Heart rate changes mediate the embryotoxic effect of antiarrhythmic drugs in the chick embryo

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Kockova R, Svatunkova J, Novotny J, Hejnova L, Ostadal B, Sedmera D. Heart rate changes mediate the embryotoxic effect of antiarrhythmic drugs in the chick embryo. Am J Physiol Heart Circ Physiol 304: H895–H902, 2013. First published January 11, 2013; doi:10.1152/ajpheart.00679.2012.—A significant increase in cardiovascular medication use during pregnancy occurred in recent years. Only limited evidence on safety profiles is available, and little is known about the mechanisms of adverse effect on the fetus. We hypothesized that drug-induced bradycardia is the leading mechanism of developmental toxicity. Embryotoxicity was tested in ovo after administration of various doses of metoprolol, carvedilol, or ivabradine. Embryonic day (ED) 4 and 8 chick embryos were studied by video microscopy and ultrasound biomicroscopy ex ovo after intramniotic injection of the drug for a period of 30 min. Stroke volume was calculated by the Simpson method and prolate ellipsoid formula. Significant dose-dependent mortality was achieved in embryos injected with carvedilol and ivabradine. In ED4 embryos, metoprolol, carvedilol, and ivabradine reduced the heart rate by 33%, 27%, and 55%, respectively, compared with controls (6%). In ED8 embryos this effect was more pronounced with a heart rate reduction by 71%, 54%, and 53%, respectively (controls, 36%). Cardiac output decreased in all tested groups but only proved significant in the metoprolol group in ED8 embryos. The number of β-adrenergic receptors showed a downward tendency during embryonic development. A negative chronotropic effect of metoprolol, carvedilol, and ivabradine was increasingly pronounced with embryonic maturity despite a downward trend in the number of β-adrenergic receptors. This effect was associated with reduced cardiac output in chick embryos, probably leading to premature death. Although standard doses of these drugs appear relatively safe, high doses have a potentially adverse effect on the fetus through reduced heart rate.

β-blocking agents; embryonic heart; embryotoxicity; pregnancy; bradycardia

ADVANCES IN PEDIATRIC CARDIAC surgery have resulted in improved survival of children with congenital heart disease, as well as improved quality of their life, which allows some mothers in whom pregnancy was previously strictly contraindicated to have their own offspring. However, surgical scars in operated hearts predispose to the development and maintenance of arrhythmias (3) that are often managed medically. In addition, the average age of pregnant women increases, we face the need of drug treatment during pregnancy more frequently. Furthermore, there is a documented increase in maternal cardiac arrhythmias during pregnancy, secondary to metabolic, hormonal, and hemodynamic changes (9).

The effects of antiarrhythmic drugs on the developing cardiovascular system are often different from the effects on the adult heart. Some (e.g., amiodarone or atenolol) are too toxic for the developing embryo, so their use in pregnancy is contraindicated (Category U.S. FDA D-positive evidence risk) (13). More often, the response to clinically relevant concentrations is blunted due to the immaturity of receptor-mediated signal transduction systems. Generally, the effects of the drugs are not fully characterized, and the recommendations for their use in pregnancy are vague (Category C: Studies in pregnant women are lacking, and animal studies are either positive for fetal risk or lacking as well; use only when benefits clearly outweigh risks) (13) and, therefore, of limited value for daily clinical practice. Only very few antiarrhythmic drugs (such as lidocaine or sotalol) are labeled as safe (Category B: No evidence of risk in pregnant women) (9). The human placental model was developed as an experimental model to test transplacental perfusion of different drugs (12). There were only four drugs with the antiarrhythmic potential (atenolol, propranolol, digoxin, labetalol) tested and the ratio of transplacental transfer mother to fetus was in the range of 0.1–1 both in the experimental model and in vivo.

The developmental toxicity of catecholamines and β-blockers was studied in the chick embryo. In sharp contrast with the most teratogenic drugs, these studies showed that the early embryos are significantly more resistant to both β-mimetic and β-blocking drugs than the fetal stages (29). We have some insight into the pathogenesis of catecholamine-induced cardio-myopathy in the chick embryo (16–19), but little is known about the mechanisms by which β-blocking drugs cause embryolethality. Because the toxicity is more pronounced at advanced embryonic stages, it is likely that the impact is on function, rather than causing malformations incompatible with embryonic survival.

