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A stereological method for estimating the total number of ventricular myocyte nuclei in fetal and postnatal hearts

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ABSTRACT

An experimental protocol is presented for obtaining efficient estimates of the total numbers of myocyte nuclei in the ventricles of human hearts from archival collections. Hearts were collected postmortem from fetuses at 31–42 weeks of gestation and infants at 7 days–9 months postnatal age. Ventricles from normal and abnormal subjects were examined. Numbers of myocyte nuclei per unit volume of myocardium were estimated separately for left and right ventricles using a design-based stereological device, the physical disector (parallel pairs of sections). Absolute numbers were calculated by multiplying nuclear packing densities by myocardial volume. The latter was estimated from ventricular mass and the percentage of ventricle occupied by myocardium. The findings, albeit preliminary, suggest that (1) myocyte number may rise during the last trimester of gestation, (2) postnatal ventricular growth is probably hypertrophic and/or interstitial rather than hyperplastic and (3) whilst absolute numbers are greater in the left ventricle, the pattern of growth is similar in both ventricles. Nuclear numbers found in 2 abnormal hearts (1 from a case of sudden infant death) tended to be lower than normal.

Key words: Heart; ventricles; stereology; myocytes; nuclei.

INTRODUCTION

Attempts have been made to estimate the number of nuclei in mammalian heart muscle in developmental, comparative, pathological and experimental studies. Most studies have relied on rat rather than human hearts and the methods used have tended to be either biochemical or morphometric (Zak, 1973, 1974; Katzberg et al. 1977; Dowell & McManus, 1978; Korecky & Rakusan, 1978; Limas & Limas, 1978; Anversa et al. 1980; Cluzeaut & Maurer-Schultze, 1986; Mandarim-de-Lacerda & Costa, 1993).

The accuracy and efficiency of morphometric estimates can be improved considerably by using a stereological counting device known as the disector (Sterio, 1984; Gundersen, 1986). This can be employed to count nuclei, and other particles, in an unbiased way because it allows particles to be sampled with equal probability in 3-dimensional (3D) space, a fundamental condition. Before the advent of the disector, nuclear counting relied on isolated 2D probes (tissue sections or section planes) but these sample nuclei with a probability which is determined by their linear dimensions perpendicular to the section plane. In an attempt to compensate for this, the earlier methods were model-based. Specifically, they relied on simplifying assumptions about the orientation, shape and size of particles, whether nuclei or cells (see, e.g. Abercrombie, 1946; Weibel & Gomez, 1962; Korecky & Rakusan, 1978; Weibel, 1979; Anversa et al. 1980).

In its original form, the disector was defined by 2 sections separated by a distance roughly one-third to one-quarter of the linear dimension of particles perpendicular to the plane of sectioning (Sterio, 1984). This approach, the 'physical disector', is employed here to estimate numbers of myocyte nuclei in human myocardia. Because this approach allows estimation of absolute number, it is preferable to those which are limited to the packing densities of nuclei or of their sectional images on tissue sections (e.g. Anversa et al. 1980; Cluzeaut & Maurer-Schultze, 1986; Mandarim-de-Lacerda & Costa, 1993). The ultimate purpose is to establish a protocol which can be employed to test for

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patterns of cardiac growth in normal and pathological human specimens. It must be emphasised that the protocol is designed for dealing with archival material and does not represent an optimal scheme for dealing, say, with hearts from experimental animals obtained for a specific purpose.

MATERIALS AND METHODS

Heart provenance

In order to establish and test a reproducible sampling protocol, 6 postmortem hearts from a collection housed in the Department of Pathology were examined. Two hearts were from apparently normal subjects and 4 from 'abnormal' subjects (see Table 1). Of the normal subjects, the first (subject 1) was a postmature individual who died at 42 wk of antepartum asphyxia with massive meconium inhalation. The second (subject 2) died at age 9 postnatal months from an accidental aortic laceration via the oesophageal wall caused by a swallowed foreign body. Of the abnormal subjects, the first (a fetus, subject 3) was obtained at 31 wk of gestation having died from septicaemia associated with maternal uterine and placental infection. The heart, great vessels and myocardium appeared entirely normal and there was no external or organic evidence of congenital malformations. The second (subject 4) died at term (40 wk of gestation), the recorded cause of death being a recent infarction of 10–15% of placental volume coupled with possible but slight postmaturity. Again, the heart, great vessels and other organs appeared normal at postmortem. The third (subject 5) died at term + 7 d and showed minor congenital abnormalities with major developmental delays despite normality of overall size and weight. There was right ventricular hypertrophy, constriction of the aortic outflow tract and probable septicaemia. The fourth abnormal subject (subject 6) was a sudden infant death syndrome (SIDS) individual (postnatal age 8 wk + 5 d) who had been born at low birth weight. There was evidence of infection (paraflu 3) and of inhalation. Except for a slightly dilated right ventricle, the heart and great vessels appeared entirely normal.

