Original Article

Cardiac Enlargement in the Chick Embryo Induced by Hypothermic Incubation Is Due to a Combination of Hyperplasia and Hypertrophy of Cardiomyocytes

(embryonic heart / cell size / cell proliferation / immunofluorescence)

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Abstract. Hypothermic incubation of chicken eggs leads to smaller embryos with enlarged hearts, originally described as hypertrophic. Over the years, however, accumulated evidence suggested that hyperplasia, rather than hypertrophy, is the predominant mechanism of cardiac growth during the prenatal period. We have thus set to re-evaluate the hypothermia model to precise the exact cellular mechanism behind cardiac enlargement. Fertilized chicken eggs were incubated at either 37.5 °C (normothermia) or 33.5 °C from embryonic day (ED) 13 onward (hypothermia). Sampling was performed at ED17, at which point wet embryo and heart weight were recorded, and the hearts were submitted to histological examination. In agreement with previous results, the hypothermic embryos were 29% smaller and had hearts 18% larger, translating into a 67% increase in the heart to body weight ratio (P < 0.05 for all parameters). The cell size was essentially the same between control and hypothermic hearts in all regions analysed. Likewise, there was no significant relationship between the cell size and heart weight; however, in the hypothermic hearts, there was a trend showing positive correlation between cell sizes in different cardiac regions and heart weight. Proliferation rate, determined on the basis of anti-phosphohistone H3 immunofluorescence, showed an overall increase in the hypothermic group, reaching statistical significance (P = 0.02, t-test) in the right ventricle. The proliferation rate was similar among different regions of the same heart. However, the correlation between the proliferation rate and heart weight was only small ($r^2 = 0.007$ and $r^2 = 0.234$ for the normothermic and hypothermic group, respectively). We thus conclude that hyperplasia is the predominant response mechanism in this volume-overload model; mechanistically, decreased heart rate at lower temperature increases the end-diastolic and stroke volume, minimizing the drop in cardiac output through the Frank-Starling mechanism.

Introduction

From the evolutionary perspective, incubation of avian eggs at a constant and elevated temperature was preceded by poikilothermic incubation in reptiles, amphibians, and fishes (Ostadal, 2013). Thus, hypothermia in the second half of incubation, when the embryo is to some degree able to generate heat, is much better tolerated then hyperthermia (Peterka et al., 1996). Hypothermic incubation of chicken eggs was already reported more than half a century ago (Merkow and Leighton, 1967; Warbanow, 1970) and was noted to result in smaller embryos with enlarged hearts. Functionally, however, these hearts showed improved contractility at normal temperature (Warbanow, 1971), at which the heart rate also returned to normal, resembling the athlete's heart (Libby et al., 2008). What is less clear is the cellular mechanism of this enlargement, the author himself calling it alternatively hypertrophy or hyperplasia, but without unequivocal evidence for either.

Biochemically, an increase of both DNA and RNA, as well as protein was found in the ventricular myocardium of hypothermic embryos (Kennedy et al., 1991), but

only the elevation in RNA concentration was statistically significant. However, without normalization for the heart weight, all three parameters were significantly increased. This argues that the hearts may be enlarged due to the presence of a higher number of cells, but changes in the water content (not reported by the authors) could also play a role.

During the first half of incubation, the mechanism of both physiological and increased loading-induced growth is represented by controlled addition of new cells, without involvement of cellular hypertrophy (Clark et al., 1989) or cell death (Sedmera et al., 2002; Krejci et al., 2016). Careful measurements in embryonic rats revealed that cardiomyocyte size does not increase during prenatal development (Knaapen et al., 1996). However, during later foetal development, myocyte hypertrophy was described in the right ventricle of lambs with experimental pulmonary artery constriction (Toussaint et al., 1998), in addition to hyperplasia reported previously (Bical et al., 1990), suggesting that both mechanisms might operate with different intensities. This dual response is a distinct possibility even after birth, as some more recent studies have shown that not all human ventricular myocytes are truly post-mitotic, and a considerable variability exists among individuals (Bergmann et al., 2009).

