Localization, cultivation and purification of sinoatrial nodes isolated from newborn rabbits**☆

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Abstract

BACKGROUND: The method of culture purified sinoatrial node cell is important in investigating its ultrastructural characteristics and autorhythmic mechanisms. However, the corresponding method has not been standardized.

OBJECTIVE: To summarize the localization, cultivation and purification of sinoatrial nodes isolated from newborn rabbits, and to study the morphological characters of primary cultured pacemaker cells.

METHODS: Hearts of the newborn rabbits (within 24 hours) were embedded in paraffin for hematoxylin-eosin staining. The location of sinoatrial nodes was observed under an optical microscope, the morphology of sinoatrial nodes cells were observed by light microscope and electron microscope.

RESULTS AND CONCLUSION: Sinoatrial nodes localized in the anterior wall of the superior vena cava and the posterior laterial wall of right atrium. There was about 0.32 mm between its lowest point and sulcus terminalis. Three distinct types of cells were observed among the cultured cells of sinoatrial nodes: spindle, spider and polygon. The spindle cells occupied the greatest proportion of the cultured cells (59.6±7.3)%. The spontaneous contraction frequency of spindle cells was the highest among the constracting cells (145±9) times per minute. The ultrastructure observation showed that myofibrils and other organelles in spindle cells were sparse and significantly decreased in number compared with triangle cells. There was no significant difference between triangle cells isolated from sinoatrial nodes and from atrial muscle. Sinoatrial nodes could be harvested along the anterior root of the superior vena cava down to the posterolateral sulcus. Among the cultured cells from neonatal rabbit sinoatrial nodes, the spindle cells with small body and fast pulse frequency are pacemaker cells.

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INTRODUCTION

For years, major problems in sinoatrial node research, which result from more reports in general and tissue level but less in cellular and molecular level, are small size, special cytoarchitecture and intricate electrophysiological activity of sinoatrial node (SAN)[1-2]. Single, spontaneously active sinoatrial node cells purified *in vitro* are indispensable for morphological and functional investigations. So far, not merely this job has been reported rarely globally, but also the results are quite different[3-9]. Based on the precise localization of SAN, this study aimed to offer consistently feasible method in single sinoatrial node cells purification for further investigation in ultrastructural characteristics and autorhythmic mechanisms.

MATERIALS AND METHODS

Design

In vitro cytological observation.

Time and setting

The experiment was performed at the Henan Provincial Key Laboratory, Henan Key Laboratory of Tissue Engineering, Xinxiang Medical University, between March 2009 and July 2009.

Materials

Totally 16 newborn New Zealand rabbits (within 24 hours), male or female, were supplied by Animal Center of Xinxiang Medical University. Protocols were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of the People's Republic of China in 2006[10].

Main reagents and instruments are as follows:

Reagent and instrument	Source
Dulbecco's Modified Eagle Medium (DMEM)	Gibco, USA
Fetal bovine serum	Hangzhou Sijiqing, China
Ethylenediamine tetraacetic acid (EDTA), hydroxyethyl piperazine ethanesulfonic acid (HEPES), 5-bromodeoxyuridine (BrdU)	Hyclone, USA
Light and Inverted phase contrast microscope	Nikon, Japan
Transmission electron microscope	Hitachi, Japan

Methods

Morphological observation of newborn rabbit SAN

A midline thoracotomy was performed on 8 rabbits after deeply anesthetized to expose the beating heart. Hearts were taken off and carefully abcised distal end of anterior vena cava, posterior vena cava and other large vessels at cardiac base. Heart tissues were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), processed for paraffin embedding and sectioned as serial sections (6 μm) in the transverse plane. After Hematoxylin-eosin (HE) staining, which was performed as follows[11]: deparaffin and rehydrate slides, slightly overstain the sections with hematoxylin 3−5 minutes, remove excess stain in tap water for 2 minutes, differentiate-restain a few seconds in acidic alcohol until sections look red, rinse in tap water, blue in bicarbonate until nuclei stand out sharply blue for 2 minutes, rinse in running tap water, dehydrate and clear, coverslip with cytoseal, the sections were observed and photographed with light

microscope to localize SAN, then measured left-to-right diameter and inner-to-outer diameter by Eyepiece Micrometer and estimates its height according to the sections from top to bottom existing SAN.

