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The effect of tumor necrosis factor-α inhibitor soon after hypoxia-ischemia on heart in neonatal rats

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ABSTRACT

Aims: Perinatal hypoxic-ischemic insult has acute and long term deleterious effects on many organs including heart. Although tumor necrosis factor alpha (TNF-α) has been reported to increase soon after hypoxia, the inhibition of this mediator has not been documented. The aim of this study was to investigate the effects of a TNF-α inhibitor (etanercept) on contractility and ultrastructure of rat heart muscles exposed to hypoxia-ischemia during neonatal period.

Main methods: Forty-five seven-day old rats divided into three groups were included in this study. The right carotid arteries of Saline and Etanercept groups of rats were ligated and kept in a hypoxia chamber containing 8% oxygen for 2 h. Immediately after hypoxia, while Etanercept group was administered 10 mg/kg etanercept, Saline group had only saline intraperitoneally. The carotid arteries of rats in Sham group were located without ligation and hypoxia. Mechanical activity of heart was recorded and tissue samples were examined by electron microscopy in the sixteenth week following the hypoxia-ischemia.

Key findings: While atrial contractile force in Etanercept group was similar to Sham group, there was significant decrease in Saline group ($p<0.001$). However, there was only non-significant decrease in ventricular contractility of Saline group comparing to Sham group ($p>0.05$). After hypoxia-ischemia, ultrastructural degenerative changes and mitochondrial damage in atriums of Etanercept group were significantly less severe than Saline group.

Significance: This study demonstrated that neonatal hypoxia-ischemia caused long term cardiac dysfunction and ultrastructural degenerative changes in the heart of rats. TNF-α inhibitor administration soon after hypoxia-ischemia may have heart protective effect.

Introduction

Hypoxic-ischemic injury during the perinatal developmental period causes various forms of pathology on many organs consisting heart. An in vivo experimental study on the fetal guinea pig heart showed that short-term in utero hypoxia (exposed to 7% O_2 for 2 h) has acute effects without having persistent cardiac damage in the postpartum period (Powell et al., 2004). On the other hand, in the isolated perfused rat heart chronic in utero hypoxia led to enhanced developed pressure and contractility (Hauton and Ousley, 2009). Thus, the influence of hypoxic and/or ischemic insult on cardiac mechanical performance to neonatal rats is poorly understood.

It has been assumed that tumor necrosis factor-alpha (TNF-α) has a dual role in cardiac injury (Sack, 2002). TNF-α released during ischemia causing apoptosis and necrosis of myocardial tissues was also reported (Li et al., 1999). On the other hand, in some experimental studies, interestingly, TNF-α was found out to protect the heart against acute ischemia or hypoxic injury (Katare et al., 2010). Therefore, the role of TNF-α on heart remains to be highly controversial and the inhibition of this mediator on the hypoxia-ischemia is not well documented. However, it has been thought that reduction of TNF-α secretion by baicalin has protected neonatal cultural rat cardiomyocytes from hypoxia/reoxygenation injury (Lin et al., 2010). On the other hand, etanercept which can also be used in humans is the most commonly preferred TNF-α inhibitor. Thus, in this study, we investigated the effects of etanercept on contractility and ultrastructure

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of heart muscles of sixteen-week rats exposed to hypoxia-ischemia during neonatal period.

Materials and methods

Seven-day-old Wistar male rat pups ($n=45$) weighing 12.0 ± 1.7 g, which were delivered spontaneously, were used in this experimental study. All procedures were approved by the Medical Faculty Experimentation Ethics Committee on Animal Research at our institution and followed the guidelines of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes.

Animal preparation and surgical procedure

The rats were allotted into three groups randomly: Saline treatment (Saline) group, Etanercept treatment (Etanercept) group and Sham group.

Rat pups in Saline and Etanercept groups were anesthetized by isofluran inhalation and the duration of anesthesia was less than 5 min. In these groups, hypoxia-ischemia was induced according to the Levine–Rice model (Rice et al., 1981). A median incision was made in the neck. Under the microscopic magnification, the right common carotid artery was dissected and ligated with a 6-zero silk suture. After the wound was sutured, the animals were allowed to have a 1 h recovery and feeding period. Except for the Sham group, rats were then placed into a plastic chamber and exposed to a continuous flow of 8% oxygen–92% nitrogen for 2 h. The carotid arteries of the rats in Sham group ($n=15$) were located without ligation and then they were placed into an open chamber without any supplemental oxygen for the same intervals. The chambers were partially submerged in a water bath at 33 ± 1 °C to maintain a constant thermal environment. No animals died in the Sham group throughout the study. However, two rats, one in the Etanercept and one in the Saline group, died during hypoxia. Immediately after hypoxia, while Etanercept group ($n=15$) was administered intraperitoneally (i.p.) 10 mg/kg etanercept (Enbrel 25 mg flakon, Wyeth) which was dissolved in saline (0.5 ml), Saline group ($n=15$) had only saline (0.5 ml) intraperitoneally. After the hypoxic period, the rats were survived in the room conditions till the sixteenth week.

