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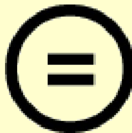

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The effect of C-reactive protein deposition on myocardial area at risk with ischemia-reperfusion injury in rats

C 반응성 단백의 경색 위험부 침착이 래트의 심근 허혈-재관류 손상에 미치는 효과

2017년 8월

서울대학교 대학원

의학과 흉부외과학 전공

오 세 진

The effect of C-reactive protein deposition on myocardial area at risk with ischemia-reperfusion injury in rats

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이 논문을 의학박사 학위논문으로 제출함

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ABSTRACT

Objective

We evaluated the effect of monomeric C-reactive protein (CRP) deposition on areas at risk (AAR) of myocardium with ischemia-reperfusion injury.

Methods

Myocardial ischemia-reperfusion injury model was produced by ligation of the left anterior descending coronary artery for 45minutes followed by 45minutes of reperfusion using female Sprague-Dawley rats. Tissue from non-ischemic areas, areas at risk, and infarct areas determined by Evans blue and 2,3,5-triphenyltetrazolium chloride staining was obtained from the sham group, the ischemia-reperfusion injury without CRP injection group (I/R only group), and the ischemia-reperfusion injury with CRP injection group (I/R+CRP group). We assessed the effect of CRP injection on infarct size, CRP deposition, CRP and IL-6 mRNA expression, the third component of complement (C3) immunodeposition, and mitochondrial structural remodeling with apoptosis by quantitative RT-PCR analyses, immunohistochemistry, direct immunofluorescence, electron microscopy, and TUNEL assay, respectively. All images were analyzed using an automated morphology tool.

Results

The infarct area significantly increased in the I/R+CRP group than in the I/R only group. The anti CRP antibody confirmed that CRP deposition occurred in both the infarct and AAR of the I/R+CRP group. The myocardium did not exhibit CRP mRNA expression, and the CRP treatment group showed a tendency for IL-6 to increase without statistical significance. Activated C3, apoptosis, and mitochondrial destruction increased on AAR and infarct area in the I/R+CRP group.

Conclusions

These results strongly suggest the active participation of the deposition of CRP on AAR in the progression of myocardial infarction following ischemia-reperfusion injury.

Keywords: myocardial reperfusion injury, C-reactive protein, myocardial infarction, mitochondria, apoptosis

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INTRODUCTION

C-reactive protein (CRP) is an acute phase protein synthesized in the liver and induced by IL-6 when inflammation, infection, or cell damage occurs [1-3]. CRP is composed of 5 identical subunits and possesses 2 major forms: pentameric CRP (pCRP) and monomeric CRP (mCRP) [4]. CRP circulates in the serum in the pCRP form. When it comes into contact with the damaged cell membrane, CRP changes into mCRP and is deposited on the activated or apoptotic cell membrane [5,6]. While pCRP has an anti-inflammatory role, deposited mCRP plays a strong inflammatory role in monocytes and produces oxygen-free radicals in monocytes, aggravating cell injury [5-7].

Elevated serum CRP levels have been found in myocardial infarction and unstable angina [8,9], and elevated serum CRP levels have been correlated with greater myocardial tissue injury, heart failure progression, and mortality rates. CRP was deposited in infarcted myocardium and aggravated myocardial infarction [10-12]. Complement activation and complement-mediated damage play an important role in ischemia-reperfusion injury and progression of myocardial infarction [13,14]. CRP is a major activator of complement systems and is deposited (with the complement systems) at tissue injury sites such as myocardial infarction and skin burns [15,16]. In ischemia-reperfusion injury, functional and structural changes in mitochondria can induce apoptosis by halting ATP synthesis and structural remodeling, releasing proapoptotic proteins, impairing calcium ionic homeostasis, and producing reactive oxygen species [17].

However, no research has been conducted regarding the deposition of CRP on the ischemic but viable area (area at risk or AAR) in ischemic reperfusion injury [18]. We hypothesized that CRP also would be deposited on AAR and that deposition would aggravate myocardial infarction in the ischemia-reperfusion injury through complement activation, mitochondrial remodeling, and apoptosis.

METHODS

Animals and Anesthesia

We constructed a myocardial ischemia-reperfusion injury model using female Sprague-Dawley rats weighing between 220 and 260 g (gestational age range, 12-14 weeks). Left thoracotomy was performed via the fifth intercostal space, and the pericardium was opened to expose the left coronary artery.

Anesthesia was achieved by the inhalation of isoflurane (4%) for induction followed by the intraperitoneal administration of Zoletil 50 (Virbac, Carros, France; 0.12 ml) and Xylazine (Rompun 2%; Bayer Korea, Gyeonggi-Do, Korea; 0.02 ml) for maintenance. Each rat was intubated with a 16-gauge intravenous catheter and connected to a ventilator. Positive-pressure ventilation using room air at a tidal volume of 2.5 to 3.0 ml (10 ml/kg) and 63 to 67 breaths/min was maintained to prevent atelectasis during the procedure.

Experimental protocols

We constructed a myocardial ischemia-reperfusion injury model using female Sprague-Dawley rats weighing between 220 and 260 g. The animals were treated according to the *Guide for the Care and Use of Laboratory Animals* (National Academy of Sciences, Washington, DC, USA). Moreover, we conducted our experimental study in accordance with the ARRIVE guideline. The study protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at the SMG-SNU Boramae Medical Center Biomedical Research Institute (approval number: 2015-0009). On the sham group (n=3), we performed left thoracotomy and pericardiotomy without manipulating of the heart to induce myocardial ischemia. After carrying out the procedures and then waiting for 90 minutes, we euthanized the rats and then performed autopsies (Figure 1A). In the ischemia-reperfusion only group (I/R only group, n=5), myocardial ischemia was produced by ligation of the left anterior

descending (LAD) coronary artery approximately 2 mm distal to its origin by snaring it with 6-0 nylon double sutures (buttressed with a small piece of plastic tube for 45 minutes) and reperfusion for 45 minutes. After reperfusion, the heart was quickly excised (Figure 1B). In the ischemia-reperfusion plus CRP injection group (I/R+CRP group, n=9), 100 μg bolus of high-purity (> 99%) human CRP obtained from human plasma (C4063; Sigma-Aldrich, Saint Louis, MO, USA) were infused via the femoral vein after the release of coronary ligation (Figure 1C).

