Chronic stress: a crucial promoter of cell apoptosis in atherosclerosis

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Abstract

Objective: Chronic stress may lead to augmented incidence rates of coronary and cerebrovascular diseases associated with atherosclerosis. However, few studies have focused on the effect of chronic stress on atherosclerosis plaque formation. Therefore, this study was designed to directly evaluate how chronic stress affects atherosclerosis.

Methods: Thirty rabbits were divided into three groups: the control group, balloon-injury operation + high-fat diet model group, and chronic stress + balloon-injury operation + high-fat diet model group. Physical and social stress were induced, and proteomic methods were applied to identify specific markers.

Results: After protein determination, the chronic stress + balloon-injury operation + high-fat diet model group exhibited significant upregulation of the following apoptosis-related proteins: UBE2K, caspase 3, caspase 9, BAX, P53, and FAS. In particular, real-time polymerase chain reaction showed that the protein expression of caspase 9 was significantly downregulated in the stress group compared with the non-stress groups. However, the other proteins showed significantly increased expression in the stress group.

Conclusion: Chronic stress may promote cell apoptosis in the physiopathologic process of atherosclerosis.

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Introduction

Atherosclerosis (AS) is the main cause of coronary heart disease,[1] cerebral infarction, and peripheral vascular disease. Cardiovascular and cerebrovascular diseases remain a main cause of death globally. AS is a multifactorial disease with a complex pathogenesis that has not yet been fully elucidated. The main risk factors are genetic factors, obesity, high cholesterol, heavy smoking, diabetes, and high blood pressure.

Chronic stress (CS) is an essential negative life event that can lead to plaque build-up in the arteries (i.e., AS). The association between stressful incidents and chronic disease is stronger than the association between stressful incidents and infectious or traumatic illness,[2] and this is true not only for adults but also for adolescents. Recent studies have shown that CS might increase the risk of AS, which again affects both adults and adolescents.[3] Some epidemiologic research has suggested that CS is an independent risk factor for the development of vascular diseases and increases the morbidity and mortality of patients with coronary artery disease.[4] CS is a nonspecific reaction to long-term repeated physical irritants (e.g., acute cardiovascular reaction to hypothalamic-pituitary-adrenal axis activation)[5,6] and psychological irritants (e.g., emotional responses). Additionally, ample evidence has shown that unpredictable mild CS can lead to depression,[7,8] and both the risk of cerebrovascular disease and higher fatality rates have been demonstrated by numerous researchers. After the first episode of depression, the risk of myocardial infarction is still high even 10 years later.[9] Because CS is also associated with AS, hypertension, visceral obesity, and an increasing incidence of insulin resistance, CS has been defined as a risk factor for cerebrovascular and cardiovascular diseases.[10]

However, most studies have mainly focused on the external relationship between CS and atherosclerotic lesions rather than on the mechanism of plaque formation and peeling. There is not enough evidence indicating that negative mood states and CS are closely related to plaque instability that mainly involves the apoptosis, proliferation, or loss of plaque cells such as smooth muscle cells (SMCs), macrophagocytes, endotheliocytes, fibrocytes, and similar cells. For instance, one study showed that plaque instability was associated with apoptosis of SMCs and loss of fibrocytes,[11] while another study showed that the absence of endotheliocytes leads to plaque instability.[12] The mechanisms underlying the connection among atherosclerotic diseases, hyperlipidemia, and physical and psychological CS have not yet been adequately elucidated. Cell apoptosis is an autonomously ordered death of cells commanded by genes to maintain the stability of the internal environment, and the phenomenon of apoptosis is widespread throughout the body at all times. One hypothesis is that loss of atherosclerotic plaques is caused by the

overexpression of certain apoptotic genes under CS.

In the present study, we tested this hypothesis by exploring the influence of a balloon-injury operation (OP) plus a high-fat diet (HD), with or without CS, on the expression of genes and proteins related to apoptosis and development of AS in rabbits. The impacts of CS, including social stress and physical stress, were evaluated with respect to behavioristics, hormonal readiness, lipid metabolism, inflammation, and AS plaque characteristics by experimental methods.

Materials and methods

Animals, diets, and groups

Thirty white New Zealand rabbits (2.5 months old) weighing 2.5 ± 0.1 kg were obtained from the Chinese Academy of Medical Sciences, Institute of Laboratory Animal Sciences, and Peking Union Medical College.[11] Upon their arrival, the rabbits were housed in three groups and permitted to adapt to their new environment for 7 days. All rabbits were housed in separate cages ($50 \times 40 \times 40$ cm) and given free access to water and food under standard laboratory conditions (temperature of $22°C \pm 1°C$; relative humidity, 60%; 12-hour light/dark cycle; light on at 07:00). The experimental scheme was approved by the Animal Care and Use Committee. All efforts were made to reduce pain and minimize suffering during the procedures.

