Toxicological and Pharmacological Evaluation of Xanthine Derivatives Using Chick Embryos As the Alternative Experimental Method

Hiroyuki Miyazaki, Takashi Sugiyama[1] and Hideyo Shimada
Division of Pathophysiology, Center for Clinical Pharmacy and Clinical Sciences, School of Pharmaceutical Sciences, Kitasato University , 9-1, Shirokane 5-chome, Minato-ku, Tokyo 108-8641 Japan

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SUMMARY

The toxicological and pharmacological effects of xanthine derivatives, caffeine, theophylline and theobromine were investigated using chick embryos. The drugs were each injected into the air sac of fertile eggs on the 2,5,8 or 16th day of incubation and LD50 values were determined from the number of the dead embryos on the 18th day of incubation. LD50 values of drugs were more higher in the late stages of incubation. The strength of toxicity was arranged in order of theobromine>caffeine>theophylline, regardless the injection ages. The LD50 values of these drugs in ICR mice i.v. route were also determined and compared with those in chick embryos. However, no good correlation was obtained from LD50 values between two animals.

Furthermore, we evaluated the effects of these drugs on the electrocardiogram tracings of chick embryos. When xanthine derivatives were injected into the air sac of fertile eggs on the 16th day of incubation, theophylline and caffeine increased as that of mammals. The effect of theobromine was different in comparison with that of other drugs.

In conclusion, lethal toxicity of xanthine derivatives in mammals which may be due to a central nervous systemic action could not predict from data of chick embryos. The heart rate of the 16th-day embryos were increased by pharmacological action which may be due to peripheral action of xanthine derivatives. From these facts, chick embryos may be very useful as an alternative animal to obtain the pharmacological effects of cardiovascular drugs .

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[1]Corresponding author; Takashi Sugiyama, Ph.D.

INTRODUCTION

Experimental studies using chick embryos has been accepted from old times as the useful screening models to estimate the pharmacological and toxicological effects of drugs or chemical compounds (Romanoff, 1967., Romauoff, 1972., Karnofsky, 1967). Recently, we proved that the LD50 values obtained from chick embryos for cardiovascular drugs may offer preliminary information on the LD50 values of mice and rats (Miyazaki et al., 1994). In addition, we reported that the stable electrocardiogram tracings in chick embryos could be recorded when a reasonable dosage of chloralose and urethane was injected into the air sac of fertile eggs, and that electrocardiogram tracings obtained from chick embryos by these pretreatment may be applicable for a method to evaluate cardiovascular drugs (Sugiyama et al, 1996., Sugiyama et al, 1997).

In this report, in order to obtain further information using chick embryos we studied on the toxicological and pharmacological effects of xanthine derivatives, caffeine, theophylline and theobromine which have two actions on the central and peripheral nervous systems in mammals.

MATERIALS AND METHODS

Animals

Eggs and incubation

Fertile eggs of White Leghorns were obtained from Ohmiya Poultry Laboratory (Ohmiya). All eggs were incubated at 37.6 ± 0.2"C at a relative humidity of about 65.5% and turned automatically every hour (Showa Incubator Laboratory, Urawa) .

Mice

Four weeks-old male ICR mice were obtained from Charles River Japan, Inc. (Atsugi). They were maintained on pellet diet (CRF-1: Charles River Japan, Inc.) and housed in plastic cages in an air-conditioned room at 24 ± 2°C with a relative humidity of about 55 %. After 1 week, animals were examined.

Drugs used

After anhydrous caffeine (Wako Pure Chemical Ind., LTD, Osaka) was dissolved in a small amount of methanol, it was diluted in physiological saline. Theophylline (neophylline, Eisai, Tokyo) and theobromine (Diuretin "Hoei", Fujisawa Astra, Osaka) were dissolved and diluted in physiological saline. Forty five mg of chloralose and 450 mg of urethane were dissolved in 1.0ml of water and a 0.1 ml of the solution

was injected into the air sac of fertile eggs to arrest the motion of embryo in egg shell.

Determination of LD50 values of xanthine derivatives in chick embryos

As previously described (Sugiyama et al, 1982., Sugiyama et al, 1985) a single injection of each drugs was made at predetermined doses into the air sac of fertile eggs on the 2nd, 5th, 8th or 16th day of incubation. All eggs were candled daily for viability. The surviving chick embryos were sacrificed on the 18th day of incubation and LD50 values were calculated from the number of dead embryos using Litchfield-Wilcoxon method.

Determination of LD50 values of xanthine derivatives in mice

Mice were intravenously administered with graded doses of drugs and LD50 values were calculated from the number of dead animals at 14 days after administration of drugs by same method described above.

