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[Original Article]

Developmental toxicity study of immunosuppressive compound, azathioprine in rats

Effects of a single treatment during the organogenesis

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The present study aimed at examining the embryolethality and teratogenic potential by azathioprine, and determining the critical period of effects by a single treatment during the organogenesis in rats. Azathioprine at 40 mg/kg was administered orally to females either on gestation day (GD) 7, 8, 9, 10, 11, 12, 13 or 14 and cesarean section was conducted on GD 21. The uterine implants were classified as live fetus, or resorbed or dead fetus. The live fetuses were weighed and examined for external or skeletal anomalies. There were significant increases in % of postimplantation losses and the corresponding decreases in number of live fetuses in the GD 7, 8, 9 and 10 treatment groups, and the critical period of fetal deaths were determined from GD 7 to 10. In the surviving fetuses, there were decreases in fetal body weights in the GD 9, 11 and 12 treatment groups. No treatment-related external or skeletal malformations were observed in any treatment groups. In conclusion, these were embryolethality with stage specificity from GD 7 to 10 but were no teratogenic potentials in this experimental conditions.

Key Words: Azathioprine, Critical period, Development, Rats, Toxicity

Introduction

Azathioprine is clinically used for the therapy of immunosuppression such as suppression of rejection after organ transplantation, inflammatory bowel disease or rheumatoid arthritis. There is a theoretical risk of teratogenicity of azathioprine, as its active metabolites can damage DNA[1]. In rats of preclinical studies[2-5], there are reports which revealed embryolethality and teratogenic potentials in excess of the human therapeutic dosage range. Skeletal abnormalities, fetal deaths and decreased fetal weights were observed in rats following oral dose during the organogenesis period in doses equivalent to up to about 10 times the human therapeutic dose[2]. No malformations occurred in the offspring of rats following intraperitoneal injection during the period of organogenesis with azathioprine in dose equivalent to up to 4 times the human therapeutic dose, but fetal deaths were increased and fetal body weights were decreased at these doses[3-5]. In other species, such as mice and rabbits, similar teratogenic and fetal deaths were reported in doses equivalent to up to 4-13 times in mice and 2-6 times in rabbits the human therapeutic doses[2,6,7]. Based on the preclinical results, azathioprine had been contraindicated to use of women of childbearing potential or pregnancy in the medical package insert in Japan. In some autoimmune diseases, common age of onset overlap with pregnancy or childbirth age in women. If the patients become pregnant, there is a concern about continuous of ongoing medication. The agent is used to treat patients who have severe illness and it is often impossible to determine if adverse effects that occur in the embryo/fetus resulted from a particular treatment, the maternal illness, or some factor[8]. Clinical guidelines in Europa and US mention that use of women of childbearing potential is not contraindication and is use of the drug in pregnant women despite potential risks with the limitation of daily dose[9]. In Japan, the medical package of azathioprine regarding to use of for women of childbearing potential was currently updated from contraindication to potential benefits which may warrant use of the drug in pregnant women despite potential risks[9], because there were no evidences which shows increased congenital anomalies by azathioprine during pregnancy based on foreign epidemiological investigations[10-14] and the clinical guidelines in Europa and US.

The present study aimed at examining the embryolethality and teratogenic potential by azathioprine, and determining the critical period of the effects by a single treatment during the organogenesis in rats since there are no published reports which are clarified the critical period of the embryolethality or teratogenic potential.

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Materials and Methods

Animals

Female Slc:SD rats at approximately 9 weeks of age were purchased from Japan SCL inc., and were housed in an animal room where the temperature (22 ± 2°C), the relative humidity (55 ± 10%) and the light and dark cycle (12 hr each) were controlled. They were allowed to have free access to Rodent Chow (Labo MR Standard, Nosan corporation) and tap water. Females at 10 weeks of age were mated overnight with mature male breeders of the same strain. The day on which vaginal plugs and/or sperms were found was designated as gestation day (GD) 0. Mated females were randomly divided into a control and 8 azathioprine treatment groups of 6 to 10 females.

The research protocol, including all experimental procedures involving animals, was approved by the animal experimentation committee of Chiba Institute of Science (18-20).

Experimental design and compound treatment

Azathioprine (Mitsubishi Tanabe Pharma Corporation) was suspended in 0.3% aqueous carboxymethylcellulose solution before treatment. The dosing volume for all females was 10 mL/kg. Azathioprine was administered orally to females either on GD 7, 8, 9, 10, 11, 12, 13 or 14 at 40 mg/kg. The dose level was referred to Fujii's report[2] and the previous our studies (data not shown). Females in the control group received 0.3% aqueous carboxymethylcellulose solution once daily from GD 7 to 14. All females were observed for physical signs daily from GD 0 to 21. Body weights were measured on GD 0 and daily from treatment day to GD 21.

