Original Paper

Antiteratogenic Effects of Caffeine on External, Visceral, and Skeletal Anomalies of Rat Foetuses

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ABSTRACT

This study reports the antiteratogenic effects of caffeine on various anomalies on Charles Foster rat foetuses. The antiteratogenic activity of caffeine was assessed against two teratogens, cyclophosphamide and tolbutamide, both having different teratogenic potentials. External, visceral, and skeletal anomalies were assessed among the foetuses of both control and treated groups. Controls showed 2.5%, 0.0% and 5% external, visceral, and skeletal anomalies respectively. The offspring of caffeine-treated females showed 2.43% and 4.36 % of external and visceral anomalies, while there were no skeletal anomalies. Foetuses of females treated with caffeine-tolbutamide combination showed 4.34% and 9.09 % external and skeletal anomalies, which were comparatively higher than the control and caffeine-treated subgroups. However, no visceral anomalies were seen in this group. The offspring from dams treated with tolbutamide showed the highest incidence of external, visceral, and skeletal anomalies (10%, 4.6% and 13.04%) respectively. This preliminary study reveals that caffeine at dose levels of 12.5mg/kg body weight has a protective effect on tolbutamide-treated foetuses. No foetuses could be obtained for the study of anomalies from the cyclophosphamide-treated dams because of total resorption.

Key Words: Teratogenicity, Antiteratogenicity, Teratogen, Visceral anomalies, Skeletal anomalies

Introduction

Environmentalists have always been concerned with the indiscriminate consumption of pharmaceutical drugs by women of childbearing age. Drug interactions during pregnancy could be the cause of some unexplained foetal anomalies. It well known that several chemicals exert teratogenic effects in the unborn child. To tackle this problem, drug and chemical consumption by pregnant women should be curbed, or compounds should be created that have an ameliorative or protective action against deleterious effects of teratogenic agents.

The reason for taking up caffeine as a possible antiteratogenic drug is because it has the ability to cross the placental barrier, and yet does not have any teratogenic risk in humans.[1,2] In fact, there are indications of an antiteratogenic effect through studies relating to the exposure of humans to a variety of widely consumed beverages and therapeutic drugs.[3] But caffeine has different activity at different dose levels, for e.g., at very high doses, it can actually be teratogenic, as demonstrated in rodents.[4] However, at lower doses caffeine has been shown to have an ameliorative effect on the teratogenic property of other agents.[5-8] Cyclophosphamide and tolbutamide are two well known teratogenic drugs,[9-10] and so it was decided to conduct this study of antiteratogenic effects of caffeine on these drugs.

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Materials & Methods

Choice of Animals and Mating

The present study was carried out on Charles Foster rats. Animals chosen for this study were sexually mature, nulliparous and pathogen-free. Rats with average age of 120 days, and weight of about 175±10g in case of males, and 165±10g in case of females, were used for the study. On a particular day, two female rats in proestrus phase at 1700 hrs were caged overnight with one male of the same stock. When spermatozoa were found in the vaginal smear the next morning at 0900 hrs, it was taken as "day 0" of gestation. The pregnant animals were kept individually in separate cages.

Study Design

Table 1

Subgroup-1	Control Group	Fed with 1 ml of distilled water
Subgroup-2	Caffeine-treated Group	Dose: 12.5 mg/kg body weight
Subgroup-3	Tolbutamide-treated Group	Dose: 500 mg/kg body weight
Subgroup-4	Cyclophosphamide -treated Group	Dose: 7 mg/kg body weight
Subgroup-5	Caffeine+Tolbutamide treated Group	Dose: 12.5+500 mg/kg body weight
Subgroup-6	Caffeine+Cyclophosphamide-treated Group	Dose: 12.5+7 mg/kg body weight

A total of 30 pregnant rats were used for the study, which were further divided into 6 subgroups, each comprising 5 pregnant rats (**Table 1**). All the pregnant rats of various sub-groups were given three doses of respective compounds by the oral route, and the procedure adopted for this study was divided into the following steps:

- Time-mated rats were treated with the particular compound during the period of major organogenesis, i.e., from day 6 to 15 of gestation.
- Throughout the whole gestation period, the dams were observed for their body weight, and food and water consumption, on days 0, 7 , 15 and 20 of gestation. A daily health check was also carried out.
- The treated rats were anaesthetized by using light, ether anaesthesia on day 20 of gestation, and the foetuses were surgically extracted.
- The visceral and uterine contents of each dam were examined, and the following observations were made:
  1. Number of corpora lutea
  2. Number of implantations
  3. Number of resorptions
  4. Number of live foetuses
  5. Number of dead foetuses
  6. A note was also made on pre- and post-implantation loss.

