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# Intermittent hypoxia exposure prevents mtDNA deletion and mitochondrial structure damage produced by ischemia/reperfusion injury *

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**Abstract**: In the present study, polymerase chain reaction (PCR) was conducted to determine $mtDNA^{4834}$ deletion, and myocardial ultrastructure was visualized by electron microscope to see whether intermittent hypoxia (high altitude) adaptation exerts some action on mitochondria against ischemia/reperfusion injury. Myocardial ischemia/reperfusion in isolated perfused rat hearts induced severe damage to the ultrastructure of myocardial mitochondria and $mtDNA^{4834}$ deletion down to 87.5% of normoxia rats. After the rats were exposed to intermittent hypoxia (5 000 m; 6 h/d for 28 d), the myocardial structure was well reserved and $mtDNA^{4834}$ deletion dropped to 28.57% of control ($P < 0.05$). It is suggested that intermittent hypoxia adaptation prevents mtDNA deletion, and preserves normal structure of mitochondria, which would be beneficial to the maintenance of normal mitochondrial function, and increases tolerance of myocardium against ischemia/reperfusion injury.

**Key words**: mitochondrial DNA; polymerase chain reaction; ischemia/reperfusion injury; intermittent hypoxia; ultrastructure

# 间歇性低氧防止缺血再灌注损伤引起的线粒体结构损伤和 mtDNA 片段缺失 *

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**摘 要： 本文用离体 Langendorff 灌流大鼠心脏造成急性心肌缺血/再灌注损伤模型，观察间歇性低氧暴露保护心肌线粒体的作用。以聚合酶链式反应（PCR）方法和电子显微镜技术，观察线粒体 DNA （$mtDNA^{4834}$）片段缺失和超微结构的变化。大鼠暴露于模拟海拔 5 000 米低氧环境（6 h/d，28 d）明显降低 $mtDNA^{4834}$ 缺失的发生率（28.57%，*vs* 常氧对照组 87.5% $P < 0.05$）；而且能够明显减轻因缺血/再灌注引起的心肌线粒体肿胀、线粒体嵴断裂、消失；较好地维持了线粒体的正常结构和形态。结果表明，间歇性低氧暴露能有效防止缺血/再灌注引起的心肌线粒体损伤和 mtDNA 的片段缺失，此作用可能是间歇性低氧心肌保护作用的机制之一。**

**关键词：线粒体 DNA；聚合酶链式反应；缺血/再灌注损伤；间歇性低氧；超微结构**
**学科分类号：**Q463；Q494

Mitochondria are vital subcellular organelles, responsible for energy metabolism in cells. Myocardial tissue is typically aerobic and its metabolism is closely dependent on oxygen viability as confirmed by the abundance of mitochondria. The high-energy requirement is almost exclusively met with mitochondrial oxidative phosphorylation. This, in turn, leads to high sensitivity of myocardial cells to oxygen deficiency. Thus, mitochondria are likely to play a central role in the molecular events that lead to tissue damage during the conditions of oxygen deprivation[1]. Myocardial ischemia is a condition when the oxygen supply is not sufficient to meet the rate of mitochondrial oxidation. Recent study has revealed that somatically acquired mutation such as deletions in mtDNA causes bioenergetic deficit leading to dysfunction of the cell[2]. Analysis of mtDNA deletions in human hearts re-

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vealed that mtDNA damage was increased in chronically ischemic hearts[3]. Similar results were also observed in the model of constriction of canine coronary arteries[4]. Mammalian mitochondrial DNA (mtDNA) is approximately a double strand DNA molecule with a size of 16.5 kb, which encodes 2 rRNA, 22 tRNA, and 13 polypeptides of the enzymes involved in oxidative phosphorylation (OXPHOS) process[5]. Mitochondria produce ATP through OXPHOS and consume more than 90% of the oxygen in a cell. The mitochondrial genome has approximately a 10-fold higher rate of mutation than the chromosome DNA, and it is much more susceptible to oxidative damage[6]. Among numerous mutations in mtDNA, 4977-bp common deletion is a most frequent mutation observed in human myocardium during disease, and thought being correlated with cardiac ischemia damage caused by coronary atherosclerotic heart disease[7]. Similar to that in human mtDNA, a 4834-bp deletion has been identified in rat mtDNA; also, the frequency of this mutation is high in animal models of cardiomyopathy[8]. Using the model of Ameroid constriction of canine coronary arteries, Marin-Garcia found that specific mtDNA deletions similar to 5 kb human mtDNA deletions occur following canine myocardial ischemia[4].

