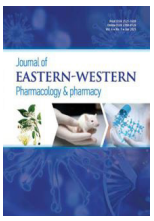

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POTENTIAL TOXICITY EFFECTS OF GURGEM INJECTION ON PRENATAL DEVELOPMENT OF WISTAR RATS

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KEYWORDS

Carthamus tinctorius; Prenatal toxicity; NOAEL

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ABSTRACT

Safflower (*Carthamus tinctorius*) has long been used to treat cardiovascular diseases. In this study, an injectable formulation of *Carthamus tinctorius* (CTI) was developed. Beyond its pharmacological benefits, potential toxicity must be thoroughly assessed. Previous studies have reported possible maternal, fetal, and teratogenic toxicities in rodents, while also suggesting fertility-enhancing properties, underscoring the need for further investigation.

Aim: This study aimed to evaluate the potential toxicity effects of CTI in pregnant Wistar (Han) rats and their developing fetuses. The research followed OECD Guideline No. 414, the international standard for prenatal developmental toxicity testing.

Methods: Pregnant rats were randomly divided into four groups (n=3 females/group): a control and three treatment groups receiving CTI at doses of 0.45 (low), 0.82 (medium), and 1.65 ml/kg (high). CTI was administered daily via intramuscular injection from gestational day (GD) 6 to 20. On GD21, cesarean sections were performed. Maternal parameters (body weight gain, ovaries with HE staining, corpora lutea count, and uterine examination) and fetal parameters (body weight, head cranium, tail length, placental weight) were evaluated.

Results: Corrected maternal weight gain and fetal body weight were significantly reduced in low- and high-dose groups compared to the control. Increased anogenital distance index and congenital abnormalities, such as hydrocephalus, were observed in some fetuses. Conversely, the 0.45 ml/kg group showed improved embryonic survival and no significant treatment-related adverse effects.

Conclusion: The study identified a monotonous dose-response curve (MDRC), showing increasing adverse effects with higher CTI doses. The lowest observed adverse effect level (LOAEL) was 0.45 ml/kg, and the no observed adverse effect level (NOAEL) was also determined at 0.45 ml/kg, suggesting limited safety margins for CTI use during pregnancy.

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INTRODUCTION

In Law of Mongolia stipulates that it is mandatory to conduct a study on the toxicity of drugs to the prenatal development before they are developed and marketed. Due to lack of equipment's and information's of prenatal development toxicity study, there is insufficient research papers about prenatal development study of drugs/ pharmaceuticals. In recent years, as the use of herbal medicines has increased, domestic drug manufacturers in Mongolia have increased their tendency to develop new herbal medicines. Such as Samples of "Gurgem" injection from the Safflower aqueous extract, provided by "Tsombo Pharm LLC".

Saffron and safflower powder are used as food seasonings and colorings, and saffron seeds are used to make cheese. It has blood-thinning properties in medicine, reduces inflammation, relieves menstrual pain[1], and accelerates bone regeneration[2]. In China, an injectable drug formulation with anticoagulant properties has been developed from the petals of Safflower (*Carthamus tinctorius*), which is widely used globally for cardiovascular diseases[3]. However, Chinese National Drug Adverse Reaction Registration Center has recorded 3,306 cases of adverse reactions caused by "Safflower" injections. Although Safflower has no reproductive or embryotoxic effects when used for food purposes, it has been reported that when Safflower is consumed at high or therapeutic doses, changes in external and internal organs and fetal resorption occur in the embryo[4].

Based on this, safflower injections pharmacological studies were conducted. In addition to pharmacological studies, it is necessary to confirm special toxicity studies, including studies on the effects on the embryo. Previous studies on Safflower have concluded that it is toxic to animals, has a fetotoxic and teratogenic impact, and may also affect the stage of fetal development in rats[6], so this study was conducted to investigate the effects of the injection on the embryos of pregnant Wistar (Han) rats.

MATERIALS AND METHODS

Animals

Wistar (Han) rats aged 8 weeks, 180-200g, were individually housed in an Individually Ventilated Caging (IVC) system of Pharmacological lab, DRI. The room was maintained under controlled conditions of 12 h light/dark periods, 50-70% humidity, and 22-25° C. The animals were provided with rat chow pellets and water ad libitum. The animal study was conducted according to the "Ethical Guidelines for the use of Animals in Biomedical Research".

Experimental design

The experiment was performed according to the Organisation for Economic Cooperation and Development (OECD) protocol guidelines, test number 414: prenatal developmental toxicity study[7].

