Vitamin C insufficiency causes the stress-induced sudden death through the induction of extensive heart injury.

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Vitamin C scavenges toxic free radicals as an antioxidant, and oxidative stress is considered as a major contributor inducing damage to cardiomyocytes in heart diseases. Also, it is well-known that stress provokes oxidative stress and induces cardiac sudden death. Here we shows the effects of vitamin C on the prevention of cardiac damage by stress in *gulo(-/-)* mice which cannot synthesize vitamin C. Vitamin C-insufficient *gulo(-/-)* mice under stress showed prominent cardiac damage and expired within 2 weeks. It was accompanied with structural changes in the heart, cardiac dysfunction and severe emphysema. These changes were caused by the elevation of pro-inflammatory cytokines, especially TNF-α, the activation of MMP-2/MMP-9, an increase in oxidative stress and a remarkable decrease in noradrenaline production. Thus, vitamin C insufficiency causes extensive cardiac damage under stress through regulating cytokine and hormone production as well as redox homeostasis, which results in stress-induced sudden death.

It is reported that 1 of the 3 American adults has one or more types of cardiovascular disease and that 35% of patients have coronary heart disease, heart failure and stroke. Cardiovascular disease is considered as a major cause of sudden death in people who are put under stress from their work. Regarding the induction and pathogenesis of cardiovascular disease, the roles of cellular antioxidants are considered to be critical in combating with ROS that induces cell death and tissue damage. Vitamin C is an outstanding water-soluble antioxidant with anti-inflammatory properties by effectively scavenging reactive oxygen and nitrogen species such as superoxide, hydroxyl radicals, peroxinitrite and nitrogen[1]. Vitamin C has proved to be beneficial to cardiac diseases through many biochemical and clinical studies. Reduction of short- and long-term oxidative stress and decreased superoxide generation by neutrophils are effectively achieved by administration of vitamin C in patients with heart failure[2]. In addition, it has been found the suppression of endothelial cell apoptosis in patients with congestive heart failure and significant improvement of endothelial dysfunction in idiopathic dilated cardiomyopathy by vitamin C administration[3, 4]. However, specific changes in the cardiovascular system by vitamin C insufficiency-closely related to sudden cardiac death−are still

unknown. So far, it is impossible to prove the *in vivo* effects of vitamin C using animal models because vitamin C is synthesized endogenously in most animals except humans and some primates. Therefore, we evaluated the effects of vitamin C on the prevention of severe heart damage and sudden cardiac death induced by stress, and the related mechanisms in *gulo(-/-)* mice *in vivo*. *Gulo(-/-)* mice cannot synthesize vitamin C endogenously because L-gulono-γ-lactone oxidase, which is an essential enzyme to the generation of vitamin C from glucose, is defective like in humans.

When mice were put under stress, corticosterone, a stress-related hormone, was remarkably increased but there was no significant difference in its level between the experimental groups (Fig. 1a). However, all of vitamin C-insufficient *gulo(-/-)* mice expired within 2 weeks after they were put under stress everyday (Fig. 1b). To clarify the cause of sudden death in vitamin C-insufficient *gulo(-/-)* mice under stress, we examined cardiac changes by immunostaining, immnoblotting and echocardiography. In *gulo(-/-)* mice, the heart size became smaller by vitamin C insufficiency. Along with small heart size and narrow left ventricular space, cardiac hypertrophy was found on the wall of left ventricle in the vitamin C-insufficient *gulo(-/-)* mice under stress (Fig. 1c and Supplementary Fig. S1). The direct evidence of cardiac hypertrophy and damage depending on vitamin C insufficiency and stress was verified with the change in the expression of atrial natriuretic factor (ANF), a marker for cardiac hypertrophy and degeneration. ANF expression was increased in the heart of vitamin C-insufficient *gulo(-/-)* mice and further increased by stress (Fig. 1d). Finally, cardiac damage induced by vitamin C insufficiency and stress was monitored by echocardiography in live *gulo(-/-)* mice. As shown in Fig. 1e, heart beats were remarkably decreased in live *gulo(-/-)* mice without vitamin C supplementation, and they became aggravated by stress (right panel), but they were completely recovered by vitamin C administration (center panel) like C57BL/6 wild type mice (left panel). In addition, the narrowness of the left ventricular space was also found as in Fig.1c. To clarify the cause of decreased heart rate in vitamin C-insufficient mice, we measured the noradrenaline level in the experimental groups because it is the principal catecholamine to regulate cardiac function under physiological conditions and acts as a main mediator of the sympathetic neural influence on the heart. Moreover, vitamin C plays an important role in the production of noradrenaline from dopamine as a

co-factor of dopamine-β-hydroxylase, which is a key enzyme in this process[5]. As expected, the production of noradrenaline was quite low in vitamin C-insufficient mice (Fig. 1f), but there were no significant differences in dopamine production between the experimental groups (Supplementary Fig. S2), suggesting that noradrenaline deficiency would happen in individuals who take insufficient amounts of vitamin C. In addition, when vitamin C-insufficient individuals are put under stress, they eventually die after extensive functional deficits develop in the heart.

Next, we investigated changes in the cardiac function by measuring the values of ejection fraction, fractional shortening, contractility index and dP/dt in each experimental group of live mice. All of the values showing cardiac functions were significantly decreased by vitamin C insufficiency in a time-dependent manner (Fig. 2a-d). However, there were no remarkably additional changes by stress (Fig. 2e-h). Besides, it is generally known that cardiac dysfunction is followed by malfunction of the respiratory system. In our study, pulmonary emphysema was observed only in vitamin C-insufficient *gulo(-/-)* mice under stress (Fig. 2i), suggesting that vitamin C deficiency increases the susceptibility to cardiac and respiratory failure induced by stress through functional and structural changes in the heart.

Free radicals may affect cardiac function through various mechanisms, including peroxidation of the lipid membrane, with subsequent perturbation of membrane-bound enzymes and receptors[6]. In addition, oxidative stress is enhanced in many cardiac diseases such as ischemia-reperfusion, cardiac hypertrophy, catecholamine-induced cardiomyopathy and heart failure[7]. Lipid peroxidation in the heart of vitamin C-insufficient *gulo(-/-)* mice under stress was more active than in C57BL/6 wild type and vitamin C-sufficient *gulo(-/-)* mice (Fig. 3a). This result was confirmed by a remarkable increase in the expression of manganese superoxide dismutase (MnSOD) which was elevated against oxidative stress (Fig. 3b). The increased oxidative stress resulted in extensive death of cardiomyocytes (Fig. 3c, d). These results imply that vitamin C supplementation can prevent cardiac death by stress through the protection of cardiomyocytes against increased oxidative stress-induced damage. In addition, the cardiac remodeling, especially the left ventricle, seems to be one of the important mechanisms of cardiac dysfunction, which is closely related to the increased expression and activity of matrix

metalloproteinases (MMPs)[8, 9]. Therefore, we examined the cardiac changes in MMP-2 and MMP-9. As shown in Fig. 3e, MMP-2 expression was slightly increased in vitamin C-insufficient *gulo(-/-)* mice without stress, but no significant differences was found under stress between the experimental groups. However, the expression and activity of MMP-9 was remarkably increased in vitamin C-insufficient *gulo(-/-)* mice by stress (Fig. 3f, g). From these results, it is inferred that vitamin C-insufficient *gulo(-/-)* mice are more susceptible to reconfiguration of the extracellular matrix in the heart through stress-induced activation of MMPs. The activation of MMPs is mediated by several pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, Interleukin (IL)-1α/β and IL-6. In addition, those cytokines are closely associated with deleterious effects on left ventricular function and accelerate the progression of heart failure and cardiomyocyte death via conventional apoptosis pathways or other cell death mechanisms[10]. Therefore, we examined the production of TNF-α, IL-1α/β and IL-6 in vitamin C-insufficient *gulo(-/-)* mice under stress. As shown in Fig. 4a, TNF-α production was remarkably increased in the sera of vitamin C-insufficient *gulo(-/-)* mice by stress. In addition, the production of IL-1α/β and IL-6 was also markedly increased upon vitamin C insufficiency and stress (Fig. 4b-d). Also, it has been reported that pro-inflammatory cytokines produced by damaged myocardium activates monocytes and subsequently impairs of myocardial function[11]. So, we examined the expression of TNF-α induced by vitamin C insufficiency and stress in cardiomyocytes. TNF-α production was increased at both transcriptional and translational levels by vitamin C insufficiency, and it was considerably increased by stress (Fig. 4 e, f and Supplementary Fig. S3). Recently, IL-32 has been identified as a novel cytokine that induces TNF-α production[12]. In our study, IL-32 production was slightly increased only by vitamin C insufficiency, and it was markedly increased by stress in vitamin C-insufficient *gulo(-/-)* mice (Supplementary Fig. S4), suggesting that increased TNF-α production in vitamin C-insufficient *gulo(-/-)* mice is achieved by IL-32 production. However, we do not exclude that the direct role of IL-32 on the induction of heart damage, and it should be further investigated. It has been demonstrated that TNF-α promotes the apoptosis of cardiomyocytes through the activation of p38 MAPK and JNK, as well as the

suppression of ERK1/2 activation[13-15]. It was examined that changes in the phosphorylation of p38 MAPK and ERK1/2 in the hearts of vitamin C-insufficient *gulo(-/-)* mice by stress. The phophorylation of p38 MAPK was up-regulated in vitamin C-insufficient *gulo(-/-)* mice by stress. In contrast, the phophorylation ERK1/2 was completely down-regulated (Fig. 4f). These results indicate that TNF-α may affect the activity of p38 MAPK and ERK1/2 in the heart in cases of vitamin C insufficiency, which is a potential mechanism of stress-induced cardiomyocytes death in such cases.

In addition to TNF-α, other soluble factors associated with death signaling are also regarded as contributors of cardiovascular disease. Fas/Fas ligand (FasL) interaction mediates extensive apoptosis of cardiomyocytes, which is an indicator of myocardial ischemia-reperfusion (IR) injury and myocardial infarction, and affects infarct size and long-term ventricular dysfunction[16-18]. Foo *et al*. reported that 50-60% reduction in infarct size is observed after IR injury in mice which have mutation on Fas[19]. We also found that soluble FasL (sFasL) was remarkably increased in vitamin C-insufficient *gulo(-/-)* mice by stress, whereas it was relatively low in vitamin C-insufficient *gulo(-/-)* mice without stress (Supplementary Fig. S5). And, the interaction between CD40 and CD40 ligand (CD40L) plays a crucial role in myocardial injury[20] and induces the production of pro-inflammatory cytokines/chemokines and the activation of MMPs in heart failure[21]. In our study, the amount of soluble CD40L (sCD40L) in mouse serum was examined in the presence or absence of vitamin C insufficiency and stress. Unlike sFasL, there was no remarkable increase in sCD40L by stress, whereas there was a significant increase in a CD40L by vitamin C insufficiency (Supplementary Fig. S6). This result means that severe cardiac damage may be induced by vitamin C insufficiency through an increase in sCD40L.

In conclusion, the results of our study suggest that vitamin C insufficiency can cause extensive inflammatory responses in the heart through the production of pro-inflammatory cytokines and oxidative stress, and may be closely related to cardiac injury through the induction of extensive structural and functional changes in the heart when vitamin C-insufficient individuals are put under chronic stress. Eventually, vitamin C deficiency seems to militate in risk of stress-induced sudden death. Thus, it is believed that vitamin C supplementation and maintenance of its blood concentration

may be necessary for the prevention of cardiac damage and sudden death by chronic stress.

< Methods>

Mice and stress

C57BL/6 wild type mice and *gulo(-/-)* mice were maintained in a specific pathogen free condition at the animal facility in the Seoul National University College of Medicine. Male g*ulo(-/-)* mice were maintained for 3-4 weeks with or without 3.3 g/L of vitamin C supplementation in water. Mice were exposed to stress for 1 hr everyday through placing in water bath containing cold (12±1^{o}C) water in a state of immobilizing in restrainers. After stress, mice were completely dried.