Chick embryonic heart rate shows a nearly linear response to variations in temperature around the physiological optimum both *in vitro* and *in vivo* (Vostarek et al., 2014). Variations in the heart rate are compensated for by changes in the stroke volume in order to maintain cardiac output even in the embryonic heart using (although within rather narrow limits) the Frank-Starling relationship (Benson et al., 1989). A chronic decrease in the heart rate at lower temperatures leads to increased end-diastolic volume, which in general is compensated for by eccentric hypertrophy (Hutchins et al., 1978; Benes et al., 2011).

We have set to investigate, at the cellular level, the mechanisms of cardiac enlargement in the chick model of hypothermic incubation in the second half of development. Taking into consideration the available evidence, we hypothesized that the increase in cell mass will be primarily due to the increased number of cells rather than the cell size. We have found that indeed, the changes in the cell width were minuscule and without statistical significance, although the trend towards larger cell size and data dispersion in the hypothermic group was clear. Labelling with antibody raised against an endogenous proliferation marker, phosphohistone H3 (pHH3), showed increased positivity in all compartments and reached statistical significance in the right ventricle. These data suggest that in the foetal chick model, end-diastolic volume overload-induced heart growth in hypothermia is still primarily based upon myocyte proliferation rather than hypertrophy.

Material and Methods

White Leghorn chicken eggs were obtained from the farm of the Institute of Molecular Genetics, Kolec, Czech Republic, and shortly stored at 15 °C prior to incubation. The eggs were incubated at 37.5 °C in a humidified incubator until ED13, and then divided into two groups. The experimental one (N = 20) was transferred to a still draft incubator set to 33.5 °C (hypothermia), while the controls (N = 10) remained in normothermia. The embryos were removed from the eggs at ED17, gently blotted on paper towels, and weighed. The hearts were then extracted from the embryos, and their wet weight (after blotting) was also recorded. The hearts were then fixed for 48 h at 4 °C in 4% paraformaldehyde in phosphate-buffered saline, rinsed, and processed into paraffin for histological analysis. Prior to dehydration, they were dissected in the transverse plane for improved penetration of the solutions.

After serial sectioning at 10 μm, guide series at 100-μm intervals were stained with Alcian blue/haematoxylin-eosin. Selected sister sections (transverse biventricular views at ventricular midportion) were incubated with mouse IgM anti-actin (α-sarcomeric) as the primary antibody (1 : 800; Sigma-Aldrich, #A2172, St. Louis, MO). The secondary antibody used was rhodamine (TRITC)-conjugated AffiniPure goat anti-mouse IgM (1 : 200; Jackson ImmunoResearch #115-025-075, West Grove, PA). Wheat germ agglutinin (WGA) Alexa Fluor 488 (1 : 50; Invitrogen #W11261, Waltham, MA) conjugate was used for detection of basal membranes and fibrous tissue. The nuclei were labelled with Hoechst (1 : 100,000; Sigma #33342). This triple staining was used for measurements of the myocyte size in optical sections across the nucleus (N ≥ 20 per each location in every heart).

The second type of labelling (to measure cell proliferation) was by rabbit polyclonal anti-phospho-histone H3 (Ser10) antibody (1 : 100; Millipore #06-570, Darmstadt, Germany). This antigen is only present during the M-phase of the cell cycle. Cy3-conjugated AffiniPure goat anti-rabbit IgG (1 : 200; Jackson ImmunoResearch #111-165-144) was used as a secondary antibody. We then used DRAQ5 (1 : 1000; LI-COR Biotechnology #928-40022, Lincoln, NE) for identification of all cell nuclei.

Consequently, two non-overlapping locations in the circular area of the left ventricle, in the papillary muscles and in the right ventricle were selected. The pictures were taken with an Olympus FluoView confocal microscope with 40× oil immersion objective. Thereafter, the images (maximum intensity projections of the entire section thickness) were analysed using Adobe Photoshop (Adobe Systems) and ImageJ (freeware). For objective comparison with other studies and normalization, the counts (usually several positive cells per field) were expressed as a number per volume of 1 mm^3 of myocardium (tissue area within the field of view multiplied by the known section thickness of 10 μm). Finally, the measured values were analysed statistically using χ^2 test (numerical parameter, MS Excel).