Vaginal fluid assessment

Vaginal fluid was obtained from the female rats using a micropipette filled with 10 µl normal saline (NaCl 0.9%), smeared on glass slide and viewed under a light microscope at × 10 magnification (Figure 1A) to determine the phase of oestrus cycle. Only females at the proestrus phase were caged overnight with a male rat on a 2:1 basis. Female rats with presence of positive to sperm in their vaginal fluid were considered to be in gestational day 0 (GD0) of pregnancy.

Administration of Gurgem injection

Twelve female Wistar rats were randomly divided into four groups (n = 3): (i) negative control, received the solvent only (saline), (ii) 4ml, (iii) 8ml, and (iv) 16ml/60kg body weight (BW) of Gurgem injection. The injection was administered (intramuscularly) daily from GD6 until GD20. The experimental doses were determined based on preliminary general toxicity studies. Dose calculation for animal study was carried out according to the following formula by the FDA: Animal equivalent dose (AED) = Human equivalent dose HED mg kg* Human conversion factor /animal conversion factor. The conversion factor for rats is 6, and for humans it is 37.

Assessment of maternal parameters

The animals were observed for signs of toxicity, such as vomiting, diarrhoea, or death. BW was measured daily throughout the experimental period until the day of euthanisation. Corrected maternal BW gain was calculated by subtracting the BW of the pregnant rat at GD21 from that at GD0 and the weight of its gravid uterus.

Caesarean hysterectomy

Pregnant dams were anaesthetised at GD21 with intramuscular (IM) injection of ketamine (50 mg/kg) and xylazine (5 mg/kg). A midline abdominal incision was made to expose the gravid uterus (Figure 1B), which was then carefully harvested, weighed, and incised to determine the number of live foetuses. The offspring were examined for external malformation such as shortened or absent limbs and malformations of hands and feet, labelled accordingly, weighed, and then euthanised with an overdose of ketamine and xylazine. Then, the placentae were delivered and weighed. Besides, foetal gender was determined based on the anogenital distance (AGD), which is usually

longer in males than in females. Further confirmation by dissection was done later to determine the presence of testicles in the male foetuses. Alternate foetuses (according to the position in the uterus) were allocated for blade sectioning of the head.