Echocardiography (ECG) and hemodynamic measurement

Mice were anesthetized with zoletil (25 mg/kg) and xylazine (10 mg/kg), and ECG was performed with VIVID-i ultrasound machine (GE Medical) by using 12 MHz probe. A two-dimensional short-axis view of the left ventricle was obtained at the level of the papillary muscles, and two-dimensionally targeted M-mode tracings were recorded. Followed by ECG, a microtip catheter transducer was inserted into the apex of left ventricle under pressure control. After stabilization, the pressure signals were recorded continuously with an ARIA pressure-volume conductance system coupled with a Powerlab/4SP A/D converter. PVAN software (Millar Instruments) was used for subsequent analysis of pressure-volume loops.

Enzyme immunoassay (EIA)/ Enzyme linked immunosorbent assay (ELISA)

Blood was collected from intraorbital plexuses of mice with capillary tube, and readily centrifuged with 14,000 rpm for 30 min at 4^{o}C. The level of corticosterone (Cayman), noradrenaline and dopamine (Labor Diagnostika Nord) was detected by EIA kit. The concentration of IL-1α, IL-1β, IL-6, TNF-α, sFasL and sCD40L (R&D system) was measured using ELISA kit. EIA and ELISA were performed according to the manufacturer's instructions.

Tissue staining

Hearts were freshly isolated and fixed in 4% paraformaldehyde at 4°C. Paraffin sections with 4 μm thickness were immunohistochemically stained with anti TNF-α antibody (R&D system), and counterstained with hematoxylin. Apoptotic cells were visualized with TUNEL staining kit (Roche) and nuclei were counterstained with DAPI. Masson's Trichrome staining was performed according to the manufacturer's instructions (Thermo scientific).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from frozen hearts using TRisol Reagent (Invitrogen) according to the manufacturer's instruction. Total RNA (2 μg) was reverse transcribed to cDNA and amplified with a primer set specific for TNF-α : forward, 5'-ATGAGCACACAGAAAGCATGATC-3' and reverse, 5'-TACAGGCTTGTCACTCGAATT-3'. PCR products were separated on 1.5% agarose gel, stained with ethidium bromide and visualized under UV light.

Immunoblotting

Extracted cardiac proteins were separated by electrophoresis and incubated with antibodies against MMP-2, IL-32, ERK, phospho-ERK, GAPDH (Santa Cruz), MMP-9 (abcam), TNF-α (R&D system), p38 MAPK, phospho-p38 MAPK (cell signaling), MnSOD (assay designs), ANF (Millipore) or β-actin (Sigma). Products were quantified using a densitometry analysis program (SCION Image program).

Gelatin zymography

To examine activities of MMP-2 and MMP-9, cardiac protein (10 μg/lane) was loaded onto 10% gelatin minigel with 0.75 mm space. After electrophoresis, gel was washed with 2.5% Triton X-100 buffer contained 50 mM Tris (pH 7.4), 5 mM $CaCl_2$ for 1 hr at room temperature. After brief washing with DW, gel was incubated with incubation buffer contained 50 mM Tris (pH 7.5), 200 mM NaCl and 20 mM $CaCl_2$ for 16 hrs at 37 °C. Gel was stained with 0.2% coomassie blue for 1 hr and

destained with 30% methanol and 10% acetic acid mixture for 2 hrs. Digested regions, white bands on a blue background, were quantified using a densitometry analysis program (SCION Image program).

TBARS (thiobarbituric acid-reactive substance) assay

Lipid peroxidation in the heart was measured by TBARS assay kit (Cayman) according to the manufacturer's instructions. The final concentration of malondealdehyde (MDA) was standardized with cardiac protein quantified with BCA method.

Statistics

Data were expressed as mean±s.d. of each group in independent experiments. For comparison of three or more groups, data were analyzed by one-way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison test. A value of $p<0.05$ was considered statistically significant. Statistical tests were carried out using GraphPad InStat (GraphPad Software).

< Figure legend >

Figure 1 Decreased survival of vitamin C-insufficient *gulo(-/-)* mice by stress accompanied with structural changes on heart. **a,** The change of corticosterone levels by stress (*n*=10 for each group). **b,** Survival rate of WT (*n*=19), vitamin C-sufficient *gulo(-/-)* mice (*n*=19) and vitamin C-insufficient *gulo(-/-)* mice (*n*=22) for 25 days of stress. **c,** The morphological changes of heart upon vitamin C insufficiency and stress were examined by hematoxylin and eosin (H&E) staining. Scale bar, 2 mm. **d,** The expression of ANF in the heart was determined by immunoblotting. **e.** Analysis of structural and functional changes on heart upon vitamin C insufficiency and stress by echocardiography. Ended line indicates the left ventricular space. **f,** After mice were put under stress for 8 days, the production of noradrenaline was monitored by EIA (n=4). Results are presented as mean±s.d.; single asterisk denotes P<0.05 versus vitamin C-sufficient *gulo(-/-)* mice group without stress, and triple asterisk indicates P<0.001 versus vitamin C-sufficient *gulo(-/-)* mice group with stress.

Figure 2 The changes on cardiac function and pulmonary structure in vitamin C-insufficient *gulo(-/-)* mice by stress. **a-d,** After vitamin C was depleted for 11, 23, and 35 days, the changes of hemodynamic indexes upon vitamin C insufficiency were monitored by ECG and hemodynamic measurement. Asterisk denotes P<0.05 versus vitamin C-insufficient *gulo(-/-)* mice group at 23 days. **e-h,** The functional changes in the heart upon stress. Triple asterisk indicates P<0.001 versus vitamin C-sufficient *gulo(-/-)* mice group without stress. Triple cross indicates P<0.001 versus vitamin C-sufficient *gulo(-/-)* mice group with stress. (**a, e**) Ejection fraction (%), (**b, f**) Fractional shortening (%), (**c, g**) Contractility index and (**d, h**) dP/dt (mmHg/sec). Results are presented as mean±s.d. **i,** Structural change on lung was examined by H&E staining. Scale bars, 200 μm. Higher magnification views are shown in the bottom-left panel. Scale bars, 50 μm.

Figure 3 Induction of oxidative stress, activation of matrix metalloproteinase, and extensive apoptosis

of cardiomyocytes in heart from vitamin C-insufficient *gulo(-/-)* mice by stress. **a,** The concentration of malondealdehyde (MDA), a product of lipid peroxidation, in the heart was measured by TBARS assay, and normalized with MDA (nM) per 1 μg of cardiac protein. Asterisk denotes $P<0.05$ versus vitamin C-sufficient *gulo(-/-)* mice with stress. **b,** The compensatory expression of MnSOD against oxidative stress in the heart was examined by immunoblotting. **c,** Images of apoptotic cardiomyocytes. Apoptotic signals were localized in nucleus of cardiomyocyte (green) in vitamin C-insufficient *gulo(-/-)* mice under stress. Scale bar, 50 μm. **d,** Apoptotic cells were represented as percentage of TUNEL/DAPI positive cells (n=6-11). **e-f,** Immunoblotting of (**e**) MMP-2 and (**f**) MMP-9 in the heart upon vitamin C insufficiency and stress. **g,** The changes on enzymatic activity of MMP-2 and MMP-9 in heart upon vitamin C insufficiency and stress were determined by gelatin zymography.

Figure 4. Increase of pro-inflammatory cytokines, especially TNF-α, production and its related signaling pathways by stress in vitamin C-insufficient *gulo(-/-)* mice. **a-d,** Mice were put under stress for 8 days, and the levels of pro-inflammatory cytokines were monitored by ELISA (n=6-8). Triple asterisk indicates $P<0.001$ versus vitamin C-sufficient *gulo(-/-)* mice with stress. (**a**) TNF-α, (**b**) IL-1α, (**c**) IL-1β, (**d**) IL-6. **e,** Immunostaining of TNF-α (brown) in the heart upon vitamin C deficiency and stress. Scale bar, 30 μm. **f,** With elevated production of TNF-α, the activation of p38 MAPK and inactivation of ERK1/2 in the heart in vitamin C-insufficient *gulo(-/-)* mice under stress were examined by immunoblotting.

Supplementary Figure 1 Hypertrophic change on cardiomyocytes upon vitamin C insufficiency and stress. Heart sections were subjected to Masson's Trichrome staining. Scale bars, 50 μm. Higher magnification views are shown in the bottom-left panel. Scale bars, 15 μm.

Supplementary Figure 2 The comparison of dopamine levels upon vitamin C insufficiency and stress. After C57BL/6 wild type mice (n=5), vitamin C-sufficient *gulo(-/-)* mice (n=10) , and vitamin

C-insufficient *gulo(-/-)* mice (*n*=10) were exposed to stress for 8 days, the levels of dopamine were determined by EIA. Results are presented as mean±s.d.

Supplementary Figure 3 The effects of vitamin C insufficiency and stress on TNF-α mRNA in the heart. The change of the expression of TNF-α mRNA transcripts upon vitamin C insufficiency and stress was examined by RT-PCR. β–actin was used as a loading control.

Supplementary Figure 4 The expression of IL-32 in the heart upon vitamin C insufficiency and stress. The changes of IL-32 expression in the heart was examined by immunoblotting. GAPDH was used as a loading control.

Supplementary Figure 5 The changes of sFasL production upon vitamin C insufficiency and stress. Mice were exposed to stress for 8 days, and the levels of sFasL in the sera were measured by ELISA (*n*=3). Triple asterisk indicates P<0.001 versus vitamin C-sufficient *gulo(-/-)* mice with stress. Results are presented as mean±s.d.

Supplementary Figure 6 The changes of sCD40L production upon vitamin C insufficiency and stress. Mice were exposed to stress for 5 days, and the levels of sCD40L in the sera were measured by ELISA (*n*=4-5). Double asterisk indicates P<0.01 versus vitamin C-sufficient *gulo(-/-)* mice without stress. Single asterisk indicates p<0.05 versus vitamin C-sufficient *gulo(-/-)* mice with stress. Results are presented as mean±s.d.