Mechanogram

Body weights of all the animals were measured in the sixteenth week following the hypoxia-ischemia, just before the mechanical activity recordings of the hearts were made. Prior to the mechanical activity recordings of the heart, the rats were anesthetized with 50 mg/kg ketamine hydrochloride (Ketalar; Eczacibasi-WL, Istanbul, Turkey), which were administered intramuscularly. All the rats were placed in supine position during the mechanical activity recordings of the heart. Then the neck and the thorax region were shaved. An incision was made to divide the midabdominal line, starting from the inferior portion of the abdominal cavity. In order to reach the heart, the left and the right sides of the sternum were incised (to avoid cardiac perforation). A silk ligature was attached to the ventricular apex and connected to the force displacement transducer (MAY-FDT-10) to measure cardiac contractility. The transducer output was connected to a differential amplifier module (MAY-GTA-200). The signals were digitized with a 16-bit analog-to-digital converter at a sampling rate of 500 samples/s. BIOPAC Acknowledge Analysis Software (ACK 100 W5.7 version) was used to measure atrial and ventricular contractile forces.

Sample preparation for electron microscopy

For transmission electron microscopic (EM) evaluation of the heart, tissue samples were fixed with 2.5% gluteraldehyde (Cat#16400,450 ml EMS), postfixed with 1% osmium tetroxide (Cat#19110 10x1g, EMS), dehydrated in graded alcohol series, cleared with propylene oxide (Serva Lot 100109, 500 ml), and embedded in epon. Thin sections (50–70 nm) were cut by a microtome (Leica UCT-125) and contrasted with uranyl acetate and lead citrate. Sections were examined and photographed under an electron microscope (JEOL JEM-1011).

Ultrastructural changes of mitochondria

Electron micrographs were taken at 10,000 magnification randomly from atrium and ventricle for all three groups. A total of 10 different fields for each biopsy and 20 mitochondria in every field were evaluated. Totally, 200 mitochondria per sample were graded. Images were analyzed by one experienced investigator unaware of the sequence of sampling. Mitochondrial damage was scored assigning a numerical value of 0 through 3 to each mitochondrion. This mitochondrial score modified from the score, used in Milei et al. (1992). Grading scale was the following: 0—normal, 1—initial swelling (separation of cristae, decreased matrix density), 2—more marked swelling than in grade 1 and architectural disruption, 3—findings as in grade 2 plus rupture of inner and outer mitochondrial membranes. The average obtained was expressed for each grade as a percentage of the total number of mitochondria counted per sample.

Statistical analyses

Descriptive statistics (mean ± standard deviation) were calculated in each group for all mechanical activity parameters of the heart. Descriptive statistics of the results are shown in Table 1. Shapiro–Wilks test was used to determine whether all parameters were normally distributed. All parameters were in normal distribution.

One-way analysis of variance (ANOVA) was used to test the mean differences between the groups for all the parameters of mechanical activity of the heart. The data were analyzed by using the MedCalc v.11.0 Statistical Packet Program. Following these processes, an S–N–K (Student–Newman–Keuls) post hoc test was used to determine the significant differences between pair-wise groups. The results were considered statistically significant if p values were less than 0.05. The error bars were used for graphics.

Ultrastructural data were analyzed by using the MedCalc v.11.0.1 and Statistica 6.0 Packet Program. Shapiro–Wilks test was used to determine whether ultrastructural data were normally distributed.

Table 1
Mechanogram findings of the groups.

Groups	Statistic parameters	Force of contraction in atrium (g)	Force of contraction in ventricle (g)	Total contraction time (s)
Sham	Mean	0.32	1.31	0.289
	95% CI	0.27–0.37	0.91–1.72	0.26–0.32
	SD	0.09	0.70	0.05
	Minimum	0.19	0.47	0.22
	Maximum	0.55	2.33	0.39
Saline	Mean	0.21[a]	1.23	0.330
	95% CI	0.18–0.23	1.04–1.43	0.29–0.37
	SD	0.04	0.34	0.07
	Minimum	0.14	0.61	0.20
	Maximum	0.28	1.66	0.42
Etanercept	Mean	0.32	1.38	0.354
	95% CI	0.27–0.38	0.85–1.90	0.29–0.41
	SD	0.07	0.63	0.07
	Minimum	0.24	0.82	0.27
	Maximum	0.43	2.79	0.47

[a] Significantly different from sham group: $p<0.05$.

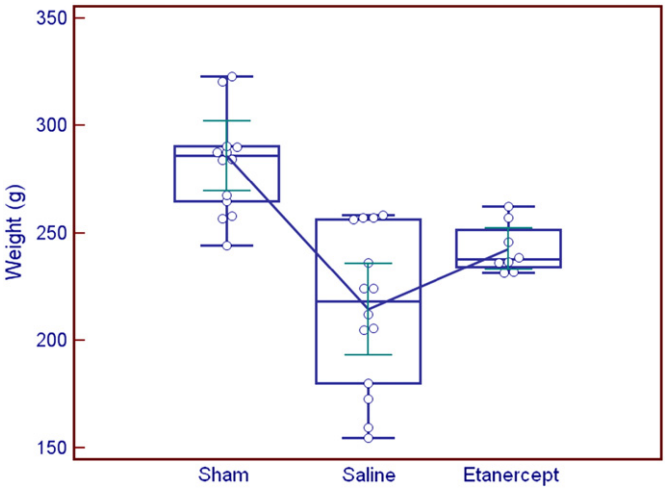

Fig. 1. Rat weights of the groups in the sixteenth week following the hypoxia-ischemia.