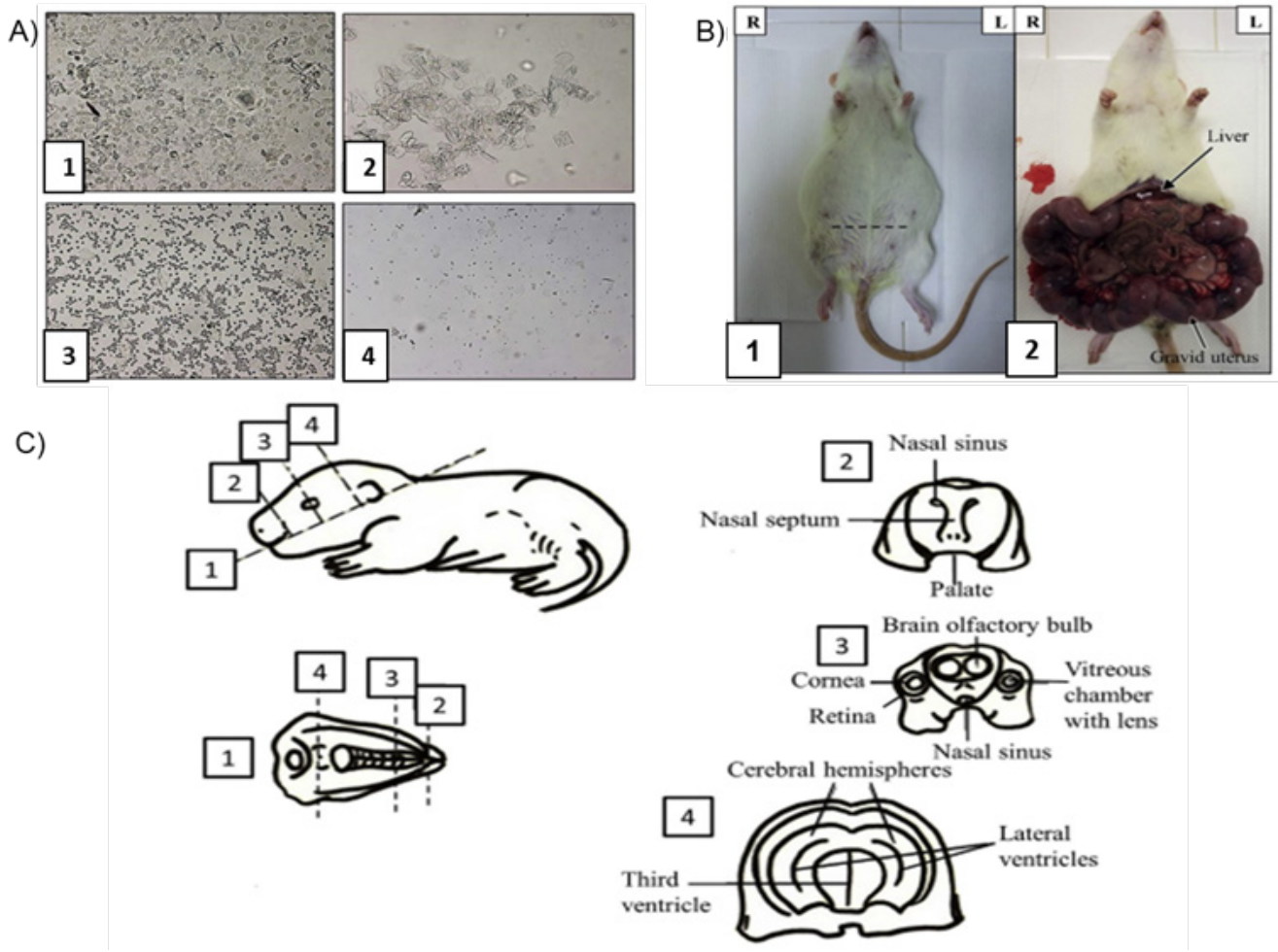

Figure 1. A): Phases of oestrus cycle in rats (1) proestrus, indicated by predominance of epithelial cells, (2) oestrus, indicated by predominance of cornified cells, (3) diestrus, indicated by predominance of leukocytes, and (4) metestrus B): Photograph of (1) a pregnant rat and the dotted line in photograph indicates the site of transverse incision made during the procedure. R: right, L: left (2) exposed gravid uterus in situ at GD20. C): Level of sectioning for foetal head examination

Foetal head examination by Wilson's techniques

Foetal heads were sectioned into four different sagittal planes (Figure 1C) using a sharp blade (Wilson, 1965). The sectioned heads were immersed in Bouin's solution and examined under a stereomicroscope according to Wilson's technique (1965)[8].

Statistical analysis:

Statically analysis were done by Two-way ANOVA of GraphPad Prism

RESULT

Among animals that were breeded, vaginal plug positive females were selected, isolated and the day of breeding was marked as day 0 of gestation, and Gurgem injection was started to administered on G6 day. The weight of the animals was recorded at each infusion, and the dose indicated in Table 1 was calculated based on the weight of the animals in each group. The initial weight before breeding, the weight on the first day of breeding, the weight on the 15th day of gestation, and the weight difference from the weight taken over 14 days are shown in Table 2.

Table 1. Dosage for each group

Experimental groups	Human equivalent dose	Animal equivalent dose
Experimental control	-	0.2 ml
Experimental group 1	4 ml	0.09 ml
Experimental group 2	8 ml	0.164 ml
Experimental group 3	16 ml	0.33 ml

Maternal body weight gain

The weight of healthy rats increases by an average of 41% during pregnancy[9]. The total weight of some animals in experimental group 1, 2 and 3 increased from their pre-breeding weight, but did not increase after the start of the study, which was performed on GD6 and these animals were resorbed at autopsy. As for the corrected maternal weight gain, significant BW reduction was observed in gurgem treated groups compared to the control group (Figure 2A).

Table 2. Maternal body weight gain

Groups	n	Before mating weight	Weight (g) of gestational sixth day (G6)	Weight (g) of gestational twentieth day (G20)	Weight difference G0-G20	Weight difference G6-G20
Control	1	200	218	346	146	128
	2	200	224	308	108	84
	3	234	258	354	120	96
Experimental group 1	1	190	204	204	12	0
	2	202	224	228	26	4
	3	214	240	280	66	40
Experimental group 2	1	198	226	216	18	-10
	2	198	208	194	-4	-14
	3	202	196	206	4	10
Experimental group 3	1	178	184	212	34	28
	2	196	212	202	6	-10
	3	200	212	218	18	6

Explanation: Statical analysis and p value is shown in Figure 2A.

Table 3. The fetal parameters in Wistar rats were treated with a Gurgem injection.

