Effect of Digoxin on Chick Embryos with Hyperthyroidism Induced by L-Thyroxin

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Abstract
The objective of this study was to demonstrate that the chick embryo with hyperthyroidism induced by treatment with L-thyroxin (TRX) was a feasible model as an alternative to animal model and to examine the pharmacological effects of digoxin using this model. When 0.5 or 1.0 µg/egg of TRX was injected into the eggs, the 16th day-chick embryos showed some characteristic changes including an increased heart rate (tachycardia), an increased relative heart weight (cardiac hypertrophy) and relative thyroid weight (thyroid hypertrophy), and an increased concentration of plasma T3 and T4. These findings suggest that TRX-treated chick embryos may be a model of the heart diseases associated with hyperthyroidism. When 50 µg/egg of digoxin was injected into the TRX-treated eggs, negative chronic action reduced. This fact suggests that digoxin decreased the sensitivity to TRX in the heart of chick embryos with hyperthyroidism as well as in dog and man. In conclusion, the TRX-treated chick embryos may prove to be an alternative to animal experiments to predict unexpected effects of cardiovascular drugs.

Key words: Chick embryo; hyperthyroidism; thyroxin; digoxin; electrocardiogram

Introduction
Many patients with hyperthyroidism show tachycardia associated with congestive heart failure (Shirani et al, 1993). Digoxin is used as a therapeutic drug in these patients (Larsen, 1988). It has been reported that digoxin at a higher dose is usually administered to obtain therapeutic effects in patients with hyperthyroidism because of the decreased sensitivity to digoxin (Huffman et al, 1977). Accordingly, to predict the unexpected pharmacological responses to cardiotonics, such as digoxin, experimental animals with peculiar sensitivity of heart such as hyperthyroidism or hypothyroidism are required. For these purposes, a hyperthyroid animal model by administering repeated doses of L-thyroxin (TRX) to rats and dogs was reported (Sanford et al, 1978, Klein, 1988, Suga et al, 1991). The hyperthyroidism with tachycardia and cardiac hypertrophy is induced by frequent administration of TRX in rats and dogs, and many cardiovascular drugs have been evaluated using this model (Skelton, 1982, Niizoe et al, 1984, Angeras et al, 1987).

With the recent concern for animal rights, experimental studies using mammals have been limited in number and methods. Thus, based on social acceptance, experimental studies using chick embryos have drawn attention. In order to develop alternative methods, we have studied the biological effects of drugs on the cardiovascular system of chick embryos using physiological techniques (Yoshiyama et al, 2005, 2004a, 2004b, 2003, Sugiyama et al, 1996). And we have also reported that the chick embryonic model of hypothyroidism induced by treatment with thiamazole can be used to examine the pharmacological and toxicological...
effects of cardiovascular drugs (Sugiyma et al, 2000).

Since the thyroid gland is present in all vertebrates, a convenient abnormal cardiac model would be beneficial for examining the pharmacological actions and toxicity of cardiotonics (Turner, 1971).

In this study, we examined to produce a hyperthyroidism model in chick embryos, which resulted from abnormalities in the thyroid gland by an injection of TRX, and to know whether this model can be used as an alternative to animal experiments to examine the pharmacological effects of cardiotonics.

Materials and Methods

Materials

L-thyroxin sodium salt (TRX), urethane and \( \square \)-chloralose were purchased from Sigma Chemical Inc. (St. Louis, MO, U.S.A.). Digoxin was from Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan). Amerlex-MAB free-T3 kit and free-T4 kit were from Oso Clinical Diagnostics (Tokyo, Japan). TRX was dissolved to the desired concentration with physiological saline and sterilized through a membrane filter (0.2 \( \mu \)m pore size, Tokyo Roshi Kaisha, Ltd., Tokyo, Japan). Digoxin was diluted to the desired concentration with sterilized saline. Urethane (450 mg/ml) and \( \square \)-chloralose (45 mg/ml) were dissolved in physiological saline.