Mated females were euthanized by a mixture anesthetic (medetomidine, midazolam and butorphanol[15]), and cesarean-sectioned on GD 21. The uterus of each female was examined to determine pregnancy status. Uterine implants were counted, and each was classified as a live fetus, or resorbed or dead fetus. The resorbed or dead fetuses were further classified as early resorption, placental remnant, macerated fetus or dead fetus to assume the timing of deaths from treatment. All live fetuses were weighed and examined for the external anomalies. The fetuses were euthanized by overdose of the mixture anesthetic[15]. After the evisceration, fetal skeletons were stained with Alizarin red S and Alcian blue, and examined for the skeletal anomalies.

Statistical analyses

Statistical significance for the numbers of resorptions and dead fetuses/litter, the percent of postimplantation loss, the number of live fetuses/litter and live fetal weights between the control and each treatment group was analyzed by Turkey-Kramer test. Chi-square test was applied to compare the incidences of external malformations and skeletal malformations or variations between the control and each treatment group. The significant levels were 1% or 5%.

Results

Maternal examination

No maternal deaths occurred in any females. There were no treatment-related physical signs in the treatment groups. Maternal body weights in each treatment group were temporally and slightly decreased next day of treatment but recovered to normal thereafter.

Cesarean-section Examination (Table 1 and Fig. 1)

There were significant increases in the percent of postimplantation losses and decreases in the number of live fetuses in the GD 7, 8, 9 and 10 treatment groups. The postimplantation loss was 100% in the GD 7, 8, and 10 treatment groups and 85.0% in the GD 9 treatment group. The average number of live fetuses in the GD 9 treatment group was 2.5 with significant difference. In the surviving fetuses, there were significant decreases in male and female fetal body weights (11% to 33% below controls) in the GD 9, 11 and 12 treatment groups. The average fetal body weights in the GD 13 treatment group were significant decrease (5% below controls) in males and slight decrease (4% below controls) in females.

Figure 1 shows the incidence of % of postimplantation losses classified into early resorption, placental remnant, macerated fetus or dead fetus. In the GD 7 and 8 treatment groups, all resorptions were classified as early resorptions. In the GD 9 treatment group, resorptions included early resorptions (10%) and placental remnant (71.1%). In the GD 10 treatment group, these included placental remnant (66.2%), macerated fetuses (26.0%) and dead fetuses (5.7%). The incidences of early resorptions or placental remnant observed in the GD 11 and 14 treatment groups were within those in the control group.

Fetal Examinations (Table 2)

Five fetuses from a litter had malformation of shortened tails in the GD 11 treatment group and the incidence of fetuses with the shortened tails (5.4%) was statistically significant. No other external malformations were observed in any group. No skeletal malformations were observed in any treatment groups. Skeletal variations in the thoracic body and supernumerary rib were observed in some fetuses in the control and treatment groups but the incidences of the variations in the treatment groups were no significant differences from controls.

Discussion

A single oral administration of azathioprine at 40 mg/kg induced

Table 1. Results of cesarean-sections

Groups	Control	Treatment day							
		GD7	GD8	GD9	GD10	GD11	GD12	GD13	GD14
No. of pregnant females	10	9	9	10	7	7	6	7	8
No. of implants	14.9 ± 1.6[a]	16.0 ± 1.1	14.2 ± 2.9	15.1 ± 1.0	11.0 ± 4.3*	14.6 ± 1.7	12.5 ± 1.4	12.1 ± 3.9	12.8 ± 3.4
No. of resorptions and dead fetuses	1.4 ± 1.6	16.0 ± 1.1**	14.2 ± 2.9**	12.6 ± 3.9**	11.0 ± 4.3**	1.3 ± 1.1	0 ± 0	0 ± 0	0.1 ± 0.4
Postimplantation loss (%)[b]	9.4 ± 10.9	100 ± 0**	100 ± 0**	85.0 ± 24.4**	100 ± 0**	5.8 ± 0.2	0 ± 0	0 ± 0	1.1 ± 3.2
No. of live fetuses	13.5 ± 2.3	0 ± 0**	0 ± 0**	2.5 ± 3.6**	0 ± 0**	13.3 ± 1.4	12.5 ± 1.4	12.1 ± 3.9	12.6 ± 3.5
Live fetal weights (g) Male	5.38 ± 0.25	-	-	3.98 ± 0.58**	-	4.01 ± 0.89**	4.78 ± 0.37**	5.09 ± 0.22*	5.35 ± 0.40
Live fetal weights (g) Female	5.09 ± 0.26	-	-	3.39 ± 0.63**	-	3.95 ± 0.84**	4.48 ± 0.43**	4.88 ± 0.14	4.99 ± 0.37

[a]: Values are given as mean ± SD (based on litter mean).

[b]: (No. of resorptions and dead fetuses/number of implants) x 100.