Groups	n	Foetuses number	Placent weight(g)	Ovary(g)		Corpora lutea(n)	
				Right	Left	Right	Left
Control	1	11	3.9	0.1	0.05	7/9	8/5
	2	8	2..24	0.04	0.08	6/6	9/6
	3	7	3.95	0.09	0.04	5/5	7/8
Experimental group 1	1	-	0.7	0.03	0.04	6/12	5/11
	2	8	2.03	0.06	0.02	6/4	5/9
	3	6	2.84	0.05	0.05	5/10	5
Experimental group 2	1	-	0.66	0.04	0.06	5/3	8/8
	2	1	0.98	0.02	0.02	7	6/2
	3	-	0.6	0.03	0.02	6/6	7/8
Experimental group 3	1	7	2.57	0.08	0.04	6/4	3/7
	2	-	0.5	0.08	0.05	5/11	5/4
	3	2	2.59	0.06	0.1	5/5	7/4

On the 20th day of gestation, the animals were not injected by exerimental drugs. They were humanely anesthetized by the ethical standards of the Medical Association for the use of experiments. A transverse incision was made in the abdominal cavity, and the ovaries and foetal with placet were removed through the fallopian tube. The weight of the removed ovaries and the number of corpora lutea are shown in Table 3. The number of fetuses, placentas, and fetal status in all animals is shown in Table 4.

The average weight of a healthy fetus is 5-7g, which can vary slightly depending on the mother's body weight and the number of births [10]. Among four groups of animals in the study, the weight of the control group was 4.5 to 5.3 g, which is a normal weight. The weight of the second animal in the 4ml/60kg dose group 1 was 6.38 g, which is healthy, while the weight of the third animal was 3.43 g, which is abnormally small weight. The weight of the second animal of Experimental group 2, which was injected with a treatment dose, or 8ml/kg, was too abnormally small weight . The weight of the second animal in Experimental group 3 was abnormally small.

Table 4. The number of fetuses, the number of placentas, and fetal status.

Groups	n	Right uterus				Left Uterus				Median weight	
		Foetuses number	Fetal condition	Implantation number	Planck number	Foetuses nimber	Fetal condition	Implantation number	Planck number	Placent (g)	Foetuses (g)
Control	1	7	alive	7	7	4	alive	4	4	0.71	4.59
	2	0	-	0	0	8	alive	8	8	0.86	5.19
	3	7	alive	7	7	2	alive	2	2	0.85	5.3
Experimental group 1	1	-	-	-	-	-	-	-	-		
	2	6	alive	6	6	3	alive	4	3		6.38
	3	2	alive	3	2	4	alive	4	4	0.87	3.43
Experimental group 2	1	-	-	-	-	1	-	3	-		
	2	-	-	-	-	1	alive	3	1	1.71	2.98
	3	-	-	-	-	-	-	-	-		
Experimental group 3	1	4	dead	4	4	3	Dead	3	3	0.39	0.89
	2	-	-	-	-	-	-	-	-		
	3	2	alive	3	3	0	-	5	-	0.69	3.4

(-) Preimplantation loss. Explanation: Due to number variations, it is unavailable to perform statical analysis

The fetuses were kept in Bouin's solution for 10-14 days, and the brains of the fetuses were dissected as shown in Figure 2B to observe whether there were any changes in the brain, nasal cavity, and eyes (Figure 2B). No abnormalities were found in all the control group pups, and the morphological structure of the palate, brain, and nasal cavity was standard (Figure 2B-a). Three selected pups from the second animal of Experimental group 1 showed (Figure 2B-b) no abnormalities (abnormal brain development or accumulation of cerebrospinal fluid) in the brains, the nasal cavity was normal, and the palate was fully developed. However, the nasal canal was observed to be closed. The brains of the third animal of Experimental group 1 showed that the palate was fully developed and the nasal cavity was normal. However, a slight depression in the third ventricle was observed. The nasal cavity of 1nd foetal from animal 2 of Experimental group 2 was not fully developed (Figure 2B-c), and the third ventricle was down-slung with abnormal brain development.