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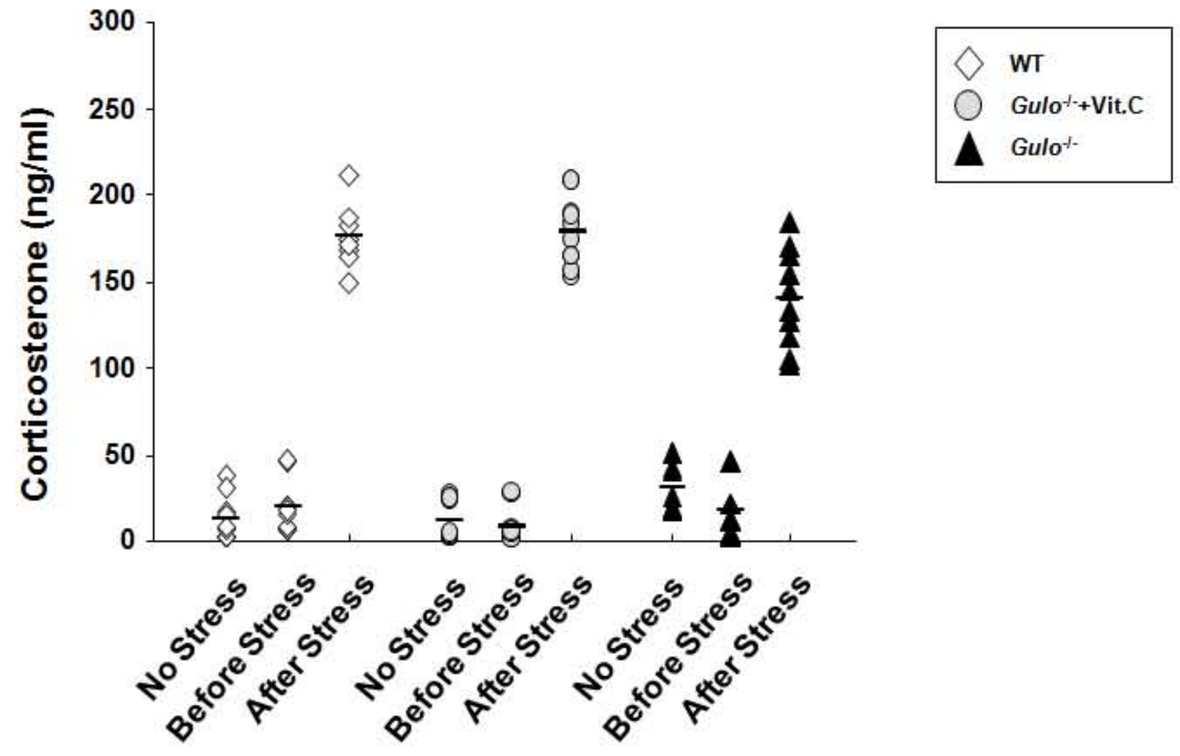
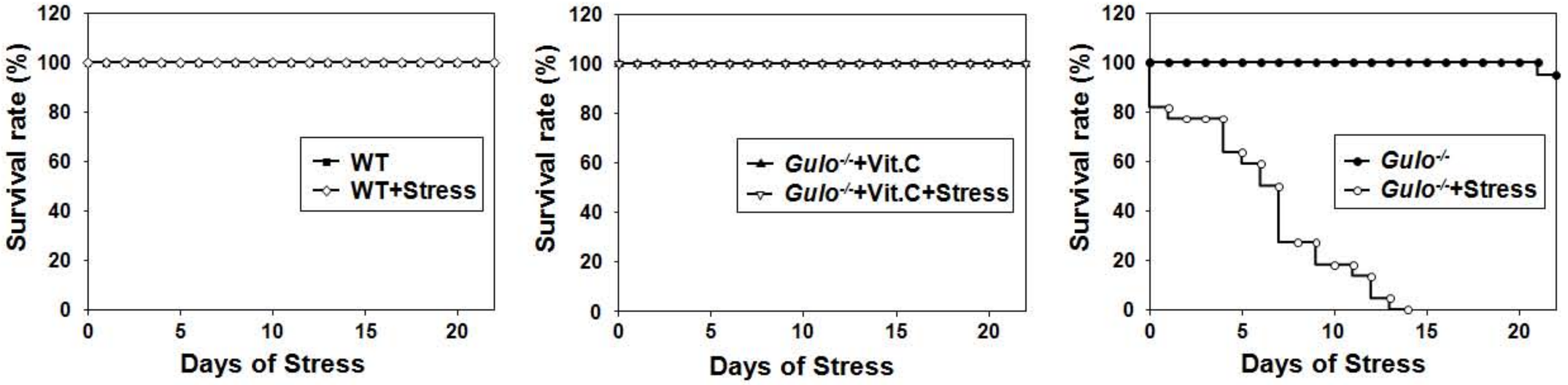
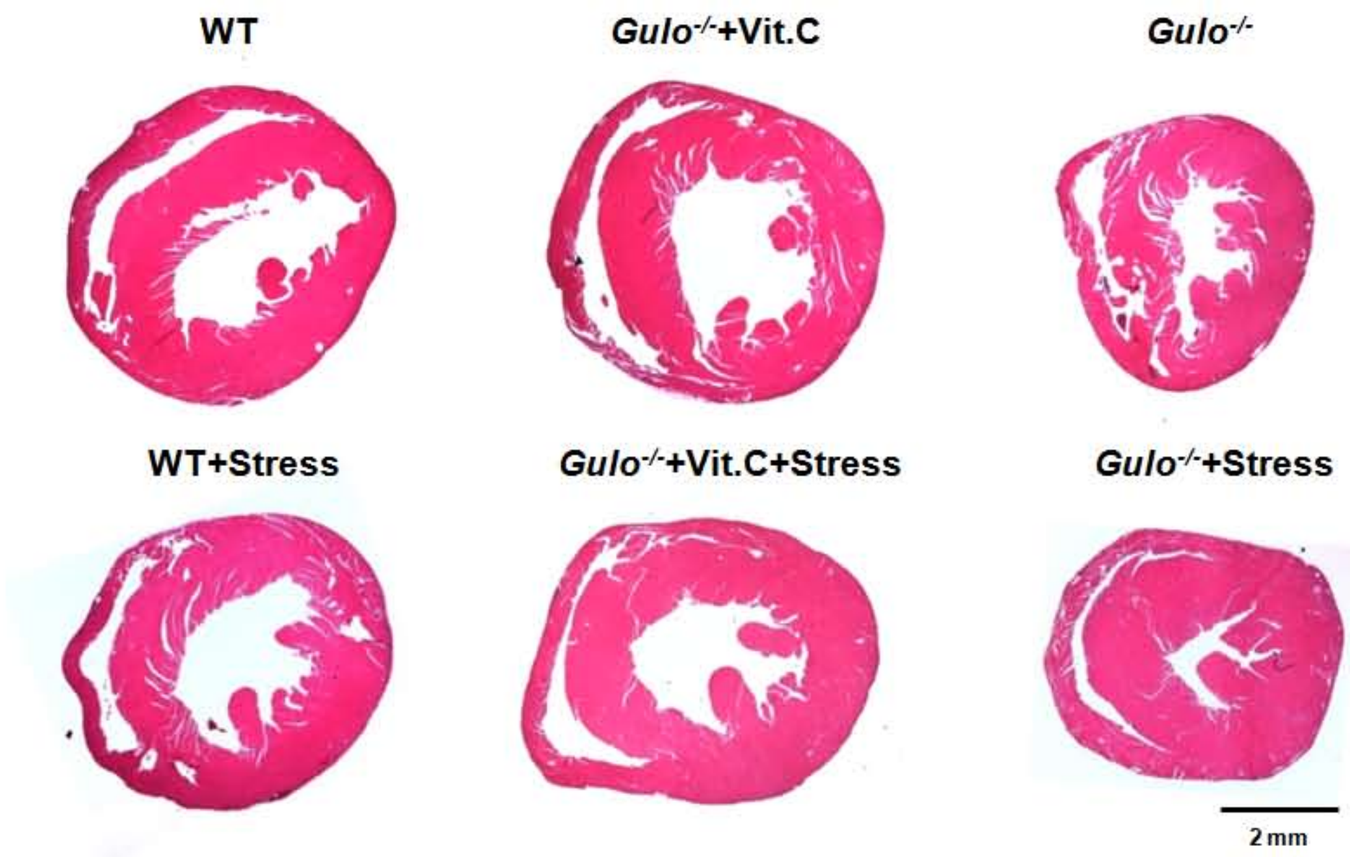
A.


Corticosterone (ng/ml)
300
250
200
150
100
50
0
WT
Gulo-/-+Vit.C
Gulo-/-
No Stress
Before Stress
After Stress
No Stress
Before Stress
After Stress
No Stress
Before Stress
After Stress

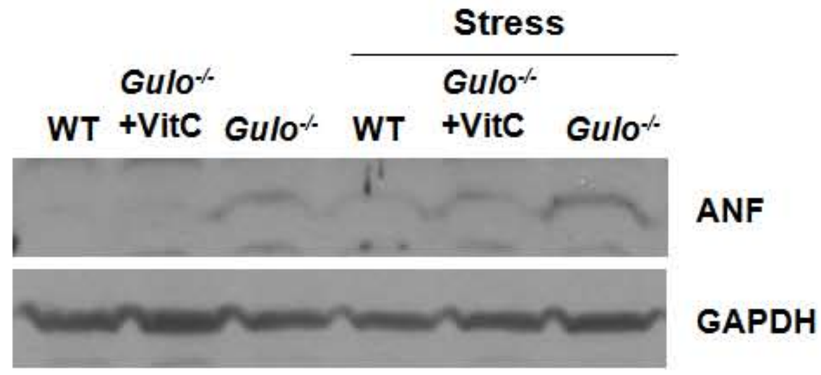
B.


Survival rate (%)
120
100
80
60
40
20
0
0
5
10
15
20
Days of Stress
WT
WT+Stress
Gulo-/-+Vit.C
Gulo-/-+Vit.C+Stress
Gulo-/-
Gulo-/-+Stress

C.


WT
Gulo-/-+Vit.C
Gulo-/-
WT+Stress
Gulo-/-+Vit.C+Stress
Gulo-/-+Stress
2 mm

D.


Stress
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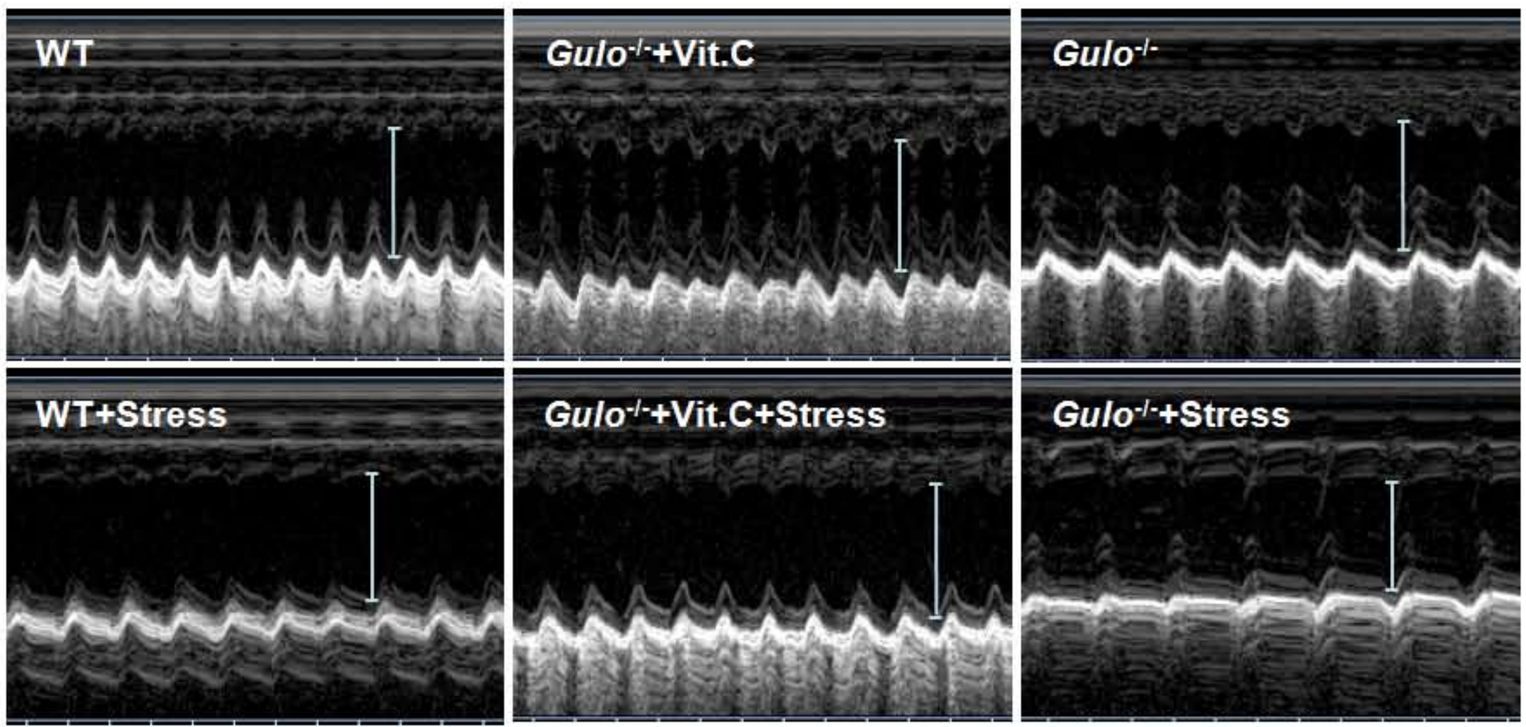
Gulo-/- +VitC
Gulo-/-
WT
Gulo-/- +VitC
Gulo-/-
ANF
GAPDH