Diets were purchased from Beijing Keao Third-Feed Co. (Beijing, China). After 7 days of acclimatization, the 30 rabbits were randomly divided into 3 groups. The first group was the control group (CG, n = 10), which was fed standard chow (46% carbohydrates and 4.1% saturated, 4.5% monounsaturated, and 2.3% polyunsaturated fat, totaling 11.5% of kcal from

fat). The second group was the OP + HD model group (OP+HD group, n = 10), which was fed high-fat chow (90.45% basic feed and 5% lard, 2% sugar, 2% cholesterol, 0.35% cholate, and 0.2% propylthiouracil) after the OP. The aim of the OP+HD group was to establish an atherosclerotic animal model, and comparison between the CG and OP+HD group indicated whether the model had been successfully established. The third group was the OP + HD + CS group (OP+HD+CS group, n = 10), which was fed 90.45% basic feed and 5% lard, 2% sugar, 2% cholesterol, 0.35% cholate, and 0.2% propylthiouracil after the OP under an environment of CS for 8 weeks. In this group, we applied the CS intervention based on an atherosclerotic animal model to explore the effect of CS on the development of AS.

Abdominal aortic OP

The rabbits in the OP+HD+CS and OP+HD groups were fasted for 12 hours but allowed unlimited drinking. The OP was then performed after induction of anesthesia with 3% sodium pentobarbital solution. The surgeon opened the skin along the right femoral artery, separated the subcutaneous tissue layer by layer, and freed the femoral artery (2–3 cm). The femoral artery was punctured, a 4-Fr vessel sheath was inserted, the balloon was expanded and pulled back three times, the right femoral artery was ligated, and the skin was sutured. The animals were administered 40,000 U of penicillin for 5 days postoperatively.

CS procedures

CS refers to the nonspecific systemic reaction that occurs when the body is stimulated by various internal and external environmental factors for a long time.[13] Because no mature stress model exists for

the rabbit, social stress combined with physiologic stress (Table 1) was adopted in the OP+HD+CS group. The program of social stress mainly involved the creation of an unstable social environment by replacing the rabbits' cage companions starting in the fifth week and continuing for 8 weeks as shown in Table 1. The time that the rabbits spent in the other cage (as an intruder) and in the home cage was balanced during the entire experiment. In addition, after 4 hours of social stress, physical stress was induced and included overnight illumination, white noise (80 dB), stroboscopic illumination, and foot shock (1 mA). This stress regimen was started at the end of the fifth week and continued for 8 weeks.

Assessment of CS model

Water intake assay. Water intake was evaluated the day before the end of the experimental procedure, and the measurement results were adjusted by the rabbit's body weight. The rabbits were closely observed after induction of CS. All rabbits were recorded on video for 60 minutes (from 08:00 to 09:00) to obtain information on the impact of the stress or on the rabbits' drinking behavior in its natural state. Drinking behavior was recorded three to four times every week. The individual drinking assessments of the rabbits in the same group were summed to provide a total score at the end of the experiment. The drinking activity of the three categories for each rabbit was scored as the percentage of the 60-minute observation period. The total percent time in each group was calculated after stress exposure (at week 12).

Inactivity and activity assay. Inactivity was defined as sitting quietly or curled up in the rabbit cage with no visible body movements. Activity (movement) was used as an indicator of the rabbits' behavior and

Table 1. Steps involved in establishment of social stress and physical stress in the balloon-injury operation + high-fat diet + chronic stress group.

Time	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
Week 5	No. 1→No. 2 then OI 12 h	No. 2→No. 1 then WN 2 h	No. 1→No. 2 then SI 2 h	No. 2→No. 1 then FS 5 times	No. 1→No. 2 then No PS	No. 2→No. 1 then No PS
Week 6	No. 1→No. 3 then OI 12 h	No. 3→No. 1 then WN 2 h	No. 1→No. 3 then SI 2 h	No. 3→No. 1 then FS 5 times	No. 1→No. 3 then No PS	No. 3→No. 1 then No PS
Week 7	No.1→No.4 Then OI 12h	This protocol was continued until Week 12.				

Annotation: The above process was begun after 4 weeks of the experiment (Week 5) and continued for a total of 8 weeks. For example: The No. 1 and No. 2 rabbits were placed in the same cage (the No. 2 cage) for 4 hours on Monday. The No.1 rabbit is then placed in the No.1 cage, and the rabbits remain in the No. 1 cage for 4 hours on Tuesday. The No. 2 rabbit is placed in the No. 2 cage, and the process continues. OI, overnight illumination; WN, white noise (80 dB); SI, stroboscopic illumination; FS, foot shock (1 mA); PS: physical stress including OI, WN, SI, and FS. After 4 hours of social stress, physical stress was carried out.

included walking, stretching the limbs, biting the rabbit cage, moving the head vertically up and down, shaking the body, licking the claws and fur, and cleaning the face, ear, hip, and cage with the claws. The time arrangements and records were similar to those described above. The test was conducted in a soundproof room with no human disturbance to eliminate bias due to scaring of the rabbits by the researchers. Two independent observers assessed the rabbits' motivational behavior in a blinded manner. The mean value of the results was statistically evaluated by checking the inter-observer reliability.

