0031-3998/90/2706-0557$02.00/0
PEDIATRIC RESEARCH

Vol. 27, No. 6, 1990
Printed in U.S.A.

Effect of Atrial Natriuretic Peptide on Hemodynamics of the Stage 21 Chick Embryo

MAKOTO NAKAZAWA, FUSAE KAJIO, KAZUO IKEDA,[1] AND ATSUYOSHI TAKAO

Department of Pediatric Cardiology, The Heart Institute of Japan, Tokyo Women's Medical College, Tokyo, Japan

ABSTRACT. **Atrial natriuretic peptide (ANP) is important in the modulation of hemodynamics and fluid balance in the mature subject, but its hemodynamic effects at early stages of morphogenesis are not defined. We studied the effect of rat atriopeptin III on hemodynamics in chick embryos at Hamburger-Hamilton stage 21. The cardiovascular system is not yet innervated, nor is the kidney formed in these embryos. The vitelline arterial and venous blood pressures were measured with a servo-null, micro-pressure system and the dorsal aortic blood flow was measured with a 20 MHz pulsed Doppler velocity meter. The peptide was infused into the vitelline vein with a microinjector at doses of 0.1, 1.0, and 10 ng. Doses normalized by body wt of embryos averaged 0.003, 0.035, and 0.32 ng/mg ($n = 61$), respectively. Vitelline arterial blood pressure decreased in a dose dependent manner [$y = 55.8 - 9.9x$; $r = -0.49$; $p < 0.01$ (y = % of baseline, x = log ng/mg)], and dorsal aortic blood flow, a measure of cardiac output, decreased similarly ($y = 39.6 - 16.2x$; $r = -0.47$; $p < 0.01$). Heart rate did not change. Ten ng of ANP increased the vitelline venous diameter, determined directly under a microscope, from 125 ± 47 (SD) μm to 139 ± 49 μm ($n = 11$; $p < 0.01$), and decreased vitelline venous pressure from 0.34 ± 0.05 mm Hg to 0.10 ± 0.07 mm Hg ($n = 5$). We conclude that ANP exerts its hemodynamic effect by direct venodilation in the noninnervated and anephric circulation. We speculate that ANP modulation of vascular tone and volume could be a mechanism for the regulation of the preinnervated embryonic cardiovascular system. (*Pediatr Res* 27: 557–560, 1990)**

Abbreviations

ANP, atrial natriuretic peptide

ANP is important in the regulation of hemodynamics and fluid balance (1–3). In mature subjects, the hemodynamic actions of ANP can be influenced by both neurohumoral reflexes and diuresis (4–7); therefore, its precise mechanism of action has not been completely defined. During the early stages of development, the cardiovascular system is not innervated (8) and the kidneys are not developed (9). Thus, an investigation of the effect of ANP in this unique model would provide information on the direct vascular effects of this cardiovascular peptide. In addition, it is not known whether the cardiovascular system responds to this potent vasoactive substance during the early period of morphogenesis. Therefore, we studied the effect of ANP on blood pressure and cardiac output in chick embryos at early developmental stages. We found that the atrial natriuretic factor had a dose dependent effect on embryonic hemodynamics, decreasing cardiac output and arterial blood pressure through direct venodilation and venous pooling.

Received June 12, 1989, accepted February 1, 1990.
Correspondence: Makoto Nakazawa, MD., Department of Pediatric Cardiology, The Heart Institute of Japan, Tokyo Women's Medical College, 8-1 Kawada-cho, Shinjuku-ku, Tokyo, 162 Japan.
[1] Present address: Department of Pediatrics, Sapporo Medical College, Sapporo City, Hokkaido, Japan.

