HMGB1-activatied NLRP3 inflammasome induces thrombocytopenia in Heatstroke Rat

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Background. Thrombocytopenia, an early common complication in heatstroke (HS), has been widely considered as a mortality predictor of HS. The mechanism underlying thrombocytopenia in HS remains unknown. It is not known whether NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome is activated in HS platelet, which, in turn, induces platelet activation and thrombocytopenia. The study tried to clarify the activation of the NOD-like receptor signaling pathway under HS conditions and investigate its roles in mediating HS-induced thrombocytopenia.**Methods.** Rat HS models were established in a certain ambient temperature and humidity. Platelets, isolated from blood, were counted and CD62P, an index of platelet activation, was measured by flow cytometry in all rats. The colocalization of NLRP3 inflammasome in platelet was detected by confocal fluorescence microscopy. Mitochondrial-derived reactive oxygen species (ROS) was detected using the molecular probes. Plasma HMGB1 and IL-1β levels were measured by ELISA.**Results.** Platelet activation, showed by upregulated CD62P, and thrombocytopenia were observed in HS rats. HS activated the NLRP3 inflammasome, which was induced by elevated levels of ROS, while the upregulated CD62P and thrombocytopenia triggered by NLRP3 inflammasome were attributed to the high mobility group box protein 1 (HMGB1) in plasma. Moreover, inhibition of the NOD-like receptor signaling pathway in rats with HS suppressed platelet activation and the decline of platelet count. Similar results were obtained when the receptor toll-like receptor 4 (TLR4) / advanced glycation end product (RAGE) was blocked. **Conclusions.** The NOD-like receptor signaling pathway induces

platelet activation and thrombocytopenia in HS rats. These findings suggested that the NLRP3 inflammasome might be the potential target for HS treatment.

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27   **Abstract**

28   **Background.** Thrombocytopenia, an early common complication in heatstroke (HS), has been
29   widely considered as a mortality predictor of HS. The mechanism underlying thrombocytopenia
30   in HS remains unknown. It is not known whether NOD-like receptor family pyrin domain
31   containing 3 (NLRP3) inflammasome is activated in HS platelet, which, in turn, induces platelet
32   activation and thrombocytopenia. This study tried to clarify the activation of the NOD-like
33   receptor signaling pathway under HS conditions and investigate its roles in mediating HS-
34   induced thrombocytopenia.

35   **Methods.** Rat HS models were established in a certain ambient temperature and humidity,
36   Platelets, isolated from blood, were counted and CD62P, an index of platelet activation, was
37   measured by flow cytometry in all rats. The colocalization of NLRP3 inflammasome in platelet
38   was detected by confocal fluorescence microscopy. . Mitochondrial-derived reactive oxygen
39   species (ROS) was detected using the molecular probes. Plasma HMGB1 and IL-1β levels were
40   measured by ELISA.

41   **Results.** Platelet activation, showed by upregulated CD62P, and thrombocytopenia were
42   observed in HS rats. HS activated the NLRP3 inflammasome, which was induced by elevated
43   levels of ROS, while the upregulated CD62P and thrombocytopenia triggered by NLRP3
44   inflammasome were attributed to the high mobility group box protein 1 (HMGB1) in plasma.
45   Moreover, inhibition of he NOD-like receptor signaling pathway in rats with HS suppressed
46   platelet activation and the decline of platelet count. Similar results were obtained when the
47   receptor toll-like receptor 4 (TLR4) / advanced glycation end product (RAGE) was blocked.

48   **Conclusions.** The NOD-like receptor signaling pathway induces platelet activation and
49   thrombocytopenia in HS rats. These findings suggested that the NLRP3 inflammasome might be
50   the potential target for HS treatment.

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Background

60 Heatstroke (HS) is a life-threatening condition characterized by a core body temperature over
61 40.5℃ (*Epstein & Yanovich, 2019*). which was often complicated with multiple organ
62 dysfunction syndrome (MODS) and even multiple organ failure (MOF), including acute hepatic
63 failure (AHF), acute renal failure, disseminated intravascular coagulation (DIC), (*Hifumi et al.,*
64 *2018*). Every few years, the deadly heat wave occurs and leads to thousands of deaths from HS
65 (*Gaudio & Grissom, 2016*).

66 During the past few decades, evidences suggest that multi-organ damage correlates with the
67 development of systemic inflammatory response syndrome (SIRS) that is stimulated by
68 cytokines and other immune modulators in HS (*Epstein & Yanovich, 2019*). Platelets, mainly
69 involved in hemostasis and thrombosis, also play important roles as inflammatory cells in innate
70 and adaptive immune responses, which is possibly involved in the SIRS in HS (*Hifumi et al.,*
71 *2018;Vieira-de-Abreu et al., 2012; Semple et al., 2011*). Importantly, thrombocytopenia, one of
72 the most common complications frequently occuring early within 24 h after HS onset, has been
73 taken as a good predictor of HS-related mortality (*Fan et al., 2015*). However, how about the
74 pathophysiological responses of platelet and the pathogenesis of thrombocytopenia are to be
75 elucidated in HS.