Biochemical analysis. Blood samples were collected under chloral hydrate anesthesia from 07:00 to 09:00 every 4 weeks, after 12 hours of fasting, and centrifuged at 3500 rpm for 15 minutes by a cryogenic centrifuge. The serum was then transferred into a separate vial and stored at 4°C. An automated biochemical analyzer (Beckman Coulter, Brea, CA, USA) was used to measure the levels of total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and homocysteine (HCY) by enzymatic assays. An enhanced immuno-turbidimetric assay was used to measure high-sensitivity C-reactive protein (hs-CRP). A commercially available enzyme-linked immunosorbent assay was used to measure interleukin-6 (IL-6) and monocyte chemotactic protein-1 (MCP-1).

Histology of abdominal aorta. The rabbits were killed and the abdominal aorta was anatomized and cut into parts. Part of the aorta was quick-frozen in liquid nitrogen and stored at −80°C for RNA extraction, western blot analysis, and proteomic analysis; the remaining parts of the aorta were stored in 4% polyformaldehyde solution and inserted into Tissue-Tek OCT compound (Sakura, Tokyo, Japan) for frozen section. After fixation in 4% polyformaldehyde solution embedded in paraffin wax, serial 6-μm-thick sections were collected on slides and visualized by hematoxylin and eosin staining. The total intima-media thickness, intima thickness, media thickness, and wall thickness were measured on hematoxylin- and eosin-stained sections of the abdominal aorta under a microscope (Axio Zoom.V16; ZEISS, Oberkochen, Germany) using Image Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, USA). Mean values were calculated by areas measured on two parts (six replicates for each section) per artery.

Proteomics. The rabbits' abdominal aortic tissues were ground in liquid nitrogen and lysed by protein extraction buffer (8 M urea, 0.1% sodium dodecyl sulphate) containing additional protease inhibitor cocktail (Roche, Basel, Switzerland) and 1 mM phenylmethylsulfonyl fluoride (Beyotime Biotechnology, Shanghai, China) on ice for 30 minutes and then centrifuged at 14,000 rpm for 15 minutes at 4°C. The supernatant was gathered and the protein concentrations were measured with a Pierce bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, Waltham, MA, USA). The cell lysis was stored at −80°C before further processing. Samples were tested for quality using a BCA quantitative kit (Table 2). Isobaric tags for relative and absolute quantitation (iTRAQ; AB Sciex, Framingham, MA, USA) with different reporter ions (113–121 Da) were applied as isobaric tags for relative quantification. iTRAQ labeling was conducted according to the manufacturer's instructions. C18 chromatographic column sample classification was carried out. In total, 40 fractionations of labeled peptides were further concatenated into 20 fractions, vacuum-dried, and deposited at −80°C until further liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis, which was

Table 2. Quantitative results of bicinchoninic acid assay.

Order	Group	Sample volume (mg)	Concentration (μg/μL)	Total protein (μg)
1	CG	210	3.686	1179
2	OP+HD	244	3.542	779
3	OP+HD+CS	214	3.800	1406

CG, control group; OP+HD, balloon-injury operation + high-fat diet group; OP+HD+CS, balloon-injury operation + high-fat diet + chronic stress group.

Table 3. Primers for real-time polymerase chain reaction.

Gene	Forward (5'→3')	Reverse (5'→3')	GenBank no.
BAX	TCCTCTCCTACTTCGGGACC	AGTAAGAAAAACGCCTGTGTCC	XM_002723696.3
UBE2K	TTCCGTCACAGGGGCTATTT	GCATCTTGTGGGTCATCTGG	XM_002709395.3
CASP3	GGTAGCGACAGAGTTCGAGT	TGAGAGGGAAGCAGAGTAACAG	XM_008273924.2
CASP9	TGTTTCCGAGCGAGGGATTT	CGCAGGAAGGTTTTGGGGTA	XM_008249762.2
p53	ATGCCTACCTCACGGGGTCT	AGGGTAGGGAACCAGCACCAT	NM_001082404.1
FAS	CTTTGCACGGGTTGTTGGTA	TGGTAGGGAGAGGGGCTTAT	NM_001081995.1

performed with a Q Exactive mass spectrometer (Thermo Fisher Scientific). The National Center for Biotechnology Information RefSeq rabbit protein sequence database was searched using the SEQUEST algorithms with Proteome Discoverer software, version 1.4 (Thermo Fisher Scientific).

Measurement of mRNA expression by quantitative real-time polymerase chain reaction. Under an ice bath, the RNA in the abdominal aorta was extracted using Trizol reagent (Invitrogen, Beijing, China). The primer sequences are listed in Table 3. Agarose gel electrophoresis was performed to confirm the quality of the samples. The optical density ratio (A260/A280) of 260 nm and 280 nm (1.8–2.0) was measured by an ultraviolet spectrophotometer, and real-time polymerase chain reaction (PCR) was performed. The following formula was used to calculate the relative mRNA levels: $2 - \Delta c$ [average ct of target gene − average ct of housekeeping gene].