SAN isolation and purified culture

Under the stereomicroscope and aseptic conditions, the sinus node region near the junction of the right atrium (RA) free wall and the atrial appendage near the crista terminalis, approximately 1−2 mm in diameter, was surgically removed according to the location of SAN from 8 newborn New Zealand rabbits. The margins of these sinus node portions would unavoidably include cells from the proximal superior vena cava and the crista terminalis. After the completion of dissection, tissue fragments were rinsed in Hank's solution and minced with fine dissecting scissors into smaller fragments in DMEM solution. The method of isolating single SAN cell was adapted from procedures used previously by Wang *et al*[5]. The medium was replaced every 24 hours, Bromodeoxyuridine (BrdU) was added at the early 5 days, and the changes were observed at the same time every day. Ventricular myocardial cells were also cultured for purposes of comparison.

Morphological observation of cultured SAN cells

Calculate cell size and pulse frequency of single SAN cell under invert microscope. Observe glass coverslips containing SAN cell which were fixed in ethanol and dyed with HE-staining under light microscope. Cultured cells were digested by 0.08% trypsin into the cell suspension, centrifuged, and the pellet was fixed with 2.5% glutaraldehyde and sent to electron microscope room for dehydrating, embedding and slicing.

Main outcome measures

SAN location, size, pulse frequency, as well as the morphology of SAN cells were observed by light microscope and electron microscope.

Design, enforcement, and evaluation

This study was designed by the first author, performed by all authors, and evaluated by the third author. All authors received professional training.

RESULTS

Morphological observation of newborn rabbit SAN

SAN stain less intensely than cardiac myocytes. Under high-power field, densely and uniformly distributed SAN cells exhibited small volume, round shape, large nucleus and light staining cytoplasm (Figure 1). Serial section observation shows the whole SAN resembled inverted triangle with the superior part surrounds the anterior vena cava root and occupied about half of the superior vena cava perimeter (0.68±0.23) mm and the inferior part descended posteriorly and laterally along sulcus (1.51±0.38) mm. The distance from SAN in the vein to angulus between the superior vena and the Right atrial appendage was (0.49±0.17) mm. The lowest point located 0.32 mm from posteriolateral sulcus and spreaded from the epicardial to endocardial with (0.07±0.02) mm thickness.

Figure 1 Sinoatrial node of newborn rabbit was stained weakly compared with peripheral atrial myocardium (×40)

Morphological observation of purified single SAN cells

Invert microscopic observation: Cells were round or rod-shaped at first. Thirty minutes later, some cells started to grow adherent. They were processed differential attachment twice, 48 hours later inverted microscope observation showed various frequency beating cells, most of which were small, spindle-shaped. Three days later, cells began to emerge 3−5 projections of different length originating from the central body. The purified SAN cells were spindle, spider, and polygonal-shaped in morphology whereas atrial myocytes were spider and polygonal-shaped (Figures 2, 3). The spindle cells comprised the greatest proportion and exhibit fastest beating frequency. Spider cells were differentiated from spindle cells in having bigger volume, more projections, and contracting less vigorously. The least proportion polygonal cells had less projections, larger bulk, lighter cytoplasm and no-beating function.

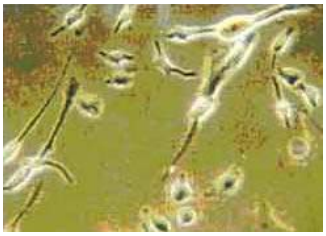

Figure 2 Types of cultured cells at 6 d, more spindle cells, fewer spider and polygonal cells (×200)

Figure 3 Observation of cultured cells at 6 d (×200)

Light and transmission electron microscopic observations: There were 3 kinds of different morphological characteristics of SAN cells: ①Spindle cells: medium sized nucleus, round in shape, located in the center. Cytoplasm stained lightly. Electron microscope showed infrequent mitochondria, undeveloped endoplasmic reticulum and Golgi apparatus,

immature myofibril, non-sarcomere (Figure 4). ②Spider cells: medium sized nucleus, elliptic in shape, also located in the center. Cytoplasm was rich in mitochondria, endoplasmic reticulum, Golgi apparatus and well-developed myofibril paralleling along long axis. ③Irregular cells: abundant mitochondria, Golgi apparatus, non-myofibril.