Results

There were seven live embryos in the normothermic group (70 %), while only 12 live embryos (one with hydrops) were obtained from the hypothermic group (60 %; P = 0.89, Yates Corrected χ²). The wet embryo and heart weights are shown in Fig. 1. The hypothermic embryos were notably and significantly smaller, but without any obvious external or internal malformations. In combination with a significantly increased heart weight, this translated to a highly significant (P < 0.001) increase in the heart to body weight ratio of 67 %.

At the macroscopic level, the heart sections showed cardiac enlargement with a slight but insignificant trend towards thickening of both left and right ventricle in the hypothermic group; the 2 : 1 ratio between the left and right ventricular wall thickness was preserved. This suggests that the increase in heart weight was due to eccentric rather than concentric hypertrophy. Analysis of cell width showed fairly uniform distribution across different heart regions (Fig. 2) with virtually no differences between the normothermic and the hypothermic group. The cell diameter of the left ventricular free wall Purkinje fibres was significantly smaller than that of the neighbouring working myocytes in the normothermic group, and the same trend was present in the hypothermic one. Like in the previous locations, there was an insignificant increase in cell diameter in the hypothermic group (Fig. 3).

Analysis of the number of proliferating cells showed increased numbers of pHH3-positive nuclei in all examined locations of the hypothermic hearts (Fig. 4). The numbers reached statistical significance in the right ventricle only, in part due to considerably higher spread of values in the hypothermic group.

Discussion

Growth of the prenatal heart is primarily based on an increase in the cell number, and the same mechanisms were found to operate during experimentally altered loading conditions (Clark et al., 1989; Sedmera et al., 2002). We thus hypothesized that the same mechanism would play a dominant role in the hypothermic model of cardiac enlargement, alternatively referred to as hypertrophy or hyperplasia (Warbanow, 1970, 1971), without unequivocal evidence for either. Even biochemical analysis performed by Kennedy et al. (1991) in a slight modification of the original model (32 instead of 33.5 °C, starting at ED11 instead of ED13) did not clearly distinguish between these two possibilities, as both increased DNA (+18 %) and protein (+15 %) were found in the hypothermic hearts.

However, both were statistically insignificant when normalized to grams of cardiac tissue (the weight of which was increased significantly by 67 %). Nevertheless, an earlier study (Boehm et al., 1987) was more spe-
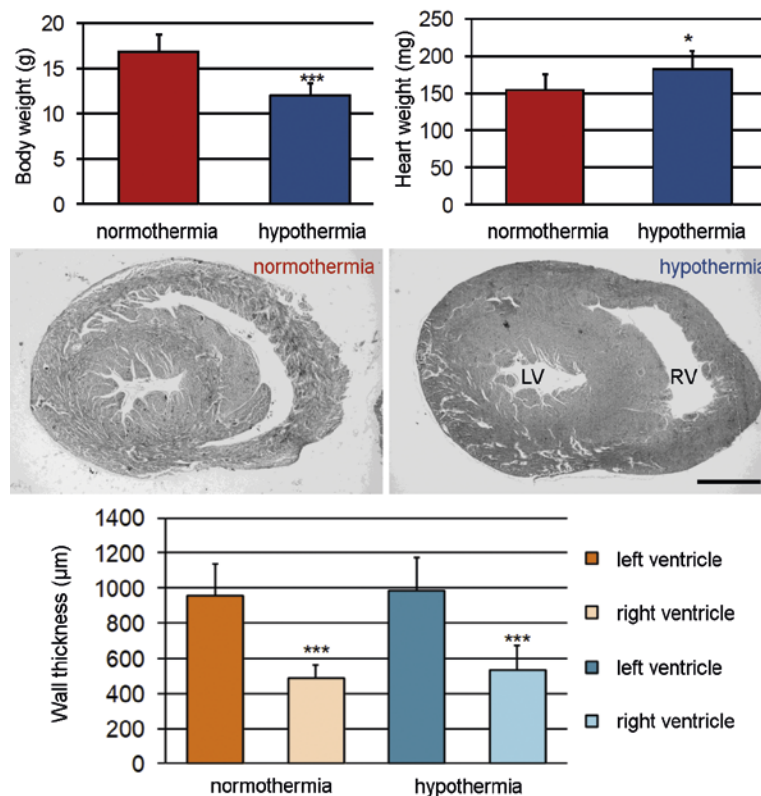