Statistical analyses. The experimental results are shown as mean ± standard deviation. Statistical analyses were performed using SPSS Statistics for Windows, version 21.0 (IBM Corp., Armonk, NY, USA). Student's t-test was used to analyze two groups, and one-way analysis of variance was used to analyze three or more groups. A P value of <0.05 was considered to indicate a statistically significant difference.

Results

Evaluation of the effectiveness of CS management by depression indicators

All rabbits showed the same physical and social states at the beginning of the experiment. When the experiment ended at week 12, one-way analysis of variance revealed a remarkable main effect of CS in the drinking and behavior tests. The rabbits' water intake was significantly lower in the OP+HD+CS than OP+HD group ($P < 0.05$). The rabbits were monitored from 08:00 to 09:00 to determine whether

the CS procedure could lead to variation in their behavior. The rabbits from the OP+HD+CS group exhibited significantly more inactivity than those in the CG and OP+HD group ($P < 0.01$ and $P < 0.05$, respectively), and the inactivity level was significantly greater in the OP+HD group than in the CG ($P < 0.05$). However, the locomotor behavior was significantly greater in the OP+HD group than in the CG ($P < 0.05$). The rabbits in the OP+HD+CS group showed significantly less locomotor behavior than those in the CG ($P < 0.05$) and OP+HD group ($P < 0.05$) (Figure 1(a)).

Changes in lipid metabolism under CS

The concentrations of TC and LDL-C were significantly higher in both the OP+HD and OP+HD+CS groups than in the CG ($P < 0.01$). However, there was no obvious difference in HDL-C. The mean serum TC and LDL-C levels were significantly higher in the OP+HD+CS than OP+HD group ($P < 0.05$). These statistical data indicate that CS and an HD have crucial impacts on lipid metabolism (Figure 1(b)).

Impact of CS on HCY level

HCY is formed when the amino acid cysteine gains an extra methylene (-ch2-) in front of the mercaptan (-sh) of the side chain. There is a hypothesis that hyper-homocysteinemia can lead to atherosclerotic vascular disease.

At week 12, the mean serum HCY level was significantly higher in the OP+HD+CS than in the OP+HD group and CG ($P < 0.05$ for both). The HCY level was also significantly higher in the OP+HD group than in the CG ($P < 0.05$) (Figure 1(b)).

Enhancement of inflammation under the condition of CS

The concentrations of IL-6, hs-CRP, and MCP-1 were significantly higher in both the OP+HD and OP+HD+CS groups than in the CG ($P < 0.05$ for both). In addition, the concentrations of hs-CRP, IL-6, and MCP-1 were significantly higher in the OP+HD+CS than OP+HD group ($P < 0.05$) (Figure 1(b)).

iTRAQ-coupled two-dimensional LC-MS/MS assay for abdominal aortic plaque protein profiling