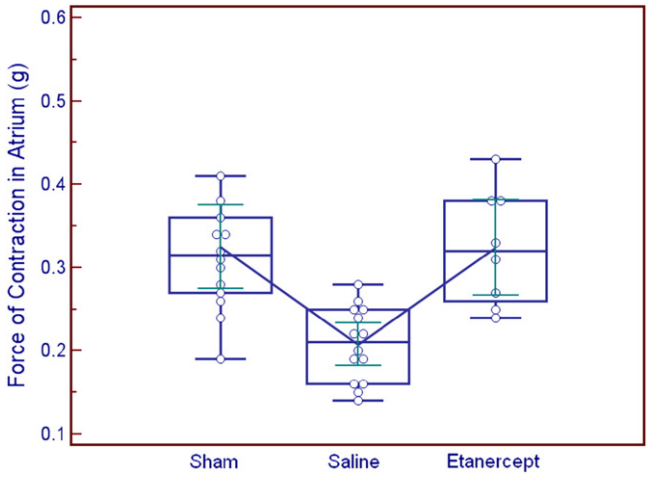

Fig. 3. Force of contraction in atrium of all the groups in the sixteenth week following the hypoxia-ischemia.

All parameters were not in normal distribution. Ultrastructural data were compared using the Kruskal–Wallis test. A value of $p<0.05$ was considered statistically significant. The error bars were used for graphics.

Results

Body weight

Rats in all three groups were weighed in the sixteenth week following the hypoxia-ischemia. The mean body weight of Sham group was 285.98 ± 27.94 g; the Saline group was 214.62 ± 36.84 g; and the Etanercept group was 242.64 ± 11.00 g. Compared to the Sham group, the Saline group and the Etanercept group showed significant weight loss ($p<0.001$). However, the mean weight of the Etanercept group rats was significantly more than Saline group ($p<0.001$). Etanercept treatment immediately after hypoxia, therefore, showed a preventive effect on the weight loss of Etanercept group (Fig. 1).

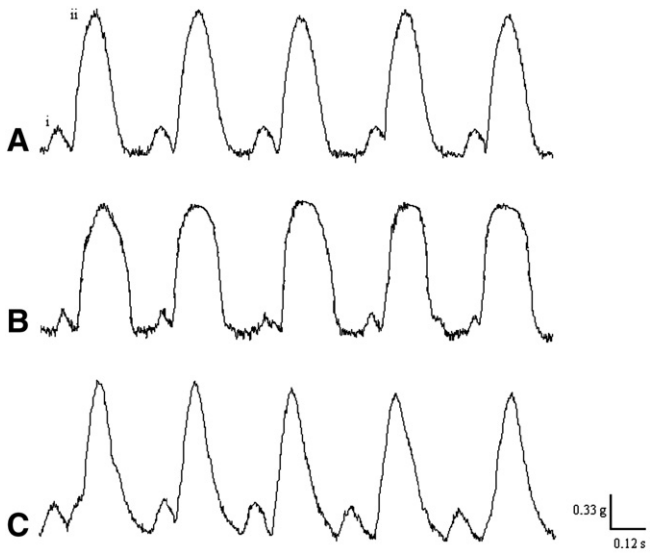

Fig. 2. Atrial and ventricular contraction recordings in the sixteenth week following the hypoxia-ischemia. (a) Sham, (b) Saline, (c) Etanercept (i: atrial contraction, ii: ventricular contraction).

Mechanogram data

Atrial and ventricular contractility, and total contraction time were measured by the mechanogram (Fig. 2). When compared to the Sham group, a significant decrease in atrial contractility in the Saline group was found (Table 1, Fig. 3). However, there was a slight reduction (6%) in the mean value of ventricular contractility in the Saline group compared to the Sham group, which was not statistically significant. As seen in Table 1, etanercept treatment prevented the decrease in atrial contractility. Total contraction time did not show significant differences between groups. In the Sham group, atrial and ventricular contractile forces (g) were 0.32 ± 0.09 and 1.31 ± 0.70,