Table 1. *Case details of the subjects used in the present study*

Gestational age (wk)	Postnatal age (wk)	Weight (kg)	Growth arrests	Cause of death, etc.
		Normal subjects (1, 2)		
42	0	4.85	No	Antepartum asphyxia Meconium aspiration Postmaturity
40	40	7.46	No	Aortic laceration Impacted foreign body
		Abnormal subjects (3–6)		
31	0	0.69	Yes	Intrauterine infection Intrauterine growth retardation
40	0	3.06	Yes	Placental infarction Developmental arrests
40	1	3.86	Yes	Minor malformations Major arrests Maternal smoking
40	8	3.56	Yes	Sudden infant death Low birthweight with poor weight gain

Sampling

Following removal at autopsy, the heart was examined by a standard procedure. Each was opened by two lateral incisions in order to examine morphology and evacuate all blood clots. The heart, attached to the pericardium, was then fixed in 5% buffered calcium-formalin solution. After fixation, the ventricles were dissected free of atria, vessels and fat and then weighed following the method of Fulton et al. (1952). The free border of the right ventricle was removed first and weighed. The left ventricle and the residual septal portions of both ventricles were then weighed separately. For present purposes, the weight of the left ventricle included that of the interventricular septum.

From the bank of fixed hearts of known weights, ventricles were cut into smaller pieces from which random samples (3–5 pieces per ventricle selected by the lottery method) were taken for embedding in JB4 acrylic resin. Blocks (1 block per tissue piece, 3–5 blocks per ventricle) were selected at random in similar manner. Using Latta-Hartmann glass knives (10 mm wide), they were cut on a Reichert-Jung Autocut microtome at a nominal thickness setting of 3 μm to provide ribbons of 4–5 serial sections. This thickness satisfied the requirement that the separation of paired section planes of physical disectors should be less than the smallest linear dimension of the particles being counted (Sterio, 1984; Gundersen, 1986). Ribbons were mounted on glass microslides and stained with haematoxylin and eosin (Bancroft & Stevens, 1990).

Light microscopical fields were selected in a systematic random fashion (Gundersen & Jensen, 1987) from sites on 'reference' sections and matching sites on consecutive 'look-up' sections. Each reference and look-up section constituted a pair of parallel sections separated by one section thickness, i.e. a

physical disector (Sterio, 1984; Gundersen, 1986; Mayhew, 1992). Colour slide transparencies were made from the recorded fields and projected onto a white screen at a linear magnification of $\times 2580$ using a stage micrometer scale as a calibration standard. Flat projection of images was checked using the circle which surrounded the micrometer scale.

Pairs of transparencies were viewed side-by-side using double-projection. In each case, the reference section was projected onto a piece of stiff white card on which was drawn a systematic set of unbiased 'forbidden line' counting frames (Gundersen, 1977; Simpson et al. 1992) each of which sampled a tissue subfield of area 81 cm^2 (equivalent to an area of 1217 μm^2 on the scale of the specimen). Between 10 and 24 (mean 15) subfields were sampled from all the section pairs drawn from a given ventricle.