As shown in Figure 2, the BCA quantitative result, sodium dodecyl sulphate polyacrylamide gel electrophoresis gradient electrophoresis map, polypeptide graded elution gradient, and liquid chromatography elution gradient parameters exhibited credible reproducibility. The numbers of different proteins in the three groups are shown in Table 4. On the basis of gene ontology analysis, the proteins were mainly involved in cellular processes, metabolic processes, single-organism processes, and regulation ($P < 0.0001$, OP+HD vs. OP+HD+CS). With respect to cellular components, the cell elements, cell parts, organelles, and organelle parts were highly enriched ($P < 0.0001$, OP+HD vs. OP+HD+CS). The top molecular functions were binding, catalytic activity, transporter activity, and molecular function regulation ($P < 0.0001$, OP+HD vs. OP+HD+CS) (Figure 3(a)). Analysis of the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway showed that apoptosis, the p53 signaling pathway, and metabolic pathways were the significantly enriched pathways ($P < 0.0001$, OP+HD vs. OP+HD+CS) (Figure 3(b)). Similar results were obtained in the analysis of CG vs. OP+HD+CS (Figure 3(c), (d)) and OP+HD vs. CG (Figure 3(e), (f)). These findings indicate
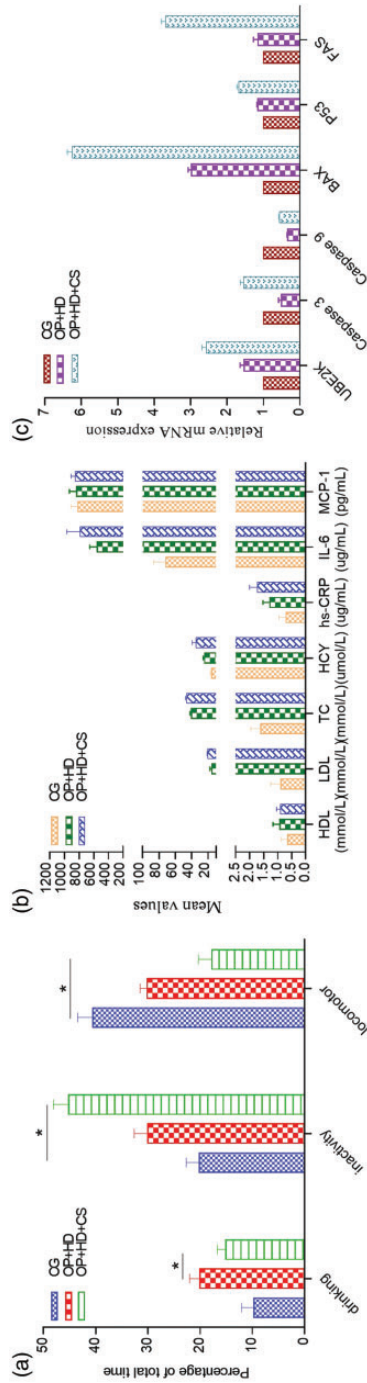
Figure 1. (a) Chronic stress exposure brought about depression-like symptoms in a rabbit model (b) Effect of chronic stress on lipid metabolism, the HCY level, and inflammation (c) Relative mRNA expression of several proteins connected with cell apoptosis.
CG, control group; OP+HD, balloon-injury operation + high-fat diet group; OP+HD+CS, balloon-injury operation + high-fat diet + chronic stress group; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TC, total cholesterol; HCY, homocysteine; hs-CRP, high-sensitivity C-reactive protein; IL-6, interleukin-6; MCP-1, monocyte chemotactic protein-1.
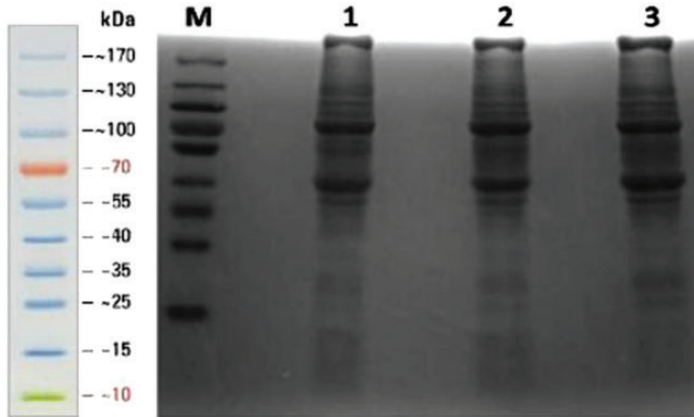
Figure 2. Sodium dodecyl sulphate polyacrylamide gel electrophoresis gradient electrophoresis map. M, marker; 1, control group; 2, balloon-injury operation + high-fat diet group; 3, balloon-injury operation + high-fat diet + chronic stress group.

Table 4. Numbers of different proteins.

	CG vs. OP+HD	CG vs. OP+HD+CS	OP+HD vs. OP+HD+CS
A (number)	239	334	163
B (number)	179	236	92

A, number of different proteins without any limits; B, number of different proteins under the limits. (≥2 specific polypeptides and ≥1.5 times). CG, control group; OP+HD, balloon-injury operation + high-fat diet group; OP+HD+CS, balloon-injury operation + high-fat diet + chronic stress group.

that CS has a certain impact on cell apoptosis, p53, and molecular function regulators. Therefore, from the database of distinct proteins, we selected BAX, UBE2K, caspase 3, caspase 9, p53, and FAS for verification because of the strong correlation between apoptosis and these six proteins.

Stimulative effects of CS on cell apoptosis

To demonstrate the impacts of CS on cell apoptosis, we examined the mRNA expression of BAX, UBE2K, caspase 3, caspase 9, p53, and FAS in the abdominal aorta using real-time PCR. Compared with the CG, the OP+HD+CS group exhibited enhanced mRNA expression of UBE2K, caspase 3, BAX, p53, and FAS ($P < 0.01$) but showed an opposite effect on caspase 9 ($P < 0.05$). The OP+HD group showed elevated mRNA expression of UBE2K, BAX, p53, and FAS ($P < 0.01$) and reduced mRNA expression of caspase 3 and caspase 9. We also found that CS seemed to have strong stimulative effects on cell apoptosis as shown by the fact that the mRNA levels of all six proteins were higher in the OP+HD+CS than OP+HD group ($P < 0.05$ for all) (Figure 1(c)).