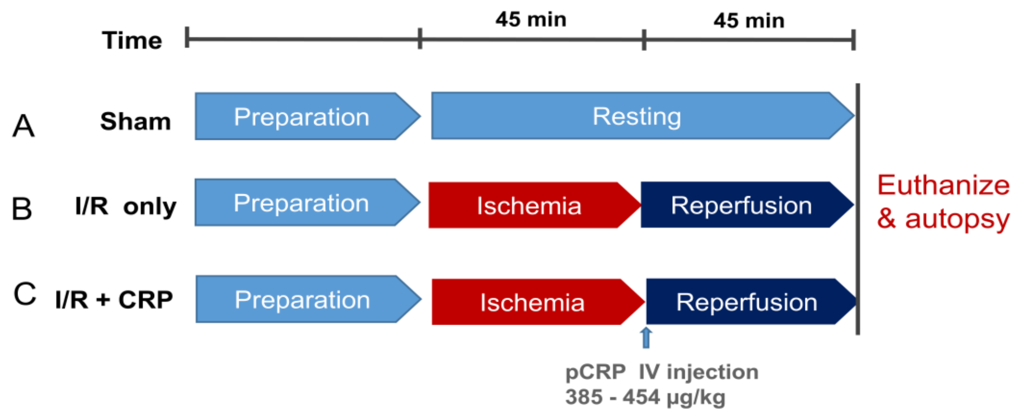

Figure 1. Schematic illustration of experimental protocols

Evans blue and 2,3,5-triphenyltetrazolium chloride (TTC) staining to determine infarct area and AAR

One milliliter of diluted heparin solution (2500 IU heparin/mL) was infused via the coronary ostia while the ascending aorta was clamped. The LAD, which had been occluded for infarct production, was again occluded with a 6-0 nylon suture, and 1% Evans blue was injected to stain the perfused myocardium. The AAR and infarct (whole ischemic area) were left unstained. The left ventricles were cut from the apex to the base in 4 transverse slices. For the sliced sections of the myocardium, the mid parts of the left ventricle at the same cross section were used to measure the ischemia and infarct size. Selected mid portions were then cut into 4 mm slices, one of which was incubated in TTC (Sigma Chemical, Saint Louis, MO) that had been dissolved in a 100 mmol/L phosphate buffer for 15 minutes.

Preparation for Electron microscopy

The heart samples were cut into millimeter-sized portions of the blue, red, and white regions, fixed in 2.5% glutaraldehyde, and postfixed in osmium tetroxide. Epon-embedded samples were cut into 70-nm sections and stained with uranyl acetate and lead citrate for final viewing.

Histopathologic analysis and immunohistochemistry

The sectioned heart was fixed in 10% buffered formalin and embedded in paraffin. The 4-μm-thick tissue sections were stained with hematoxylin and eosin (H&E). We performed immunohistochemical staining using an OptiView DAB immunohistochemical detection kit (Roche Diagnostics, Mannheim, Germany), and a Benchmark XT autoimmunostainer (Ventana Medical Systems, Inc, Tucson, Arizona). The heart sections were immunostained using rabbit polyclonal anti-CRP antibody (ab32412; Abcam, Cambridge, United Kingdom; 1:250 dilution) and human

monoclonal anti-CRP antibody (C1688; Sigma-Aldrich, Saint Louis, MO; 1:400 dilution). Although ab32412 cannot distinguish between pCRP and mCRP, we point out that anti-CRP antibody C1688 recognizes an epitope located on the 24 kD subunit of CRP and that it can detect the monomeric form of CRP.

Immunofluorescence study of complement and electron microscopy

One section of heart was embedded in an OCT compound (Tissue-Plus; Scigen Scientific Inc, Gardena, California) and frozen. Serial sections 4 μm thick were obtained using a cryomicrotome (HM550; Thermo, Germany). The sections were air-dried at room temperature for 20 minutes. The frozen heart tissue sections were stained with FITC anti-C3, C4, C1q, IgG, and IgM (Ventana Medical Systems, Tucson, AZ). Images of tissue sections were captured using a Zeiss LSM 780 confocal microscope (GmBH1997-2015; Carl Zeiss, Germany) and fluorescence microscopy.

Ultrathin sections were examined with a transmission electron microscope (model GEM-1400; JEOL, Tokyo, Japan). The diameters of 50 mitochondria per sham, I/R only, or I/R + CRP group rat were measured to assess the severity of mitochondrial injury and degree of morphological change.

Analysis of CRP mRNA levels in rat myocardium

CRP total RNA was isolated from rat myocardium using a miRNeasy Mini Kit (Qiagen, Hilden, Germany). Quantitative RT-PCR analyses of CRP were performed using a TaqMan Gene Expression Assay (Rn000567307_g1; Applied Biosystems, Carlsbad, California). The ACTB (Rn00667869_m1; Applied Biosystems, Foster City, California) was used as an endogenous control for normalization purposes.

IL-6 RNA expression assay

RNA was prepared using a miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The extracted RNA (1 μg) was reverse transcribed using a Reverse Transcription System (Promega, Madison, Wisconsin), and cDNAs were amplified using a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). qRT-PCR analysis of IL-6 was done using TaqMan Gene Expression Assays (Rn01410330_m1; Applied Biosystems, Foster City, CA) and a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). The rat ACTB (Rn00667869_m1; Applied Biosystems, Foster City, CA) endogenous control was used for normalization purposes.

Determination of cardiomyocyte apoptosis with TUNEL (Terminal deoxynucleotide transferase dUTP Nick End Labeling) assay

Adjacent serial sections in dewaxed slides were stained using TUNEL assay (ApoBrdU-IHC DNA Fragmentation Assay Kit, BV-K403; Biovision, Milpitas, California) to label DNA breaks and detect apoptotic cells through immunohistochemistry. The nuclei of the apoptotic cells exhibited brown staining, whereas those of nonapoptotic cells and the negative control were stained blue. The number of apoptotic cells in each group was counted, and the apoptotic index (number of apoptotic cells / total number of cells x 100%) of the nonischemic areas, the AAR, and the infarct in each specimen was calculated [19].