For our experiments we specifically chose three drugs that are frequently used in cardiology daily practice. A negative chronotropic effect is typical for β-blocking agents and ivabradine, although they present two different groups of cardiovascular drugs. The indications for β-blocking agents (metoprolol, carvedilol) include the treatment of arrhythmias, arterial hypertension, heart failure, ischemic heart disease, and thyrotoxicosis. Ivabradine is mainly used for the treatment of ischemic heart disease and arrhythmias. Metoprolol does cross the placental barrier, whereas carvedilol shows only low transfer (http://polaros.com/carvedilol-pharmacokinetics.html). Ivabra-
dine, the newest of the drugs tested, is not recommended in pregnancy, since embryotoxicity was observed in rats even in standard clinical dosing (European Medicine Agency; http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Scientific_Discussion/human/000598/WC500035338.pdf).

We focused our investigations on the effects of commonly used anti-arrhythmic drugs on the function of the developing chick heart as a determinant of embryonic survival. Because the heart rate is the most important determinant of cardiac output in the embryonic heart (7), we hypothesized that drug-induced bradycardia is the leading mechanism of heart failure. We found that β-blockers caused stage-dependent reduction of heart rate, translating in decreased cardiac output despite some Frank-Starling compensation (24). Similar findings were obtained with ivabradine, a new drug supposed to decrease heart rate by blocking the so-called If current (20). Therefore, we concluded that the heart rate is the most important determinant of cardiac performance in development. These results suggest that any drug causing significant bradycardia (7) should be considered embryotoxic.

### MATERIAL AND METHODS

Embryonic heart rate was measured non-invasively using Ex ovo videomicroscopy. ED4 chick embryos cultured ex ovo were studied by videomicroscopy to calculate cardiac volumes using prolate ellipsoid formula (25) (Fig. 1). Ten-second loops were recorded every minute for 30 min with a Canon G5 CCD camera mounted on a Leica SMZ16 dissecting microscope. The embryos in ex ovo culture dishes (14) were maintained at 37.5°C using a metal container filled with preheated Bath Armor dry medium placed on an ECHOTHERM chilling/heating plate.

Additional group of embryos was studied at ED5 in ex ovo 24 h after injection of the drugs (Fig. 1). Because of less clear view of the heart due to more advanced stage of development and turning of the embryo, only heart rate and regularity were evaluated.

Ex ovo ultrasound investigation. Ultrasound biomicroscopy ex ovo (Fig. 1) was performed on a Vevo 760 setup, adapted for chick embryos (14), after acute intra-amniotic injection of 200 µl of metoprolol, 50 µl of carvedilol, or 200 µl of ivabradine or 200 µl of normal saline for a period of 30 min. A lower dose of carvedilol was used because acute mortality was too high with the 200 µl dose. In the ED8 embryos, stroke volume was calculated from the long parasternal short-axis view, using the Simpson method in ED8 embryos. Cardiac output was then calculated from the following equation: cardiac output (in µl/min) = stroke volume (in µl) * heart rate (in beats/min).

### Table 2. Results from treatment at ED8

<table>
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<th>ED8 and dose, µg</th>
<th>N</th>
<th>Dead</th>
<th>Mortality, %</th>
<th>Embryo weight, g</th>
<th>Heart weight+, g</th>
<th>HBWR, g/g</th>
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<td></td>
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<td>1.388 ± 0.120</td>
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<tr>
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Values are means ± SE. ND, not determined. *P < 0.05; +average calculated from pooled hearts.

Doses of antiarrhythmic drugs. For the functional studies, we used 200 µl of metoprolol per egg (57 g on average), which represents a dose 16 times higher than the maximum recommended dose for human (70 kg) with respect to the body weight and its pharmacokinetics. Administration of 50 µl of carvedilol represents a dose four times higher than the maximum recommended dose for humans; 200 µl of ivabradine is seven times higher. The maximal acute effective concentration in the embryo itself with the intra- amniotic application is ~20 times higher considering the gradual distribution to the entire volume of one egg. We chose these high doses of the three drugs for our experiment because there was no effect noticed on chick embryonic heart rate at the lower doses recommended for humans. We tested different drug doses in the increasing fashion, and finally we chose doses with obvious effect on embryonic heart rate. The results thus reflect potential toxic effect of accidental overdose, rather than the effects occurring in the usual clinical setting.