MATERIALS AND METHODS

A total of 84 white Leghorn chick embryos at Hamburger-Hamilton stage 21 (10) were used. Hemodynamic parameters were obtained by the method reported by Clark and Hu (11). Briefly, the vitelline artery or vein was punctured with a microglass pipette that was connected to a servo-null micro-pressure system (W-P Instruments, Inc., New Haven, CT, Model-900) to measure the blood pressure. The blood flow velocity at the dorsal aorta was measured with a 20 MHz pulsed Doppler ultrasound velocity meter (University of Iowa), and the flow was calculated from the velocity and diameter of the aorta. The pressure and flow studies were done in separate groups of embryos. Environmental temperature was maintained at 37–38°C throughout the study. We injected rat atriopeptin III (Peninsula Laboratories, Inc., Belmont, CA) into the vitelline vein in doses of 0.1 ng, 1 ng, and 10 ng diluted in 100 nL of chick Ringer solution over a 1-min period using a microinjector (nanoliter pump, WPI Model-1400). Separate embryos were used for each injection: 12 embryos for the dose of 0.1 ng, 10 for 1 ng, and 7 for 10 ng in the pressure study, and 11, 11, and 10 in the flow study, respectively. We used rat atriopeptin because it induced a significant hemodynamic change in mature chickens (12) and because chick ANP was not available. We have already found that there is no hemodynamic effect of the 100 nL volume load using an identical procedure (13). Dosage corrected for body wt of embryos was 0.0035 ± 0.0015 (SD), 0.035 ± 0.011, and 0.32 ± 0.08 ng/mg, respectively.

To elucidate direct effects on the vein, we measured the diameter and pressure of the vitelline vein before and after 10 ng atriopeptin infusion. The diameter of the third or fourth order of the vitelline vein was measured by using a microscope high speed video system (HSV-400, NAC, Tokyo, Japan), with which we recorded the vessels at a frame rate of 200/s before and after drug infusion. The vitelline venous pressure was measured as described above. We considered that these results, even if they were in the extreme due to the high dose, could give us better understanding of the mechanism of action of ANP.

The effects of atriopeptin became stable 3 to 5 min after the end of injection (Fig. 1); thus, we used data taken at 3 min after injection. The data for the arterial blood pressure and dorsal aortic flow were tested by regression analysis and those of the venous diameter and pressure were tested by the paired t test. A p value less than 0.05 was considered statistically significant.

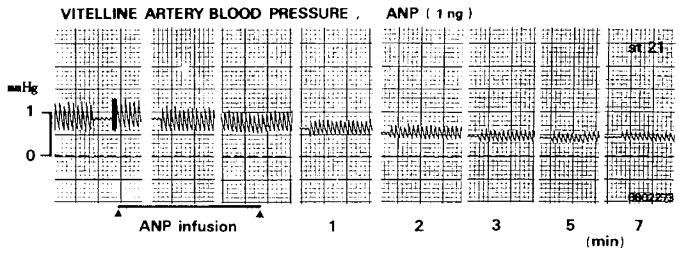

Fig. 1. The vitelline arterial blood pressure decreased with atriopeptin infusion. The change started during or immediately after the end of infusion and became stable 3 to 5 min after the end of infusion. *1, 2, 3, 5,* and *7,* min after the end of infusion of atriopeptin (ANP).

Table 1. *Changes in mean blood pressure (mm Hg) (mean ± SD)*

Dose (ng)	*n*	Control	3 min (%)
10	7	0.73 ± 0.06	0.42 ± 0.05 (59 ± 8)
1	10	0.98 ± 0.11	0.67 ± 0.05 (70 ± 6)
0.1	12	0.80 ± 0.08	0.63 ± 0.15 (76 ± 11)

Table 2. *Changes in mean blood flow (mm^3) (mean ± SD)*

Dose (ng)	*n*	Control	3 min (%)
10	10	0.64 ± 0.06	0.31 ± 0.05 (46 ± 8)
1	11	0.51 ± 0.07	0.28 ± 0.06 (56 ± 7)
0.1	11	0.61 ± 0.07	0.49 ± 0.04 (82 ± 3)