Image analysis

All images of Evans blue and TTC staining and CRP immunohistochemistry were acquired using a Vectra automated imaging system (PerkinElmer, Waltham, MA). The infarct area (white zone after the Evans blue and TTC staining) and the AAR area (red zone after the Evans blue and TTC staining) were automatically calculated by inForm (PerkinElmer, Waltham, Massachusetts) imaging analysis software. The size of the infarct was expressed in terms of the ratio of infarct to the whole ischemic area (infarct / (AAR + infarct)). The areas exhibiting CRP immunopositivity were expressed

in terms of the ratio of CRP-immunostained area to the whole ischemic area (CRP-immunostained area / (AAR + infarct)).

Statistical analysis

Data was expressed and plotted as the mean with standard error of the mean. The normality of all of the parameters was tested using the Kolmogorov-Smirnov test. To compare 2 groups, a 2-tailed *t* test was used. For comparison of 3 groups, 1-way ANOVA and Tukey's post hoc analysis were used. In cases where normality was excluded, the nonparametric Mann-Whitney *U* test was used to compare 2 groups. A P value <.05 was considered to be statistically significant. Analysis of data was performed using GraphPad Prism software version 5.0 (GraphPad Software, San Diego, California) or SPSS V.18.0 (SPSS, Chicago, Illinois).

RESULTS

Histopathological Analysis, CRP immunohistochemistry, and CRP mRNA expression

After H&E staining, myocytes in the infarct area showed contraction bands with intensely eosinophilic intracellular stripes, which are representative of ischemia-reperfusion injury (Figure 2B). Moreover, cardiomyocytes in the infarct area showed intense cytoplasmic immunostaining with both anti-CRP antibodies (ab32412; Figure 2B, C1688).

After Evans blue/TTC staining, the non-ischemic area was stained blue, the AAR was stained red, and the infarct was stained white (Figure 2A). The ratio of infarct to the whole ischemic area was much higher in the I/R+CRP group (38.5±4.2%) than in the I/R only group (23.9±3.4%, *P*=.012; Figures 2A and C). On the other hand, the ratio of the whole ischemic area to the whole myocardium was not statistically different between the 2 groups (I/R only group: 45.2±3.8%, I/R+CRP group: 42.3±3.3%, *P*=.592; Figure 2D). These results demonstrated that the myocardial infarct size increased in the CRP-deposited myocardium, and that the infused CRP did not affect the perfused area (outside of the LAD coronary artery territory), but affected the ischemic area.

In the I/R only group, a small amount of CRP immunopositivity was detected in the infarct area. On the other hand, in the I/R+CRP group, CRP was extensively immunostained with the ab32412 and C1688 antibodies (Figure 2A) not only in the infarct area (white zone), but also in the AAR (red zone). In the whole section of the heart, the anti-CRP antibody positive area (ab32412) was significantly larger after infusion of CRP (I/R only group: 6.4±3.4%, I/R+CRP group: 44.3±6.4%, *P*=.001; Figure 2E). The anti-mCRP antibody positive area (C1688) was also significantly larger in the I/R+CRP group (I/R only group: 43.8 ± 7.0%, I/R+CRP group: 86.8 ± 14.7%, *P*=.042; Figure 2F). In sham group, there was no ischemia, infarction and CRP deposition.

The CRP mRNA was not expressed in rat myocardium using quantitative RT-PCR analyses in any group. These results support the notion that CRP immunostaining was a consequence of deposition rather than CRP production in ischemic reperfusion injury in rat myocardium.