Eggs and incubation

Fertile eggs from White Leghorn chickens were obtained from Ohmiya Poultry Science (Saitama, Japan) and incubated at 37.5 \( \pm \) 0.2 °C at a relative humidity of about 65.5%, and turned automatically every hour (P-1 type, Showa Incubator Laboratory, Saitama, Japan).

Injection of TRX and digoxin into fertile eggs

TRX (0.5 or 1.0 \( \mu \)g/egg) was injected into the albumen of eggs on the 9th day of incubation according to a method previously reported (Sugiyama et al, 1985). Sterilized physiological saline (0.2 ml/egg) was used as control. Digoxin (25, 50 or 75 \( \mu \)g/egg) was injected into the air sac of control or TRX-treated eggs on the 16th day of incubation.

Electrocardiogram (ECG) recording systems from chick embryos

Anesthetic injection of urethane (\( \{45 \text{ mg/egg}\}^{+} \square \)-chloralose [4.5 mg/egg]) was performed into the air sac of eggs to record the stable ECG waves in 16-day-old embryos in which RR intervals could be clearly analyzed. ECGs were recorded 0 to 60 min after the drug injection, and heart rate was determined from RR intervals. Data represented the percentage of changes in the heart rate from each 0 time. Four small holes every 90 degrees on "the equator", one small hole on "the south pole" and one small hole on "the north pole" were made on each fertilized egg by an electric drill and sealed with Paraffin (m.p. 60 \( \square \)). Specially designed needle electrodes were inserted into the appropriate holes of "the equator" and "the south pole". The two needles on "the equator" were used as a bipolar lead of the embryonic heart, and the needle on "the south pole" was used as a ground lead. These needles were connected to the memory oscilloscope (VC-11, Nihon Koden Co., Tokyo, Japan). ECGs were recorded as bipolar waves between two needles on a recorder (PowerLab System, ADInstruments Japan Co., Tokyo, Japan) (Sugiyama et al, 1996).

Observations in the embryos treated with TRX

After recording of ECG waves, blood samples were collected from vitelline vein using a heparinized disposal syringe (Fig. 1). Next, embryos were removed from eggshells, and body, heart and thyroid were removed and weighed. The collected blood was centrifuged at 3000 rpm for 10 min and plasma was used to determine the concentrations of free-T3 and free-T4, and to measure
TRX-treated eggs (0.5 or 1.0 µg/egg) on the 16th day of incubation, ECG waves were recorded from 0 to 30 min after injection.

**Statistical analysis**
Statistical significance was evaluated by analysis of variance followed by Dunnet’s multiple comparison when the effects of different concentrations of drugs were examined, while Student’s t-test was employed when two groups were compared. P-values less than 0.05 was considered significant.

**Results**
Changes of the HR and organ weights in chick embryos treated with TRX

As shown in Fig. 2, the HR in chick embryos treated with 0.5 or 1.0 µg/egg of TRX significantly increased compared with that of TRX-untreated embryos. The weights of the body and the relative weights of heart and thyroid gland to body weight are shown in Fig. 3. The body weight in TRX-treated embryos decreased dependently on doses but was not significantly different from TRX-untreated embryos. The relative weights of the heart and thyroid gland to body weight at 0.5 and 1.0 µg/egg of TRX were significantly increased compared with those of TRX-untreated embryos.
Yoshiyama Y et al., Effect of Digoxin on Chick Embryos with Hyperthyroidism Induced by L-Thyroxin, AATEX 11(2), 105-111, 2005

**Plasma free-T3 and T4 concentration in chick embryos treated with TRX**
Both Free-T3 and T4 concentrations in plasma in chick embryos treated with 0.5 or 1.0 µg/egg of TRX significantly increased compared with those of TRX-untreated embryos (Fig. 4).

**Effects of digoxin on the HR in control and TRX-treated embryos**
The HR in chick embryos treated with digoxin alone decreased dependently on doses (Fig. 5). And digoxin induced arrhythmias including AV block 30 min after injection (Fig. 6). When 50 µg/egg of digoxin was injected into the TRX-untreated eggs, the ECG waves show a negative chronotropic action. However, when 50 µg/egg of digoxin was injected into the TRX-treated eggs, negative chronic action reduced (Fig. 7).