Fig. 1. Embryo and heart weights and gross morphology in normothermic and hypothermic hearts. Mean ± SD, *P < 0.05, ** P < 0.01, *** P < 0.001 (*t*-test). LV – left ventricle, RV – right ventricle, scale bar 1 mm.
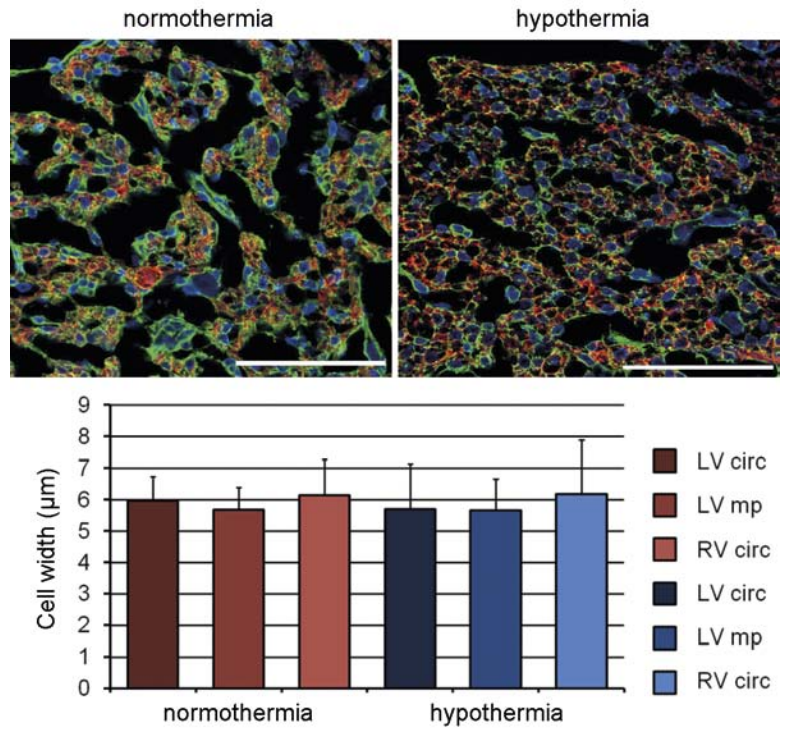
Fig. 2. Typical examples of triple-stained heart sections of normothermic and hypothermic left ventricle and cell size measurements. No significant differences were found among different heart regions, although there is a slight trend towards larger myocyte width in the hypothermic hearts. Circ – circular layer, LV – left ventricle, mp – papillary muscles, RV – right ventricle, scale bar 50 μm.
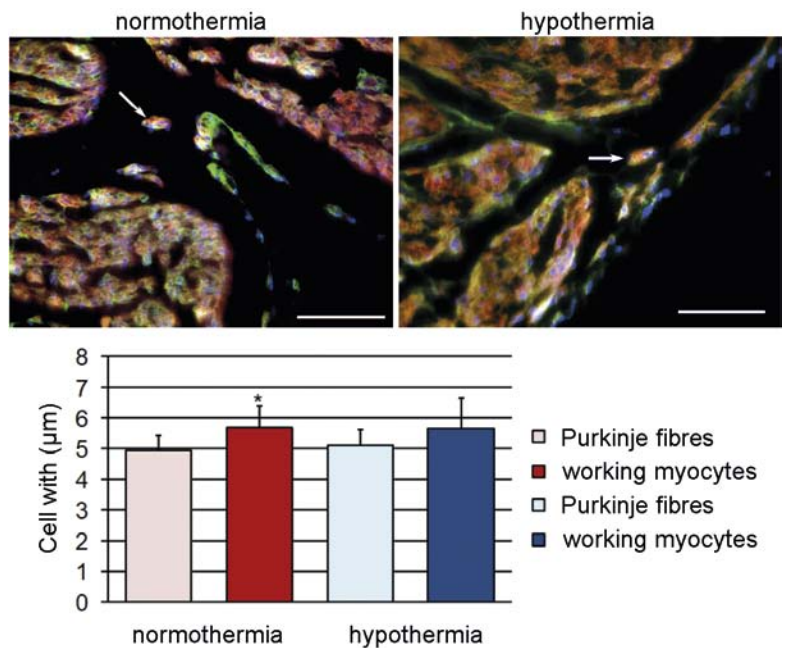