Electrocardiogram recording systems for chick embryos

Chloralose and urethane (CU) solution were injected into the air sac on the 16th day of incubation. After 20 minutes of CU solution pre-treatment, a single injection of varying doses of drugs was made into the same site of eggs. ECG waves in chick embryos were recorded O to 40 minutes at 10 minutes intervals as following; Two diagonal holes on the "equator" and one hole in the "south pole" of egg were made using electronic drill and immediately were closed with paraffin. The specially designed needle-electrodes were inserted into three small holes. Two needles on the "equator" were used as a bipolar lead for the embryonic heart and one needle at the "south pole" was used as a ground lead. These needles were connected to the electronic box which linked to electrocardiograph equipment (Nihon Koden AVB-21, Tokyo). ECGs were recorded as bipolar waves between needles with a paper speed of 25 mm/sec on a thermal array recorder (Nihon Koden PTA-1100M, Tokyo) and heart rate was calculated from R-R intervals.

Statistical analysis

All the results are given as mean ±S.E.M. The data were analyzed Bartlett' s test for homogeneity of variance. They were then analyzed by one way analysis of variance (ANOVA) when the variance was homogeneous, or by Kruskal-Wallis' s test when it was not homogeneous. If there was a significant difference among the groups, a mean or rank multiple comparison test was conducted by Durmet's test. The fiducial limit of 0.05 or 0.01 two tails, was used as the criterion for significance.

RESULTS

Lethal toxicity of xanthine derivatives in chick embryos and mice

LD50 values of xanthine derivatives in chick embryos at the different injection day are shown in Table 1. LD50 value for caffeine was 3 mg/egg when caffeine was injected on the 2nd day of incubation. When the caffeine was done on the 5, 8, 10 or 16 day of incubation, LD50 values gradually increased, resulting in 4.50, 6. 62 and 16.0 mg/egg, respectively. LD50 values of theophylline and theobromine in chick embryos gradually increased with ages of injection as that of caffeine. Compared with three drugs in chick embryos, toxicity of xanthine derivatives was the highest in the theobromine treatment, second in theophylline and the lowest in caffeine. On the other hand, LD50 values of xanthine derivatives in mice are shown in Table 2. Lethal toxicity of xanthine derivatives in mice was the strongest in caffeine and the weakest in theobromine, and theophylline was middle between three drugs. These LD50 values in mice which administered by intravenous (i. v.) route did not show any large difference between experimental data and those for rodents obtained from published references (Doris, 1987).

Pharmacological effects of xanthine derivatives in chick embryos

Fig. 1 shows the changes in heart rate of chick embryos for 40 min after injection of drugs. No change in heart rate was observed by treatment of 3.3 mg/egg of caffein. However, it increased gradually from 5 min to 20 min after treatment of 7.5 mg/egg and thereafter remained relatively constant. When the highest dosage,15 mg/egg was injected, heart rate increased at 5 min and a peak occurred at 10 min after

Table 1
LD50 values in chick embryos treated with caffeine, theophylline or theobromine at the different days of injection

	Days of injection (Day)			
Drugs	2	5	8	16
Caffeine	3.00	4.50	6.62	16.00
Theophylline	1.80	3.43	4.07	7.74
Theobromine	1.16	1.75	3.02	5.29

(mg/egg)

Each drug was injected into the air sac of fertile eggs on the different incubation days. The LD50 values were calculated using Litchfield-Wilcoxon method on the 18th day of incubation.

Table 2
LD50 values of xanthine derivatives in mice

Drugs	LD50 values mg/kg		
	Reference data		Experimental data
	i.v.	p.o.	i.v.
Caffeine	62	127	39
Theophylline	136	252	186
Theobromine	No data	837	345

Reference data from "Registry of toxic effects of chemical substances 1985-1986." The experimental LD50 values were calculated using Litchfield-Wilcoxon method as shown in "Matetrials and Methods".

treatment. However, thereafter it decreased rapidly including arrhythrnia with A-V block like. Injection of theophylline showed increase of heart rate accordingly dose-dependent manner (Fig.2, 4B).