Procedure for Observing Various Anomalies:

Young, live rats were examined externally, and were then preserved in 70% ethyl alcohol. After 5 to 7 days, half of the foetuses were examined for visceral anomalies by slicing method as described by Wilson and Warkeny, and the remaining half of the foetuses were examined for skeletal anomalies by staining them with Alizarin Red stain as described by Dawson.[11]

The following anomalies were looked for in the foetuses:

Gross

1. Inverted claw
2. Clubbing of limb
3. Wrist drop
4. Curly tail
5. Open eye
6. Oligodactyly
7. Agnathia
8. Microstomia
9. Imperforate anus
10. Amelia
11. Craniofacial anomaly
12. Others

Visceral

1. Cleft palate
2. Ectopic kidney
3. Cardiac hypertrophy
4. Hydrocephaly
5. Ectopic heart
6. Cardiac defects
7. Hydropic ureter
8. Agenesis of organ
9. Others

Skeletal

1. Non ossification of skull bones
2. Ribs bent inside
3. Intercostal space
4. General lack of ossification
5. Wavy ribs
6. Twisted clavicle
7. Branched/missing ribs
8. Acephaly
9. Anencephaly
10. Exencephaly
11. Divided/fused sternum
12. Others

Techniques for Skeletal Study

Procedure

1. Preparation of specimens for clearing: Prior to processing, the specimens were preserved and fixed in 70% ethanol, then eviscerated by making a nick over the abdomen.
   - Preliminary Clearing: The specimens were placed in 2% aqueous potassium hydroxide until the flesh was sufficiently clear to render the skeleton visible. The solution was changed when it became discoloured.
   - Staining: The specimens were then placed in Mall's solution. To this solution, Alizarin red S (alizarin-sodium monosulphate) was added to give it a deep purplish-red colour. The solution was gently agitated a few times per hour. The specimens were left in solution until the bones were stained to the desired extent. Specimens were then again passed through Mall's solution for removal of overstaining, and passed successively through 25%, 50%, and 80% glycerol. They were then finally preserved permanently in 90% glycerol solution. One or two crystals of thymol were added as preservative.
2. **Wilson's soft tissue sectioning technique**

   Foetuses used for the Wilson's soft tissue sectioning technique were first fixed in Bouin's solution, which is a mixture of saturated picric acid, formaldehyde, and glacial acetic acid. The purpose of the fixative is to fix the tissues, harden the soft tissues, and soften the bones in order to preserve the specimens, and make it possible to slice them cleanly with a razor blade into sections of approximately 1mm (or less) thickness. One drawback to this fixed-tissue technique is that the original colouration of the tissues gets lost, and all tissues acquire a shade of yellow, with the exception of blood, which appears brown, and the liver, which appears olive green. The foetuses were allowed to remain in the fixative for a period of two weeks minimum, and then rinsed in alcohol prior to slicing. Since the formaldehyde and acetic acid fumes from the fixative are irritating to the eyes and respiratory tract, and present a carcinogenic danger, it is recommended that the rinsing and slicing be done under a fume hood or in a well-ventilated area. As Bouins solution stains tissues, workers handling these specimens should wear latex gloves (preferably two pairs).

The foetuses were sliced with a sharp razor blade held in a Lipshaw or Pathco handle. The blades were changed frequently in order to ensure even slices with smooth face, and care taken to slice with as few strokes as possible. Excessive downward pressure with the blade, or squeezing of the foetus in the grip, will cause the internal organs to be pushed out resulting in uneven slices, and slices which fall apart into pieces.

The identity of the foetus was first verified, after which it was given an external examination. The forelimbs were removed with either the razor blade, or a sharp pair of scissors, and the head sliced into four to eight slices, the minimum being a slice at the beginning of the nasal passage just behind the nares, a slice at a point just before the eye bulges to examine the nasal passages (turbinates), the palate, and the nasal septum, a slice through the eye bulges to allow the examination of the lenses and upon removal of the lenses the retinas, and a slice behind the eye bulges at the level of the frontal/parietal suture in order to examine the lateral and third ventricles of the brain. If only these four slices are taken, it is recommended that the brain be carefully removed from the last section to allow an examination of the cerebellum and meninges. If eight slices are taken, then the final slice should allow examination of the fourth ventricle of the brain. Following these slices, the bottom of the head was removed from the foetus by slicing through the neck, and the structures of the inner ear were examined.