The present study was designed to define the existence of $mtDNA^{4834}$ mutation induced by ischemia/reperfusion rat hearts through the method of polymerase chain reaction (PCR). The incidence of $mtDNA^{4834}$ mutation was compared between normal rat and rat exposed to intermittent hypoxia. The ultrastructure changes in rat hearts subjected to ischemia and reperfusion injury were also observed.

# 1 MATERIALS AND METHODS

1.1 *Animal models of intermittent hypoxia exposure*

Male Sprague-Dawley (150 ~ 180 g) rats were used in the experiments grouped randomly. Intermittent hypoxia exposure: the hypoxia group was put in a hypobaric chamber (simulating 5 000 m altitude over sea level, $PO_2$ 11.3 kPa) 6 h/d for 28 d (IH, $n = 7$) at temperature of 22 ~ 24℃. Two other normoxia groups were fed outside the chamber just as the rats subjected to hypoxia treatment. Some of the rats in the group were subjected to myocardial ischemia and reperfusion injury (IR, $n = 8$), and the rest served as normal control (control, $n = 5$). All animals were maintained under a 12 h light : 12 h darkness cycle.

1.2 *Isolated rat heart perfusion* The rat heart was rapidly removed and put into ice-cold Krebs-Henseleit (K-H) solution. Excess tissue was cut out, and the aorta was cannulated. The heart was then mounted on a Langendorff apparatus and retrogradely perfused with K-H solution (in mmol/L: NaCl 120, KCl 5.9, $MgSO_4$ 1.2, $CaCl_2$ 1.8, $NaHCO_3$ 25, glucose 11; aerated with 95% $O_2$ + 5% $CO_2$, pH 7.4 at 37℃). Perfusion pressure was maintained at 90 cm $H_2O$. After a perfusion of 15 min for stabilization, ischemia was induced and kept for 30 min by clamping the perfusion line. Reperfusion, achieved by restoring flow of K-H buffer at 90 cm $H_2O$ pressure, continued for 30 min. Autopsy was obtained for ultrastructure analysis. Then the left ventricle of heart was excised freshly for extraction of mtDNA.

1.3 *mtDNA extraction and purification* mtDNA was isolated from fresh tissue sample to avoid rupture of the intracellular organelles[9], and mtDNA was extracted by phenol : chloroform : isoamyl alcohol.

To estimate mtDNA damage, the $mtDNA^{4834}$ deletion (nucleotide pairs [np] 8203 through 12937) in total mtDNA was investigated by PCR amplification. The conservative region (where rare deletion occurred) of mtDNA (region ND-1 and 16-sRNA) was also amplified to qualify the PCR reaction system.

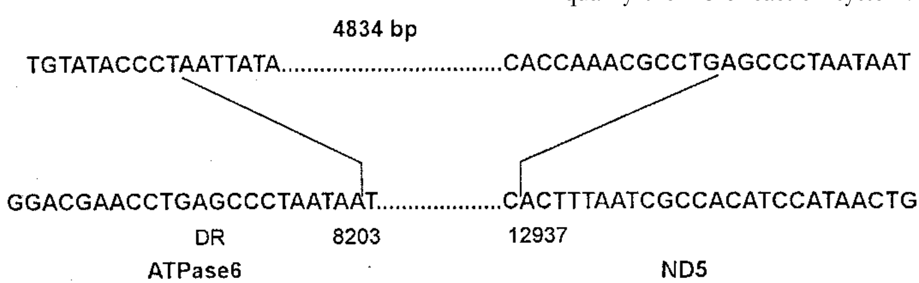


Fig.1. Detailed map of mtDNA deletion region which is amplified.

Table 1. PCR primer setting

| Region | Sequence | Predicted size |
|---|---|---|
| Conservative | P1 GCCTATCGAGCTTGGTGATA (1421-1440) | |
| | P2 GACCGTGCAAAGGTAGGATA (2003-2022) | 601 bp |
| Deletion | P3 GCGAAGCTTAGAGCGTTAAC (7682-7701) | |
| | P4 GCAGGCTTCCTTATCTCACT (13110-13129) | 597 bp |

The reaction of PCR was carried out separately, because the size of the products from the two sets of PCR amplification was similar. Both amplifications were applied to each mtDNA sample from one heart. The PCR mixture (50 ml) contained 25 pmol of each primer, 100 mmol/L of each dNTPs, 1 × PCR buffer (in mmol/L: KCl 50, TrisHCl 10, pH 8.4, $MgCl_2$ 2.5) and 2 U Ampli Taq DNA polymerase (Perkin Elmer Cetus, USA). PCR amplification was carried out for 35 cycles of 30 s at 95℃, 45 s at 54℃ (51℃ for conservative fragment amplification), and 45 s at 72℃ performed in a Perkin Elmer 9600 Thermal Cycler. The PCR products were electrophoresed on 2% agarose gel, visualized by ethidium bromide (EB) staining, scanned by a gel scan system (GDS 8000, Gene Co., USA), and analyzed with software GelWork 3.01 and Grab-It 2.59.