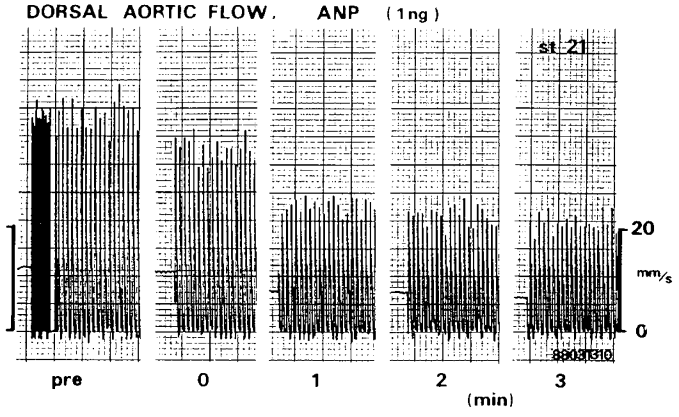

Fig. 2. The dorsal aortic blood flow was also decreased by injection of atriopeptin. Illustrated is a case in which 1 ng of the drug was given. *Pre,* baseline; *0, 1, 2,* and *3,* min after the end of ANP infusion.

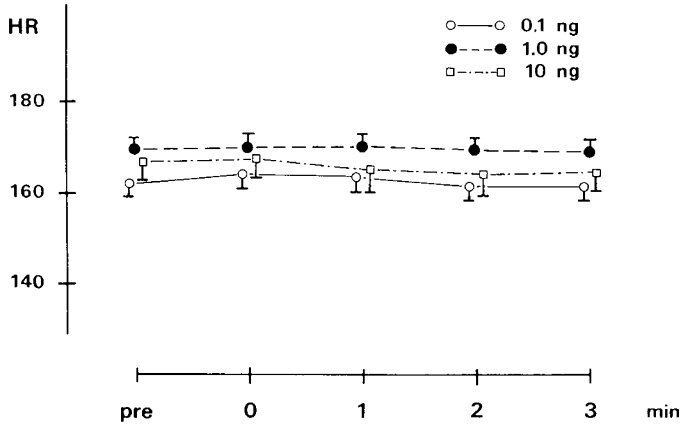

Fig. 3. Heart rate was not affected by ANP.

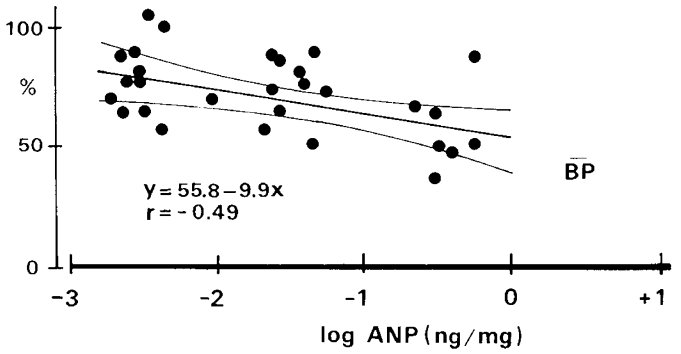

Fig. 4. Mean vitelline arterial blood pressure (*BP*) was decreased in a dose-dependent manner. Values of the pressure, taken 3 min after the end of injection, are expressed as percent of baseline. The dose of atriopeptin is normalized for body wt of embryos and is expressed in logarithm. *Lines* indicate mean and 95% confidence intervals.

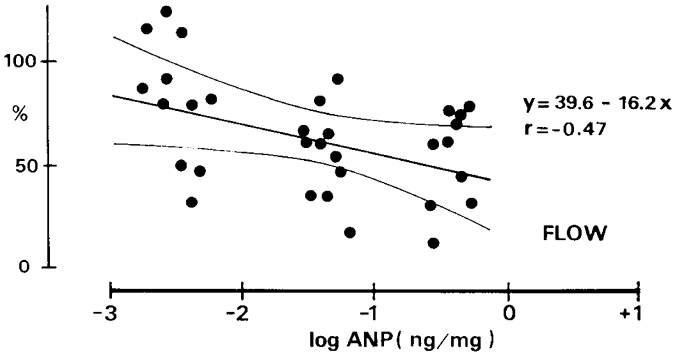

Fig. 5. The decrease of mean dorsal aortic blood flow was also dose-dependent. The data were expressed as in Figure 4.