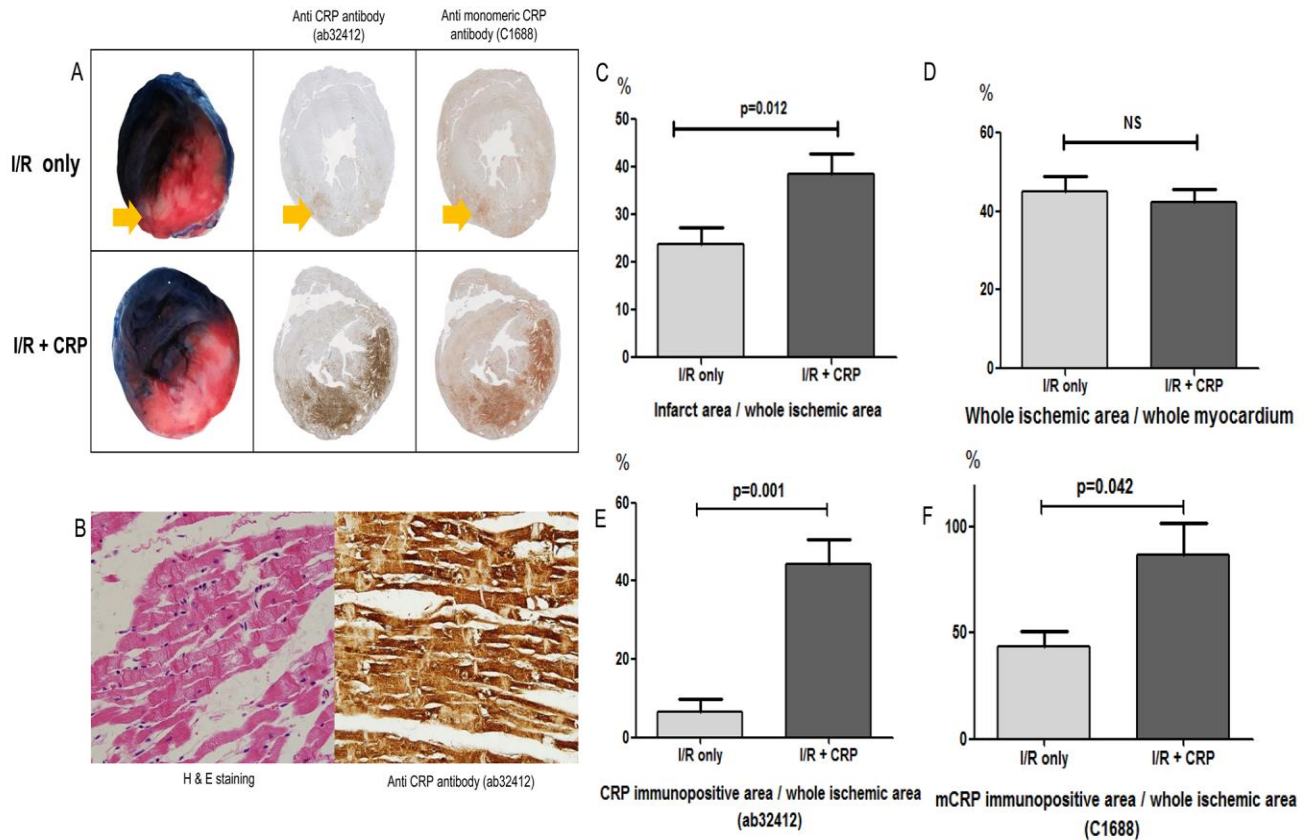

Figure 2. TTC and Evans blue staining and CRP immunohistochemistry

(A) White myocardium represents infarct area, red myocardium represents area at risk, and blue myocardium represents non-ischemic area. In the I/R only group, only infarct area (arrow) was faintly immunostained by both anti-CRP antibody (ab32412) and anti-mCRP antibody (C1688). In contrast, the I/R + CRP group showed that both areas at risk and infarct area (red and white zone) were strongly immunostained by both antibodies. (B) Representative H&E and CRP immunostaining of damaged myocardium in the I/R + CRP group. Cardiomyocytes showed contractile bands typical of ischemia-reperfusion injury. The damaged myocardial cells showed diffuse cytoplasmic immunostaining by anti-CRP antibody (magnification x400). (C) The ratio of infarct area to whole ischemic area was significantly larger in CRP injection group. (D) However, the ratio (whole ischemic area to whole myocardium) was not significantly different. (E) CRP-immunostained area (ab32412) increased more in the I/R + CRP group than in the I/R only group. (F) Anti-mCRP antibody–immunostained area (C1688) also increased more in the I/R + CRP group than in the I/R only group.

IL-6 expression increased after CRP injection

CRP is induced by IL-6, and consequently, the deposited monomeric form of CRP is known to induce proinflammatory reaction. In this study, the IL-6 expression pattern did not showed statistically significant difference but it showed tendency to increase after CRP injection (P =.076; Figure 3).

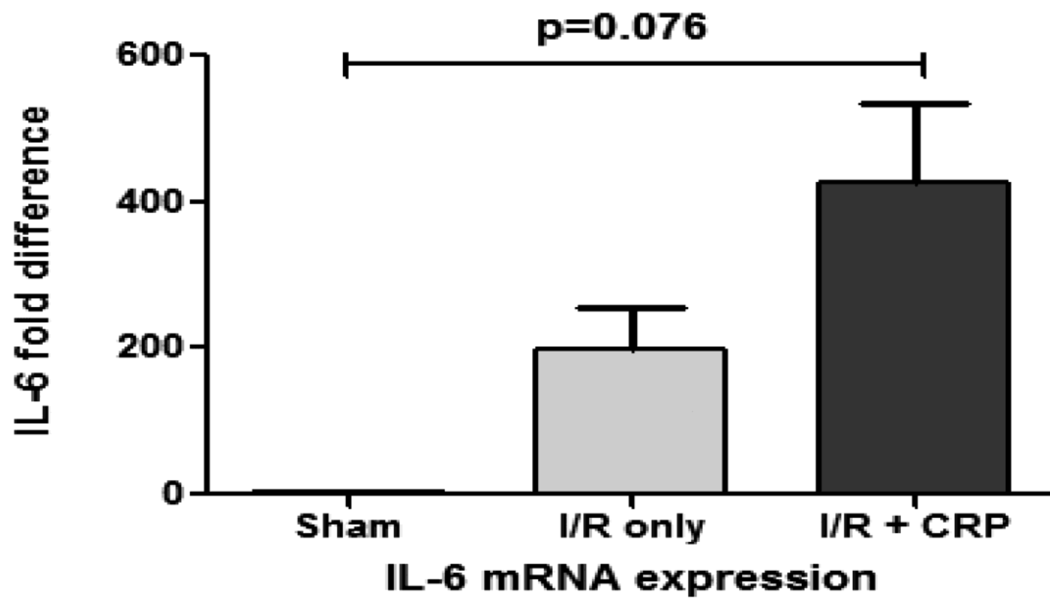

Figure 3. IL-6 mRNA expression

IL-6 mRNA expression showed tendency to increase after CRP injection. However, there was no statistical significance (P = .076)

C3 activated in the AAR and infarct area in the I/R + CRP group

C3 deposition was mainly deposited in the infarct area in the I/R only group (Figure 4A), whereas it was deposited more extensively in both the AAR and infarct area in the I/R+CRP group (Figure 4B). Interestingly, the complement was co-localized in the CRP-immunodeposited area in the AAR (Figure 4C) in the I/R+CRP group. IgG was deposited mainly in the contraction band of myocardial cells in the infarcted area in both groups (Figures 4A(d) and B(h)). C1q and IgM were nonspecifically deposited, and C4 was not detected in either group (data not shown).