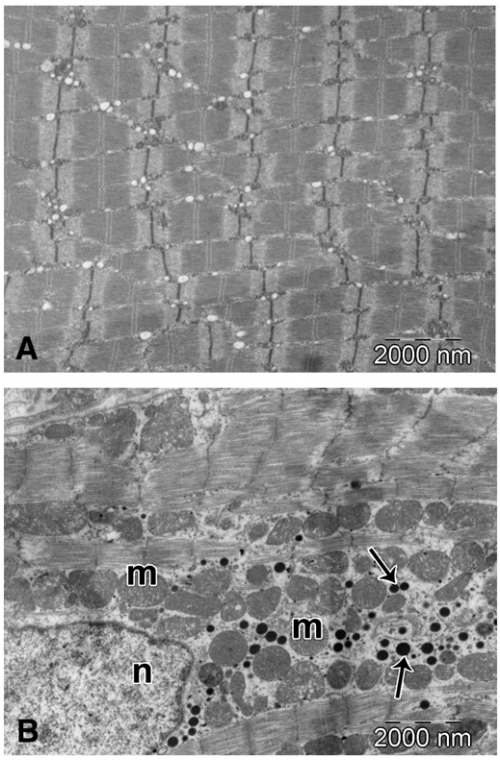

Fig. 4. A, B. Atrium in Sham group. The heart muscle cells have normal morphological characteristics. Mitochondrion (m), nucleus (n), atrial specific granule (arrow).

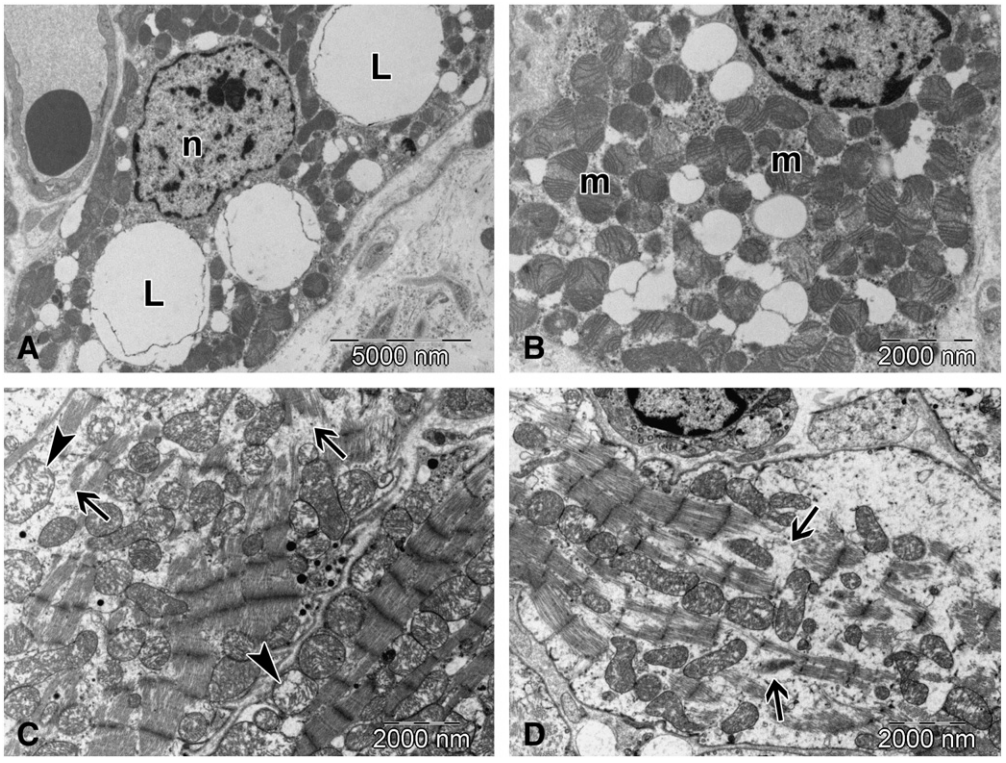

Fig. 5. Atrium in Saline group. A. Large lipid vacuoles (L) observed in some heart muscle cells. Nucleus (n). B. A great number of mitochondria (m) observed. C, D. Degeneration in myofibrils (arrow) and degenerative alteration in mitochondria (arrow head) followed.

respectively. In the Saline group, atrial and ventricular contractile forces (g) were 0.21 ± 0.04 and 1.23 ± 0.34, respectively. When these parameters in the Saline group were compared with those of the Sham group, the mean decreases in atrial and ventricular contractile forces were 36% and 6%, respectively.

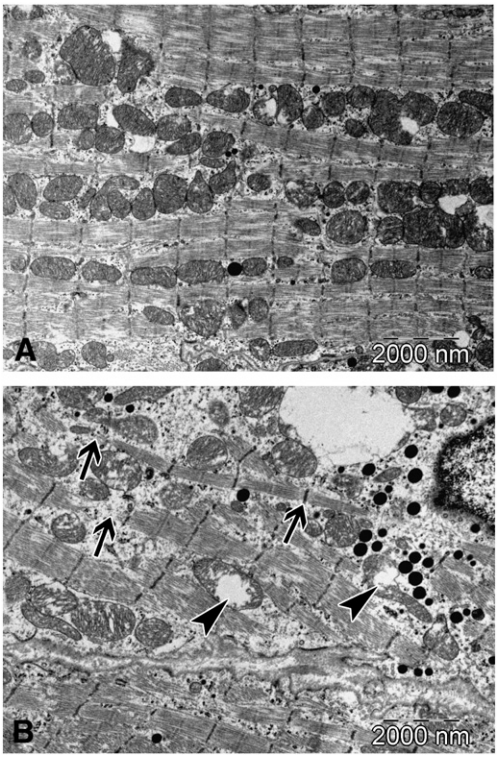

Fig. 6. Atrium in Etanercept group. Some heart muscle cells have normal morphological characteristics (A). Degenerative alteration in mitochondria (arrow head) and degeneration in myofibrils (arrow) observed in some heart muscle cells (B).