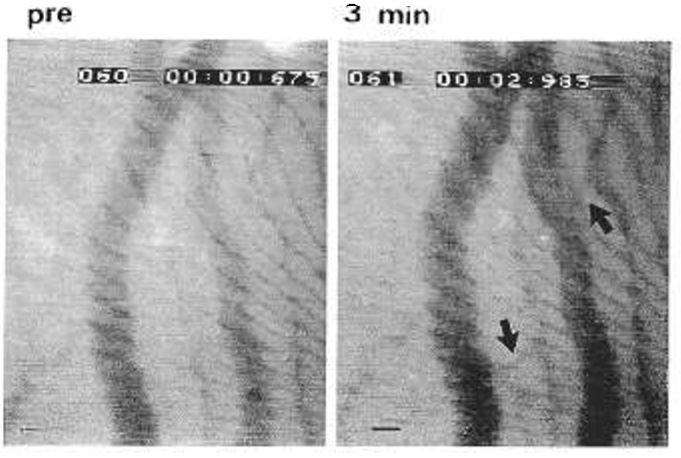

Fig. 6. The vitelline vein was dilated by atriopeptin. Vessels at control, (*left*) became larger 3 min after atriopeptin infusion (*right*). In addition, the small connecting veins were also dilated by the drug (*arrows*). *Bar* indicates 100 μm.

RESULTS

The mean vitelline arterial blood pressure and the mean dorsal aortic blood flow at baseline were not different from the normal data (11, 13) (Tables 1 and 2).

Vitelline arterial blood pressure and dorsal aortic blood flow decreased on ANP (Figs. 1 and 2, Tables 1 and 2), although the heart rate was not affected (Fig. 3). The change in mean blood pressure, expressed as percent of baseline, was inversely correlated with the dose of ANP, expressed as the logarithm of dose normalized for body wt ($p < 0.01$; Fig. 4). The flow was also

inversely correlated with the dose of ANP ($p < 0.01$; Fig. 5). In some embryos, the pressure and/or flow registered as zero, although the heart continued to beat and blood flow was seen through the microscope.

The diameter of the vitelline vein increased by 10 ng of atriopeptin from 125 ± 47 (SD) μm to 139 ± 49 μm (+13 ± 11%; $n = 11$; $p < 0.01$; Fig. 6), whereas it did not change from volume load alone (123 ± 23 μm to 118 ± 19 μm; $n = 7$). We also observed that the flow became slower in all embryos after atriopeptin infusion. The vitelline venous pressure decreased on ANP infusion from 0.34 ± 0.05 mm Hg to 0.10 ± 0.07 mm Hg ($n = 5$; $p < 0.01$).

DISCUSSION

Our study has clearly demonstrated that ANP has a circulatory effect in these stages of cardiovascular development and that the decreases in cardiac output and arterial pressure were similar to the response reported in mature animals (1–3). The basic mechanism of action of the peptide, however, probably differs from that in the mature subjects, because the effects would be altered by neurohumoral reflexes and diuresis in the mature subject.

The hemodynamic effects observed could not be attributed to depressed cardiac function because the venous pressure would be elevated with poor myocardial function. In fact, Natsume *et al.* (14) analyzed the effect of atriopeptin III on ventricular function in rats and found that the peptide did not have a direct negative inotropic effect. Coronary blood flow modulation through vasoconstriction has been suggested as a mechanism (15), but this cannot be the case in our experiment because there is no coronary system in these embryos.