Fig. 3. Purkinje myocytes are smaller than the working myocytes, and there is a trend towards their increased width in the hypothermic hearts. *P < 0.05, Purkinje fibres versus neighbouring working myocytes (in the papillary muscles, transversely cut) in the normothermic group. Scale bar 50 μm.

cific, noting significantly increased water content in both hypothermic embryos (87 vs. 80 %) and their hearts (87 vs. 84 %), and showed a clear increase of the protein to DNA ratio (from 50 to 118, μg/μg) in ED18 hearts incubated at 32 °C starting from ED11. The authors interpreted this finding, corroborated by a significantly increased cell volume in hypothermic hearts (measured in isolated myocytes, 641 vs. 531 μm^3) and a lack of change
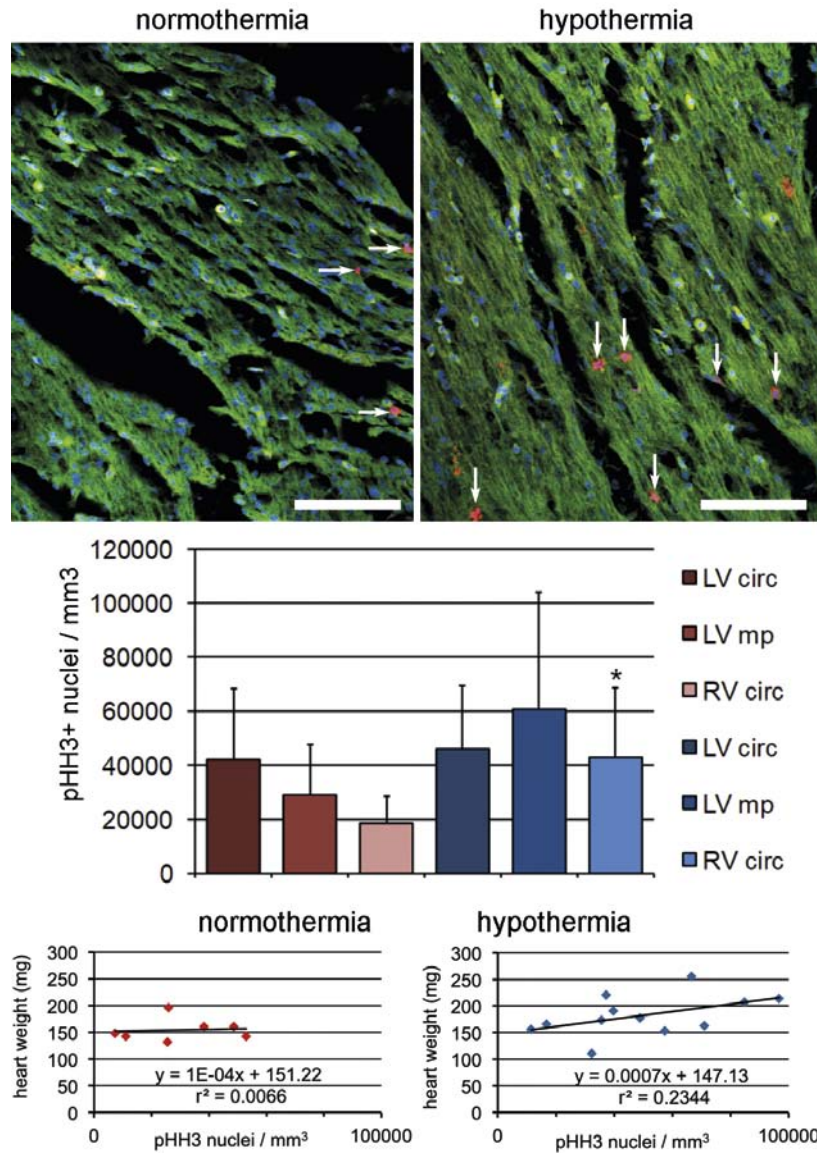
Fig. 4. Representative areas of the left ventricular free wall illustrating an increase in numbers of pHH3-positive cells (red) in the hypothermic group. The bar chart shows increased variability and a trend towards increased cell numbers (*P < 0.05 in the right ventricle) in the hypothermic hearts. The correlation plot shows little correlation between the heart weight and proliferation index in the normothermic hearts, while there is a trend towards larger hearts showing more proliferating cells in the hypothermic group. Circ – circular layer, LV – left ventricle, mp – papillary muscles, RV – right ventricle, scale bar 50 μm.