Electron microscopy studies

Qualıtatıve evaluatıon

The ultrastructures of the atrium and ventricle muscle cells were observed by an electron microscope.

Atrium

Heart muscle cells had normal morphological features in the Sham group. Myofibrils, sarcomeric structures, mitochondria and other sarcoplasmic organelles were normal. It was also observed that nucleus had normal morphological characteristics in this group (Fig. 4). However, there were large lipid vacuoles in some heart muscle cells in the

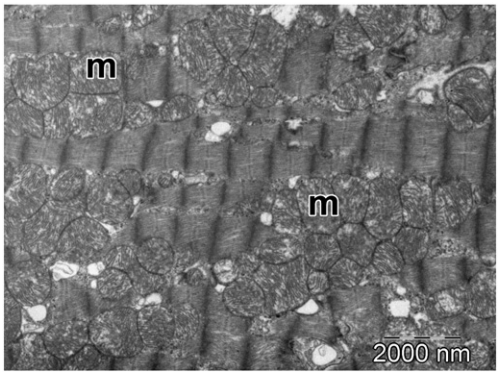

Fig. 7. Ventricle in Sham group. The heart muscle cells have normal morphological characteristics. Mitochondrion (m).

Saline group. The number of mitochondria distinctly increased in this group and degenerative mitochondrial changes were evident. Moreover, there were widespread degenerative changes in some heart muscle cells and some myofibrils thinning and breaking off were determined (Fig. 5). In the Etanercept group, although some heart muscle cells had normal morphological features, mitochondrial and myofibril degenerations were observed (Fig. 6).

Ventricle

Ultrastructurally, normal heart muscle characteristics were observed in the Sham group (Fig. 7). However, not only increase in the number of mitochondria but also a number of degenerative changes in some mitochondria, as well as destruction of some myofibrils and expansions in sarcoplasmic reticulum cisternas were determined in the Saline group (Fig. 8). On the other hand, some degenerative mitochondrial changes and increase in the number of mitochondria were evident in the Etanercept group, as well (Fig. 9).

Quantıtatıve analysıs

The mitochondrial damage of the atrium and ventricle muscle cells was evaluated. Ten fields at 10,000 magnification were scored for each biopsy.

Atrium

Fig. 10 shows the results of quantitative grading of damage obtained after examination of 3,000 mitochondria in three groups. Biopsies taken in the Sham group showed, as expected, that the large majority of mitochondria had minimal signs of injury (grade 0: 99.9 ± 0.71, grade 1: 0.1 ± 0.71). In the Saline group, mitochondrial injury developed and the proportion of mitochondria showing normal morphology decreased (grade 0: 30.1 ± 29.97%), while the number of mitochondria showing higher (i.e., worse) scores increased

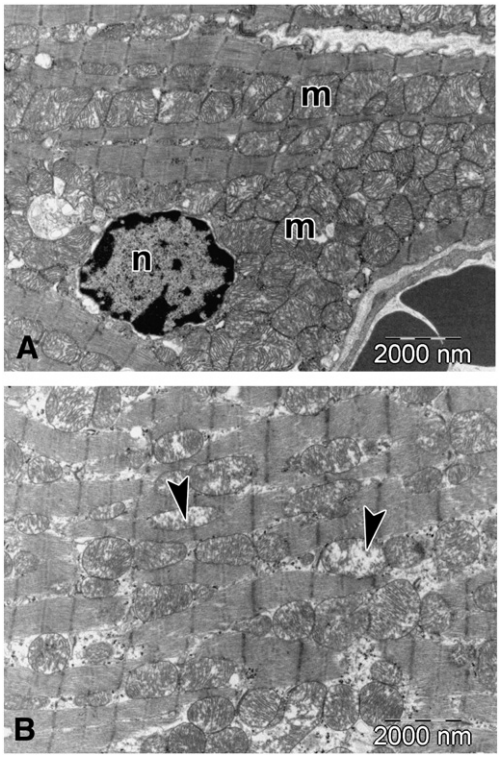

Fig. 9. Ventricle in Etanercept group. A great number of mitochondria (m) (A) and degenerative alteration in mitochondria (arrow head) (B) observed in heart muscle cells. Nucleus (n).