In the mature animal, ANP alters renal blood flow, promoting diuresis and affecting the intravascular volume (1). This mechanism does not operate in the early embryo because the renal premordia are not yet developed. Almeida *et al.* (16) measured hematocrit and plasma volume in nephrectomized rats after ANP infusion and found that hematocrit increased from 44.5 to 47.4% and plasma volume was 3.86 mL/100 g body wt, significantly less than that of control animals (4.51 mL/100 g). They have postulated that atrial natriuretic peptide shifts fluid from the intravascular to the interstitial compartment. Their data were taken at 15 to 30 min after injection of the peptide. Although we were unable to measure hematocrit or plasma volume in embryos, the hemodynamic alteration observed in the embryo began quickly—even during atriopeptin infusion—and the vitelline vein was dilated by the drug, which led us to consider the decrease of plasma volume unlikely to be a major factor.

In the previous studies (1–3), the major factor causing the decrease of cardiac output has been attributed to a decrease in venous return. *In vitro* studies (17–19) have indicated that ANP relaxes vascular smooth muscle by activating membrane-bound cGMP (18, 19). This action can decrease venous return by venodilation and pooling blood in capacitance vessels. This response is a combination of direct and indirect mechanisms (4–7), the latter of which includes autonomic reflexes. The vagal limb of the autonomic nervous system becomes functional at stage 39 (d 9) and the sympathetic limb is functional at stage 42 (d 17) (8). Thus, the responses in the embryos used in our study should not be modified by autonomic reflexes.

Trippodo *et al.* (20, 21) did not find an increase in venous capacitance upon ANP administration. Rather, they found that ANP increased the vascular resistance to venous return, indicative of vascular constriction instead of dilation. Recently, Faber *et al.* (22), using denervated skeletal muscle in microscopic observation, reported that ANP increased diameters of the large distributing arterioles and capacitance venules preconstricted by α-1 agonist, and also dilated the intact small terminal arterioles. In general, vascular constriction causes an increase of blood pressure, even if only transiently; this was not seen in our study. In addition, our direct observations that the vein dilated on ANP along with the slowing of blood flow and the decrease in the central venous pressure support the proposition that venodilation is the direct mechanism of action of atriopeptin.

In these early stage chick embryos, ANP has not been directly identified. Manasek (23) found multivesicular bodies in the stage 10 (10 somites) chick embryo and occasional dense granules by the 4th d of incubation (the stage could be from 21 to 24, although it was not clarified). If the multivesicular body were a precursor of the dense granule, and the granule were the same as the dense granule that was presented as the principal storage site of ANP by Metz *et al.* (24) and Cantin *et al.* (25), ANP may possibly have physiologic significance. At least it can be concluded that the vascular system of this stage of chick embryo possesses an ability to respond to ANP. Sequencing of amino acid of chick ANP (26) has only recently been completed, thus the presence, localization, and concentration of ANP in chick embryos will be clarified in the near future, and will define, along with the present results, its physiologic role in the developing circulation.

The dose of ANP was relatively large compared to circulating quantities measured in mature animals. We estimated the concentration of ANP from previously measured circulating blood volume of 21 chick embryos (27). The estimated concentration of 2×10^3 to 2×10^5 pg/mL was higher than that noted in mature subjects, which ranged from 50 to 2000 pg/mL, including those in congestive heart failure, hypertension, or renal failure. However, the lowest dose of ANP in the present study was not too high compared to doses used in some studies of fetal sheep (28–30). In the study by Brace *et al.* (28), ANP was injected at a dose of 8 μg/kg of body wt of the fetus as a bolus, which corresponded to 8 pg/mg. Varille *et al.* (29) used 4 μg/kg (4 pg/mg) over 30 s. In a study by Robillard *et al.* (30), 0.1 μg/kg/min of ANP was infused over a 60-min period, giving a total dose of 6 pg/mg. In addition, we normalized the dose for body wt of embryos, but a recent study by Hu and Clark (31) has shown that the wt of vitelline circulation averaged 163 mg at stage 21, which is approximately 4 times that of embryo wt. Using this number, the doses normalized for wt would be approximately one fifth of those presented above. The fetal sheep has its placental circulation as extrafetal (or extraembryonic) circulation, but the relative wt of the placenta to the fetus at late gestation, as in the studies described above, should be much less than the figure for the chick embryo. Thus, the lowest dose of ANP used in our study was comparable to regimens used by others in fetal studies. We then used the higher doses to show the dose-response relation to confirm that the effect was pharmacologic. We did not try lower doses because the absolute values of parameters and their changes were small enough that any lesser changes would be insignificant.