Pathological changes in abdominal aorta among the different groups

The intima was significantly thicker in the OP+HD and OP+HD+CS groups than in the CG ($P < 0.05$). However, there was no significant difference between the OP+HD and OP+HD+CS groups. Similarly, the media was significantly thicker in the OP+HD group than in the CG and OP+HD+CS group ($P < 0.05$). However, the media thickness was similar between the OP+HD+CS group and CG
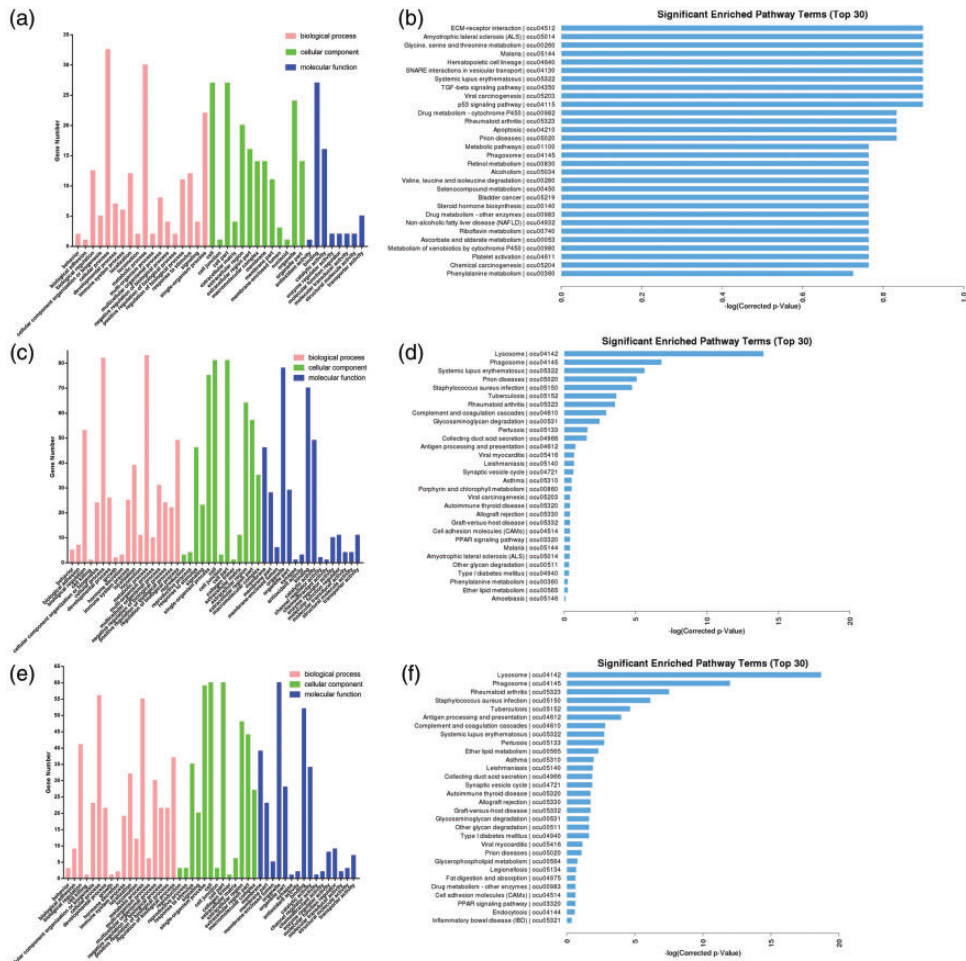
Figure 3. Analysis of gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway. (a) GO analysis for different proteins between the OP+HD and OP+HD+CS groups (b) KEGG pathway analysis for different proteins between the OP+HD and OP+HD+CS groups (c) GO analysis for different proteins between the CG and OP+HD+CS group (d) KEGG pathway analysis for different proteins between the CG and OP+HD+CS group (e) GO analysis for different proteins between the OP+HD group and CG (f) KEGG pathway analysis for different proteins between the OP+HD group and CG.
CG, control group; OP+HD, balloon-injury operation + high-fat diet group; OP+HD+CS, balloon-injury operation + high-fat diet + chronic stress group.

(Figure 4). These findings indicate that CS did not have an impact on the intimal thickness but did result in a decrease in the medial thickness. Furthermore, the OP+HD group showed an increase in the thickness of both the media and intima.

Discussion

The principle of abdominal aortic balloon injury is that mechanical strain results in the loss of vascular endothelial cells and destruction of the endometrial integrity;
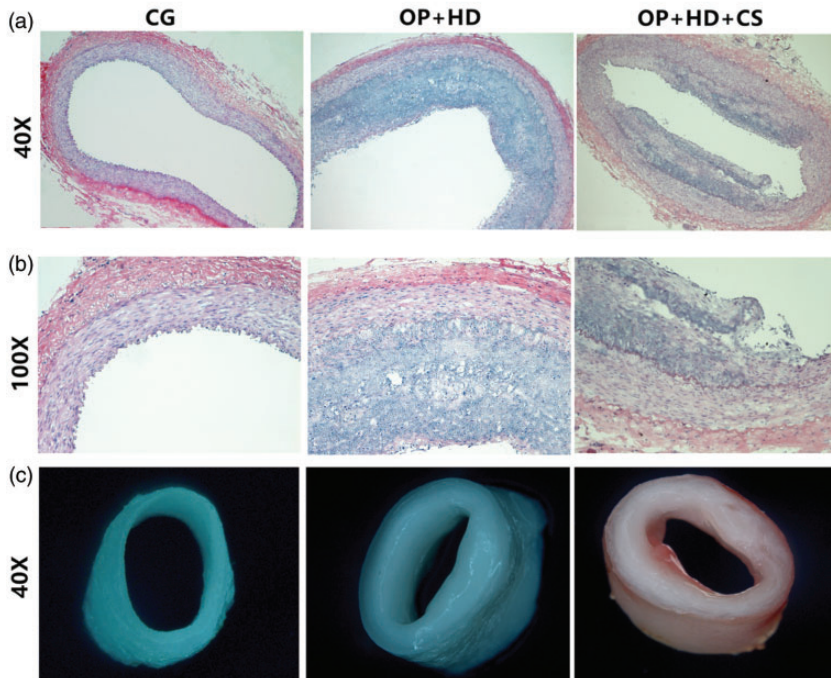
Figure 4. Histological changes in abdominal aorta by (a, b) hematoxylin and eosin staining and (c) gross examination. Hematoxylin and eosin staining was used to analyze the development of atherosclerotic lesions in all groups (CG, OP+HD, and OP+HD+CS). (a) 40× magnification (b) 100× magnification (c) original magnification.
CG, control group; OP+HD, balloon-injury operation + high-fat diet group; OP+HD+CS, balloon-injury operation + high-fat diet + chronic stress group.