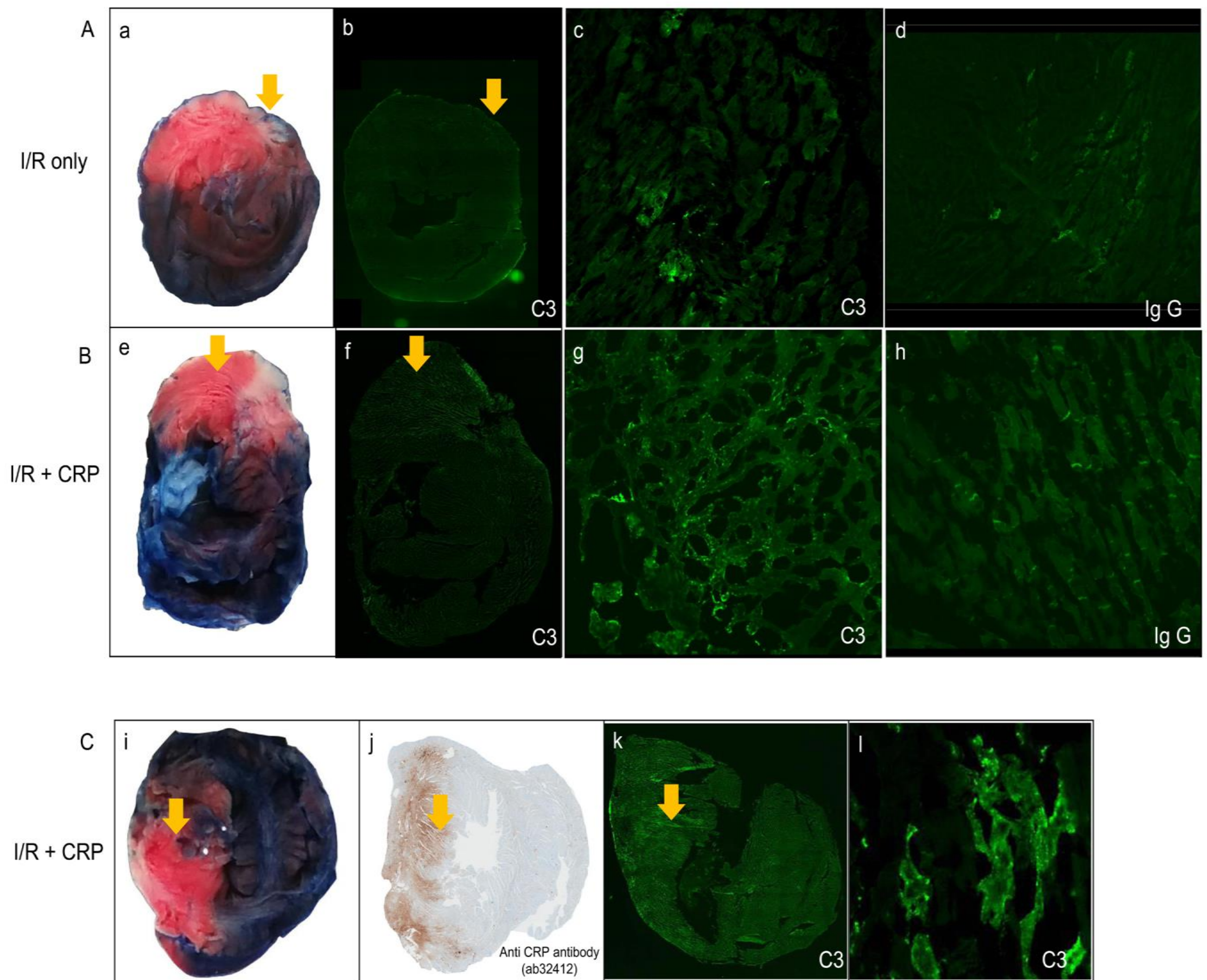

Figure 4. Relationship among infarct area, AAR, C3, and IgG deposition

(A) The I/R only group demonstrated that only infarct (arrow) showed C3 immunofluorescence (b, x100 tile scan, c, x200) and only contraction band of infarct showed IgG positivity (d, x200). (B) The I/R + CRP group showed more extensive infarction (e), and both infarct area and AAR (arrow) showed diffuse C3 positivity (f, x100 tile scan, g, x200). As in the I/R only group, IgG was deposited only in the contraction band of infarct area (h, x200). (C) Relationship among AAR, CRP (ab32412) immunopositivity, and C3 immunofluorescence in the I/R + CRP group. Arrows indicate AAR. AAR in the CRP infusion group showed intense CRP deposition, and this region correlated with the C3 activation site (k, x100 tile scan, l, x400).

Mitochondria swollen and extensive autophages production in AAR in the I/R + CRP group

Electron microscopy showed that the mitochondria of the non-ischemic area still had normal architecture with multiplicity of cristae in their native configuration and inner/outer mitochondrial membranes (Figure 5A). In contrast, the mitochondria in the AAR in the I/R+CRP group were swollen and exhibited cristae disorganization. Abundant autophages were produced to a greater extent in the I/R+CRP group than in the I/R only group (Figure 5B, arrow). The mitochondrial diameters in the AAR were significantly greater in the I/R+CRP group than in the I/R only group (1169.3±31.1 vs 921.9±30.9 nm, $P<.001$; Figure 5C). In the infarct, both the I/R only group and the I/R+CRP group showed amorphous matrix densities, which are signs of irreversible ischemic injury (Figure 5D, arrow). The mitochondria were more shrunken and condensed in the I/R+CRP group than in the I/R only group (1028.0±24.3 vs 1140.2±38.3 nm, $P=.026$; Figure 5E).