in the percentage of nuclei in S-phase (determined by 3-h pulse of [³H]-thymidine, 25 vs. 26 % of labelled nuclei), as hypertrophy. This is contrary to our findings, which showed a minimal increase in the cell transverse diameter, and could be explained by different methodology (cell volume vs. cell width). In this type of hypertrophy, induced by the increased ventricular end-diastolic volume, the myocytes react more by elongation (eccentric hypertrophy), as was shown in rats with aorto-caval fistula (Benes et al., 2011).

We likewise did not notice any significant thickening of the left ventricular wall, while about 35% increase was noticed by Boehm et al. (1987), possibly explainable by a different state of ventricular contraction at the time of fixation, as hypothermic hearts show better contractility (Warbanow, 1971). The discrepancy between the cell proliferation data could also be possibly explained by different methodology (measurements of S-phase vs M-phase of the cardiac cycle), but the data of Boehm et al. (1987) are also in contrast to the values obtained with large samples by previous investigators (Grohmann, 1961; Jeter and Cameron, 1971), who both noted a peak of proliferative activity in the ventricular free wall at ED4 followed by a gradual decline until hatching. This is in a sharp contrast to the findings of higher rates at ED18 (26 %) than at ED14 (18 %) by Boehm et al. (1987). It is also not clear from how many biological repeats the proliferation index values were derived, as the authors did not mention explicitly the number of hearts used for this particular measurement

and only noted in the text that autoradiography was performed on two sections, raising potential concerns. Further suspicious data (probably recorded visually from embryos removed from the eggs at room temperature) are the reported heart rates of 58 and 69 beats per minute for the normothermic and hypothermic group, respectively ($P < 0.005$).

The physiological adaptations of the cardiovascular system in the hypothermic embryos are significant, as they form the mechanistic explanation for the structural remodelling. It is noted that the embryonic heart rate is highly dependent on temperature (Vostarek et al., 2016). Lowering the temperature leads to bradycardia, causing increased heart filling during the diastole and thus increased stroke volume through the Frank-Starling relationship. This compensation is present already in the pre-septation heart (Benson et al., 1989), where it is, however, limited by the rich trabecular network resulting in relatively high ventricular stiffness (Buffinton et al., 2013). This partly explains why hypothermia of 32 °C is poorly tolerated by embryos prior to ED10, when trabecular compaction occurs (Sedmera et al., 1997). Such hearts are structurally more similar to the adult ones and capable of a wider physiological range of chamber volume changes. The compensation is not always sufficient, resulting in cardiac failure manifesting as increased water content (Boehm et al., 1987) in hypothermic embryos and hearts and foetal hydrops in the most severe cases (1 of 12 of our survivors, and several of the dead ones).

From the phylogenetic perspective, cardiac enlargement is reported in cold-acclimated fish, and the underlying cellular mechanism seems to be a combination of hyperplasia and hypertrophy (Ostadal, 2013), although the question is still unresolved. This is due to increased oxygen demands at higher temperatures, which might pose a limit on these adaptive changes that include heart rate changes (Ostadal, 2013).

True prenatal cellular hypertrophy based on the enlargement of cardiomyocytes was only reported in an abstract from the ovine model of pulmonary artery atresia (Toussaint et al., 1998), while a published, peer-reviewed study in the same model, where the right ventricle was challenged by pulmonary artery banding (Bical et al., 1990), did not notice any changes in the myocyte size. With mounting evidence from the studies performed in different foetal animal models (reviewed by Sedmera (2016)), it seems that indeed, the major cellular mechanism of prenatal heart growth and adaptations is represented by myocyte hyperplasia, with hypertrophy only participating in later foetal stages or under very severe challenges.

In our study, we observed clear evidence of hyperplasia (increased amount of M-phase cell nuclei) as well as a trend (not statistically significant) towards increased transverse cellular diameter. We thus conclude that the cardiac enlargement in this model is primarily based on the increased number of cells (hyperplasia) rather than hypertrophy.

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