos survived, 13 females and 7 males. Three control eggs from this study were analyzed for sex, these eggs contained two males and one female.

In the second study of N = 10 randomly selected embryos exposed to a middle dose (1.00 mg/kg) from Incubation Day 8 to 19 were sexed. These eggs included 6 females, 1 male and 3 dead specimens which were not sexed. There were also more female than male controls in this condition, six to one.

Overall the sex ratio of surviving embryos exposed to methadone during development was more than 2:1 in favor of females. Fig. 1 shows agarose gel electrophoresis for specimens from each of the two studies. Table 2 identifies the dose for embryos from the first study by sex.

If most of the embryos not sexed because they died and degraded early in development were males, the sex ratio would more approximate the pattern for this species of 1:1. Table 2 identifies conditions for specimens shown in Fig. 1 for high (1.75 mg/kg) and low dose (0.456 mg/kg) conditions. Table 3 identifies specimens from the second study and controls.

Results in Table 2 above include embryos exposed to methadone for different durations of time over development and are thus not directly comparable to results in Table 3 in which all embryos were exposed from the same length of time. Because of the sample sizes and high number of deaths in some cells, it was not possible to statistically analyze the dose by duration interactions across the two studies.

All doses of methadone in this study (1.75 mg/kg, 1.00mg/kg, and 0.485 mg/kg) killed embryos if administered prior to Incubation Day 12. The sex ratio of survivors was more than 2:1 in favor of females, a finding which strongly suggests a disproportionately high rate of death in male embryos.

Chi squared for the observed sex ratio for observed vs. expected in exposed specimens was 3.85, p = 0.049.

Table 1. Percentage of dead embryos by dose and duration of methadone exposure study 1

	Incubation days days 5 to 19	**Incubation days days 9 to 19**	**Incubation days days 12 to 19**
1.75mg/kg	47%	36%	14%
0.458mg/kg	35%	43%	14%

Table 2. Sex of embryos by high (1.75 mg/kg) or low (0.456 mg/kg) dose conditions

	High dose 1.75mg/kg	**Low dose 0.456mg/kg**	**No dose 0.00 mg/kg**
Females	HD 3,4,5,7,9	LD 1,2,3,4,5,6,8,9	Cc10
Males	HD 1,2,6,8,10	LD 7,10	Cc9,11