Counting myocyte nuclei

The myocardium contains nuclei from different sources which may be classified, for convenience, as myocytic and interstitial. The interstitial cell compartment include endotheliocytes, fibroblasts, mast cells, mononuclear phagocytes and smooth muscle cells (Long et al. 1991; Weber & Brilla, 1991). Here, only the numerical density of nuclei belonging to ventricular myocytes was estimated. The estimation device used was the disector (see Sterio, 1984; Gundersen, 1986; Simpson et al. 1992; Mayhew, 1992; Mayhew et al. 1994). The myocyte nucleus was adopted as a convenient counting unit and nuclei were considered eligible for counting if they met the first condition that they should not touch the forbidden lines (or their extensions) of the counting frames. Of those meeting this condition, we actually counted only those which appeared on reference sections but *not* on the paired look-up sections. This number, symbol Q^-, is contained in a tissue volume determined by the product $A \times t$, where A is the area bounded by the unbiased counting frame (1217 μm^2) and t is the mean distance between section planes (equivalent to mean section thickness, see below). Therefore, nuclear number per unit volume, N_v, is estimated (Sterio, 1984) as follows:

$$\text{est}\, N_v = Q^-/(A \times t).$$

Estimates of Q^- and A for each ventricle were summed over all disector subfields. To estimate the mean distance between disector section planes, we used a method described elsewhere based on the difference in height of presectioned and postsectioned resin blocks measured with a precision steel micrometer screw gauge (Simpson et al. 1992). Using this method, the mean thickness of sections cut at the microtome was estimated to be 2.87 μm. This distance may not be equal to the thickness of section on the microslide but any relative bias is common to all hearts and so comparisons remain valid.

Numbers of nuclei per ventricle were estimated by multiplying N_v by the volume of the corresponding myocardium. The latter was computed after first estimating the relative volume of ventricle occupied by myocardium and multiplying this by ventricular volume. The former was estimated by test point counting (see Mayhew, 1992) using sections from the set cut for the nuclear counts. Ventricular volume was determined from ventricular mass assuming a specific gravity of 1.05 g/cm^3.

Values of total nuclear number obtained in this way are biased because ventricular mass was measured on fixed but not processed tissue whilst N_v was determined for fixed and processed tissue. Again, since the relative errors are common, the numbers have real comparative worth. Finally, the number of nuclei is equal to myocyte number only if each myocyte has, on average, one nucleus. However, there is evidence that some cardiac myocytes may be binucleate in vivo (Katzberg et al. 1977; Anversa et al. 1980) and in vitro (Chacko, 1973).

Statistics

For each ventricle, individual quantities were estimated where necessary after summing over all subfields. The coefficient of error (CE, equal to the standard error expressed as a fraction of its mean) for the estimated numerical density of nuclei in myocardium, $CE(N_v)$, was calculated by the method described in Braendgaard et al. (1990), assuming independent sampling. On 2 ventricles, repeat estimates of total nuclear number were made so that we could assess the reproducibility of estimation. Again, this was expressed as the coefficient of error, $CE(N)$. All data were handled and analysed using the Unistat statistical software package (version 4.50, Unistat Ltd, London) on a Viglen Genie MiniTower 4DX266 PC.

RESULTS

Weights and relative volumes

For normal subjects, body weights were 4.85 kg (42 wk of gestation) and 7.46 kg (9 months postpartum). Corresponding ventricular weights were: left – 14.4 g and 18.7 g; right – 8.0 g and 6.3 g, giving

Table 2. *Numbers of myocyte nuclei (× 10⁹) in left and right ventricles of 6 subjects*

Age (wk)*	Right ventricle	Left ventricle	Both ventricles
Normal subjects			
42	2.61	7.45	10.06
40+40	2.58	6.84	9.42
Abnormal subjects			
31	0.37	1.09	1.46
40	2.13	4.60	6.73
40+1	2.70	3.28	5.98
40+8	1.59	4.56	6.15

* The age is the gestational age + postnatal age in weeks. Where no second figure is given, the child was stillborn.

a left/right weight ratio of 1.8–3.0 g/g. The fraction of ventricular volume occupied by the myocardium was 91–96% (mean 94%) on the left and 91–95% (mean 93%) on the right.

In the abnormal subjects, body weights were 0.69 kg (31 wk gestation), 3.06 kg (term), 3.86 kg (7 d postpartum) and 3.56 kg (8 wk postpartum). Left ventricles weighed 1.5 g, 8.1 g, 11.7 g and 13.7 g whilst the weights of right ventricles were 0.84 g, 5.1 g, 7.2 g and 4.8 g, respectively. The corresponding weight ratios fell in the range 1.6–2.9 g/g. The volume fractions of myocardium tended to be lower than in normal subjects, i.e. 77–94% (mean 86%) on the left and 75–93% (mean 86%) in the right ventricle. The implication is that total myocardial volume per ventricle tended to be smaller in these hearts.