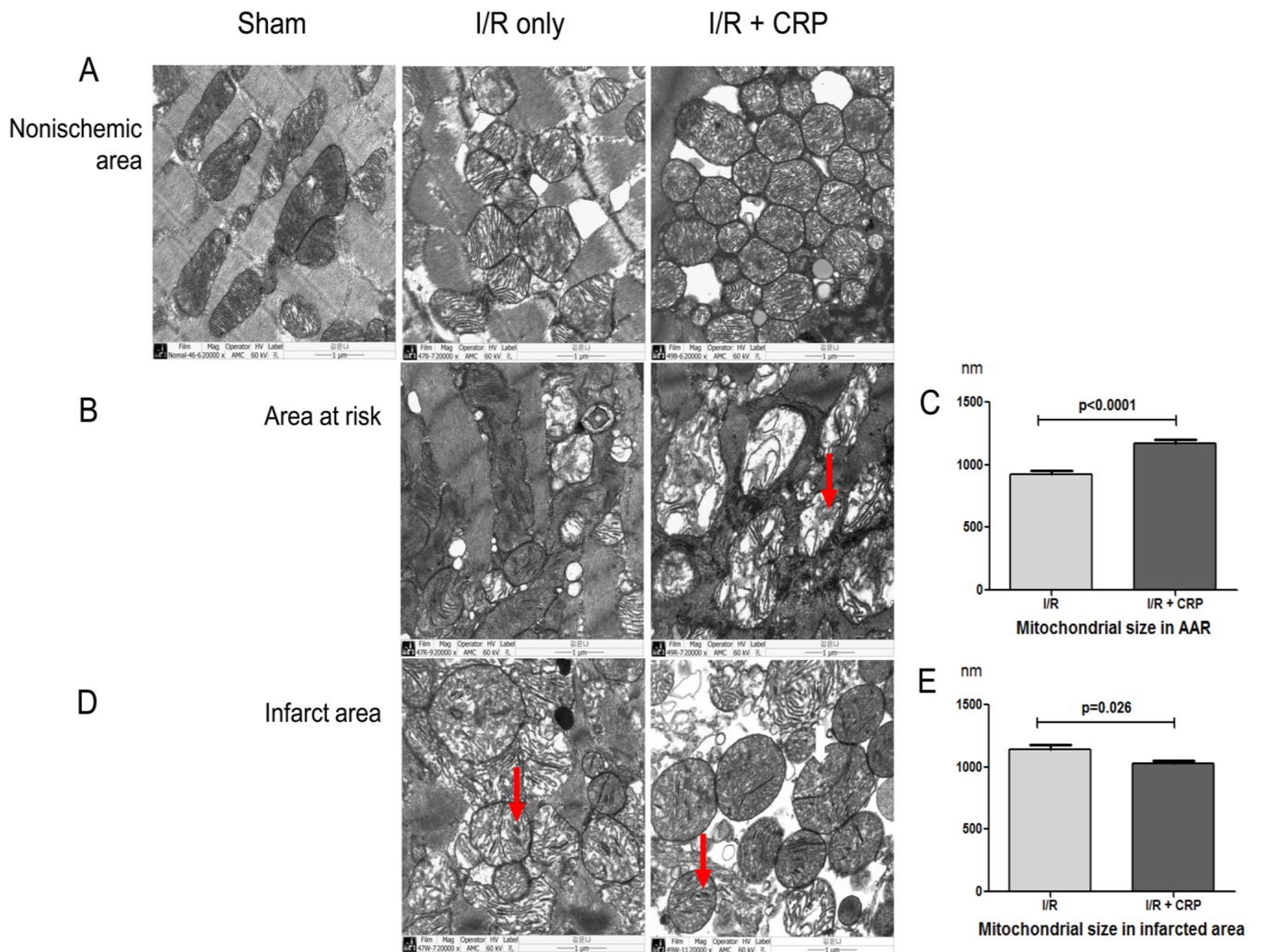

Figure 5. Transmission electron micrographs of non-ischemic areas, areas at risk, and infarct area in myocardium in each group

(A) Non-ischemic areas in all groups showed normal mitochondria with tightly packed cristae and electron-dense matrices. (B) Mitochondria in areas at risk. In the I/R only group, some mitochondria maintained normal architecture with multiplicity of cristae in their native configuration. In contrast, the CRP infusion group showed that almost all mitochondria had swollen and exhibited disorganization of cristae with abundant autophages formation (arrow) and (C) mitochondrial diameters in AAR were significantly greater after CRP infusion (P < .001). (D) Infarct area. Both groups showed amorphous matrix densities that are signs of irreversible ischemic injury. (E) Mitochondria were more condensed and smaller on AAR in the CRP infusion group (P = .026).

Apoptosis increased in the AAR in the I/R+CRP group

Representative TUNEL assay results are shown in Figures 6A-C. In all areas of the sham group and the non-ischemic area of the I/R only group and the I/R+CRP groups, most myocardial cells were non-apoptotic (Figure 6A). In the AAR, TUNEL staining showed a significant increase in the cardiomyocyte apoptotic rate in the I/R+CRP group compared to that in the I/R only group (60.1±6.0% vs. 31.2±7.7%, P=.013; Figures 6B). In contrast, there was no statistical difference in the cardiomyocyte apoptotic rate in the infarct area in the 2 groups (I/R only group: 75.5±4.6%, I/R+CRP group: 70.2±6.8%, P=.373; Figure 6C).