E.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Gulo-/-
WT+Stress
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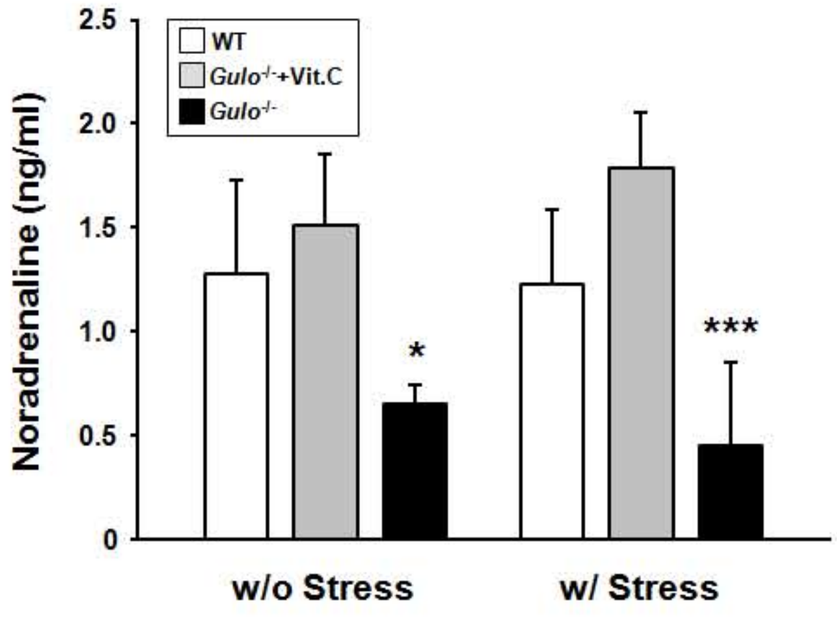
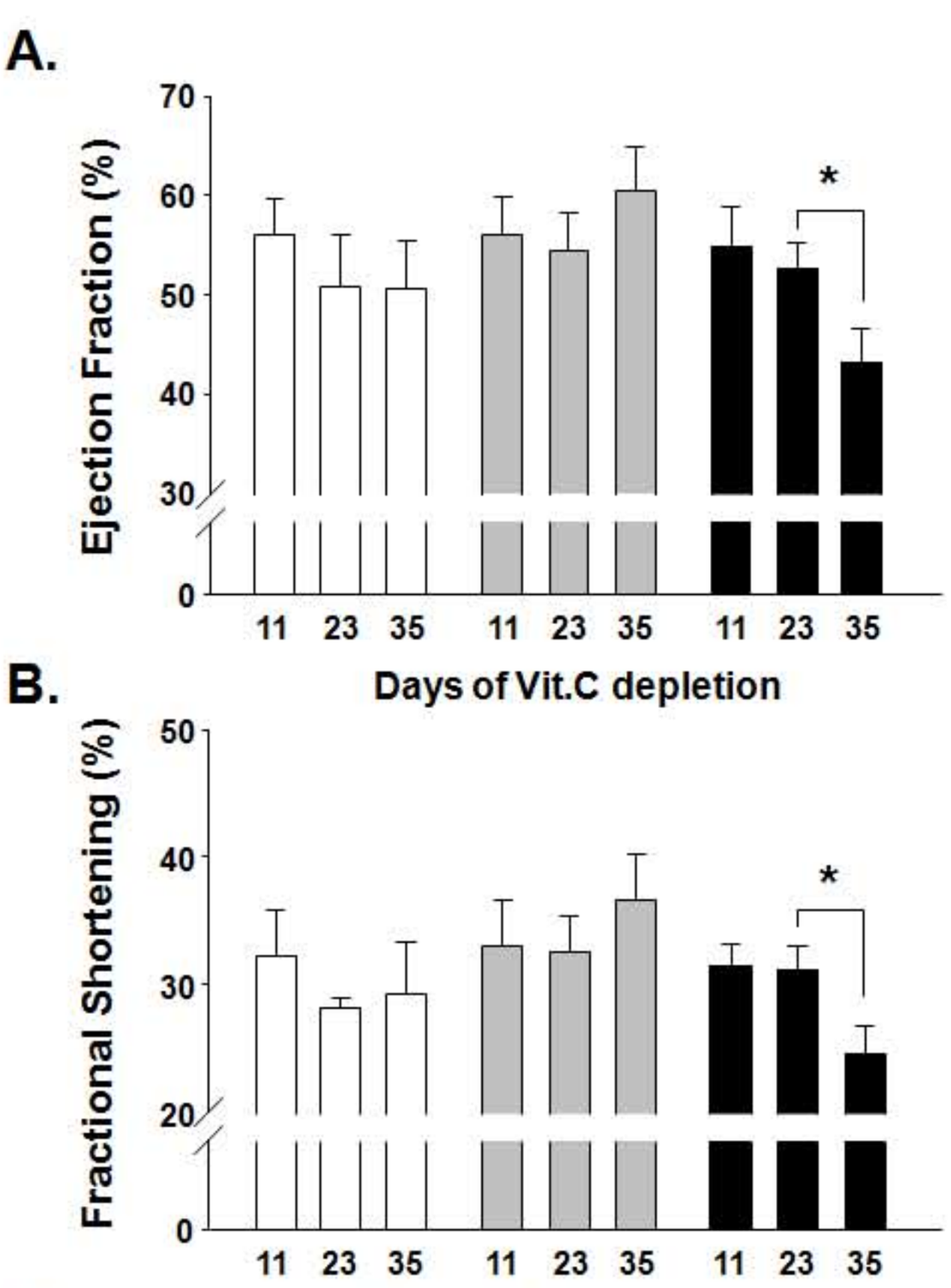
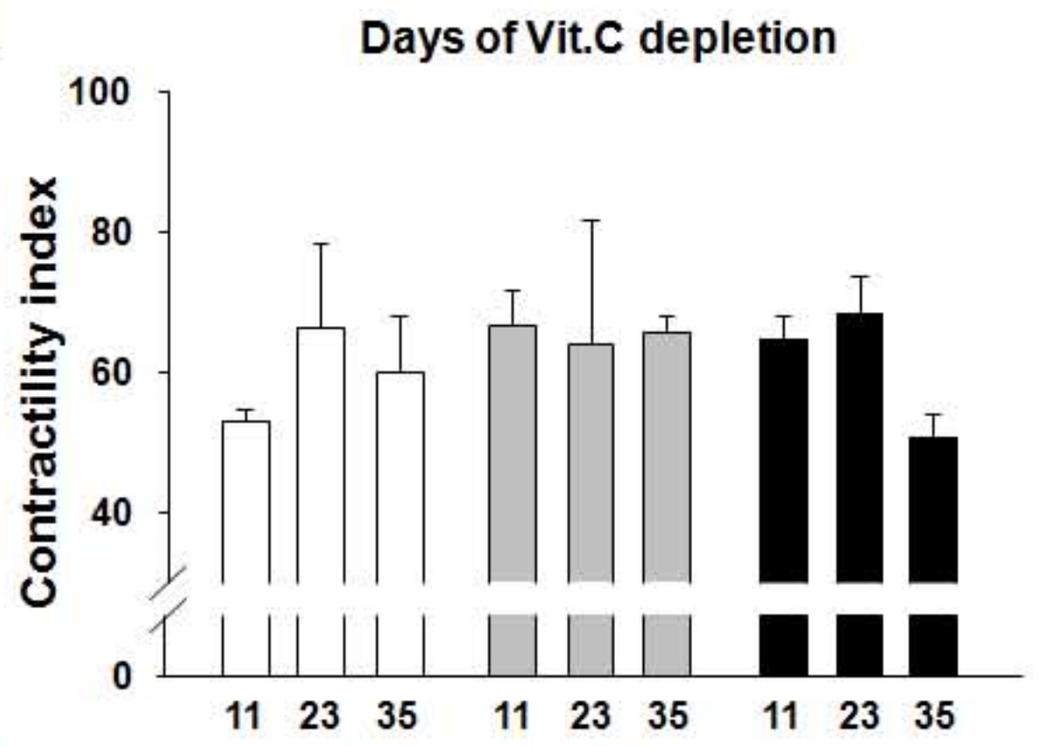
Gulo-/-+Stress

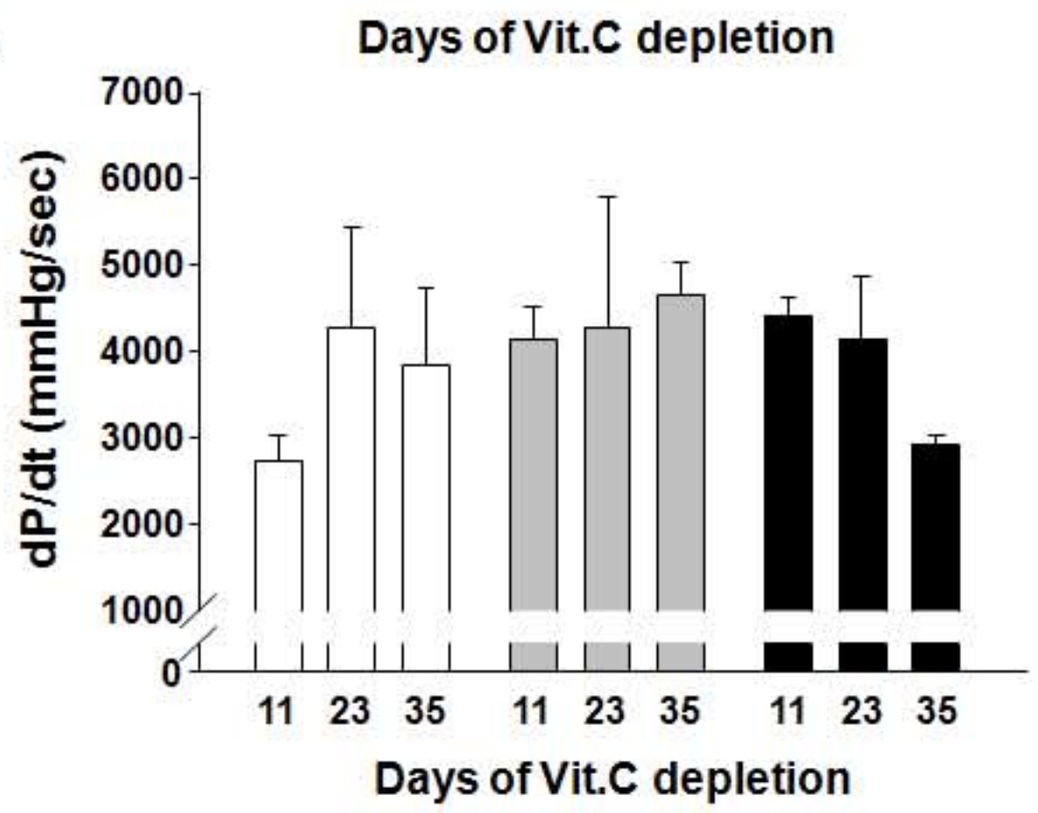
F.