combination of this injury with an HD results in lesions similar to those of human AS plaques. This model is used in the research of atherosclerotic disease, and some of its advantages include a more mature approach, a high success rate, and a less time-consuming process. Studies have shown that the degree of balloon injury is related to the diameter of the balloon.[14] A balloon with a large diameter can cause serious injury to the internal elastic plate and middle membrane. Properly decreasing the diameter of the balloon can reduce the pressure in the balloon and exert slight tension on the vessel wall, resulting in only desquamation of endothelial cells and leaving the internal elastic plate and medial membrane relatively intact; this can better simulate the pathological process of human atherosclerotic disease. However, the balloon diameter is too small to injure the endothelial cells. Therefore, choosing the most appropriate balloon is crucial. After a preliminary experiment in which 4-Fr and 5-Fr vaginal sheaths were compared, the 4-Fr vaginal sheath was selected for the OP. A Chinese study demonstrated that a sustained HD increases lipid deposition and induces endothelial damage.[15] Normal feeding is extraordinarily important for the first 4 weeks after surgery because it can help to form a fibrous cap and the lipid core, similar to the human atherosclerotic plaque.[16]

The evaluation of a CS model consists of three aspects: water intake, inactivity, and locomotion. CS affects animals' food and water intake, thereby delaying normal weight gain. During long periods of exposure to CS, the rabbit's mood changes and depressive episodes are noted, including increased inactivity and decreased locomotion. However, OP+HD does not affect the water intake and induce an obvious depression-like state. Additionally, ample evidence indicates that unpredictable mild CS can lead to depression,[7,8,17–19] and strong relationships between moderate depression and both the risk of cerebrovascular disease and higher fatality rates have been demonstrated by numerous researchers. More obvious apoptosis (with changes in Bcl-2 family expression), worsened oxidative and nitrosative damage, and infarction have been observed in models of moderate depression than in the normal group; thus, the vessel's sensibility to protective conditioning stimuli is attenuated in response to depression.[20–22]

In the present study, the serum TC and LDL-C levels were 25 times higher in the OP+HD group than in the CG. Furthermore, the effects of CS were indicated by the higher levels of TC and LDL-C in the OP+HD+CS than OP+HD group. However, the HDL-C level did not change in either the OP+HD or OP+HD+CS group. High levels of LDL-C and TC can harm to the body; in contrast, HDL-C is beneficial. Therefore, increases in the LDL-C and TC levels are manifestations of a lipid metabolism disorder. Studies have shown that the main mechanisms of CS-induced lipid metabolism disorders are as follows. (1) Under CS, the liver produces more cholesterol. (2) Elevated stress hormones affect the activity of lipoprotein lipase, hepatic lipase, and other hormone-sensitive lipases, facilitating fatty tissue to release fatty acids to provide a substrate for the synthesis of triglycerides and very-low-density lipoproteins. (3) The increase in sympathetic activity restrains insulin secretion and stimulates the secretion of glucagon, both of which can greatly induce adipose tissue decomposition. (4) Throughout this process, the rate of exogenous lipid clearance decreases.[23] Research has also indicated that the development of AS may be associated with dyslipidemia.[24,25] However, a novel insight is that neuropeptide Y, a mediator of vascular lipid metabolism disorders in CS, is a stress-induced risk factor for lipid metabolic syndrome and AS. An understanding of how neuropeptide Y and its homologous receptors regulate lipid metabolism can offer meaningful insight into future therapies for stroke.[26,27]

HCY is an important risk factor for cardiovascular disease. A high HCY level indicates an increased concentration of both HCY and mixed sulfide in plasma, which is caused by a metabolic disorder of methionine. Increased HCY in the blood stimulates damage to arterial walls, causing inflammation and formation of plaque and finally resulting in blocked blood flow of the heart; thus, high HCY is an independent and significant risk factor for coronary heart disease.[28] Additionally, in the present study, CS significantly increased the concentration of HCY, further illustrating that stress is an pivotal risk factor for AS.[29] An important reason for the high HCY level secondary to CS is decreased activity of cysteine in liver cells and renal cells.