β-Adrenergic receptors analysis. The preparation of myocardial membranes and determination of β-adrenergic receptors was performed as follows. The chick hearts (normal at various developmental stages, and ED9 with and without treatment with β-blockers at ED4
Fig. 1. Experimental outline, morphology of embryonic day (ED)4 and ED8 embryos, and demonstration of methods for data acquisition and analysis. Top: schematically the experimental design of treatment and sampling with evaluation using various methods. Videomicroscopy at ED4 chick embryonic stage with the heart rate recordings (A–E) shows a negative inotropic effect of ivabradine. Microphotograph and ultrasound B mode and M mode in ED8 chick embryonic stage (F–J) show a negative chronotropic and dromotropic effect of metoprolol. A, atrium; FL, fore limb; HL, hind limb; LA, left atrium; LV, left ventricle; OT, outflow tract; RA, right atrium; RV, right ventricle; V, ventricle. The yellow dot shows the region of interest from which the rhythm strip (D and E) was generated. Bpm: beats/min.
and/or ED8) were homogenized in TMES buffer containing (in mM) 20 Tris-HCl, 3 MgCl₂, 1 EDTA, a 250 sucrose (pH 7.4) containing a complete protease inhibitor cocktail (Roche Diagnostics), using a Potter-Elvehjem glass-teflon homogenizer. Coarse cell debris and nuclei were removed by low-speed centrifugation (600 g, 10 min), and membranes were then pelleted by centrifugation at 50,000 g for 30 min at 4°C. The pellets were suspended in TMES buffer and stored in aliquots at −80°C.

β-Adrenergic receptors were determined by a single-point binding assay using [³H]CGP12177, a nonselective β-adrenergic antagonist. Myocardial membranes (60 μg) were incubated with 3 nM [³H]CGP12177 in incubation buffer containing (in mM) 50 Tris-HCl, 10 MgCl₂, and 1 ascorbic acid (pH 7.4) for 60 min at 37°C in a total volume of 0.5 ml. The reaction was terminated by the addition of 3 ml of ice-cold washing buffer containing 50 mM Tris-HCl and 10 mM MgCl₂ (pH 7.4) and immediate filtration through Whatman GF/C filters presoaked with 0.3% PEI. Nonspecific binding was assessed by incubating the samples with radioligand in the presence of 10 μM L-propranolol. The radioactivity retained on filters was measured by scintillation counting in 5 ml of CytoScint (ICN Biomedicals). Receptors in all samples were determined in 3–5 independent experiments, each done in triplicate. The results are expressed as the mean, with error bars denoting standard deviations.

Statistics. The embryotoxic effect was tested using a Yates corrected χ²-test. Embryo and heart weights were compared using an unpaired Student’s t-test.

Longitudinal comparisons of functional parameters before and after drug administration were performed using both a paired Student’s t-test (for changes in time within group) and the ANOVA and Scheffe’ test for multiple comparisons between the saline and drugs groups. P value below 0.05 was considered as significant in all tests.

RESULTS

Embryotoxic effects. In the control group injected with 200 μl of normal saline the background mortality was 14% for ED4 embryos and 6% for ED8 embryos. No significant increase in mortality was observed in ED4 embryos injected with different doses of metoprolol, and 39% mortality was achieved in ED8 embryos injected with 200 μl of metoprolol. A significant mortality of 86% was observed in ED4 embryos injected with 200 μl of carvedilol; this effect was less pronounced with only 10% mortality at the same dose (Table 2). Wet and dry embryo and embryonic heart weights were not significantly different among the groups (Tables 1 and 2). No external or internal malformations, growth retardation, or edema were noted in the survivors.