1.4 *Myocardium ultrastructure* A autopsy of rat myocardium was cut into 1 ~ 2 $mm^3$ and then fixed with 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer (pH 7.4), postfixed in 1% $OsO_4$, then dehydrated, infiltrated, and embedded in Spurr's resin. Ultrathin sections of 70-nm thick were made, stained by uranyl acetate and lead citrate for ultrastructural study and observed under a transmission electron microscope (Zeiss EM-902, German).

1.5 *Data analysis and statistics* Incidence of mtDNA deletion of normoxia group and intermittent hypoxia group was tested by *chi-square* test. Statistical significance was defined as $P < 0.05$.

# 2 RESULTS

## 2.1 Identification of myocardial mtDNA

mtDNA from rat myocardium of normoxia group (both IR and control group) and intermittent hypoxia group were of the same size around 16.5 kb. No contamination of genomic DNA and RNA was observed (Fig.2).

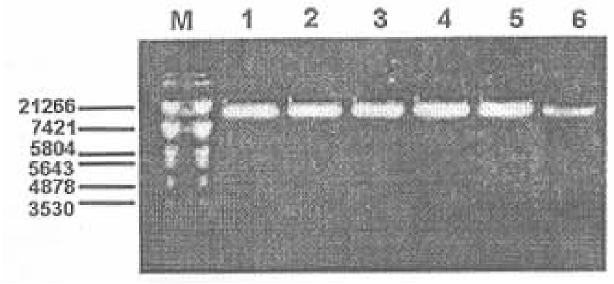


Fig.2. Total myocardial mtDNA from rat heart. M: DNA molecular weight marker (λDNA/Ecorl); 1,2,3: normoxia rats; 4,5,6: intermittent hypoxia rats.

## 2.2 mtDNA deletion induced by ischemia/reperfusion injury

Primer P3 and P4 flank the 16 bp direct repeated sequence (Fig.3) which would produce an approximately 5.5 kb PCR product from normal mtDNA genomes and a 597 bp product from deleted mtDNA. Reducing the PCR extension time will favor short products over long ones. Actually only short fragments (597 bp) instead of long ones (approximate 5 kb) were observed in the present study. Amplified products from mtDNA conservative region were achieved in all samples from different groups. No deletion product was found in rats without being subjected to global ischemia (Fig.4*A*). Obvious deletion amplified fragments were present in 7 (87.5%) out of total 8 normoxia rats subjected to ischemia reperfusion injury.

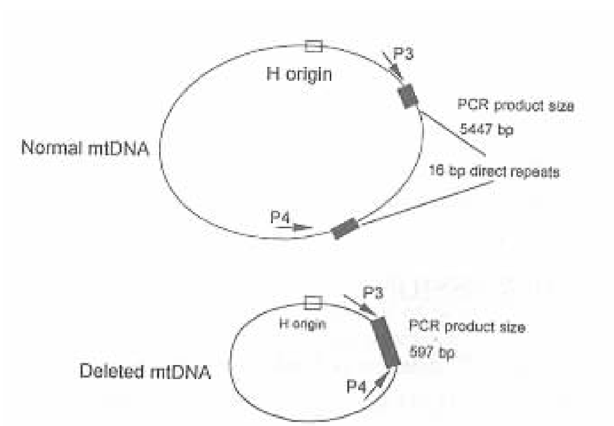


Fig.3. Strategy for preferential amplification of deleted versus normal mitochondrial DNA. Primer P3 and P4 flank the 16 bp direct repeated sequence (shaded rectangles) and would produce an approximately 5.5 kb PCR product from normal genomes and a 597 bp product from deleted genomes. Reducing the PCR extension time will favor short products over long ones.