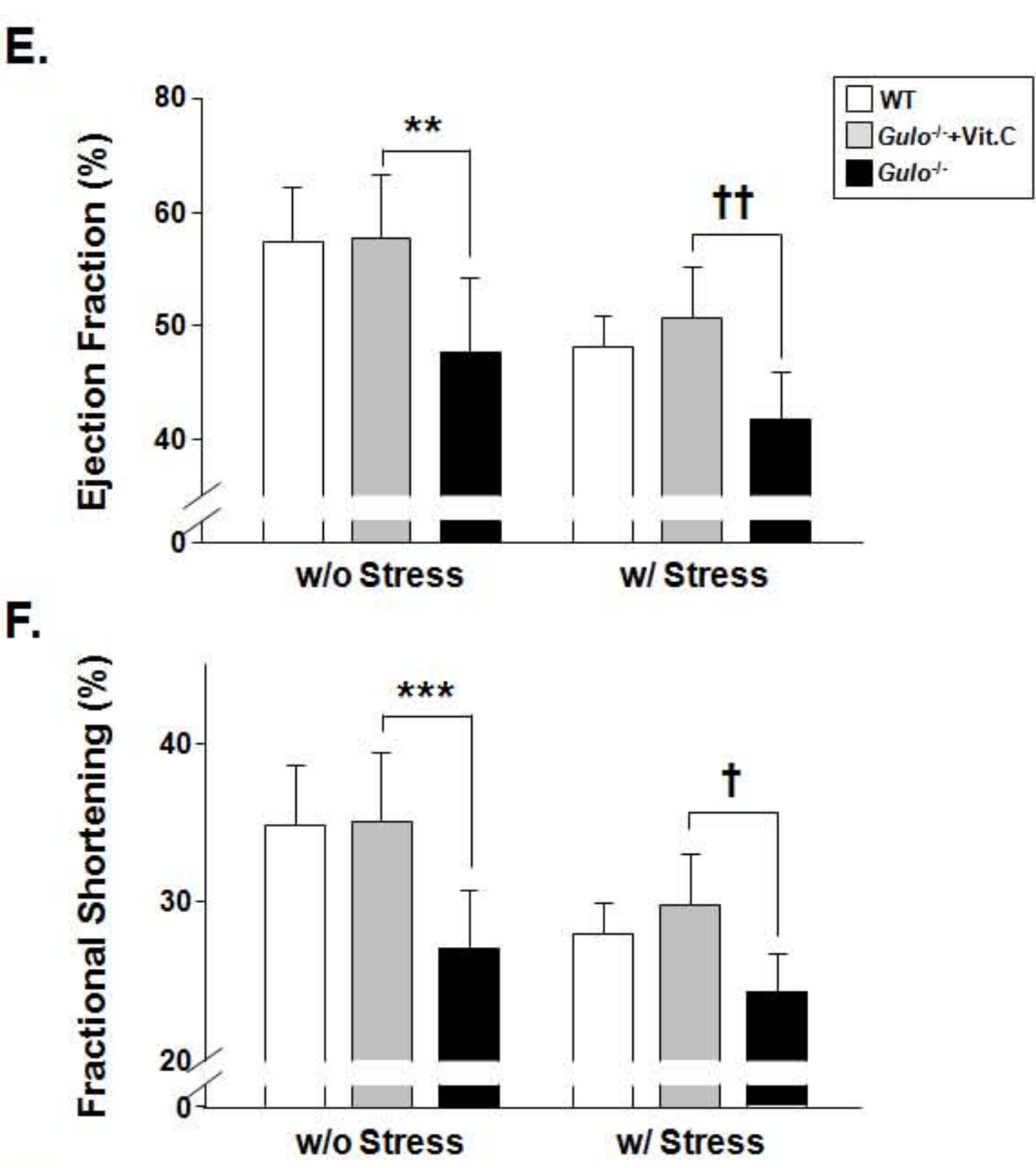

Noradrenaline (ng/ml)
2.5
2.0
1.5
1.0
0.5
0
WT
Gulo-/-+Vit.C
Gulo-/-
*
***
w/o Stress
w/ Stress

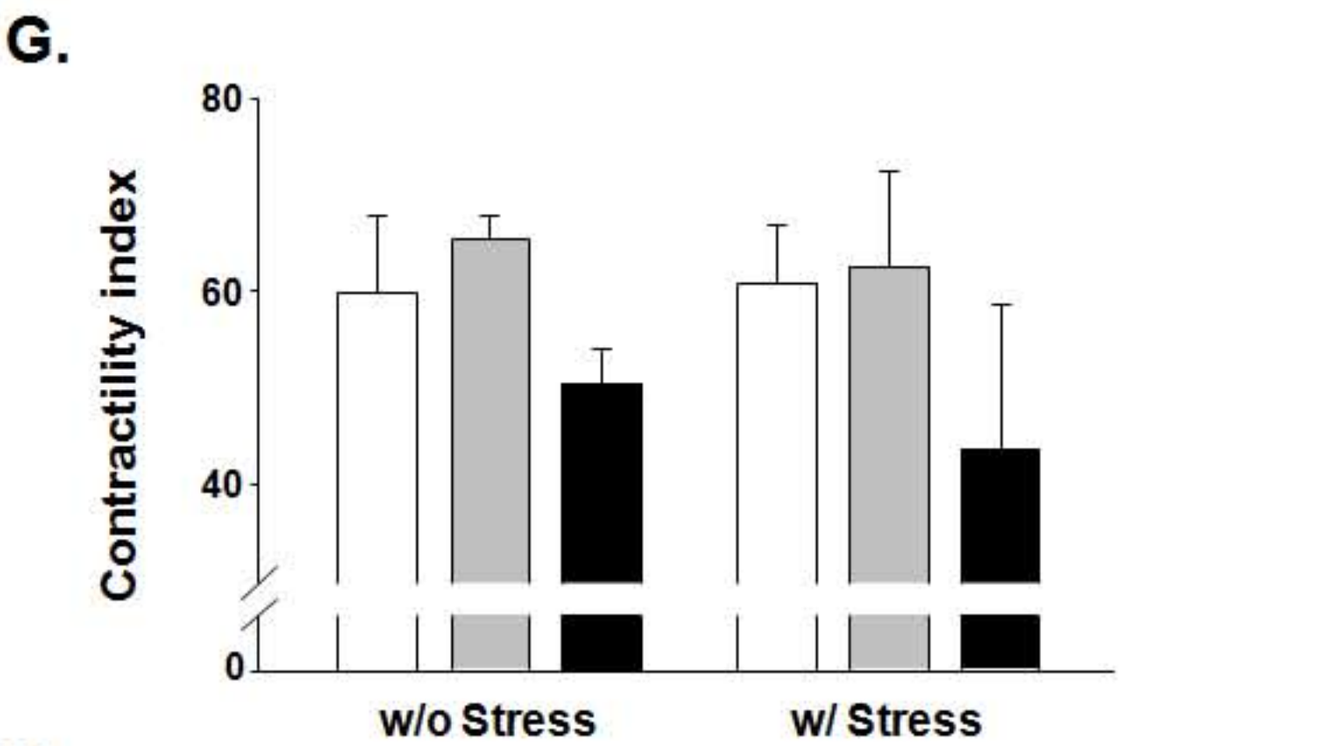

A.
Ejection Fraction (%)
70
60
50
40
30
0
*
11 23 35 11 23 35 11 23 35
Days of Vit.C depletion

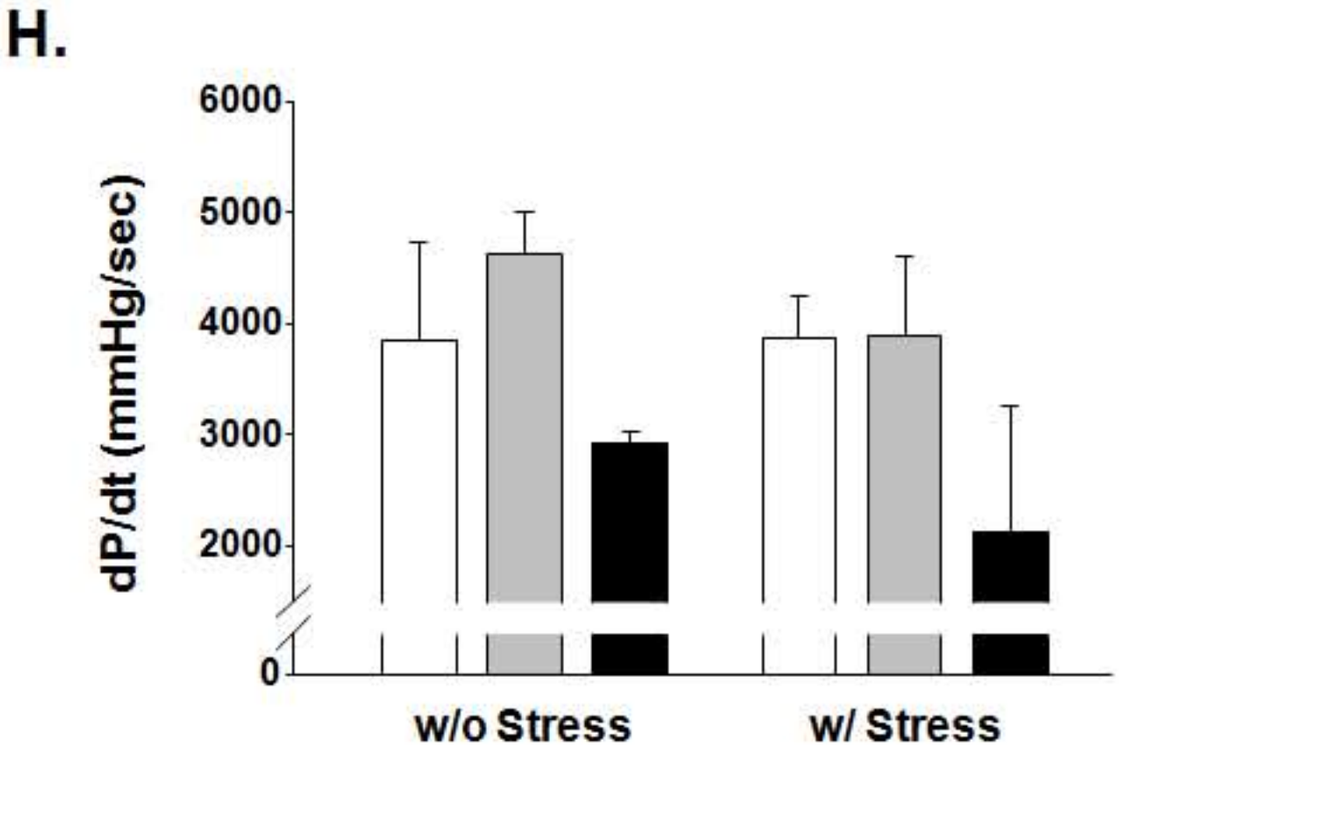
B.
Fractional Shortening (%)
50
40
30
20
0
*
11 23 35 11 23 35 11 23 35
Days of Vit.C depletion