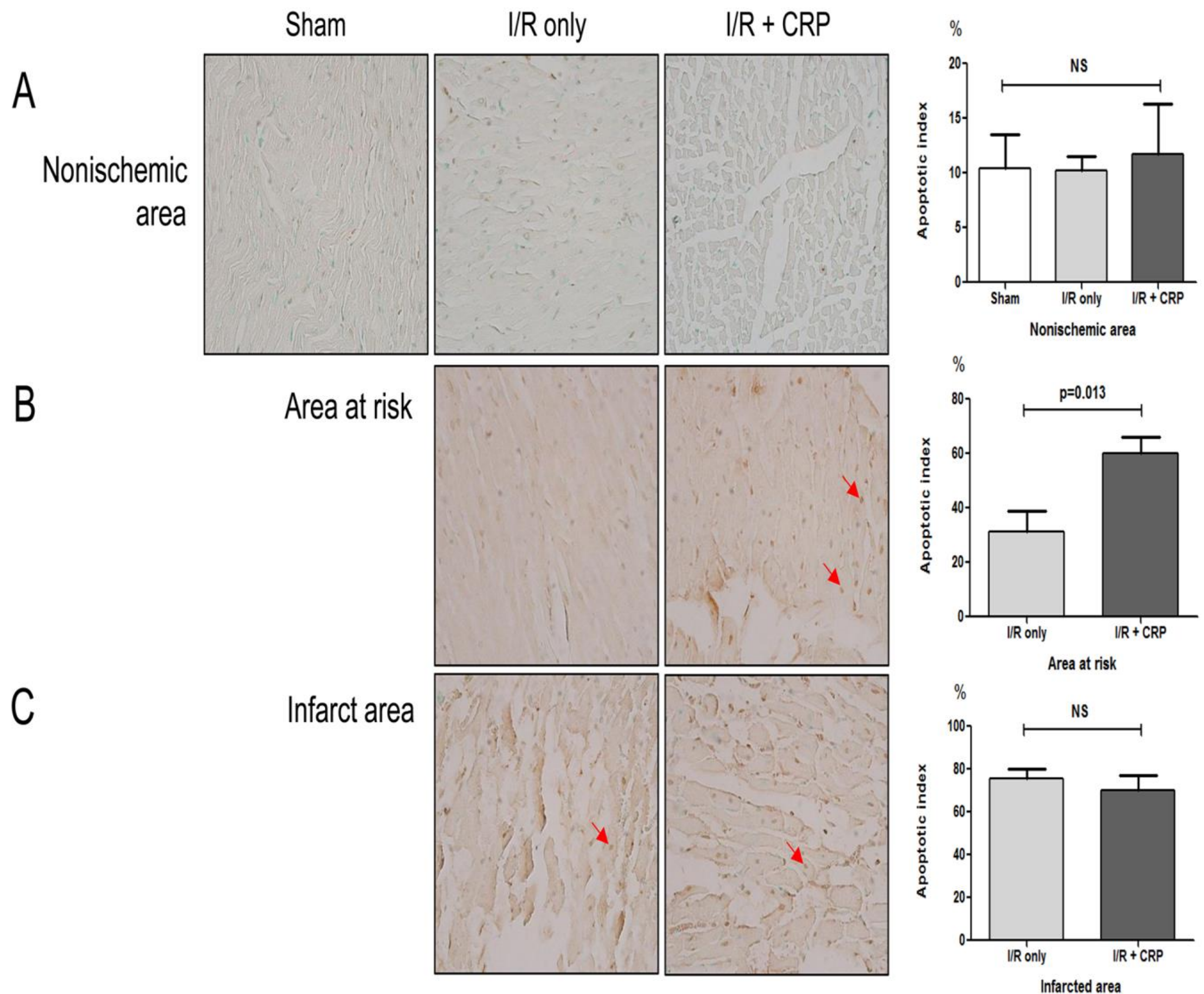

Figure 6. Representative apoptotic cells determined with Terminal deoxynucleotide transferase dUTP Nick End Labeling and quantitative apoptotic indices

Arrows indicate apoptotic cells. Apoptotic nuclei showed brown staining (magnification x 400). (A) In all groups, most myocardial cells in non-ischemic areas were nonapoptotic. (B) In areas at risk, the CRP infusion group showed increased apoptotic indices (*P* = .013). (C) However, in infarct, there was no significant difference between the 2 groups (*P* = .373).

DISCUSSION

The present study demonstrated 3 main findings. Firstly, myocardial infarct size was increased after CRP injection, and the effect of CRP deposition on ischemia-reperfusion injury model in rat myocardium occurred in both the infarct area and AAR. Secondly, C3 was also immune-deposited in both the infarct area and AAR after CRP injection, and was co-localized in the CRP deposited area. Thirdly, the region of AAR showed mitochondrial destruction, autophagy formation, and increased apoptosis.

Results of the present study are consistent with those of previous studies showing that the presence of serum CRP at the time of myocardial infarction increases the entire myocardial infarct size for human autopsy specimens with acute myocardial infarction [15] and a rat ischemia-reperfusion model. These studies evaluated CRP deposition only in the irreversibly infarcted area. In our study, however, we re-perfused sufficiently after ischemia to simulate AAR, which is viable but prone to irreversible infarct, and evaluated the effect of CRP deposition in both the infarct area and AAR. To the best of our knowledge, the present study may be the first to show the effect of CRP deposition in the AAR induced by ischemia-reperfusion injury.

In this study, the CRP treatment group showed a tendency for IL-6 to increase without statistical significance. As IL-6 is an upstream inducer of CRP [20], an increase in IL-6 might enhance endogenous CRP production in the rats' livers. However, rat CRP does not activate rat complement, whereas human CRP potently activates both rat and human complements [21]. Using human CRP is a good method for controlling complement activation by endogenous CRP [10]. Therefore, we used human CRP as it was done in previous studies [22]. We used anti-human CRP antibodies to detect CRP deposition.

Vilahur et al showed that short periods of myocardial ischemia are related to increased mCRP mRNA expression in infiltrated macrophages [23]. In addition, Slevin et al showed that ischemic brain tissue and peri-infarcted brain tissue have mCRP expression [24]. We sought to determine whether

CRP immune-positivity is a result of myocardial cell-origin mCRP mRNA expression. However, we found no mCRP mRNA expression in the non-ischemic area, infarct area, or AAR of the myocardial tissues in both the I/R only group and I/R+CRP groups. We therefore concluded that mCRP immune-positivity was the result of immune-deposition rather than CRP production in myocardium.

CRP is known as one of the main activators of classical complement pathways. In previous studies, CRP was co-localized with complement, and activation of the complement aggravated myocardial infarction [12,15]. In addition to the myocardium, ischemic brain tissue showed co-localization with CRP and complement [24]. After removal of the complement, the CRP failed to aggravate the degree of myocardial infarction [15]. Moreover, the complement activated by CRP deposition has an inflammatory function [12]. While previous studies demonstrated that the complement was activated only in the infarct area, we showed that complement C3 was co-localized to the AAR as well as to the infarct area and that the presence of complement C3 was associated with an increase in myocardial infarct size. Thus, we inferred that CRP immunodeposition on the AAR might also deteriorate ischemia-reperfusion injury through complement activation. As the classical complement pathway is also activated by IgG or IgM, we studied IgG and IgM. IgG was found only in the infarct area with contraction bands in both groups, and IgM was nonspecifically immunostained. The C3 activation in the AAR in the I/R+CRP group therefore appears to be a consequence of CRP rather than IgG or IgM in the classical complement pathway activation.