The relatively high concentration of ANP required for an apparent physiologic response compared to the mature subject may reflect the immaturity of the ANP system. Like the development of the autonomic nervous system (8), the ANP system may progressively establish its function throughout development. Thus, it would be possible that ANP receptors may be less sensitive to the peptide in the early stages than in later or mature stages. The low sensitivity was also suggested by the fact that serum level of ANP in the lamb at late gestational days was five or more times that in the maternal level (32). The dynamic effects of ANP suggest that this peptide may play a role in regulating the circulation in the early preinnervated and prenephric embryos. We speculate that as the autonomic nervous system becomes functional and the kidneys form, the role of ANP shifts in response to the changing pattern of control mechanisms. Integrated studies of the ANP system in the chick embryo will increase our understanding of the ontogeny of hemodynamic control and the action of the peptide.

REFERENCES

1. Ballermann BJ, Brenner BM 1986 Role of atrial peptides in body fluid homeostasis. Circ Res 58:619–630
2. Pegram BL, Trippodo NC, Natsume T, Kardon MB, Frohlich ED, Cole FE, MacPhee AA 1986 Hemodynamic effects of atrial natriuretic hormone. Fed Proc 45:2382–2386
3. Kleinert HD, Volpe M, Odell G, Marion D, Atlas SA, Camargo MJ, Laragh JH, Maack T 1986 Cardiovascular effects of atrial natriuretic factor in anesthetized and conscious dogs. Hypertension 8:312–316
4. Sasaki A, Kida O, Kangawa K, Matsuo H, Tanaka K 1986 Involvement of sympathetic nerve in cardiosuppressive effects of alpha-human atrial natriuretic polypeptide in anesthetized rats. Eur J Pharmacol 120:345–349
5. Lappe RW, Smith TFM, Todt TA 1985 Failure of atriopeptin II to cause arterial vasodilation in the conscious rat. Circ Res 56:606–612
6. Volpe M, Cuocolo A, Vecchione F, Mele AF, Condorelli M, Trimarco B 1987 Vagal mediation of the effects of atrial natriuretic factor on blood pressure and arterial baroreflexes in the rabbit. Circ Res 60:747–755
7. Holtz J, Sommer O, Bassenge E 1987 Inhibition of sympathoadrenal activity by atrial natriuretic factor in dogs. Hypertension 9:350–354
8. Pappano AJ 1977 Ontogenetic development of autonomic neuroeffector transmission and transmitter reactivity in embryonic and fetal hearts. Pharmacol Rev 29:3–33
9. Bulter H, Jurlink BHJ 1987 An Atlas for Staging Mammalian and Chick Embryos. CRC Press, Boca Raton, FL.
10. Hamburger V, Hamilton HL 1951 A series of normal stages in the development of the chick embryo. J Morphol 88:49–92
11. Clark EB, Hu N 1982 Developmental hemodynamic changes in the chick embryo from stage 18 to 27. Cir Res 51:810–815
12. Gregg CM, Wideman Jr RF 1986 Effects of atriopeptin and chicken heart extract in Gallus domesticus. Am J Physiol 251: R543–R551
13. Nakazawa M, Ohno T, Miyagawa S, Takao A 1989 Hemodynamic effects of acetylcholine in the chick embryo and differences from those in the rat embryo. Teratology 21:555–561