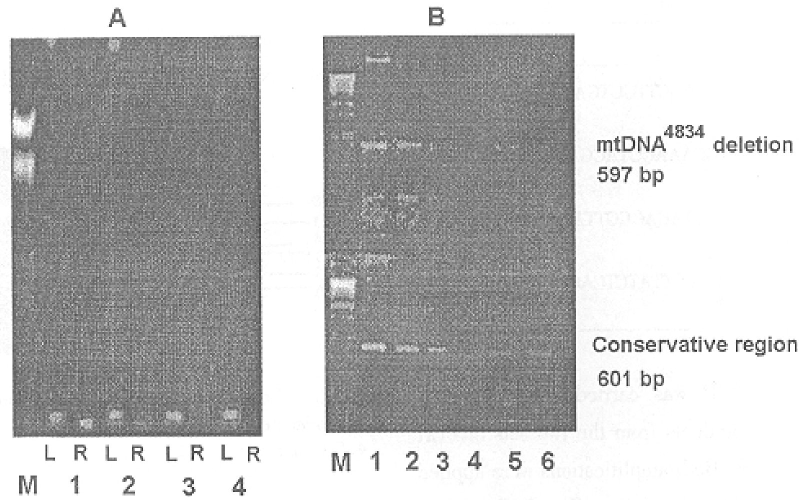


Fig.4. Detection of mtDNA[4834] deletion in rat heart. *A*. Comparison between normoxia rats. M: DNA molecular marker (λDNA/Hind); 1,2: rats subjected to ischemia/reperfusion; 3,4: rats of normal control; left lane (L): PCR product of conservative region; right lane (R): PCR product of deleted region. *B*. Comparison between normoxia rats and intermittent hypoxia exposed rats subjected to ischemia/reperfusion injury. 1,2,3: normoxia rats; 4,5,6: intermittent hypoxia exposed rats.

While for the rats with intermittent hypoxia training for 28 d, deletion was present in only 2 out of 7 rats (28.57%, $P < 0.05$ compared with normoxia group, Fig.4*B*).

### 2.3 Morphological change in myocardial ultrastructure

In normoxia IR rats, no flow ischemia and subsequent reperfusion induced severe damage to the ultrastructure of myocardium. The mitochondria swelling and interstitial edema were observed. The number of cristae was markedly reduced in mitochondria, and the empty bulbs of mitochondria increased (Fig.5*A*). In the samples from rats of IHA28 group, no significant damage in mitochondria was observed. The mitochondrial swelling and interstitial edema were extenuated, and the empty bulbs of mitochondria decreased obviously (Fig.5*B*).

## 3 DISCUSSION

mtDNA[4834] deletion is flanked by two 16 bp repeats (CCTGAGCCCTAATAAT) in normal mitochondrial genome mapping ATPase 6 and ND 5 (NADH) genes respectively at a distance of 4.8 kb. These repeats, positioned at nucleotide 8103 ~ 8118 and 12937 ~ 12952, encompasses a region that, both in the size and gene content, is analogous to that deleted in human mitochondrial genome[10]. The deletion region includes the coding sequence of subunits of ND 3, 4, 5 and 4L, of ATPase 6, and of a subunit of enzyme cytochrome oxidase (subunit Ⅲ). This region is essential for energy supply and cytochrome oxidase is the major site of the reduction of molecular oxygen in normal cells. The complete deletion critically affects the cell's energy metabolism[11]. It has been reported by Wallace that coronary artery heart disease is associated with dramatically elevated mtDNA4977 deletion (40 ~ 45 fold increase over normal control) in human[3]. A similar result was obtained in animal models subjected to ischemia induced by constriction of canine coronary arteries[4]. mtDNA deletion affects the process of OXPHOS, thus increases the production of free oxygen radicals (FOR). In turn FOR aggravates the deletion of mtDNA. This vicious feedback between mtDNA and OXPHOS affectes not only the energy supply system, but also the morphology and structure of myocardium[12]. Our study showed that ischemia and reperfusion damaged the structure of myocardial mitochondria and induced mtDNA[4834] deletion. When the rat hearts were preadapted to intermittent hypoxia, the incidence of mtDNA[4834] deletion was reduced significantly.