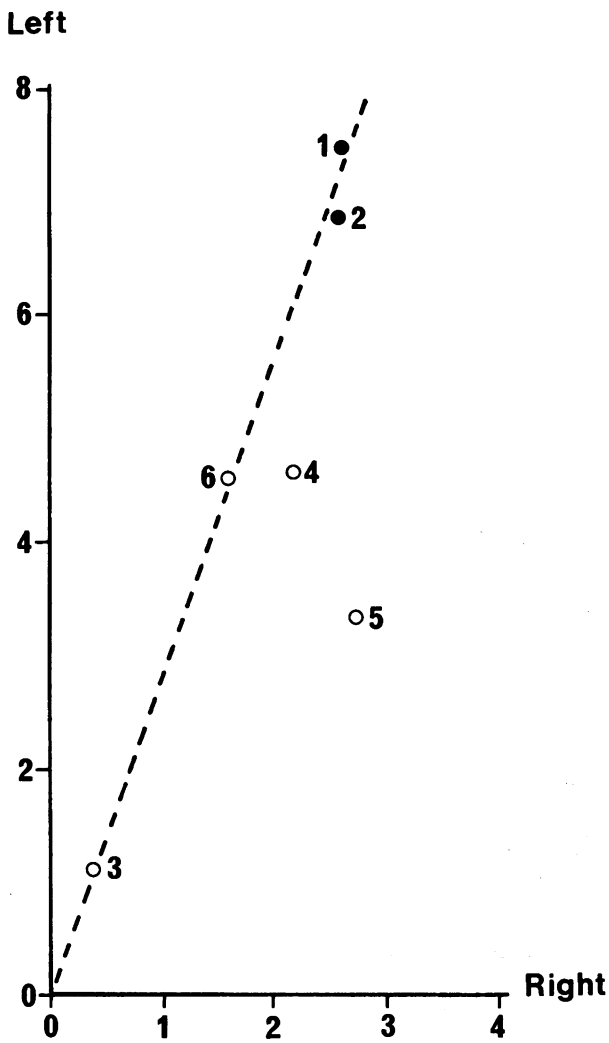

Fig. 1. Relationship between numbers of myocyte nuclei (× 10⁹) in left and right ventricles of 2 normal (1, 2) and 4 abnormal (3–6) subjects. Purely for the sake of illustration, the indicated line is drawn through the origin to the mean values for the 2 normal subjects (left ventricle 7.15×10^9 nuclei; right ventricle 2.60×10^9 nuclei). Subject 3 died of intrauterine infection. Subject 4 showed developmental arrests associated with placental infarction. Subject 5 showed minor congenital malformations and several major developmental arrests. Subject 6 died of sudden infant death. Subjects 4 and 5 have smaller left:right ratios. In the latter case, there was evidence of right ventricular hypertrophy at autopsy.

Nuclearity

Absolute numbers of myocyte nuclei per ventricle are summarised in Table 2 and Figure 1.

In normal hearts, the density of nuclei in fixed and processed myocardium was 4.0–6.0 (mean 5.0) $\times 10^8$ per cm^3 in the left and 4.1–4.7 (mean 4.4) $\times 10^8$ per cm^3 in the right ventricle. The apparently higher packing density in the left myocardium was not significant (paired $t = 0.44$ for 1 degree of freedom). However, absolute numbers were greater on the left (paired $t = 15.69$; $P < 0.05$). The mean (S.E.M.) difference between ventricles amounted to 4.6 (0.29) $\times 10^7$ nuclei in favour of the left. The ranges of nuclear packing densities in abnormal hearts were 4.3–8.2 $\times 10^8$ (left) and 4.5–5.2 $\times 10^8$ (right).

Figure 2 illustrates the relationship between age and absolute nuclear content (expressed as log N). The data are consistent with the notion that proliferation of myocyte nuclei ceases at, or before, term and that the subsequent increase in myocardial volume occurs by hypertrophic and/or interstitial growth. In addition, numbers of nuclei in two of the abnormal subjects (subject 5 – left ventricle only, and subject 6 – both ventricles) tended to be lower than might be expected on the basis of age alone. Subject 5 showed minor congenital abnormalities with major developmental arrests as assessed by gross examination and histology of various organs. In addition, there was probable septicaemia. Subject 6 was a previously low birthweight sudden infant death at 8–9 postnatal wk showing evidence of paraflu virus infection.