14. Natsume T, Kardon MB, Trippodo NC, Januszewicz A, Pegram BL, Frohlich ED 1986 Atriopeptin III does not alter cardiac performance in rats. J Hypertens 4:477–480
15. Wangler RD, Breuhaus BA, Otero HO, Hastings DA, Holzman MD, Saneii HH, Sparks Jr HV, Chimoskey JE 1985 Coronary vasoconstrictor effects of atriopeptin II. Science 230:558–561
16. Almeida FA, Suzuki M, Maack T 1986 Atrial natriuretic factor increases hematocrit and decreases plasma volume in nephrectomized rats. Life Sci 39:1193–1199
17. Garcia R, Thibault G, Cantin M, Genest J 1984 Effect of a purified atrial natriuretic factor on rat and rabbit vascular strips and vascular beds. Am J Physiol 247:R34–R39
18. Winquist RJ, Faison EP, Waldman SA, Schwartz K, Murad F, Rapoport RM 1984 Atrial natriuretic factor elicits an endothelium-independent relaxation and activates particulate guanylate cyclase in vascular smooth muscle. Proc Natl Acad Sci USA 81:7661–7664
19. Waldman SA, Rapoport RM, Murad F 1984 Atrial natriuretic factor selectively activates particulate guanylate cyclase and elevates cyclic GMP in rat tissue. J Biol Chem 253:14332–14334
20. Trippodo NC, Cole FE, Frohlich ED, MacPhee AA 1986 Atrial natriuretic peptide decreases circulatory capacitance in areflexic rats. Circ Res 59:291–296
21. Chien YW, Frohlich ED, Trippodo NC 1987 Atrial natriuretic peptide increases resistance to venous return in rats. Am J Physiol 252:H894–H899
22. Faber JE, Gettes DR, Gianturco DP 1988 Microvascular effects of atrial natriuretic factor: interaction with alpha-1 and alpha-2 adrenoceptors. Circ Res 63:415–428
23. Manasek FJ 1969 The appearance of granules in the Golgi complex of embryonic cardiac myocyte. J Cell Biol 43:605–609
24. Metz J, Mutt V, Forssmann WG 1984 Immunohistochemical localization of cardiodilatin in myoendocrine cells in the cardiac atria. Anat Embryol 170:123–127
25. Cantin M, Gutkowska J, Thibault G, Milne RW, Ledoux S, Minli S, Chapeau C, Garcia R, Hamet P, Genest J 1984 Immunocytochemical localization of atrial natriuretic factor in the heart and salivary glands. Histochemistry J 80:113–127
26. Miyata A, Minamino N, Kangawa K, Matsuo H 1988 Identification of a 29-amino acid natriuretic peptide in chicken heart. Biochem Biophys Res Commun 155:1330–1337
27. Rychter Z, Kopechy M, Lemez L 1954 On the circulating blood volume from the 2nd day of incubation till hatching. Cesk Morfol 3:11–25
28. Brace RA, Cheung CY 1987 Cardiovascular and fluid responses to atrial natriuretic factor in sheep fetus. Am J Physiol 253:R561–R567
29. Varille VA, Nakamura KT, McWeeny OJ, Matherne GP, Smith FG, Robillard JE 1989 Renal hemodynamic response to atrial natriuretic factor in fetal and newborn sheep. Pediatr Res 25:291–294
30. Robillard JE, Nakamura KT, Varille VA, Andresen AA, Matherne GP, VanOrden DE 1988 Ontogeny of the renal response to natriuretic peptide in sheep. Am J Physiol 254:F634–F641
31. Hu N, Clark EB 1989 Hemodynamics of the stage 12 to 29 chick embryo. Circ Res 65:1665–1670
32. Cheung CY, Gibbs DM, Brace RA 1987 Atrial natriuretic factor in maternal and fetal sheep. Am J Physiol 252:E279–E282