Functional effects at the early embryonic stage. Metoprolol is a drug with a strong negative chronotropic effect leading to a 33% decrease of heart rate measured 30 min after administration in ED4 ex ovo embryos compared with a nonsignificant 6% reduction from the baseline of 150 ± 13 beats/min (mean ± SD) in the normal saline group (Fig. 2). The difference was statistically significant with P = 0.009. Cardiac output in ED4 embryos decreased by 1% from the baseline of 26,072 ± 17,791 μl/min (mean ± SD) in the control group and by 16% in the metoprolol group (Fig. 2). Within the metoprolol group there was no statistically significant difference compared with the baseline values (P = 0.930) as well as compared with the controls (P = 0.503). In more complex figures showing the time trends of normalized values, error bars were omitted for clarity of presentation.

The maximum negative chronotropic effect of 50 μl carvedilol dose injected to ED4 ex ovo embryos was achieved already after 6 min, when the heart rate significantly decreased by 27% (P = 0.003), with no further change after 30 min (Fig. 2). The difference in heart rate between the control and carvedilol groups was statistically significant at 6 and 30 min, with P = 0.001 and P = 0.047, respectively. There was no statistically significant difference in cardiac output between the groups (Fig. 2).

Ivabradine injection to ED4 embryos resulted in a decrease of heart rate after 30 min by 55%, whereas in the controls there was only a 6% decrease (Fig. 2). This difference was highly statistically significant with P < 0.001. In the ivabradine group cardiac output decreased significantly against the baseline by 43% at 30 min (Fig. 2), whereas in the controls the difference at 30 min did not reach statistical significance (P = 0.986).

To obtain further insight into the durability of these effects, a separate group of embryos injected with the highest doses tested was studied by in ovo videomicroscopy 24 h after injection at ED5. No effect on heart rate and regularity was noticed with the β-blockers (controls: heart rate 161 ± 12,
mean ± SD, N = 14; metoprolol 155 ± 9, N = 8; carvedilol 160 ± 11, N = 13). However, there was a significant reduction in baseline heart rate in the ivabradine group (141 ± 18, N = 15, \( P = 0.003 \)), where we also observed in the majority of embryos periods of even more profound bradycardia and brief asystole over the 30-min recording period, during which 2 of 15 embryos actually died.

Functional changes at the fetal stage. In more mature ED8 embryos, the negative chronotropic effect of metoprolol was even more pronounced (Fig. 3), with the heart rate decreasing by 71% (\( P < 0.001 \)) compared with the controls where the heart rate decreased only by 36% (\( P < 0.001 \)) from the baseline of 205 ± 28 beats/min (mean ± SD). The difference between the metoprolol group and the controls was significant at 30 min with \( P = 0.011 \). In both groups the cardiac output decreased significantly concomitantly with the heart rate by 61% in the metoprolol group and by 36% in the control group from the baseline of 37,442 ± 12,826 μl/min (mean ± SD; Fig. 3). The difference between the control and metoprolol groups was statistically significant (\( P = 0.001 \)).

In ED8 embryos injected with 200 μl of carvedilol, we observed pronounced periods of asystole and embryonic death (4 embryos out of 5; \( P = 0.004 \) in a \( \chi^2 \)-test). Therefore, a reduced dose of 50 μl was used afterward. Administration of 50 μl of carvedilol caused a negative chronotropic response resulting in a decrease of heart rate by 54% from the base line (\( P < 0.001 \)) at 30 min. When compared with that of the controls, the difference was statistically significant with \( P = 0.037 \) (Fig. 3). In the carvedilol group we also noticed a statistically significant increase in stroke volume at 6 and 30 min that was sufficient to keep the cardiac output decreasing only slowly with the decreasing heart rate. This compensatory effect resulted in no difference in cardiac output between the carvedilol and control groups. In both groups, cardiac output decreased by 36% and 33% in 30 min (Fig. 3).

The same, more pronounced, negative chronotropic effect in more mature embryos was also observed after ivabradine treatment. The heart rate decreased significantly by 53% in the ivabradine group (\( P < 0.001 \)). There was a borderline difference between the ivabradine group and the controls with \( P = 0.052 \) (Fig. 3). There was no significant difference in stroke volume between the two groups. Cardiac output decreased by 45% in the ivabradine group and by 36% in the controls (\( P = 0.128 \)) with no significant difference between the groups (Fig. 3).