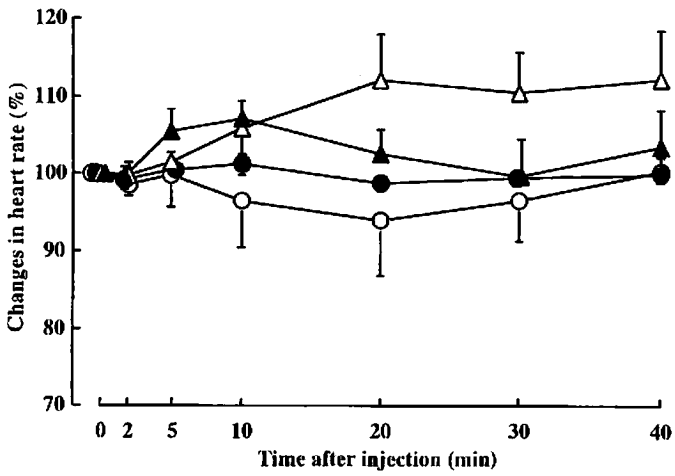

Fig. 1 Heart rate changes in chick embryos treated with caffeine. Vehicle (○) and 3.3 mg/egg (●), 7.5 mg/egg (△) and 15 mg/egg (▲) of caffeine were injected into the air sac of the 16th-day fertile eggs. Data represented the percentage of changes in the heart rate from each 0 time. All values are the mean±S.E.M. of five embryos.

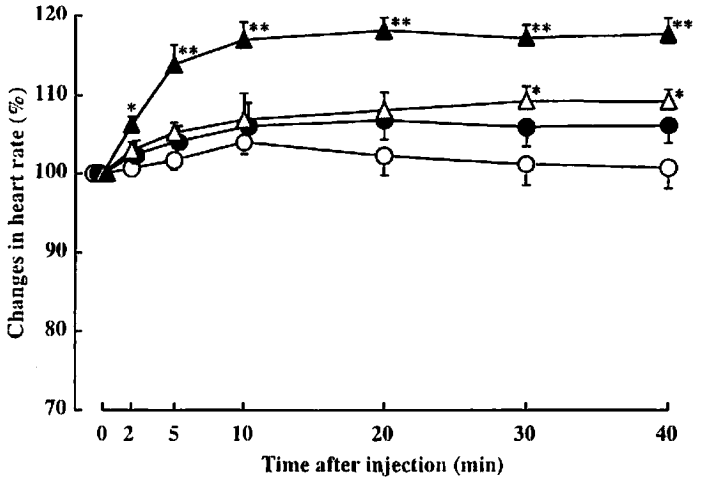

Fig. 2 Heart rate changes in chick embryos treated with theophylline. Vehicle (○) and 5 mg/egg (●), 10 mg/egg (△) and 20 mg/egg (▲) of theophylline were injected into the air sac of the 16th-day fertile eggs. Data represented the percentage of changes in the heart rate from each 0 time. All values are the mean ± S.E.M. of five embryos. *P<0.05, **P<0.01, significantly different from vehicle group on each point by Dunnett's test.

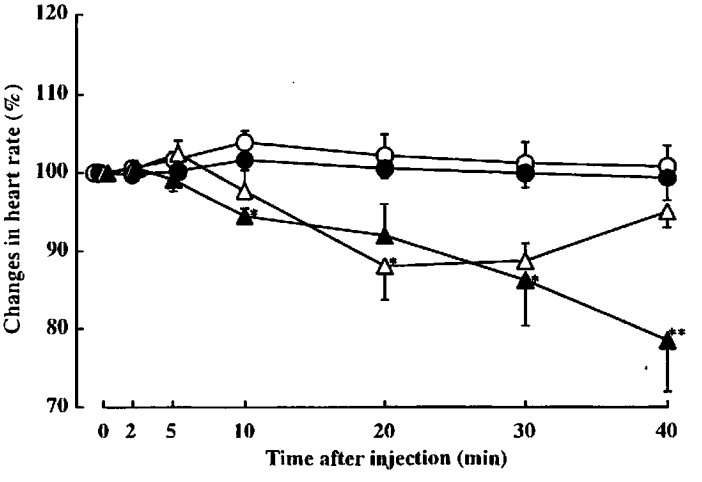

Fig. 3 Heart rate changes in chick embryos treated with theobromine. Vehicle (○) and 1 mg/egg (●), 2.5 mg/egg (△) and 5 mg/egg (▲) of theophylline were injected into the air sac of the 16th-day fertile eggs. Data represented the percentage of changes in the heart rate from each O time. All values are the mean ± S.E.M. of five embryos. *P<0.05, **P<0.01, significantly different from vehicle group on each point by Dunnett's test.