(grade 1: 32.8 ± 22.81%, grade 2: 37.1 ± 35.69%) (Fig. 10). Biopsies from the Etanercept group showed a less proportion of normal morphology versus those in the Saline group (grade 0: 12.0 ± 21.40%),

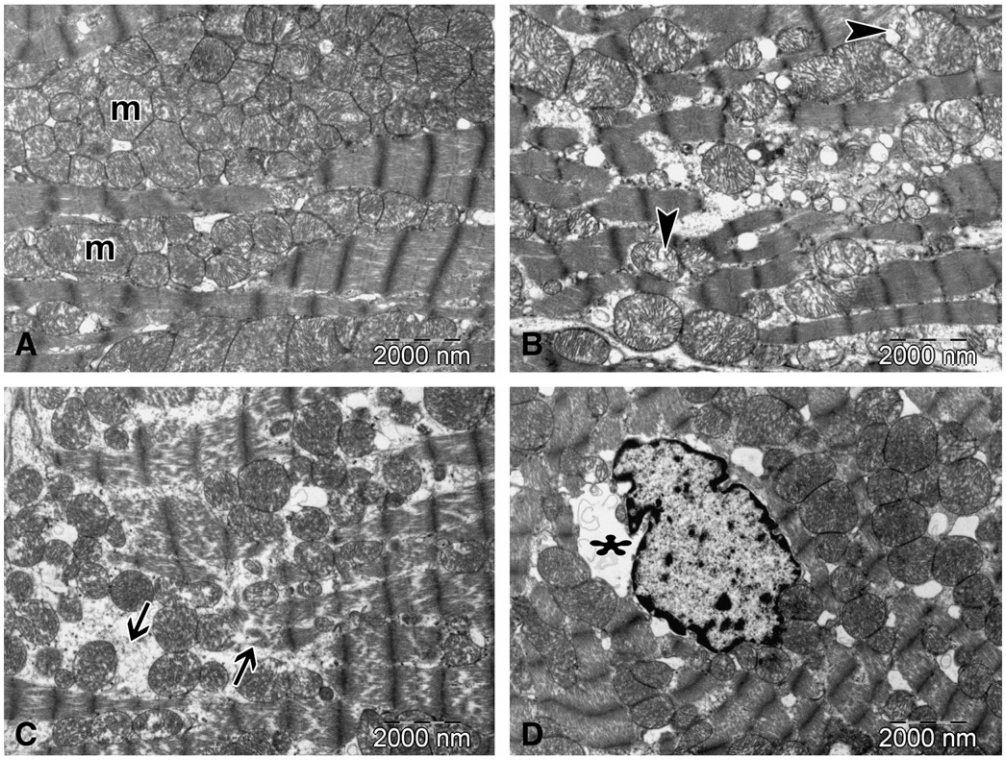

Fig. 8. Ventricle in Saline group. A great number of mitochondria (m) (A), degenerative alteration in some mitochondria (arrow head) (B), degeneration in some myofibrils (arrow) (C) and expansions in sarcoplasmic reticulum cisternas (asterisk) (D) observed in heart muscle cells.

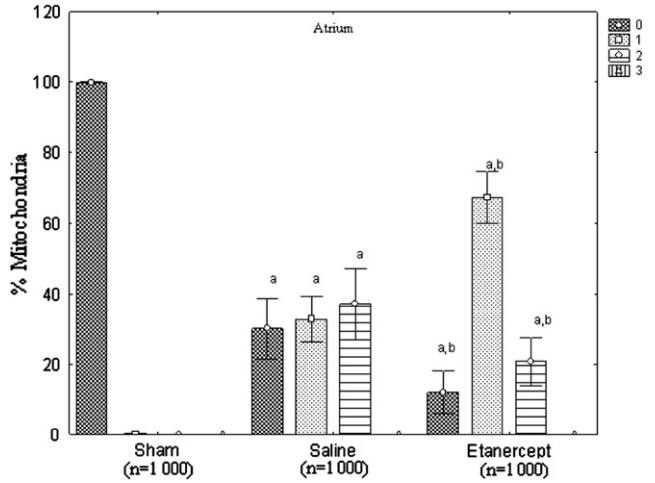

Fig. 10. Mitochondrial score of atrium biopsies taken from three groups (Saline, Etanercept and Sham groups). Grading scale is the following: 0—normal, 1—initial swelling (separation of cristae, decreased matrix density), 2—more marked swelling than in grade 1 and architectural disruption, 3—findings as in grade 2 plus rupture of inner and outer mitochondrial membranes. The average obtained is expressed for each grade as a percentage of the total number of mitochondria counted per sample. Data are mean ± sd of percent mitochondria scored. Values in parentheses are the number of mitochondria scored for each group. [a]$p<0.001$ vs. sham, [b]$p<0.001$ vs. saline.

but the number of mitochondria showing higher (i.e., better) scores decreased (grade 1: 67.2 ± 25.95%, grade 2: 20.8 ± 23.87%) (Fig. 10).