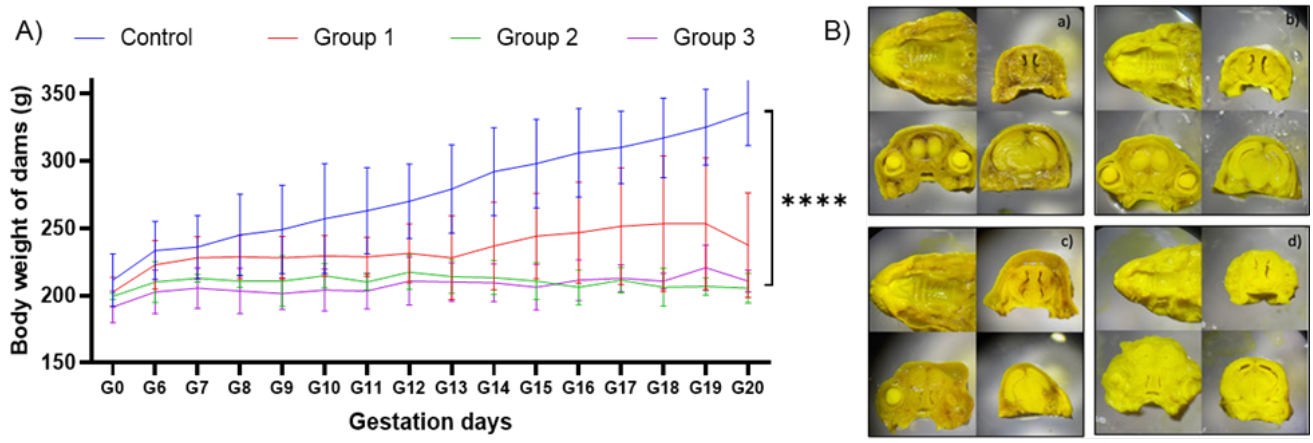

Figure 2. A): The maternal weight changes for each group are shown as G0-G20. Data are expressed as mean ± SD (n = 3). ****p < 0.0001 vs. control. B): Photographs of the foetal head. a) control group: b) Experimental group 1 (lowest dose): c) Experimental group 2 (medium dose): d) Experimental group 3 (highest dose).

In the fetuses from the first animal of Experimental group 3 (Figure 2B-d), the palate was not fully developed, the nasal mucosa did not develop into a canal, the lateral ventricle cavity of the brain was too large, and the third ventricle was too far apart. A small hole was in the front of the brain or the visual center. The brain of the seventh foetus had abnormally light weighted, and the right and left hemispheres

had abnormal lateral ventricles which caused by high pressures of cerebrospinal fluid. In the brain and nervous system of the fetuses from the third animal of experimental group 3, the palate was underdeveloped, and the nasal cavity was incompletely developed

The control group showed a healthy and steady weight gain. In comparison: The animals in experimental groups 1-2 and 3-1 had many pups but gained little weight, which is abnormal. The some animals in experimental groups 2 and 3 gained slightly in weight, although they were pregnant.

DISCUSSION

Although Safflower is not known to cause organ damage, embryotoxicity, or mutagenicity when used for food purposes (tea, flavoring, and aromatization, etc.) (Lewin, 2021), some studies have shown that it can cause embryotoxicity when used for non-food therapeutic purposes or at high doses (Qing Xia, 2017,). For example, when pregnant Balb/C mice were injected intraperitoneally with 0.7, 1.4, and 2.8 mg/kg of saffron (Carthamus Tinctorius) on days G6-G16 of embryonic development (G6-G16), the morphological structure of the placentas of animals injected with 1.4 and 2.8 mg/kg decreased the number of trophoblastic giant cells. It increased the volume of the interlabyrinthine membrane. In addition, the number of offspring of animals injected with these doses was significantly lower than that of the control group (Abbott, 2000). In another study, pregnant mice were given an aqueous extract of saffron flowers on days G0-G8 and euthanized on day G13. However, no changes were observed in the offspring of animals injected with 0.2-0.8 mg/kg. However, as the dose increased (1.2, 1.6, and 2.0 mg/kg), the fetus showed resorption and teratogenicity (M Nobakht, 2000).

In a comparison of the principles of the study, after administering drugs that are known to be toxic to the embryo, fetal head examination showed signs such as enlargement of the right and left cerebral ventricles and retinal wrinkle (Deshpande, 2017), which were similar to the changes observed in fetal head examination of animals injected with saffron.

CONCLUSION

When *Carthamus Tinctorius* injection was injected at doses of 4 ml/60 kg, 8 ml/60 kg and 16 ml/60 kg during pregnancy in rats, adverse effects such as abortion, low birth weight fetuses, pathological changes in the fetal nervous system, stillbirth, resorption, and vaginal bleeding were observed, which is consistent with other studies. This indicates that Safflower should be prohibited during pregnancy and lactation, and that dosage limits should be strictly adhered to at different times.

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