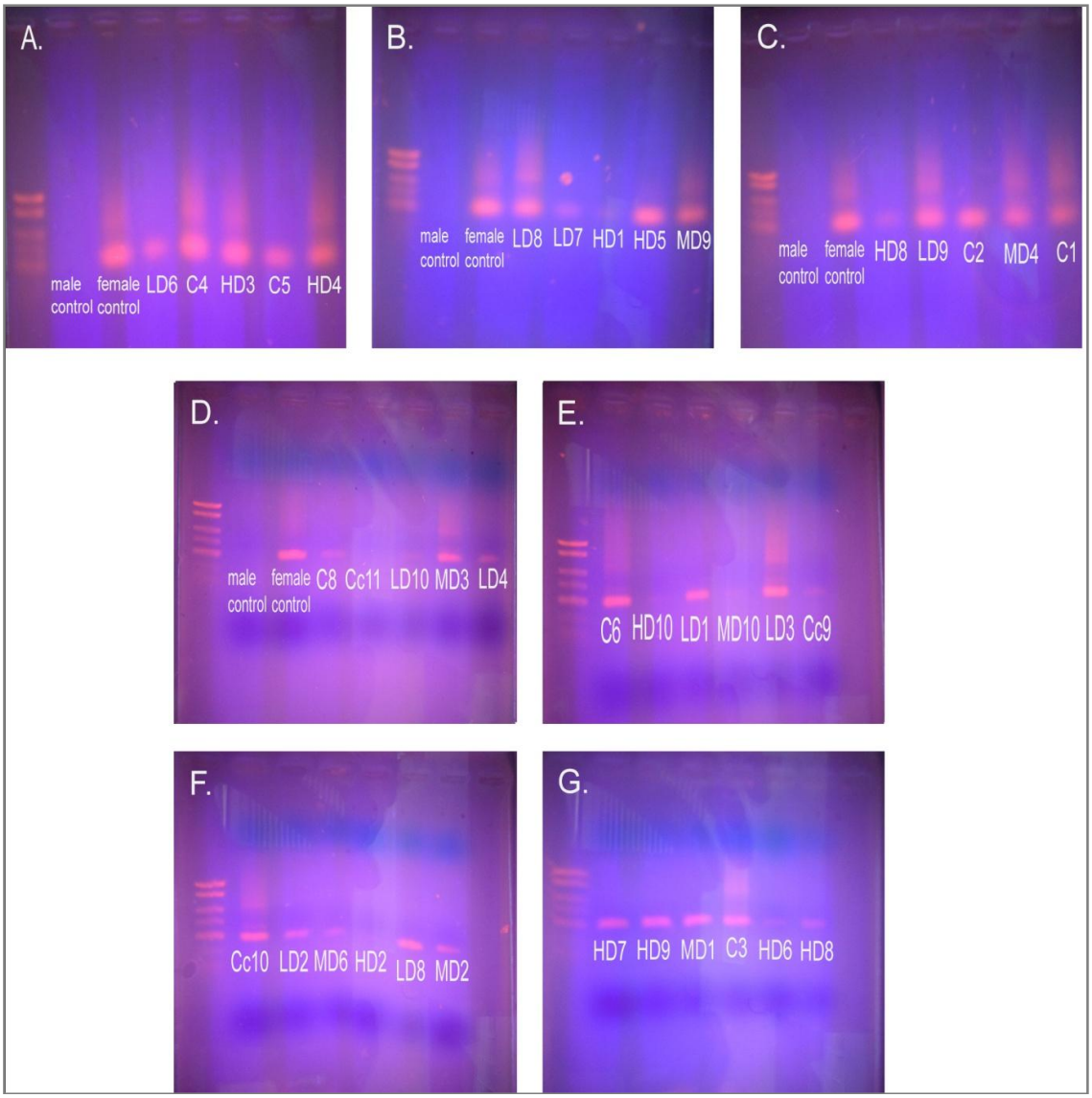

Fig. 1. Gel electrophoresis results for sex

HD = high dose, 1.75 mg/kg, MD = medium dose, 1.00 mg/kg, LD = low dose, 0.458 m/kg
Cc = no dose control Study One, C = PBS control Study Two. Numbers following dose condition identify a particular specimen. Note that specimens LD8 and HD8 appear twice

Table 3. Sex of embryos for medium dose (1.00 mg/kg) condition

	Medium dose 1.00 mg/kg	No dose 0.00 m/kg
Females	MD1,2,3,4,6,9	C1,2,3,4,5,6
Males	MD10	C8

4. DISCUSSION

As part of an ongoing research project, we are conducting histological, hematological and genotoxicity studies of the effects of methadone exposure during development. Because there is some evidence from human literature that methadone may be more harmful to males, and evidence in general that males may be more susceptible to prenatal insults, we undertook an effort to sex specimens in the dose by duration study using DNA analysis. The method we used

relied on collecting a feather when the egg was first opened on Day 20 of the study, but we were not able to collect feathers from embryos which died early in development and degraded long before the eggs were opened. We had not anticipated finding unequal numbers of males and females or the high rate of embryo deaths.

Finding the sex ratio skewed more than 2:1 when the literature reports that domestic chickens normally have a sex ratio of about 1:1 [39] was an anomaly. One study of 67,993 chicks found that 48.77 percent were males, so males have a slightly higher pre-hatching mortality rate [40]. We contacted the egg supplier, Charles River, and asked if their hens laid eggs with a disproportionally high number of females. Support personnel at Charles River were helpful but were not aware of a skewed sex ratio in their eggs and could not suggest any systematic reason for an elevated number of females. The flocks at Charles River are inbred, creating near clones, an advantage to lines of research where genetic variation between specimens is undesirable; but the skewed sex ratio we observed in this study had never been reported back to them by other laboratories using their eggs.

In birds, females are the heterogametic sex (ZW) and males are homogametic (ZZ). Sex is determined at the time of the first meiotic division, when one sex chromosome is retained in the oocyte and the other segregates to the polar body. The sex-determining division in avian meiosis occurs prior to ovulation and fertilization. Conditions in our laboratory could not have altered the sex of the embryos in the eggs but meiotic mechanisms have been suggested by which birds can alter the sex ratio of broods. For White Leghorns, there is a reported tendency to more males only in the first five eggs of the first reproductive season [41,42]. Levels of progesterone at the time of meiosis may affect the sex of the resulting egg, with high progesterone levels resulting in fewer males [43]. Although these are possible mechanisms, these are not particularly likely to be the case in this instance.

Occam's razor says that among competing hypotheses, the one with the fewest assumptions should be selected. In this instance we believe that the methadone adversely affected the survival of males more than females, especially at higher doses and earlier in development. If most of the dead embryos in the study were males, the sex ratio would be closer to the normal 1:1. In rats perinatal methadone exposure alters dopaminergic, noradrenergic, and serotonergic activity in a sex-specific manner [44]. The results of the present studies suggest methadone also has sex specific effects in chicken embryos. Sex differences in outcomes from prenatal exposure to opioids in humans were reported to be more adverse for males in one of the few studies that reported results by sex [45].

NMDA is a receptor for the excitatory neurotransmitter glutamate. MDMA/Ecstasy (3,4-methylenedioxy-N-methylamphetamine) evokes a delayed and sustained increase in glutamate [46]. A recent study found that 71 percent of pregnant women using the drug gave birth to boys [47]. This figure is skewed enough from the normal sex ratio for humans to suggest MDMA exposure reduced the odds of females surviving gestation. In this study, methadone, a glutamate antagonist [48], apparently reduced the odds of male survival.

Larger sample sizes, a greater range of doses, variation in duration of exposure, and eventually a mammalian model could improve the applicability of this line of inquiry to humans; but the death of male avian embryos from methadone exposure suggests that research is warranted to investigate sex differences from prenatal exposure in humans.

5. CONCLUSION

This study found a significantly skewed sex ratio in avian embryos after exposure to methadone during development. Death of male avian embryos from methadone exposure suggests that research is warranted to investigate sex differences from prenatal exposure in humans.

CONSENT

Not applicable.

ETHICS STATEMENT

The National Institutes of Health's Office of Laboratory Animal Welfare (OLAW) has interpreted the term "live vertebrate animal" to apply to egg-laying species only after hatching so this study did not require oversight by an institutional animal care committee. The protocol for this study was reviewed by a program officer

at the National Institute on Drug Abuse of the National Institutes of Health.

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COMPETING INTERESTS

We disclose no possible conflict of interest in the conduct and reporting of research, such as financial interests in any test or procedure or funding by a pharmaceutical company for drug research.

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