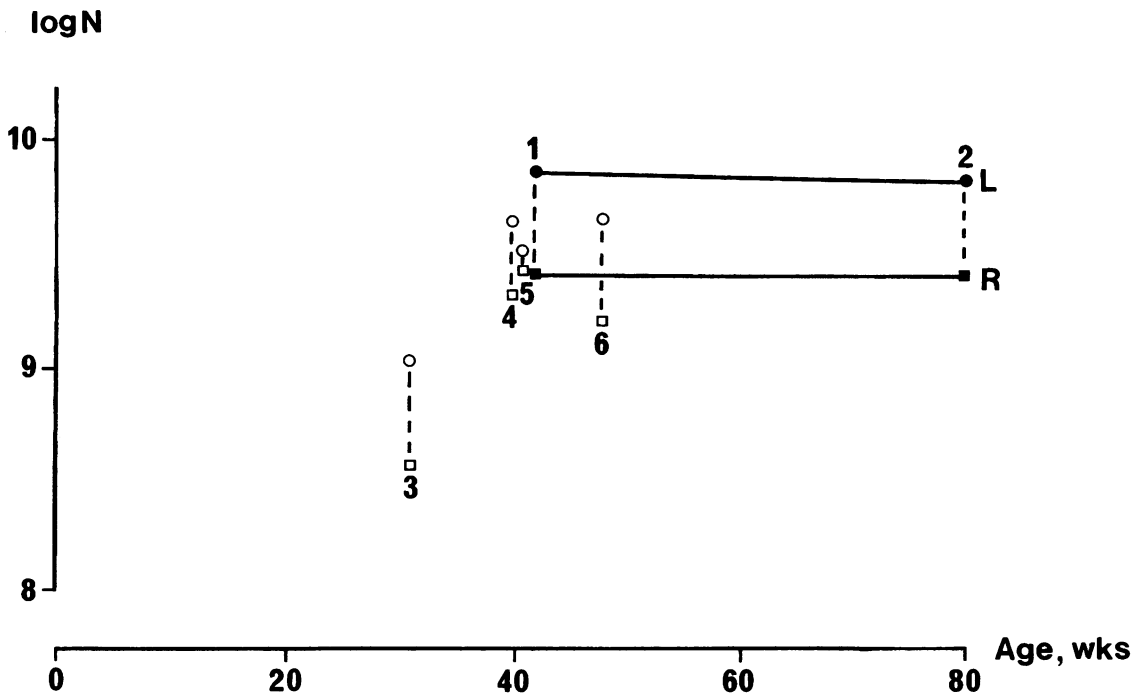

Fig. 2. Relationship between nuclear number (expressed as log N) and age (gestational + postnatal) for 2 normal (1, 2) and 4 abnormal (3–6) subjects. The continuous lines joining normal subjects indicate no postnatal increase in nuclear number in either the left (L) or right (R) ventricle. Vertical broken lines join paired ventricles (left, circles and right, squares) from the same subject. Two subjects (5, 6) appear to have reduced numbers for their age: subject 5 showed minor congenital malformations with several major developmental arrests and subject 6 was a sudden infant death.

Experimental errors

The predicted coefficient of error for nuclear numerical density, CE(N_v), varied between ventricles from 0.129 (or about 13%) to 0.224 (22%) with a mean of 0.178 (18%). The mean CE(N_v) was calculated from the following relation:

$$\text{mean } CE^2(N_v) = [CE1^2 + CE2^2 \ldots + CEn^2]/n.$$

Repeat measurements of total number were made on ventricles from 2 subjects. In the first case, the CE(N) amounted to 0.049 (5%) and in the second to 0.107 (11%), giving a predicted overall CE(N) of 0.083 (8%).

DISCUSSION

The present study has established a sampling regime for estimating the numerical density of myocyte nuclei from physical disectors. The predicted coefficient of error for numerical density estimates was about 18% but the resulting estimates of absolute number had an error of only 8%. Using this regime, packing densities of nuclei in the left and right ventricles have been found to be similar but with absolute numbers greater on the left, reflecting its greater myocardial volume. In both ventricles, numbers appeared to increase up to term (although inadequate sample size precludes a firm statement on this point) but to remain constant thereafter, the postnatal increase in myocardial mass being attributable to cell hypertrophy and/or interstitial growth. In a sudden infant death individual, the numbers of nuclei in each ventricle appeared to be smaller than normal for the age.