An interaction between CS and related diseases, such as anxiety, depression, and the inflammatory response, has been confirmed.[30–32] Inflammation is the body's defensive reaction to stress, which helps the body adapt to the environment. However, inflammation can also promote disease progression. CS can activate stress hormones; promote the body to release a large number of cytokines, such as IL-6,

acute-phase protein, MCP-1, and nuclear factor kappa B; and enhance the inflammatory response. In some studies, the hs-CRP, IL-6, and MCP-1 levels were higher in the serum of patients with than without CS.[33,34] In the present study, the serum levels of hs-CRP, IL-6, and MCP-1 were significantly higher in the OP+HD group and were more obvious in the OP+HD+CS group. Moreover, intercellular adhesion molecule-1, the acute-phase reactant CRP, and the proinflammatory cytokine IL-6 were meaningfully higher in CS-treated apolipoprotein E knockout mice than in untreated animals as reported by Kershaw et al.[35] and Chumaeva et al.[36]

Through general observation and examination of pathological sections, we found that CS had no impact on the intimal thickness but did result in a decrease in the medial thickness. However, previous studies have shown that CS can increase the total area of AS lesions.[37] The lack of a significant change in the thickness of the endometrium after 12 weeks of CS may be related to the following factors. (1) The time of CS was not long enough. (2) The OP may have induced great damage to the intima, preventing accurate simulation of human endothelial cell injury. Moreover, the structural changes in the vessel wall are due to the remodeling of blood vessels, and SMCs play an important role in the structure and function of the vessel wall. In the present study, the media was significantly thicker in the OP+HD than normal diet groups, but the thickness of the media and the elastic fibers in the blood vessels decreased after CS. Therefore, we believe that CS not only leads to a decrease in the number of SMCs in the plaques but also results in a decline in the number of SMCs in the middle membrane. One hypothesis states that a thin media may be associated with cell apoptosis, which occurs at all stages of AS, affecting the early buildup and stability of plaques.[38,39] Early macrophage apoptosis

helps to reduce plaque volume but later increases the plaque burden.[40] The balance between proliferation and apoptosis of SMCs determines the structure of the vascular wall and plays an important role in the development of AS. At the beginning of AS, the SMC proliferation activity is stronger than the apoptosis activity, leading to a significant increase in the number of SMCs in the plaques and promoting the formation and development of the fiber cap. With the development of pathological changes, cell apoptosis predominates and rupture of unstable plaques occurs. Therefore, we performed a comprehensive proteomic analysis using iTRAQ-coupled two-dimensional LC-MS/MS, gene ontology, and the KEGG pathway. Under CS, proteins or genes were enriched in cellular processes, cell and molecular function regulation, apoptosis, and the p53 signaling pathway, which is consistent with the decrease in the middle membrane cells. Furthermore, we found that rabbits with CS had upregulated expression of apoptosis-related arterial genes (BAX, UBE2K, caspase 3, caspase 9, p53, and FAS), which boosted plaque instability; conversely, the protein, mRNA, or gene levels of all six of these proteins were lower in the OP+HD than OP+HD+CS group. The expression of UBE2K increased, reflecting activation of the ubiquitin proteasome system, which is the core pathway of protein degradation in eukaryotic cells and plays a role in regulating the cell cycle, apoptosis, and the process of AS. BAX is a water-soluble protein associated with bcl-2, which is the gene that promotes cell apoptosis in the bcl-2 gene family, and the overexpression of BAX can antagonize bcl-2 and cause the cell to die. Cysteinyl aspartate-specific proteinases (caspases) play a crucial role in cell apoptosis. Caspase 9 is a major participant in the initiation of cell apoptosis, triggering the cascade response through self-splicing

activation. This activates apoptosis executors including caspase 3, the common downstream part of various apoptosis pathways, the sign of irreversible cell apoptosis, and the most important apoptosis executor. The wild-type p53 gene promotes cell apoptosis. The DNA binding protein p53 checks DNA for damage, stops cells from entering the cell cycle, and starts the DNA repair mechanism once the defective DNA is discovered. If the repair fails, the p53 protein starts the cell apoptosis program, reduces bcl-2 gene expression, and activates Bax gene expression. The FAS gene is widely expressed in human tissues and belongs to the tumor necrosis factor and tumor necrosis factor receptor superfamily. In death receptor-associated apoptosis, FAS can combine with its ligand FASL, further activate the caspase cascade reaction, and eventually lead to apoptosis. The present study showed that the effects of CS were likely to be associated with apoptosis of middle membrane cells, which can cause plaque desquamation and cardiac and cerebrovascular occlusion. These proteins or genes can provide new insights into the mechanism of atherosclerotic disease and create a new target for the prevention and treatment of cardio-cerebrovascular disease. However, further research is necessary to clarify whether the targeting of proteins or stress-related biomarkers is an effective way to reduce the harmful effects of CS.

Conclusion

CS may promote cell apoptosis in the physiopathologic process of AS.

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