**Discussion**
We have reported that the chick embryonic model of hypothyroidism induced by treatment with thiamazole can be used to examine the pharmacological and toxicological effects of cardiovascular drugs (Sugiyama et al, 2000). In this study, we demonstrated that the chick embryo with hyperthyroidism induced by treatment with L-thyroxin (TRX) was a feasible model as an alternative to animal model and examined the pharmacological effects of digoxin using this model.

When 0.5 or 1.0 µg/egg of TRX was injected into the eggs, the
16th day-chick embryos showed some characteristic changes including an increased HR (tachycardia), an increased relative heart weight (cardiac hypertrophy) and relative thyroid weight (thyroid hypertrophy), and an increased concentration of plasma T3 and T4. These findings showed high similarities between rat or human and chick embryos. The results obtained suggest that TRX-treated chick embryos may be a model of the heart diseases associated with hyperthyroidism.

However, injection of 2 µg/egg of TRX showed a tendency to decrease the body, heart and thyroid gland weights to the control and induced bradycardia, which are considered to be due to the over dose of TRX. We examined the pharmacological effects of digoxin using the chick embryo with hyperthyroidism induced by treatment with TRX 0.5 or 1.0 µg/egg.

Growth, development and differentiation on cells in various organs of mammals and chick embryos are regulated by thyroid hormone (Freeman, 1974). Thyroid gland in chick embryos is situated near the base of neck (Romanof, 1960). Romanoff reported that the first indication of thyroid was at the end of day 2 of incubation. It is important to determine the suitable period and site for the injection of drugs into fertile eggs. We already reported that the same agent can exhibit significantly different effects in chick embryos when given at different sites or on different days of incubation (Sugiyama et al, 1982). Therefore, in the preliminary experiment, a fixed dose of TRX was injected into the albumen of eggs from 5th day to 14th day of incubation. A change of HR on the 9th day of incubation was stronger than that by other injection days (unpublished). According to these findings, in this study, we injected TRX into the albumen of fertile eggs on the 9th day of incubation.

Digoxin has been widely used as a therapeutic drug in patients with congestive heart failure, atrial fibrillation or atrial flutter. However, digoxin at a higher dose is usually given to patients with hyperthyroidism than patients with hypothyroidism because of their reduced sensitivity to this drug. And we examined to know whether this fact could be proved using the chick embryo. The HR of chick embryos decreased.
dependently on doses of digoxin. When 50 µg/egg of digoxin was injected into the saline-treated eggs, the ECG waves showed a negative chronotropic action as in mammals. However, when 50 µg/egg of digoxin was injected into the TRX-treated eggs, negative chronic action reduced. It is suggested that digoxin decreased the sensitivity to TRX in the heart of chick embryos with hyperthyroidism as well as in dog and man. Marrow et al. reported that the myocardial responses of ouabain, cardiotonic alkaloid derivatives were influenced in dog with hyperthyroidism (Marrow et al, 1963).

Thyroid hormones increase protein syntheses of Na+K+-ATPase and atrial natriuretic peptide (ANP), and the activity of these proteins is higher in hyperthyroidism than in the normal conditions (Larsen, 1988). However, the cardiac glycosides, digoxin and ouabain are drugs frequency used in a cardiac insufficiency treatment. From previously reported findings, the main action mechanism of digoxin in hyperthyroidism is the suppression of the Na+-K+-ATPase activity (Forfar and Caldwell, 1985).

Although further investigations are necessary to clarify the mechanism underlying the cardiac function induced by digoxin in hyperthyroidism of chick embryos treated with T-4, this model may offer in alternative research possibilities to predict unexpected effects of cardiovascular drugs on patients with hyperthyroidism.

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Yoshiyama Y et al., Effect of Digoxin on Chick Embryos with Hyperthyroidism Induced by L-Thyroxin, AATEX II(2), 105-111, 2005

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