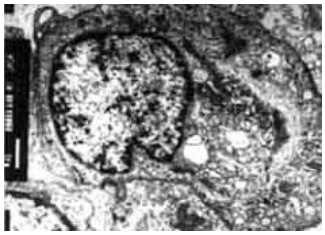

Figure 4 Undeveloped myofibrils and other organelles in cultured spindle cells at 6 d (×4 000)

DISCUSSION

At present, many researchers value morphological and electrophysiological investigation from purified single SAN cell, but they have been troubled by how to position and cut SAN accurately[12-15]. Due to dividing ability of Myocardial cells in newborn rabbits (within 24 hours) still exist, this study chose to culture SAN cells of newborn period. We found the position of SAN in newborn rabbits is higher than that of adult rabbits[16]. Newborn rabbit SAN presented inverted triangle, mostly situated in the anterior wall of the superior vena cava. It has great significance for physiological, pharmacological experiment of SAN by excisinig SAN precisely under stereomicroscope to increase the proportion of cultured sinoatrial node pacemaker cells and decrease the proportion of ordinary myocardial cells as well as fibroblasts on the other hand.

Single SAN cell culture indicate that the number and length of pseudopodium changes great during early and late cultured period. The large proportion of spindle cells *in vitro*, which were classified as pacemaker cell by their fast beating frequency, limited myofibrils and cellular organ, accord with high density of rabbit pacemaker cells in histological sections. Spider-shaped cells with well-developed myofibrils, complete sarcomere and Z line should be the myocardial cells. The least proportion polygonous cells were thought to be fibroblasts on account of features such as less projections, large bulk, plain periphery, light cytoplasm, non-beating and non-myofibril. The morphological identification of the SAN has not yet been agreed. Marvin[3, 17] take the small and fast-beating spindle cells as sinoatrial node cells, while the large, slow-beating polygonous cells as atrial myocardium. Zhang[4] consider that polygonal cell, spindle cell and triangular cell as pacemaker cell, transitional cell and ordinary atrial myocytes respectively. Nathan[18] observed cultured adult rabbit SAN cells present spindle shape and spherical shape. Different animal species, ages, experimental conditions and researchers may affect morphological observations of cultivated sinoatrial node cell on account of the unique position and intricate composition of SAN. Therefore, uniform standards about how to accurately procure SAN should be discussed so as to increase proportion of pacemaker cells in cultured cells and identify morphological feature.

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乳兔窦房结定位、取材及纯化和培养**☆

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摘要

背景：分离和纯化培养的窦房结细胞是研究其超微结构及自律性的重要条件，然而，相应的培养方法尚未标准化。

目的：建立乳兔窦房结进行定位、取材和纯化培养方法，观察并判断培养窦房结细胞中起搏细胞的形态特点。

方法：新生 24 h 内乳兔心脏连续石蜡切片，苏木精-伊红染色，光镜下判断窦房结的位置并测定，计算其大小，光镜、电镜下观察纯化培养的窦房结细胞形态。

结果与结论：乳兔窦房结位于上腔静脉根部前壁向下至界沟后外约 0.32 mm 处。培养的窦房结细胞主要有 3 种形态细胞：梭形、蜘蛛形、多边形，其中梭形细胞最多，占(59.6±7.3)%，搏动频率最快为(145±9)次/min，肌原纤维稀少，细胞器不发达。蜘蛛形和多边形细胞则和培养的心房肌细胞培养无差异。沿上腔静脉根部前壁向下至界沟后外侧可较精确地对窦房结进行取材。培养的乳兔窦房结细胞中，细胞体积小，搏动频率快，所占比率高的梭形细胞是窦房结的起搏细胞。

关键词：细胞培养；窦房结；上腔静脉；定位；乳兔

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课题的创新点：实验首先将乳兔整心从上至下作横位连续切片并进行苏木精-伊红染色。在显微镜下观察、测量窦房结左右径和内外径，并根据上下出现窦房结组织片数计算其上下高度，确定了窦房结取材的位置，培养后观察含量较高的梭形细胞为起搏细胞。

课题评估的“金标准”：实验发现在乳兔上腔静脉根部前壁向下至界沟后外约 0.32 mm 处取材进行细胞培养能获得高含量的起搏细胞。

课题的偏倚与不足：实验不足之处在于未能结合免疫组织化学及电生理方法进一步确定梭形细胞为起搏细胞。

提供临床借鉴的价值：培养纯化的窦房结细胞可为临床构建生物起博器提供细胞来源，为窦房结细胞的形态学和电生理研究提供帮助。

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