*: $p \leq 0.05$, **: $p \leq 0.01$

Table 2. Results of fetal external and skeletal examinations

Groups	Control	Treatment day				
		GD9	GD11	GD12	GD13	GD14
External examinations						
No. of fetuses examined	135	25	93	75	85	101
No. of fetuses with external malformations	0 (0[a])	0 (0)	5 (5.4)*	0 (0)	0 (0)	0 (0)
Skeletal examinations						
No. of fetuses examined	135	25	93	75	85	101
No. of fetuses with skeletal malformations	1 (0.7)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
No. of fetuses with skeletal variations						
Thoracic body of split	1 (0.7)	0 (0)	4 (4.3)	0 (0)	0 (0)	1 (1.0)
Thoracic body of dumbbell	3 (2.2)	1 (4.0)	3 (3.2)	4 (0)	1 (1.1)	2 (2.0)
Supernumerary ribs	3 (2.2)	0 (0)	4 (4.3)	1 (0)	3 (3.5)	1 (1.0)

[a]: Values in parentheses indicate the incidences (%) of malformations or variations.

*: $p \leq 0.05$

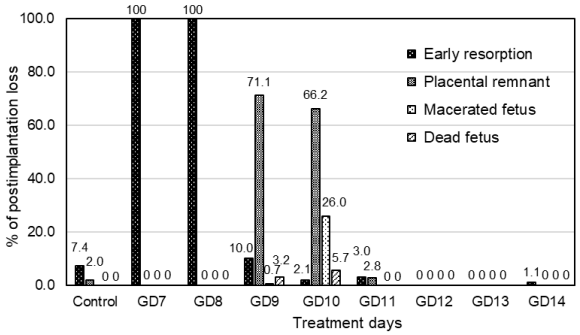

Fig. 1. Percent of postimplantation loss classified into early resorption, placental remnant, macerated fetus or dead fetus in treatment days

the entire or severe fetal deaths by treatments of GD 7 to 10, but no treatment-related external and skeletal anomalies were observed in surviving fetuses in any treatment groups. Five fetuses from a litter had external malformation of shortened tails in the GD 11treatment group. This malformation was considered unrelated to treatment by azathioprine and may be related to genetic factor because the malformed fetuses were from a single female and no such malformation was reported[2-5]. Fujii *et al*[2] reported that azathioprine at 40 mg/kg/day was orally administered to females daily from GD 10 to 15, and significant increases in skeletal malformations of thoracic deformations or split were observed but no such skeletal malformations were observed in the present study. The reasons for different results are unknown but these may be due to the criteria of malformation, or different strains of animals or production lot of the compound used. In the dose of 40 mg/kg/day, severe fetal deaths occurred and decreased fetal weights were noted[2], and the findings are in agreement with our findings. Fetal loss or growth retardation were reported at 9.4 mg/kg which are equivalent dose to 4 times the human therapeutic dose but no malformations occurred at this dose[3-5]. As shown in Figure 1, types of resorbed or dead fetus after treatment of azathioprine on GD 7 and 8 were classified as early resorptions, and those after treatment on GD 9 and 10 were mainly placental

remnant. The results observed assume that the fetuses were dead earlier after treatment. The incidences of early resorptions or placental remnant observed in the GD 11, 12, 13 and 14 treatment groups were considered spontaneous deaths because the incidences were within those in controls.

In the foreign epidemiological investigations, although controlled epidemical studies are not available, information regarding the outcome of pregnancy in women who tested with azathioprine during pregnancy has been reported in a large number of clinical series. In clinical series in US, England, France and Israel, infants delivered from women who exposed to azathioprine during pregnancy were investigated, and there were no relationships between pregnancy exposed to azathioprine and malformations in infants[10-14]. In a clinical series of Israel[13], 189 women exposed to azathioprine were compared to 230 women not exposed to any drugs during pregnancy. The incidences of women with cesarean section or premature, or low birth weight of infants in the exposure of azathioprine group were higher than those in no exposure group, but the incidences of congenital anomalies were no significant differences between them. In clinical series in Sweden[14], survey was conducted in 481 infants who delivered from mother exposed to azathioprine during early stage of pregnant. The incidences of premature birth or low birth weight in the infants exposed to azathioprine are higher than those of controls but there are no significant differences in incidence of congenital anomalies between them. Thus, there were no evidences which shows increased congenital anomalies by azathioprine during pregnancy in the foreign epidemiological investigations.

In conclusion, azathioprine had embryolethality which the critical period of fetal deaths was early pregnancy stage of GD 7 to 10 and fetal growth retardation, but no teratogenic potentials in this experimental condition. The results in the present study would be one of useful information to support the update of medical package for women of childbearing potential from contraindication to potential benefits which may warrant use of the drug in pregnant women despite potential risks.

The result of the present study was presented at the 47th annual meeting of the Japanese society of toxicology.

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