Developmental profiling of β-adrenergic receptors. We observed a clear downward trend in the number of β-adrenergic receptors (β-ARs) during chick embryonic development. The early embryonic stages ED4 had a considerably higher number of β-ARs compared with the more mature ED18 (Fig. 4). The density of β-ARs was not significantly different between ED8 embryos incubated for 24 h with metoprolol compared with ED9 controls (76 vs. 78 fmol/mg protein). However, administration of carvedilol reduced the number of β-ARs in ED8 embryos compared with the controls (56 vs. 72 fmol/mg protein). Interestingly, early stage ED4 chick embryos expressed a significantly lower number of β-ARs when incubated with metoprolol and carvedilol compared with the more mature ED8 embryos or controls. The mean myocardial β-AR density in the group of ED4 + ED8 embryos treated with metoprolol was 55 fmol/mg protein; in ED4 + ED8 embryos treated with carvedilol it fell to 37 fmol/mg protein (Fig. 5).

DISCUSSION

We studied three different antiarrhythmic drugs with a strong potential to slow down the heart rate. Metoprolol is a moderately selective β₁-blocking agent with a negative chronotropic and inotropic effect (15). Carvedilol is a nonselective β-blocker directly inhibiting both β₁- and β₂-ARs as well as...
β-ARs present in blood vessel walls (15). This wide blocking effect of adrenergic receptors results in a negative chronotropic effect, a less pronounced negative inotropic effect (5), and decreased afterload. Ivabradine is a relatively new drug with a strong potential to slow down the heart rate without negative effects on myocardial contractility (27).

The effects of β-blocking agents were studied retrospectively in pregnant women with hereditary long QT syndrome, and there were no reported fetal malformations (20). Metoprolol use for the treatment of arterial hypertension during pregnancy exhibited no abnormal effect on the fetus (22). Metoprolol is recommended for the treatment of supraventricular tachycardias during pregnancy as class I, level of evidence B, with a note that β-blocking agents should not be taken in the first trimester, if possible (4). Metoprolol has nearly a 100% effectiveness in crossing the placental barrier as its concentrations determined in blood serum were found to be close to equal in both mother and fetus according to manufacturer’s data. Although this corresponds well with our findings of no cardiac or other malformations in the chick embryos treated during the critical period for induction of developmental defects (ED4) even with the highest doses of these agents, we could not support the statement about not using these drugs in the first trimester. We found that early embryos are much less sensitive to β-blockers than older ones, despite the presence of relatively high levels of β-ARs. A negative chronotropic effect of a β-blocking agent on human newborns was also proven in a placebo-controlled trial of atenolol for the treatment of hypertension associated with pregnancy. Neonatal bradycardia was more common after atenolol, but no adverse effect on the fetus was noticed (21). We also noticed a significant negative chronotropic effect of metoprolol in chick embryos at more mature fetal stages (ED8), compared with controls. This effect was followed by a significant decrease in cardiac output because the trabecular structure and volume of the fetal left ventricle (26) are not able to increase stroke volume with the Frank-Starling mechanism as effectively as described in the adult heart. Insufficient cardiac output might be the cause of fetal death, which we observed during our experiment with ED8 chick embryos injected with 200 μl of metoprolol. As reported earlier, prolonged hypoxia as a consequence of mild to moderate bradycardia may be a mechanism for embryonic death (6).

We observed a decrease in heart rate in the control group, which was not significant in the early embryonic ED4 stage but became significant in the more mature ED8-fetal stage group. This phenomenon can be explained by the amniotic membrane drying up despite our effort to stop this process. The pressure of the ultrasound probe on the embryo might influence heart rate. The ultrasound power output itself had no influence on embryonic heart rate. The heart rate remained unchanged when we reduced the ultrasound power output by 50%. We also monitored closely the temperature in the culture dishes, which remained constant during the experiments.