The remainder of the foetus was sliced as thinly and evenly as possible, and the sections laid on the wax block with the cranial side upward. The trachea, oesophagus, and blood vessels were followed through the slices, and the spinal cord and organs of the neck were examined. The right carotid, the right subclavian, left carotid, and left subclavian should be apparent, and the right carotid and subclavian should be seen to converge to form the innominate. Care should be taken to have a cut transect

the top of the aortic arch. From the top of the aortic arch through the apex of the heart the technician should attempt to slice thinly enough to produce ten slices in which the following structures should be apparent: the aortic arch, the ductus arteriosus, the pulmonary artery, the artia, the semilunar valves, the tricuspid valve, the mitral valve, the ventricles, and the interventricular septum.

Following the slices through the heart, there are two possible techniques for continuation. The first technique involves slicing thinly through the abdomen to reveal the liver lobes, stomach, and intestines, until the level where the slices transect the kidney through the pelvis, following which the remaining viscera can be gently pulled back to internally verify the sex of the foetus. The second technique stops thoracic slicing at a point just before the diaphragm is reached, and the remainder of the lungs can be gently removed in order to examine the diaphragm for diaphragmatic hernias. Following this, a single abdominal slice can be made from the level of the umbilicus on the ventral aspect to a point just above the kidneys and adrenals on the dorsal aspect. The sections of liver lobe in the upper section were gently pulled apart and examined, following which the remaining portions of liver and the intestines were pulled out of the lower section to reveal the kidneys and sex organs. The kidneys were sliced through the pelvis and the foetal sex was internally verified.

Observations

External, visceral, and skeletal anomalies were assessed in the foetuses (control as well as treated groups). Controls showed 2.5%, 0.0% and 5% external, visceral and skeletal anomalies respectively. Offspring from caffeine-treated mother (dose level 12.5mg/kg body wt) showed 2.43% and 4.36 % of external and visceral anomalies, whereas there were no skeletal anomalies. Foetuses of caffeine- and tolbutamide-treated females showed 4.34% and 9.09% external and skeletal anomalies, which were comparatively higher than the control and caffeine-treated subgroups. However, no visceral anomalies were noticed in this group. The offspring from dams treated with tolbutamide showed the highest incidence of external, visceral and skeletal anomalies (10%, 4.6% and 13.04% respectively). This preliminary study concludes that caffeine at dose level of 12.5mg/kg body weight has a definite protective effect on tolbutamide-treated foetuses. No foetuses were available for the study of anomalies from the cyclophosphamide-treated dams because of total resorption.

Refer **Table 2** for details of observations.

Conclusions

1. Caffeine at dose level of 12.5 mg/kg body weight showed no adverse effects on the developing embryos and foetuses that could be linked to the compound.
2. Whatever anomalies were observed were just incidental findings, and were comparable to the controls.
3. Cyclophosphamide at dose level 7 mg/kg body weight resulted in 100% resorption in all the pregnant rats. But when it was given along with caffeine, some protective effect was observed in the form of delayed resorption.

Table 2

Parameters	Groups/Dose					
	Control	CF	CP	TB	CF+CP	CF+TB
Total number of animals studied	5	5	5	5	5	5
Number of corpora lutea	44	46	45	56	36	46
Number of implantations	44	43	45	56	36	46
Number of resorptions	4	2	45	5	34	Nil
Number of live births	40	41	Nil	50	Nil	46
Pre-implantation loss %	Nil	6.5	Nil	Nil	Nil	Nil
Post-implantation loss %	9	4.6	100	10.7	100	6.5
Anamolies						
a) External %	1(2.5)	1(2.43)	Nil	5(100)	Nil	2(4.34)
b) Visceral %	Nil	1(4.76)	Nil	1(4.16)	Nil	Nil
c) Skeletal %	1(5)	Nil	Nil	3(13.04)	Nil	2(9.09)

CF = ,Caffeine; CP = Cyclophosphamide; TB = Tolbutamide

4. Tolbutamide at dose level 500mg/kg body weight showed very little resorption, and few malformations. Here also, caffeine showed protective effects on the effects of tolbutamide.
5. It was observed that caffeine has more ameliorative action on the effects of tolbutamide, than on the effects of cyclophosphamide.

In conclusion, this preliminary study reveals that caffeine at dose level of 12.5 mg/kg body weight has a definite protective or ameliorative effect on the teratogenic and embryotoxic properties of cyclophosphamide and tolbutamide at their presently used doses. However, it would be desirable to study escalated doses of caffeine to reach definite conclusions.

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