Ventricle

Fig. 11 shows the results of quantitative grading of damage obtained after examination of 3,000 mitochondria in three groups. Biopsies taken in the Sham group showed, as expected, that the large majority of mitochondria had minimal signs of injury (grade 0: 98.1 ± 6.84%, grade 1: 1.9 ± 6.84%). In the Saline group, mitochondrial injury developed and the proportion of mitochondria showing normal morphology decreased (grade 0: 13.3 ± 13.42%), while the number of mitochondria showing higher (i.e., worse) scores

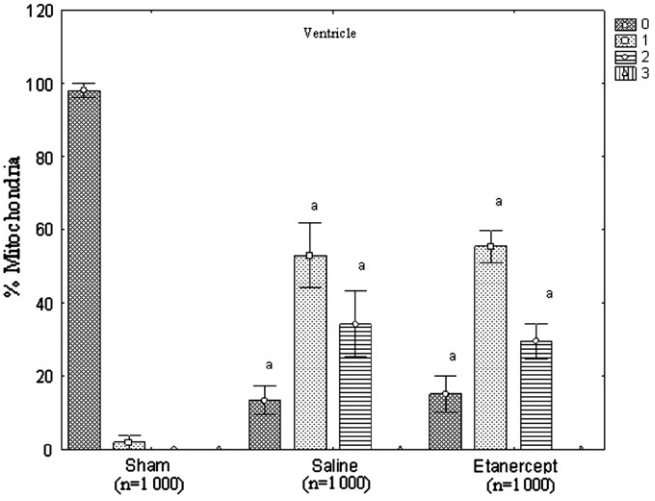

Fig. 11. Mitochondrial score of ventricle biopsies taken from three groups (Saline, Etanercept and Sham groups). Grading scale is: 0-normal; 1-initial swelling (separation of cristae, decreased matrix density); 2-more marked swelling than in grade 1 and architectural disruption; 3-findings as in grade 2 plus rupture of inner and outer mitochondrial membranes. The average obtained is expressed for each grade as a percentage of the total number of mitochondria counted per sample. Data are mean ± sd of percent mitochondria scored. Values in parentheses are the number of mitochondria scored for each set. [a]$p<0.001$ vs. sham.

increased (grade 1: 52.90 ± 31.10%, grade 2: 34.2 ± 31.51%) (Fig. 11). Biopsies from the Etanercept group did not show any significant difference compared to the Saline group (grade 0: 15.1 ± 17.45%, grade 1: 55.4 ± 15.04%, grade 2: 29.5 ± 16.57%) (Fig. 11).

Discussion

In this study, a significant decrease in atrial contractility after hypoxia-ischemia was found. Although, there was a slight reduction in the ventricular contractility in the Saline group compared to Sham group that was not statistically significant. TNF-α has a dual role in cardiac damage (Sack, 2002). Most of the studies have demonstrated that during ischemia, TNF-α is released which causes apoptosis and necrosis of myocardial tissues (Bryant et al., 1998; Li et al., 1999). Contrarily, in some experimental studies, the cardioprotective effect of TNF-α has been established (Eddy et al., 1992; Katare et al., 2010; Smith et al., 2002). Moreover, in the clinical study on the newborn with hypoxic ischemic encephalopathy, Liu et al. (2007) have evaluated cardiac and cerebral functions for 14 days after the birth (infant rats). In this study, the effect of myocardial dysfunction on the cerebral hemodynamics has been reported; however, left ventricle contractility returned to the normal values after 3 days. Therefore in our study, the late effects of hypoxia-ischemia on adulthood rats (16-weeks) were investigated on myocardial contraction and ultrastructure, whereas the early effects on heart contractility could not be taken into consideration. In our study, since the late effects of hypoxia-ischemia were investigated, the early effects on ventricle contractility could not be taken into consideration.

In another study by Caparrotta et al. (1989), the effects of hypoxia on contractile tension were investigated in isolated, spontaneously beating guinea pig atria. When two different degrees of hypoxia were induced by lowering oxygen tension from 95% O_2 (control) to 40% (moderate hypoxia) and 20% (severe hypoxia) for 30 min, contractile tension slowly decreased to 60% and 40% of control, respectively. In our study, the mean decrease in atrial contractility became 36% with the reduction of O_2 from 95% (Sham) to 8% (Saline) for 2 h after ischemia. Besides, the reduction of atrial contractility in later period of hypoxia-ischemia might be assumed to have longer existing effect compared to the study by Caparrotta et al. (1989).

In another research study by Yeung et al. (2008), the effects of chronic hypoxia (10% oxygen for 28 days) on myocardial functions were investigated in isolated perfused rat hearts. They reported that the ratio of heart-to-body weight was increased in the chronically hypoxic rats. However, in this study, since the effects of acute hypoxia (8% oxygen for 2 h) on contractility and ultrastructure of rat heart muscles were investigated, the other possible parameters appeared during the process were ignored.