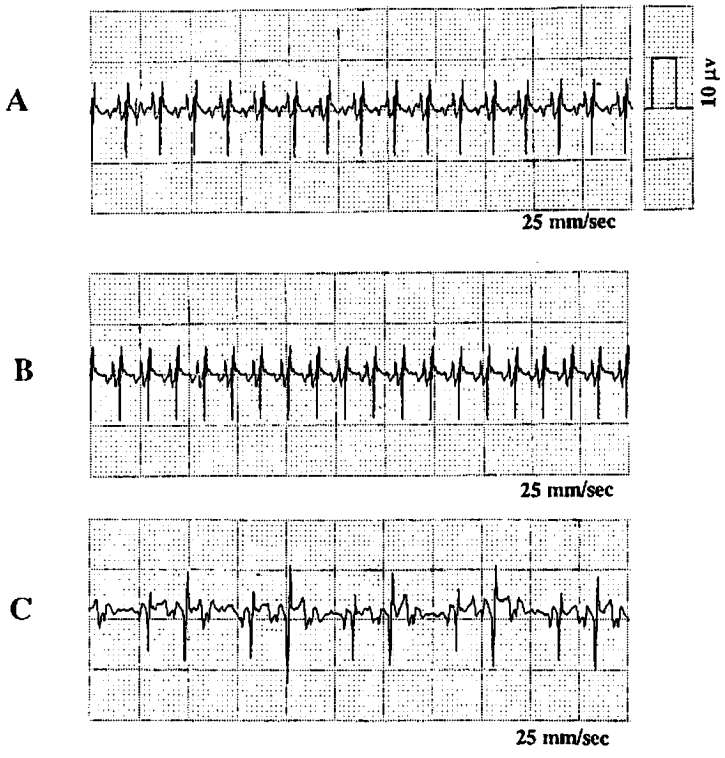

Fig. 4 ECG tracings in chick embryos treated with xanthine derivatives. A: Vehicle. B: Tachycardia was shown at 20 minutes after injection of 20 mg/egg of theophylline. C: Arrhythnria was shown at 40 minutes after injection of 5 mg/egg of theobromine.

However theobromine did not show the changes in the heart rate as that of theophylline: Heart rate decreased by dosage of 2.5 mg/egg and more. Decreased heart rate turned to the increase after 30 min in dosage of 2.5 mg/egg, but 5 mg/egg did not show any pattern of increase (Fig.3) and their ECG tracings had arrhythmia with A-V block like at 20 min of treatment (Fig.4C).

DISCUSSION

LD50 values of xanthine derivatives, theophylline, caffeine and theobromine were determined using chick embryos and mice. The strength of lethal toxicity of xanthine

derivatives in chick embryos was arranged on theobromine > theophylline > caffeine. On the other hand, it was in mice caffeine >theophylline> theobromine. Accordingly, the toxicity of xanthine derivatives was shown a clearly difference between rodent and chick embryos. It is well known that xanthine derivatives have two pharmacological effects, stimulation against central nerve and peripheral nerve systems. In general, the stimulative action against the central nerve systems is the strongest in caffeine, second in theophylline and the lowest in theobromine. On the other hand, the action against peripheral nerve system is theophylline, theobromine and caffeine in order of strength. In this experiment, the lethal toxicity of xanthine derivatives in mice was similar order to the actions of central nerve systems. From these results, it is suggested that the toxic effect of xanthine derivatives in rodent mainly due to actions of the central nerve systems.

We previously reported that LD50 values for cardiovascular drugs in rodents with i.v. route could be predicted from the LD50 values in chick embryos injected into the air sac on the 2nd day of incubation (Miyazaki et al, 1994). When the present data (LD50 values) of xanthine derivatives in chick embryos were inserted into the regression line (Y=0.98559X+0.36388) and calculated, the expected LD50 values of xanthine derivatives in mice was 3.33, 2.14 and 1.5 mg/kg in caffeine, theophylline and theobromine, respectively, and showed a large difference in comparison with data obtained from references. Accordingly, it is thought that the lethal actions of xanthine derivatives in chick embryos probably due to the peripheral actions.

Of pharmacological effects of xanthine derivatives, we observed from the ECG tracings in chick embryos. The changes in heart rate of chick embryos were not similar between three drugs. Degree of increase in heart rate of chick embryos was the strongest in theophylline, second in caffeine and the lowest in theobromine. However, when the over dosage of these drugs was injected into the air sac, ECG tracings showed bradycardia with arrhythmia. It has been reported that the overdose of theophylline in man occurred tachycardia with arrhythmia which induced by a release of endogenous catecholamine (Hall et al., 1984., Kearney et al., 1985., Levine et al., 1985., Taniguchi et al., 1989., Vestal et al., 1983).

It is thought that these sign in chick embryonic heart probably due to stimurance of peripheral nerve systems. If so, it is suggested that theobromine showed the strongest toxicological and pharmacological effects between three drugs in chick embryos.

Detail pharmacological and phannacokinetic actions of xanthine derivatives were not investigated yet in chick embryos. Although further investigation is necessary to clarify the mechanism for xanthine derivatives in chick embryos, it is concluded that chick embryos may be very useful as animal for screening test of cardiovascular drugs.

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