Clinical evidence of the adverse effect of carvedilol on the human fetus is lacking. To date, no human studies or case reports have been published. No data are available regarding the tranplacental transfer of carvedilol in humans. Low placental transfer was demonstrated in animal studies (http://polaros.com/carvedilol-pharmacokinetics.html). Based on our results we can report a significant adverse effect of high carvedilol dose (200 μl) on both (embryonic and fetal) stages. This dose, at least 16× times higher than the one used routinely in humans, led to highly significant periods of prolonged asystole and embryonic death when acute effects of this drug were tested. A reduced dose of 50 μl of carvedilol showed a much better safety profile with no significant embryotoxicity. The negative chronotropic effect of carvedilol in ED8 embryos was significant but led to a nonsignificant decrease of cardiac output.

The safety of ivabradine during pregnancy in humans is unknown; only one case report has been published, showing no adverse effect on the fetus (2). No data are available to specify how much ivabradine crosses the placental barrier. Ivabradine was found in amniotic fluid of pregnant rats and was excreted into maternal milk of rats in experiments (http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Scientific_Discussion_human/000598/WC50005338.pdf). Furthermore, teratogenicity (ectrodactylyia) and increased intrauterine demise was noticed in the rabbits. The significant embryotoxicity of ivabradine in early embryonic stages should warrant further investigations, to discern whether they represent direct teratogenic effects, or consequences of abnormal cardiac function. The pacemaking funny current is present in the developing heart from the earliest stages and becomes gradually restricted to the sinoatrial and atrioventricular nodes (1, 23). It is therefore not surprising that ivabradine has a negative chronotropic effect on the developing heart, as was shown in vitro by Sarre et al. (1, 23) and confirmed in vivo by our data.

Endogenous synthesis of catecholamines in the pacemaking area reported in the early mammalian heart by Ebert and Thompson (8) supports our findings of moderate negative chronotropic response even at ED4. We have further proved the presence of functional β-ARs in the chick embryo at ED4. In this independent experiment performed on isolated chick hearts incubated with a ramped dose of 2–20–72 μl (μg) adrenaline per 2 ml, there was a 60% increase of heart rate, compared with saline controls. It was also shown previously that the sensitivity of the heart to β-mimetic agents (isoproterenol) increases during chick development, with a peak at ED9 (17). We have confirmed these observations by finding relatively higher acute sensitivity to the same dose of β-blocker at
the fetal stage (ED8) compared with the pre-inervation embryonic stage (ED4). This cannot be attributed to a greater number of β-ARs because we found a significant downward trend in the expression of these receptors during the developmental stages from ED4 to ED18. We hypothesize that more efficient coupling of the receptors to their downstream effectors in the later developmental stages may well explain the increased sensitivity to β-mimetics, and we plan to perform systematic evaluation of downstream effectors (adenylate cyclase activity measurements) in future research focused on this experimental model. Moreover, it has been reported that sympathetic autonomic innervation reaches the developing chick heart after the parasympathetic (vagal) nerves at ED9 (11, 28). We also observed that long-lasting effect of β-blocking agents lead to the downregulation of β-ARs specifically in early embryonic stages. This effect is more pronounced in carvedilol, a nonselective β-blocking agent, rather than in metoprolol, which is a moderately selective β1-blocking agent.

Conclusions

Sensitivity to the negative chronotropic effect of metoprolol, carvedilol, and ivabradine increases with development. In agreement with our hypothesis, the embryonic heart has a limited potential to regulate stroke volume, and significant bradycardia is therefore followed by a significant decrease in cardiac output, likely leading to embryonic death. According to our animal data, metoprolol in standard doses appears to be relatively safe in pregnancy, which complies with the results of experiments; J.N., B.O., and D.S. edited and revised manuscript. R.K., J.N., and L.H. prepared figures; R.K. drafted manuscript; R.K. and

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: R.K. and B.O. conception and design of research; R.K., J.S., and L.H. performed experiments; R.K., J.S., J.N., and L.H. analyzed data; R.K., J.N., and L.H. prepared figures; R.K. drafted manuscript; R.K. and D.S. approved final version of manuscript; J.N., B.O., and D.S. interpreted results of experiments; J.N., B.O., and D.S. edited and revised manuscript.

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