The sampling design employed in this pilot investigation is not for general usage but for use with archival material. It was not optimally efficient and could be improved in terms of general utility and efficiency. For direct estimates of number from material collected expressly for the purpose, a better design would be to employ the fractionator (Gundersen, 1986; Mayhew, 1992), thereby avoiding the problems associated with section thickness estimation, volume estimation and tissue distortions during fixation and processing. Where the disector is used but volumes need to be determined without resort to weight and specific gravity, the Cavalieri principle of volume estimation could be utilised (Gundersen & Jensen, 1987; Mayhew, 1992).

In normal subjects, the observed subject-to-subject variation (expressed as a coefficient of variation, CV) in nuclear packing density amounted to about 0.209 when averaged over left (CV = 0.278) and right (CV = 0.099) ventricles. It follows that the CE(N_v) of

0.178 accounted for roughly $0.178^2 \times 100/0.209^2$ or 73% of the total experimental variance, the residuum being attributable to natural differences between subjects (i.e. biological variation). However, this biological variation is misleading since it is based on a heterogeneous mix of hearts of different ages and pathological status. In a well executed study, it is preferable to reduce estimation error so that $CE^2(N_v)$ does not exceed the biological variation, $CV^2(N_v)$. To achieve this aim, sensible future refinements might include increasing the size of sampling frames or decreasing the magnification, thereby capturing more nuclei within a given frame. In addition, it would be sensible to increase the number of frames to increase the total number of nuclei counted. These changes would help to reduce $CE(N_v)$ and, hence, $CE(N)$. Often in stereology, CE values of 5–15% can be obtained by sampling 100–200 particles per individual.

We counted 1–3 (mean 2) nuclei per disector frame and 17–56 (mean 29) nuclei per ventricle. Assuming for the sake of calculation that sampling is independent and the myocardium is homogeneous, the maximum predicted CE for a total count of 29 nuclei per ventricle is $1/(29)^{0.5}$ or about 19%. This is reasonably close to the calculated $CE(N_v)$ of 18% suggesting that the intrasubject error introduced by systematic sampling was low. Since $CE(N)$ depends on both $CE(N_v)$ and the precision of estimation of myocardial volume, it suggests further that myocardial volume was estimated with relatively high precision.

The present estimates are valid for comparative purposes and so the methods may be valuable as a means of studying banked material processed in a standardised fashion. However, such material may produce distorted estimates of actual numbers because of fixation, processing and other artifacts. Possible improvements include trying to correct for processing biases or changing to glutaraldehyde-fixed, resin-embedded tissues in which processing distortions are minimal. However, the fact remains that unbiased estimation of total number requires that values of nuclear numerical density and myocardial volume are obtained from material fixed and processed in the same way.

Present results suggest that proliferation patterns are similar in both ventricles with numbers predominating on the left. However, in the SIDS infant, numbers in each ventricle appeared to be much lower than normal for age, i.e. there was retarded proliferative growth. The same appeared to be true of the left ventricle, but not the right, in one other abnormal infant.

From myocardial volumes and nuclear numbers, it is possible to compare the relative volumes of myocardium per nucleus. In normal subjects, this volume was 1680–2490 (mean 2200) μm^3 when averaged over both ventricles. The corresponding values for the abnormal subjects were 1220–2630 (2000) μm^3. Apparent differences between ventricles were not significant. From estimates of myocyte length (80–100 μm) and diameter (15 μm) in adult human hearts, mean myocyte volume is calculated to be about 14000–18000 μm^3 (see, e.g. Leeson & Leeson, 1976; Williams et al. 1989). Our present estimates of 1220–2630 μm^3 are not strictly comparable because (1) they are distorted by tissue shrinkage during processing, (2) they are based on fetal/infant rather than adult hearts, and (3) the volume includes that of all myocardial elements and not just that of myocytes. Nevertheless, these figures are consistent with the notion that postnatal growth of myocardium is hypertrophic and/or interstitial rather than hyperplastic.

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