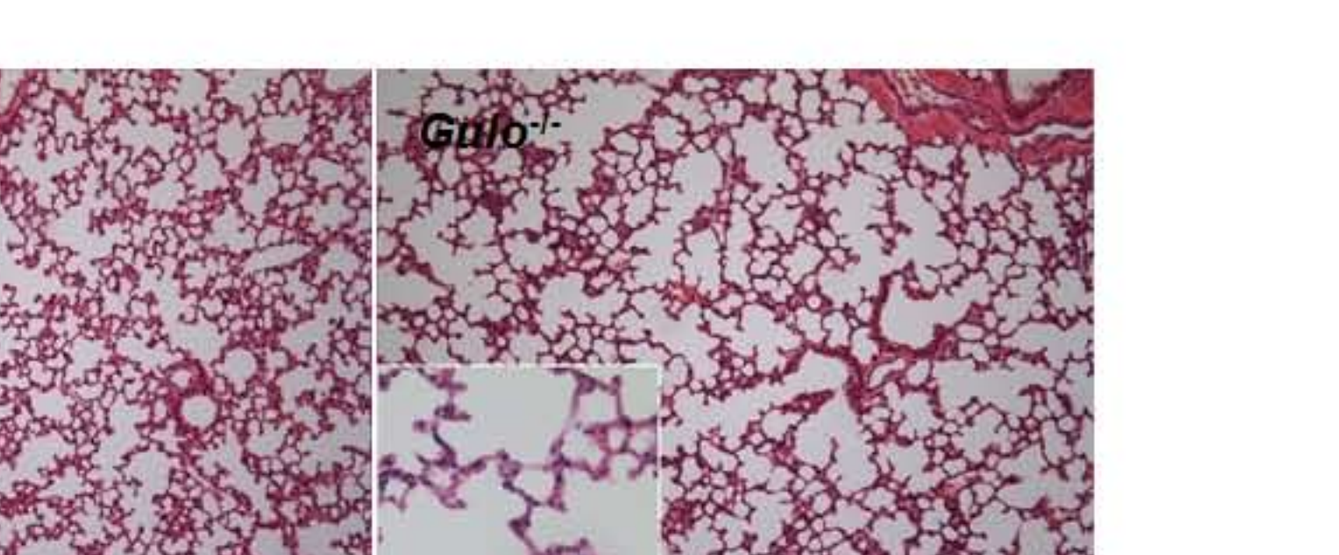

C.
Contractility index
100
80
60
40
0
11 23 35 11 23 35 11 23 35
Days of Vit.C depletion


D.
dP/dt (mmHg/sec)
7000
6000
5000
4000
3000
2000
1000
0
11 23 35 11 23 35 11 23 35
Days of Vit.C depletion


E.
Ejection Fraction (%)
80
60
50
40
0
**
††
WT
Gulo-/-+Vit.C
Gulo-/-
w/o Stress
w/ Stress

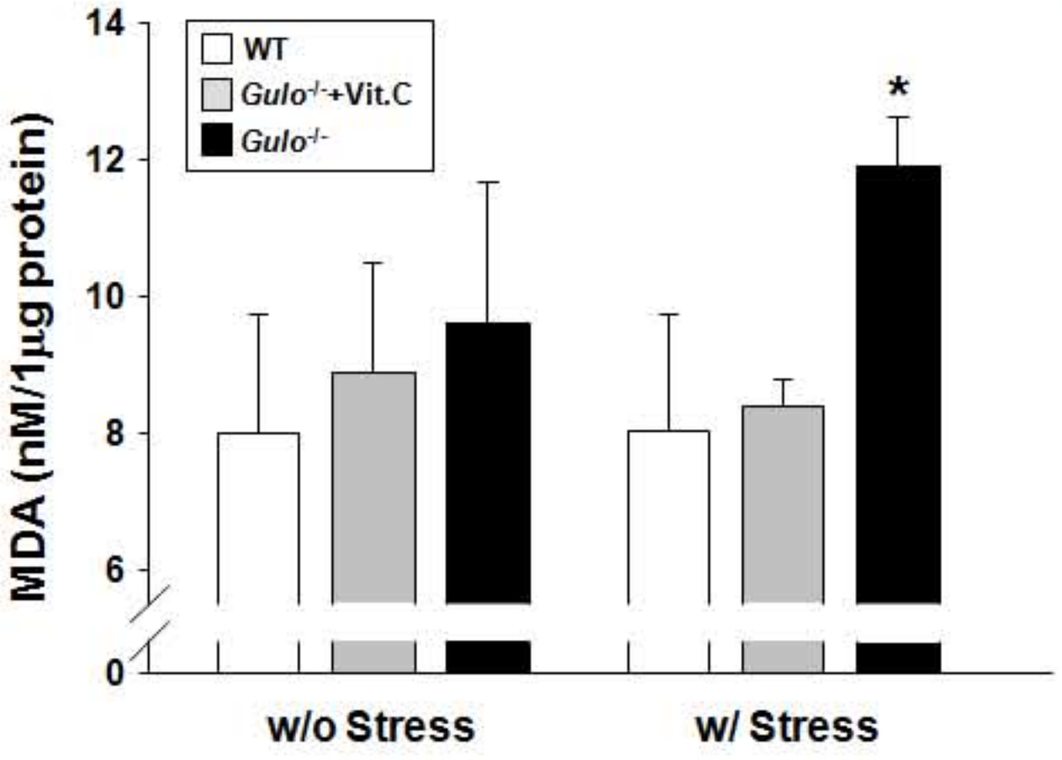

F.
Fractional Shortening (%)
40
30
20
0
***
†
w/o Stress
w/ Stress


G.
Contractility index
80
60
40
0
w/o Stress
w/ Stress

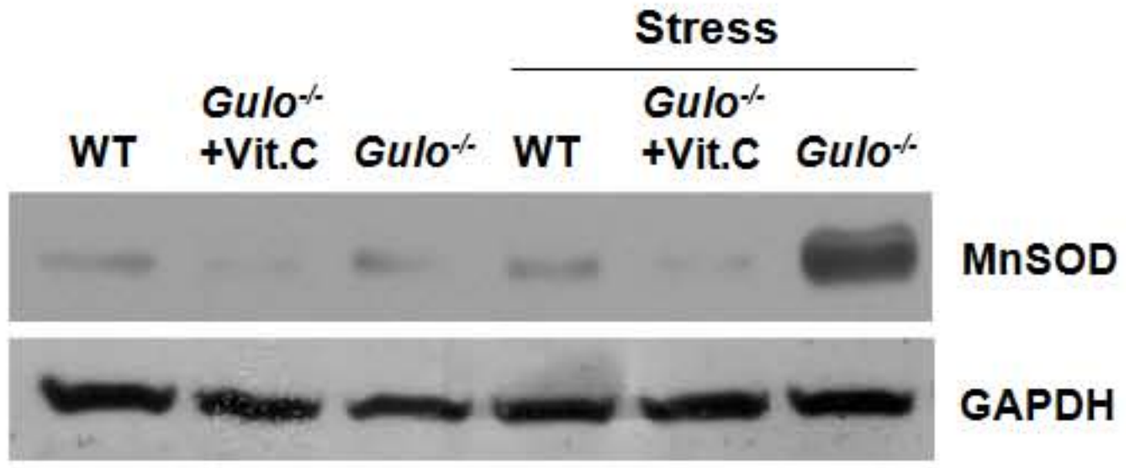

H.
dP/dt (mmHg/sec)
6000
5000
4000
3000
2000
0
w/o Stress
w/ Stress

I.
WT
Gulo-/-+Vit.C
Gulo-/-
WT+Stress
Gulo-/-+Vit.C+Stress
Gulo-/-+Stress

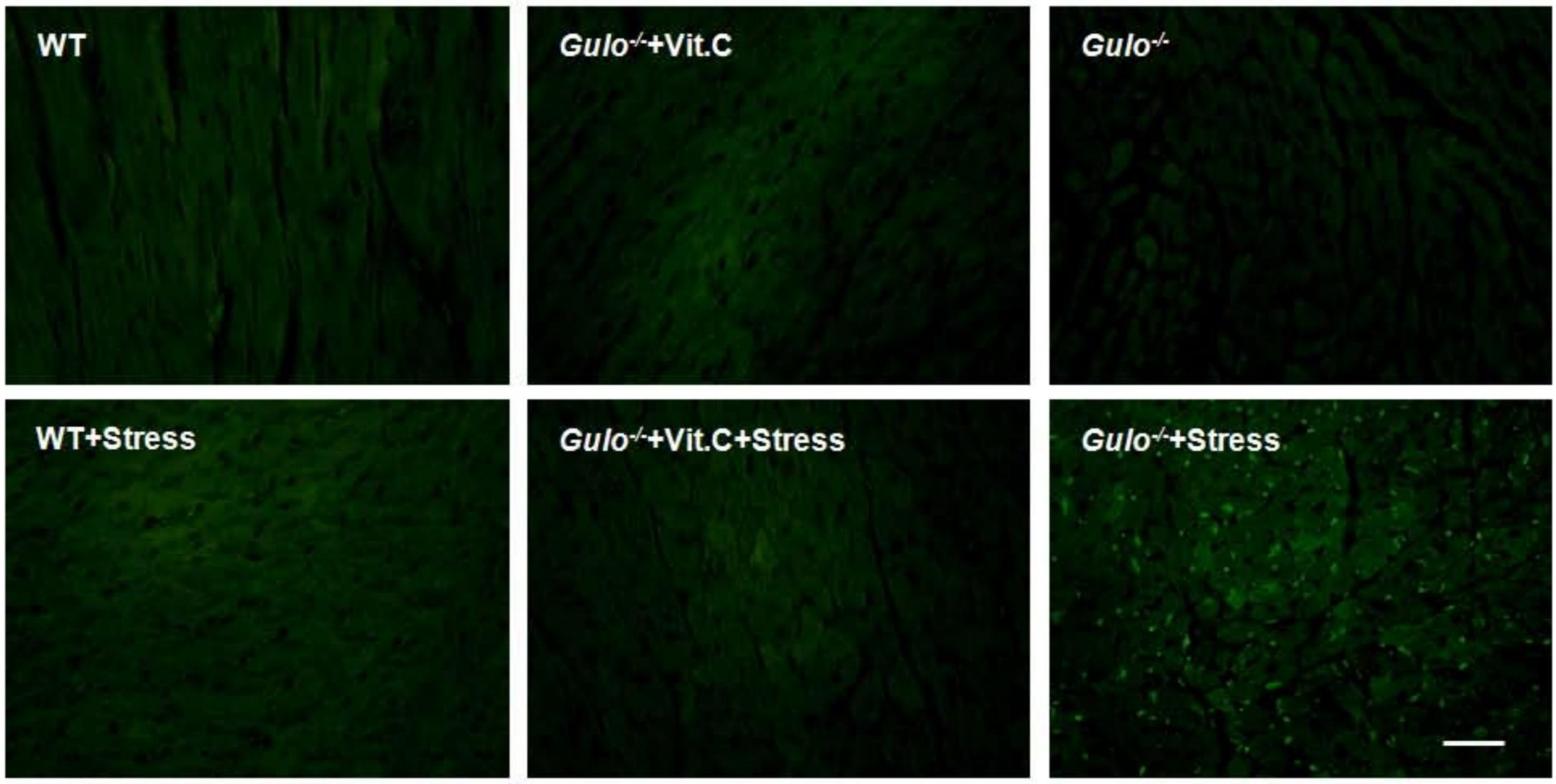
A.


WT
Gulo-/-+Vit.C
Gulo-/-
MDA (nM/1μg protein)
14
12
10
8
6
0
*
w/o Stress
w/ Stress

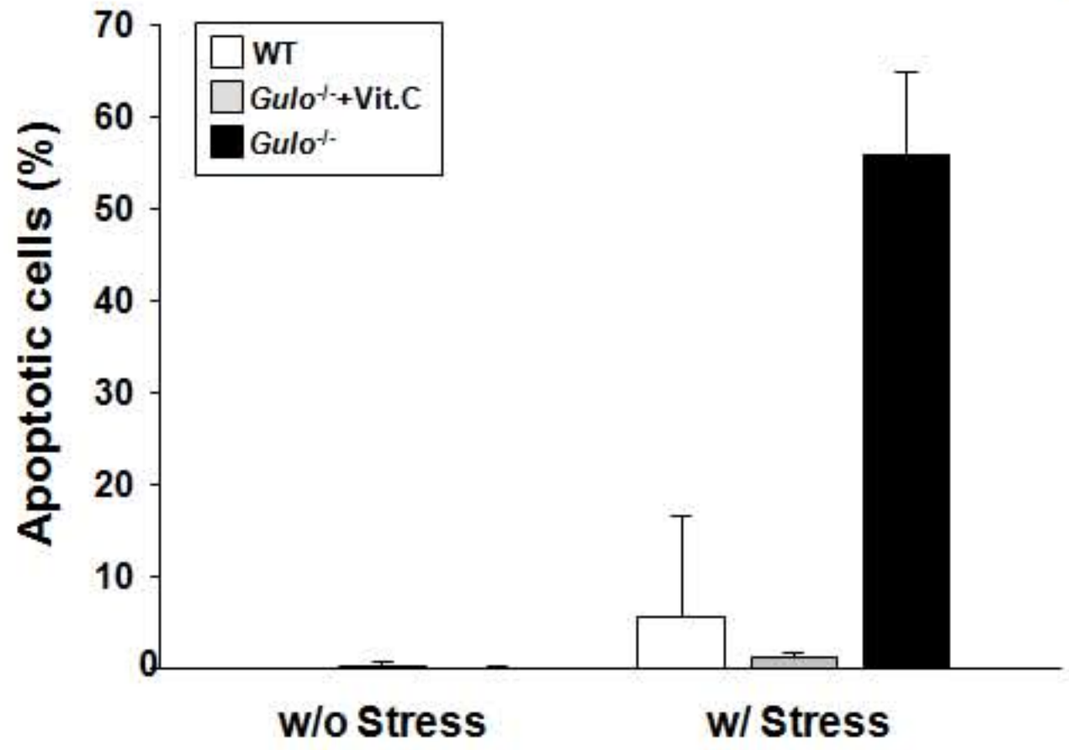
B.


Stress
WT
Gulo-/- +Vit.C
Gulo-/-
WT
Gulo-/- +Vit.C
Gulo-/-
MnSOD
GAPDH

C.