It has been well recognized that mitochondria are important modulators that decide the fate of cells such as programmed cell death, necrosis, and apoptosis [25]. Structural and functional dysregulation of mitochondria induces irreversible cell damage through the loss of ATP synthesis, the increase in ATP hydrolysis, the impairment of Ca^{2+} homeostasis, the formation of reactive oxygen species, and the release of proapoptotic proteins in ischemia-reperfusion injury [26]. Moreover, autophagy driven by mitochondria can be subjected to the extreme stress of ischemia-reperfusion injury and lead to cell death. Whereas autophagy may be protective under mild ischemia, it can contribute to damage by causing cell death under severe ischemia-reperfusion injury [27] and play a significant role in myocyte disappearance and the development of contractile dysfunction in failing hearts [28]. Therefore,

mitochondria preservation is important for myocardial protection when dealing with ischemia-reperfusion injury. We indicated that mitochondria in the AAR with CRP immunodeposition were severely damaged, lost their normal configuration and size, and induced numerous autophages. As we expected, we demonstrated that the apoptotic rate increased after CRP treatment of the AAR. This finding was consistent with those of previous studies such as an in vitro study using stimulated primary neonatal rat cardiomyocytes. In that study, hypoxia-induced cardiomyocytes with co-treatment CRP showed significantly increased apoptosis, which was related to the cytochrome c release by mitochondria into the cytosol. It is concluded that CRP could enhance apoptosis in hypoxia-stimulated cardiomyocytes via the mitochondrial dependent pathway [19]. Therefore, we can speculate that deposited CRP may have a profound effect on mitochondrial damage. As a result of mitochondrial damage, hypoxic myocardium may become more prone to apoptotic death.

Pepys et al. first proposed the inhibition of mCRP deposition for the treatment of cardiovascular diseases [13]. They used 1,6-bis(phosphocholine)-hexane as an inhibitor of CRP conformational change to reduce myocardial infarct size [10,12]. In a clinical setting, smoking, diabetes, obesity, and metabolic syndromes are major risk factors for cardiovascular diseases, and serum CRP is elevated in these clinical settings [29]. As it is almost impossible to salvage the infarcted myocardium, salvaging the AAR might be a new effective cardio-protection strategy. Inhibiting CRP deposition in the AAR may be beneficial during the immediate post-recanalization procedure in patients who have increased serum CRP. Control of CRP conformational change and inhibition of CRP deposition in such sets to undergo coronary artery revascularization could also be good preoperative strategies. In the future, sequestration of pCRP by tailored antibodies (for inhibiting transformation of pCRP into mCRP) or selective inhibition of deposition of mCRP in the injured myocardium will prove to be a promising method for minimizing ischemia-reperfusion injury in patients with elevated serum CRP.

Limitations of this study

It was reported that sodium azide (contained in human CRP for the preservation of proteins) could make an artifact in the study of vascular endothelial cells [29]. And human derived CRP could result in alloimmune reaction, and this influence apoptosis and mitochondrial changes. Therefore,
the experiment with purified endotoxin-free, azide-free recombinant CRP is being conducted. This study showed that groups having CRP injections had greater apoptotic rates and more CRP immunodeposition. Previous studies suggested that CRP binds to apoptotic cells in skin burns [6] or apoptotic lymphocytes in a Ca^{2+} dependent manner and augmented the classical complement pathway [30]. Therefore, we cannot totally exclude the possibility that the CRP injection increased apoptosis in different, unknown ways (other than mitochondrial damage), and mCRP was deposited in the apoptotic cells.

CONCLUSION

In summary, we demonstrated that CRP was deposited in the infarct area and AAR in ischemia-reperfusion injury and that monomeric CRP deposition was related to complement activation, mitochondrial injury, apoptosis, and aggravated myocardial infarction. Inhibition of CRP deposition in ischemia-reperfusion–damaged myocardium could be a new therapeutic strategy for the post-recanalization of ischemic myocardium.

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국문 초록

목적

래트의 심근 허혈-재관류 손상에 있어 심근의 경색위험부위 (areas at risk, AAR) 에 단량체 C 반응성 단백 (monomeric C-reactive protein, mCRP) 의 침착이 미치는 영향에 대해 연구하였다.

방법

Sprague-Dawley 래트를 이용하여 좌전하행 관상동맥을 45 분간 결찰 하여 허혈 손상을 가한 후, 45 분간 재관류를 시행함으로써 심근 허혈-재관류 손상 모델을 수립하였다. 실험군은 허혈-재관류 손상이 없는 sham 군과 CRP 주입없이 허혈-재관류 손상만 시행한 I/R only 군, 허혈-재관류 손상에 CRP 를 주입한 I/R+CRP 군으로 하였다. 그리고 Evans blue 및 2,3,5-triphenyltetrazolium chloride 염색을 이용하여 심근 조직을 비허혈 부위, 경색위험부위 및 경색 부위로 구분하였다. Quantitative RT-PCR 분석, 면역조직화학염색, 직접 면역형광법, 전자현미경 분석 및 TUNEL assay 를 통해 CRP 주입으로 인한 심근 경색 크기 및 CRP 침착을 확인 및 측정하고 심근세포 내 CRP 및 IL-6 mRNA 발현을 관찰하였으며, C3 보체의 면역침착 및 미토콘드리아의 구조적 변화와 아포토시스 (apoptosis) 를 확인하였다.

결과

I/R only 군에 비해 I/R+CRP 군에서 경색 부위 크기가 통계적으로 유의하게 증가하였다. I/R+CRP 군에서는 CRP 항체를 이용한 면역조직화학 염색법을 통해 경색 부위 및 경색위험부위 모두에서 CRP 가 세포질에 침착되는 것을 확인할 수 있었으나, 심근 세포내 CRP mRNA 발현은

나타나지 않음으로써 외부에서 주입한 CRP 만이 세포에 침착 되었음을 확인하였다. 또한 CRP 를 주입한 군에서 심근의 IL-6 가 증가하는 경향을 보였다. C3 보체 활성화, 심근세포의 아포토시스 및 미토콘드리아 손상은 I/R+CRP 군에서 경색 부위 및 경색위험부위 모두 다른 군에 비해 유의하게 증가하였다.

결론

본 연구를 통해 래트의 심근 허혈-재관류 손상에 있어 CRP 의 경색위험부위 침착은 보체 활성화 기전 및 미토콘드리아 변화를 동반함으로써 심근 경색의 진행에 관여함을 확인할 수 있었다.

주요어: 심근 재관류 손상, C 반응성 단백, 심근 경색, 미토콘드리아, 아포토시스

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