Some experimental studies established that over expression of TNF-α in transgenic mice developed cardiac dilatation, abnormal calcium hemostasis, increased apoptosis, ventricular arrhythmias and early death (Kubota et al., 1997; Li et al., 2000). In addition, the tissue-specific production of TNF-α by cardiac myocytes in vivo is sufficient to cause severe cardiac disease and this supports a causal role for this cytokine in the pathogenesis of human cardiac disease (Bryant et al., 1998). This paves the way to the clinical studies on the relation between TNF-α and heart failure. Thus, it has been determined that there is a relation between TNF-α level and the degree of heart failure in some experimental studies (Feldman et al., 2000; Torre-Amione et al., 1996). Furthermore, the usage of TNF-α blockers in treatment has become the main topic. Anti-TNF-α treatment was initially used in animal models of septicemia which was effective in reversing the depressed myocardial contractility (Porat et al., 1995). Subsequent studies in the TNF-α transgenic mice have shown that neutralizing fragments of the TNF-α receptor attenuate certain features of the heart failure phenotype (Henriksen and Newby, 2003). Early trials of TNF-α blockade in patients with heart failure were

encouraging. A pilot study of etanercept, which is a TNF-α blocker, led to significant increases in quality of life scores and ejection fraction in patients with heart failure (Deswal et al., 1999). Nevertheless, widespread clinical trials of anti-TNF-α therapy to treat heart failure were not successful (Mann et al., 2004). On the other hand, the effects of inhibition of TNF-α on the hypoxia-ischemia are not well documented. Apart from the previous studies on heart failure, our study investigated the protective effect of etanercept against hypoxia-ischemia on myocardial tissue and found that TNF-α blocker treatment made no significant protection in the left ventricle contractility.

Myocardial ischemia/reperfusion injury is not only related to reactive oxygen formation, but also to inflammation (Frangogiannis et al., 2002). On the basis of the fact, it has been thought that reduction of TNF-α secretion by baicalin has protected neonatal cultural rat cardiomyocytes from hypoxia/reoxygenation injury related to anti-inflammatory activity (Lin et al., 2010). In our study, etanercept was preferred as a TNF-α inhibitor since it can be used in humans, as well. Moreover, it was demonstrated that etanercept prevented the decrease in atrial contractility. In addition, various atrial ultrastructural changes such as large lipid vacuoles in some heart muscle cells and an increase in the number of mitochondria and degenerative mitochondrial changes were found in the hypoxia-ischemia group (Saline group), whereas no such changes exist in the Etanercept group. Since the heart relies almost exclusively on the mitochondria for energy, contraction, and ion transport, it is considerable that defects in mitochondrial energy production would preferentially affect the heart (Wallace, 2000). Due to the fact that, mitochondria evidently play a crucial role in cardiac contractility, they are a common subcellular target of cardiotoxicity (Lu, 1996). Mitochondria have also been implicated as regulators of cytosolic Ca^{2+} concentration. On the basis of our study, the findings suggested that hypoxia-ischemia caused an increase in the amount of mitochondria for protection mechanism, but even so, a lot of mitochondria have shown degenerative alteration and this may be one of the reasons for reduced atrial contraction force. On the other hand, in this study, it was found that hypoxia-ischemia did not cause statistically significant decrease in ventricular contractility. However, ultrastructural changes and detailed analysis of 3,000 mitochondria in ventricle tissue apparently do not agree with the findings using quantitative scoring. A statistically significant difference in the degeneration of mitochondria between the Sham and the Saline groups has also been found (Fig. 10). Probably, in spite of hypoxia-ischemia, in presence of normal contracting ventricle areas caused ventricular contractility to continue normally. In the study with baicalin, the results verify that baicalin has a protective effect on hypoxia/reoxygenation injury soon after pretreatment, and the structures of mitochondria, the nuclear membrane and myofilament were protected (Lin et al., 2010). Although the protective effect of baicalin has been explained with TNF-α inhibition, the contribution of the blockage of other cytokins also cannot be ignored.

In patients with hypoxic ischemic encephalopathy, it is well known that the more cardiac dysfunction the patients have, the more severe cerebral problems they tend to have (Liu et al., 2007). It is a fact that the results of cerebral hypoxic effects can be seen in long time period. Since atrial contractility is not as important as ventricular contractility clinically, it is not used in routine echocardiographic measurements. Hypothetically, if there is an echocardiographic parameter based on atrial contractility in routine, decrease on atrial contractility may be demonstrated in clinical studies. It is very difficult to obtain results from the patients who cannot be applied echocardiographic examination in early period after hypoxia-ischemia, due to the ventricle contractility effect, which immediately occurs and returns to normal afterwards. This also causes us not to be able to get the info about the cardiac and indirectly cerebral status.

There is a number of limitations in the present study that need to be addressed in future research. The first being that, it was not possible to verify the ultrastructural data with some biochemical experiments (the mitochondrial copy number by qPCR and mito protein levels by Western blotting experiments) due to the limited resources.

Secondly, only clinical therapeutic effect of etanercept after hypoxia was investigated in the study. Since perinatal hypoxic-ischemic insult is a commonly existing event, hypoxic-ischemic injury could not be predicted in infants. As a result, the clinical therapeutic effect of etanercept before hypoxia was not taken into consideration. On the other hand, the study would be more enriched if the TNF alpha inhibitor was applied before hypoxia-ischemia in the other group. Finally, the necessity of another group of hypoxia without operation was not predicted at the beginning of the study.

Conclusion

On the basis of the results, it is assumed that if a reliable echocardiograghic parameter related to atrial contractility is provided, some predictive information of cerebral functions especially in the late period after hypoxia-ischemia can be required. Since this is an experimental study, the results may possibly not reflect the data of clinical studies. Thus, more clinical trials are necessarily required.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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