WT
Gulo-/-+Vit.C
Gulo-/-
WT+Stress
Gulo-/-+Vit.C+Stress
Gulo-/-+Stress

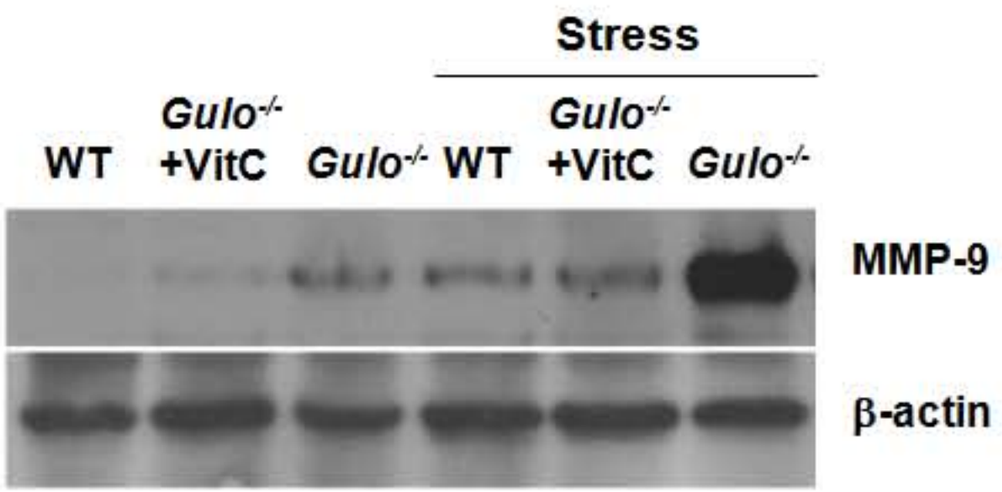
D.


WT
Gulo-/-+Vit.C
Gulo-/-
Apoptotic cells (%)
70
60
50
40
30
20
10
0
w/o Stress
w/ Stress

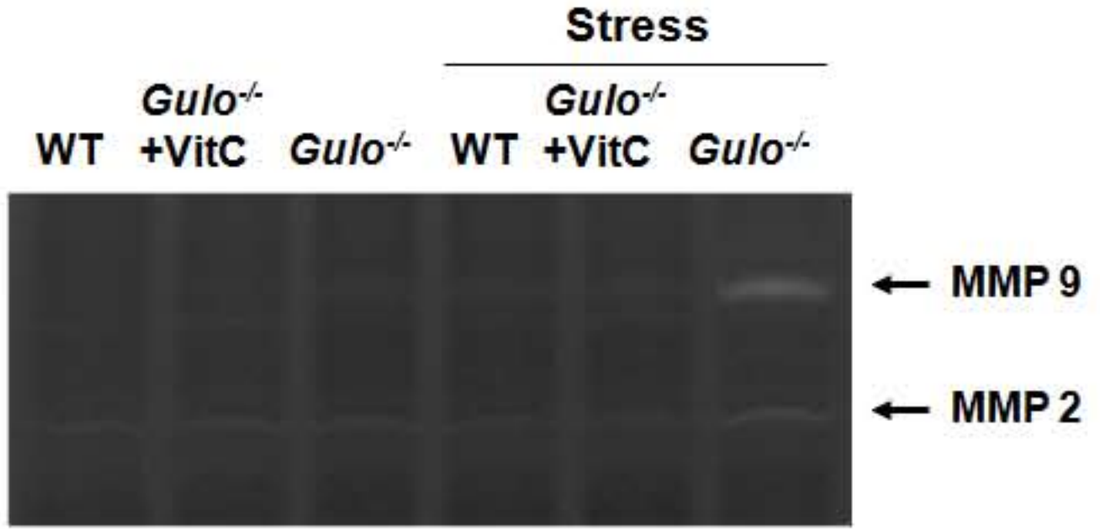
E.

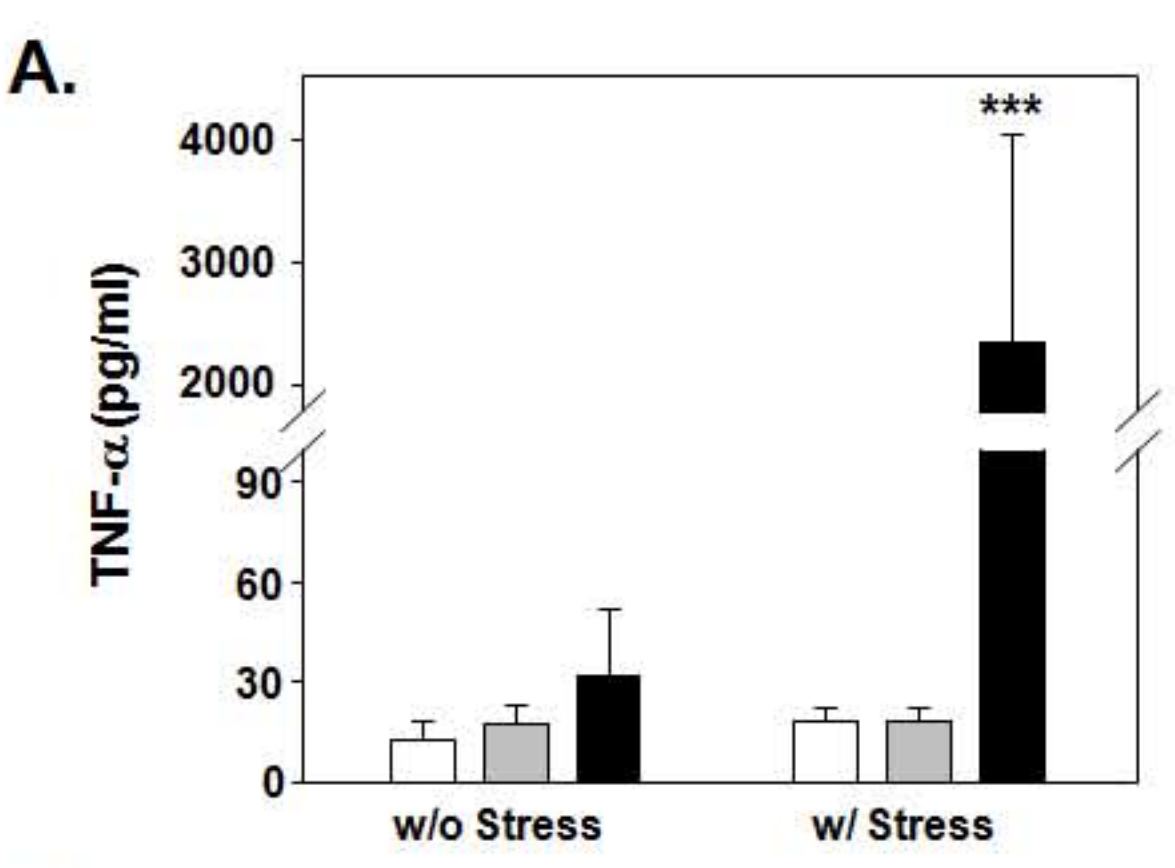
Stress
WT
Gulo-/- +VitC
Gulo-/-
WT
Gulo-/- +VitC
Gulo-/-
pro MMP-2
active MMP-2
GAPDH

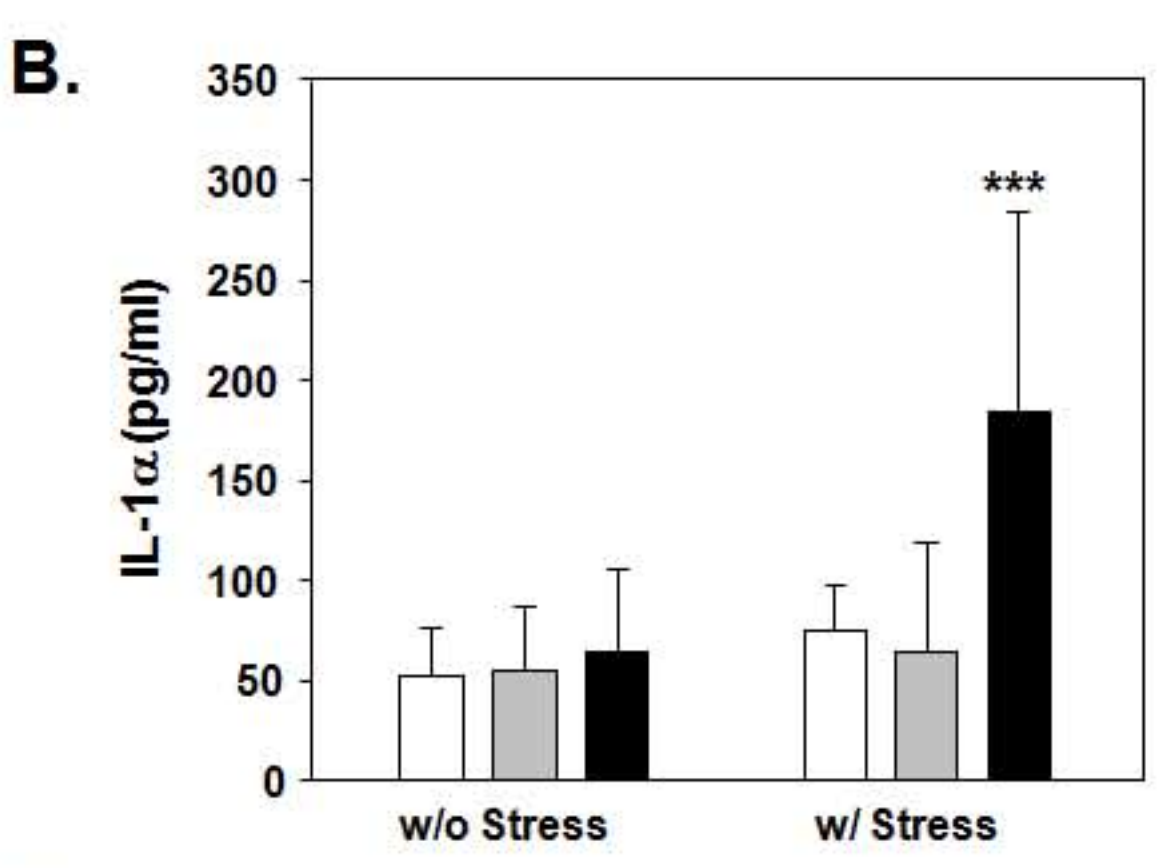
F.