It has also been noticed presently that the structures of myocardial mitochondria are well preserved in preadapted

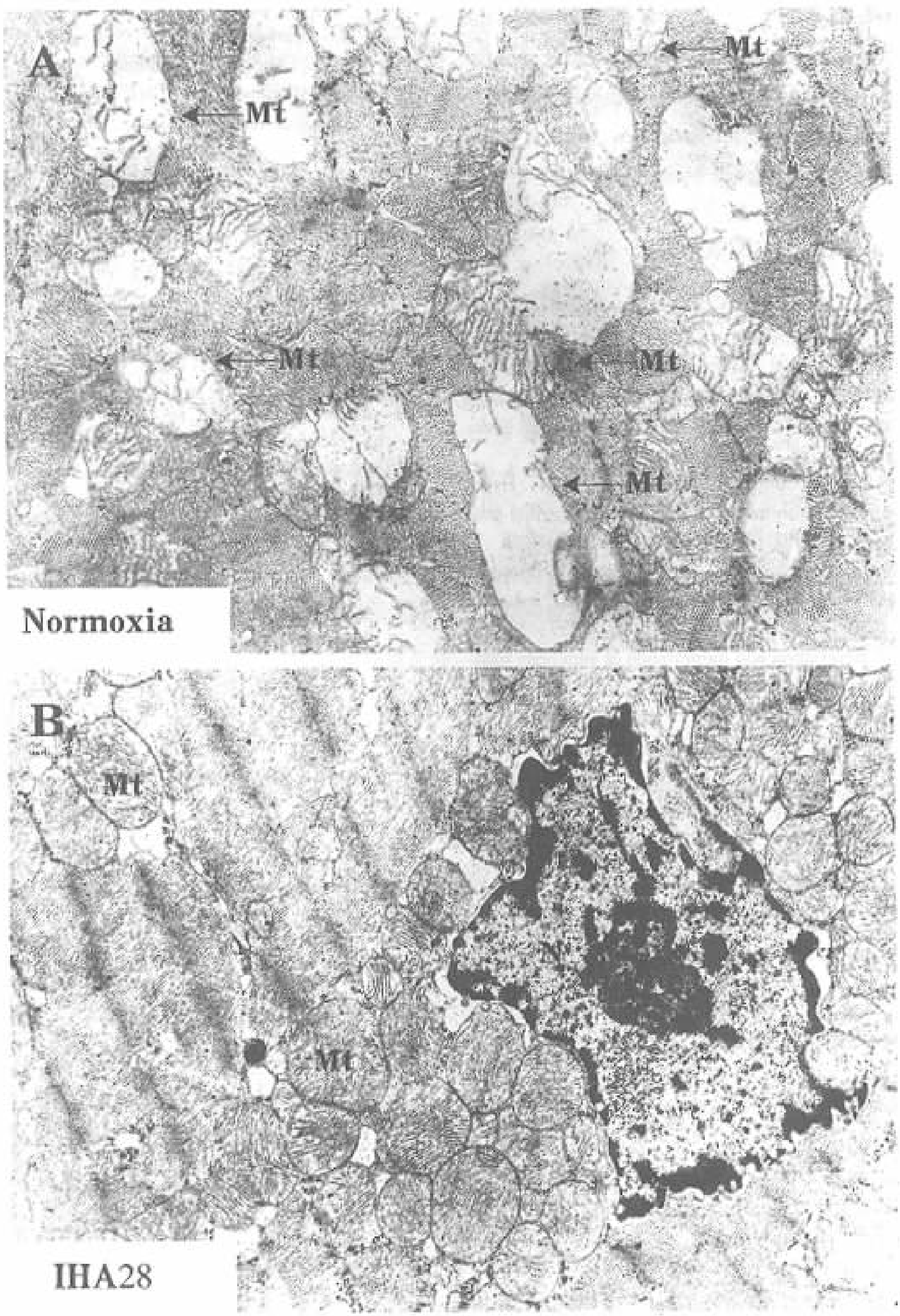


Fig.5. Ultastructure of rat myocardium. *A*. Ischemia/reperfusion induced ultrastructure damage in normoxia rat myocardium ( × 10000). Mt : mitochondrium. *B*. Intermittent hypoxia exposure (for 28 days) reserved the structure of rat myocardium subjected to ischemia reperfusion injury ( × 10000). Mt : mitochondrium.

animals subjected to ischemia/reperfusion. The high-energy requirement of myocardium is almost exclusively met with mitochondrial oxidative phosphorylation, and mitochondrial function is likely to play a central role in the molecular events that lead to the tissue damage occurring under the condition of oxygen deprivation. Not only the function of mitochondria was subdued, but also the structure altered. Long-term intermittent hypoxia adaptation is associated with a variety of adaptive changes in myocardial energy production and utilization enabling the heart to work more economically and protecting myocardium from lack of oxygen[13]. In our observation the preservation of normal structure of mitochondria and the low incidence of mtDNA deletion were definitely related to the reservation of mitochondrial function, which would help the heart to maintain normal energetic status.

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