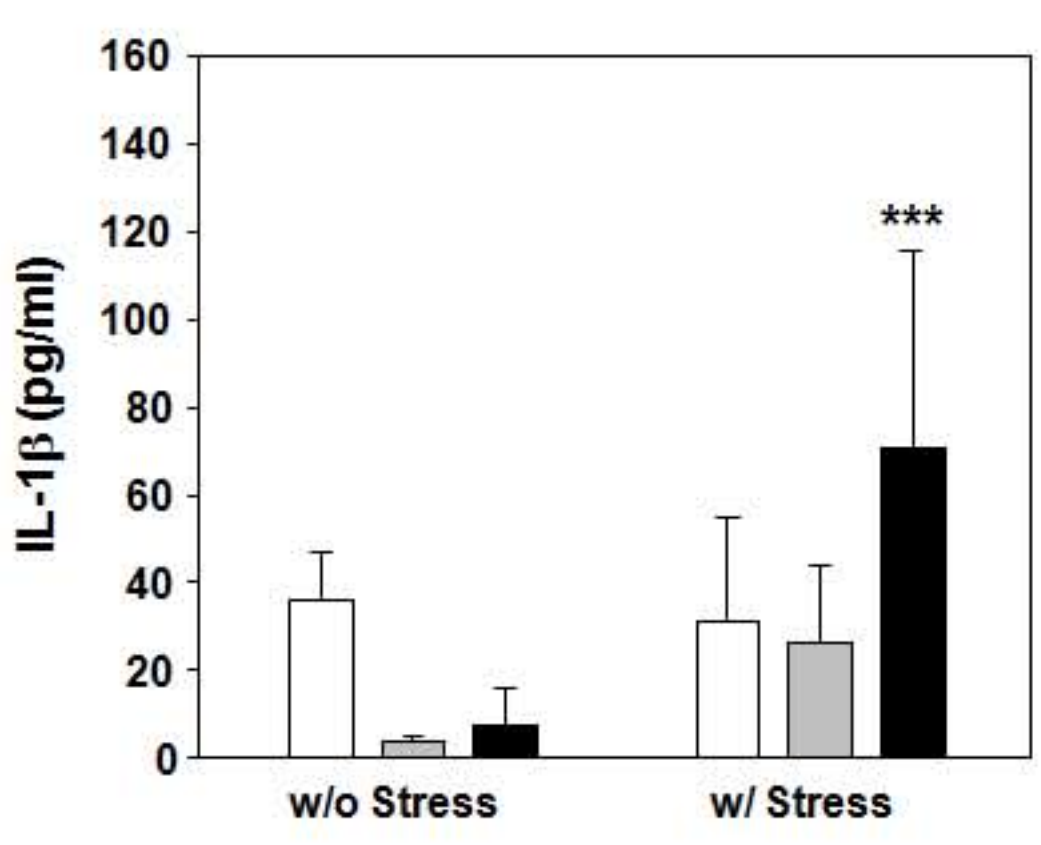

Stress
WT
Gulo-/- +VitC
Gulo-/-
WT
Gulo-/- +VitC
Gulo-/-
MMP-9
β-actin

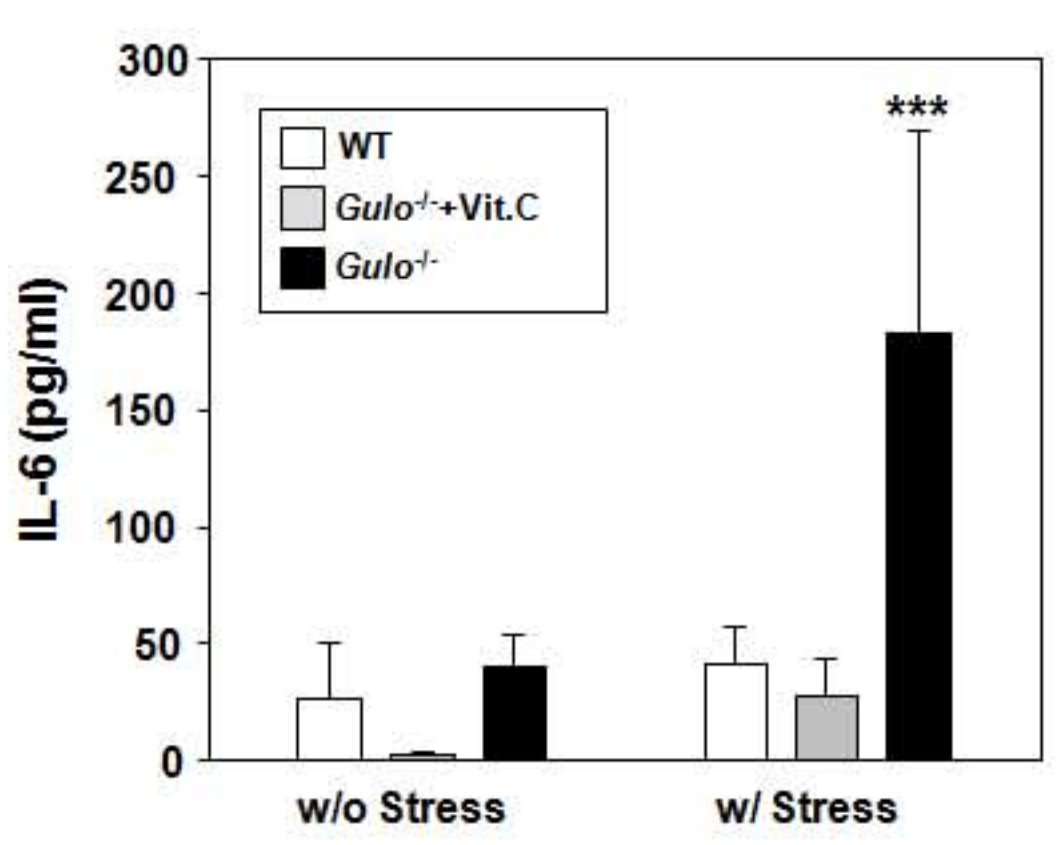
G.

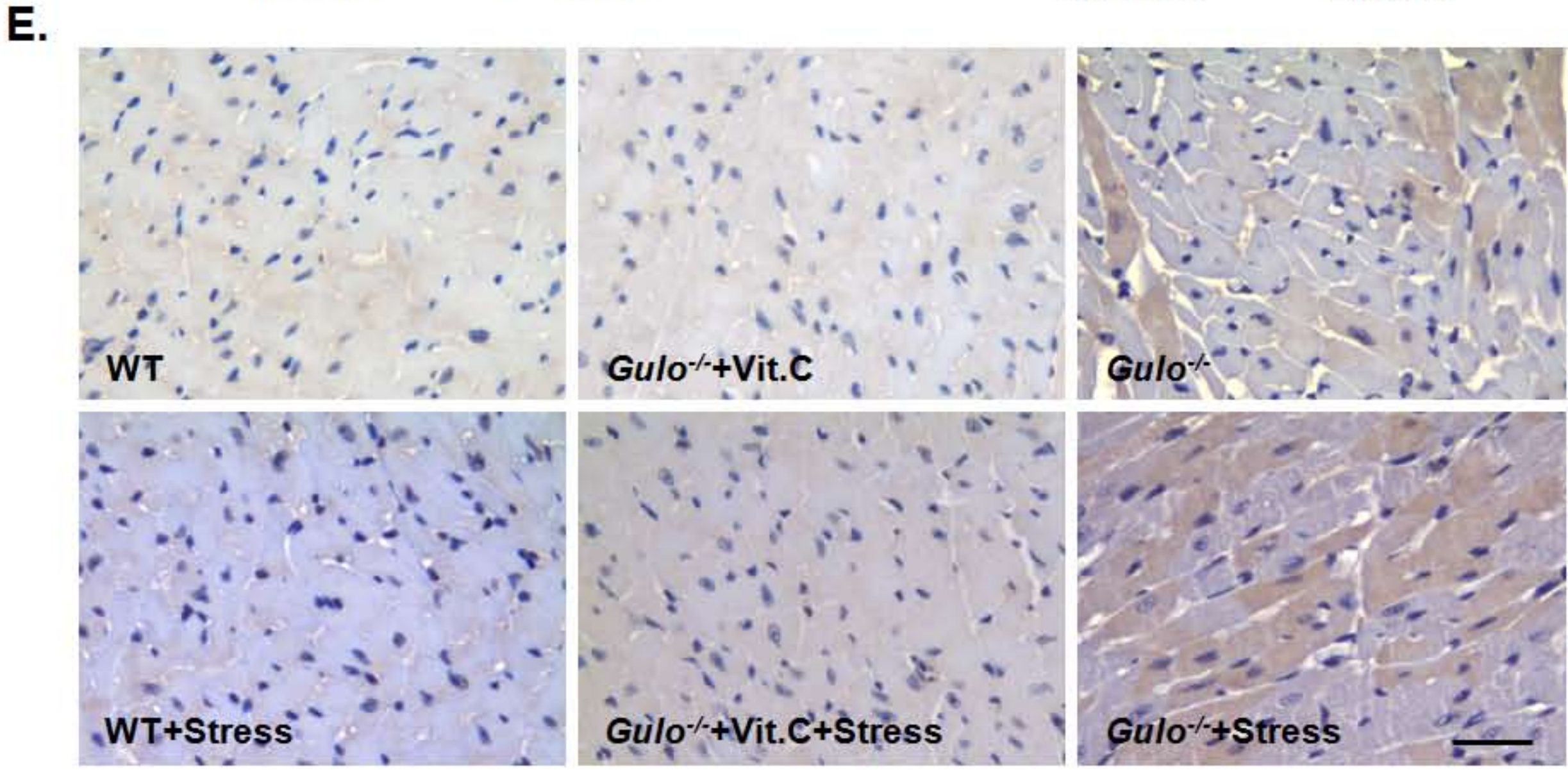

Stress
WT
Gulo-/- +VitC
Gulo-/-
WT
Gulo-/- +VitC
Gulo-/-
MMP 9
MMP 2

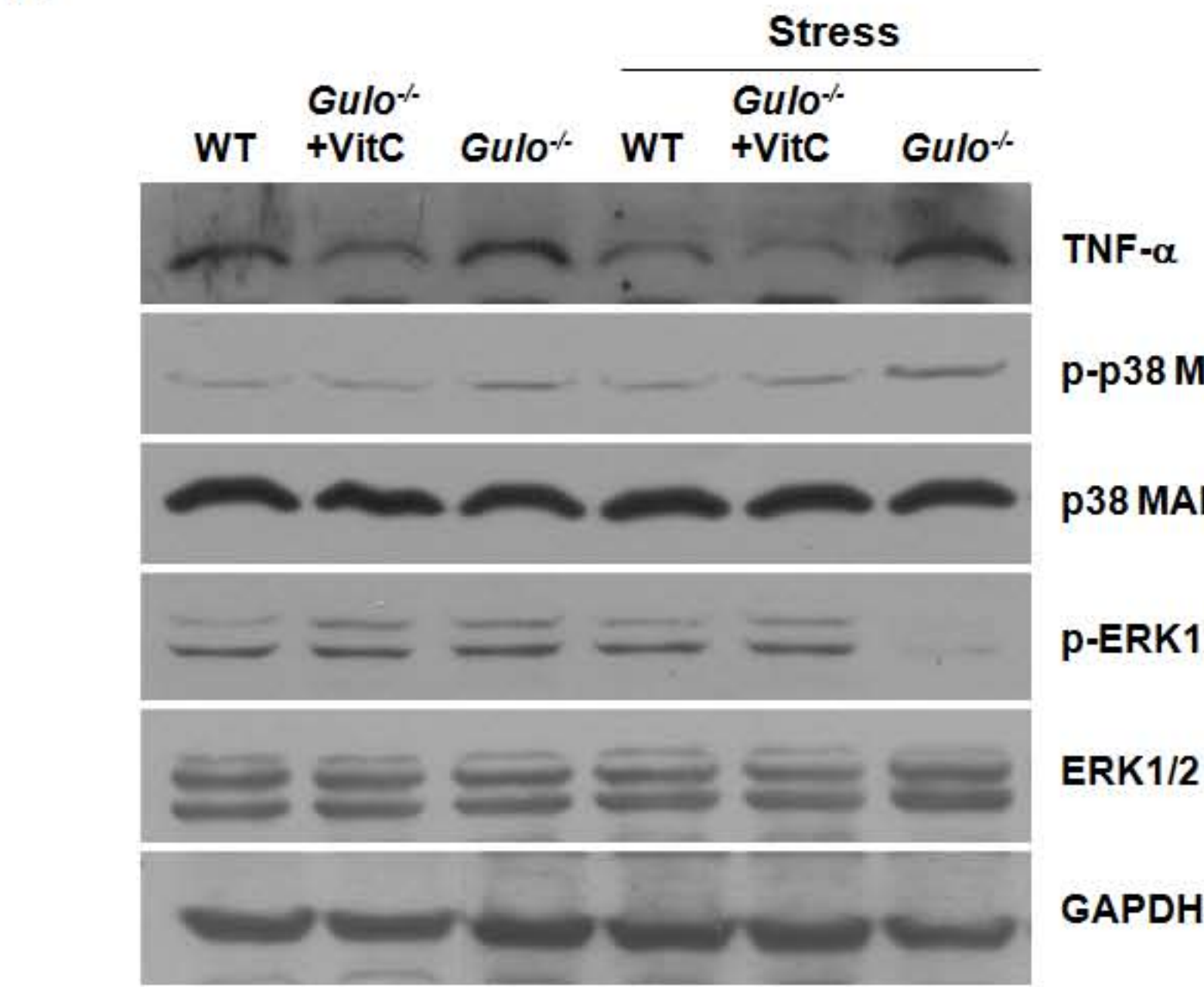

A.
TNF-α (pg/ml)
4000
3000
2000
90
60
30
0
***
w/o Stress
w/ Stress


B.
IL-1α (pg/ml)
350
300
250
200
150
100
50
0
***
w/o Stress
w/ Stress


C.
IL-1β (pg/ml)
160
140
120
100
80
60
40
20
0
***
w/o Stress
w/ Stress


D.
IL-6 (pg/ml)
300
250
200
150
100
50
0
WT
Gulo-/-+Vit.C
Gulo-/-
***
w/o Stress
w/ Stress


E.
WT
Gulo-/-+Vit.C
Gulo-/-
WT+Stress
Gulo-/-+Vit.C+Stress
Gulo-/-+Stress


F.
Stress
WT
Gulo-/- +VitC
Gulo-/-
WT
Gulo-/- +VitC
Gulo-/-
TNF-α
p-p38 MAPK
p38 MAPK
p-ERK1/2
ERK1/2
GAPDH