76 NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome has been
77 extensively investigated in the processes of dysbalanced inflammation and coagulation cascade
78 (*Qiao et al., 2018*). NLRP3 inflammasome contributed to platelet activation and aggregation, and
79 thrombosis via activating caspase-1 within inflammasome complexes (*Murthy et al., 2017; Qiao*
80 *et al., 2018*). Hottz et al. found NLRP3 inflammasome was activated to release increased
81 interleukin-1β (IL-1β) in activated platelets, with increased expression of P-selectin (CD62P),
82 which is associated with thrombocytopenia in patients with dengue fever (*Hottz et al., 2013*). IL-
83 1β, an important inflammatory factor mediated by NLRP3-dependent caspase-1 activation, is
84 linked to increased endothelial permeability (*Bozza et al., 2008*), thrombosis, and dysregulated

85   hemostasis in dengue fever (*Qiao et al., 2018*). What's more, significantly increased IL-1β levels
86   have been found in HS (*Geng et al., 2015*).

87   High mobility group box protein 1 (HMGB1), a typical damage-related molecular pattern
88   molecule, is often transferred from the nucleus to the cytoplasm and can be extracellularly
89   released under many stimuli (*Kang et al., 2014*). We have previously demonstrated that early
90   elevated plasma levels of HMGB1 prognosed the severity and mortality in patients with HS
91   (*Tong et al., 2011*). More importantly, Gen et al. found that HS induced liver dysfunction via
92   HMGB1-induced activation of NLRP3 inflammasome and IL-1β release (*Geng et al., 2015*). It is
93   well known that HMGB1 plays roles in regulating platelet activation, microparticle secretion and
94   adhesion, and thrombus formation (*Vogel et al., 2015; Vallance et al., 2017*). Moreover, mice
95   lacking HMGB1 in platelets exhibited increased bleeding time, reduced thrombus formation,
96   platelet aggregation, inflammation, and organ damage during experimental trauma/hemorrhagic
97   shock (*Vogel et al., 2015*). Unfortunately, whether HMGB1-induced activation of NLRP3
98   inflammasome is involved in platelet activation and thrombocytopenia in HS is to be elucidated.

99   In this study, using a rat model of HS, we tried to investigate whether HMGB1-induced
100   activation of NLRP3 inflammasome occurs via its receptors including toll-like receptor 4 (TLR4)
101   and the receptor for advanced glycation end product (RAGE) signaling pathway, and contributes
102   to platelet activation and thrombocytopenia.

103   **Materials & Methods**

104   *Experimental animals*

105   Because estrogen has certain effects on HS pathogenesis and organ injury (*Chen et al.,*
106   *2006*), only adult male, pathogen-free Sprague-Dawley (SD) rats (Experimental Animal Center
107   of the General Hospital of Southern Theatre Command, license number for animal
108   experimentation: SCXK, GuangDong 2016-0041), weighing 220 to 250 g, were used in this
109   study. The rats received humane care according to the criteria outlined in the Guide for the Care
110   and Use of Laboratory Animals (National Institutes of Health publication 86-23, 1985 revision).
111   We adhered to the guidelines for animal care of the General Hospital of Southern Theatre
112   Command. The animal ethics committee of the General Hospital of Southern Theatre Command
113   of PLA approved this study.

114   *Rat model of HS*

115   Rats housed for 6 h at ambient temperature (25℃ ± 0.5℃) with a humidity of 35% ± 5%,

116   126 rats were randomly divided into 21 groups, with 6 rats per group. Animals have free access

117   to standard food and water. To induce HS, rats were placed in a prewarmed incubator maintained

118   at 39.5℃ ± 0.2℃ with a relative humidity of 60.0% ± 5.0%. The rectal temperature (Tr) was

119   monitored at 10-min intervals using a thermocouple (BW-TH1101; Biowill, Shanghai, China),

120   which was inserted 5.5 cm into the rectum of each rat. The time point at which the Tr reached 43℃

121   was considered a reference point of HS onset (*Geng et al., 2015*). The rats were removed from

122   the incubator after HS onset, transferred to room temperature (22.0℃ ± 0.5℃), and fed with

123   adequate food and water. The rats in control group were sham-heated at a temperature of 25℃ ±

124   0.5℃ and a humidity level of 35% ± 5%. Ethyl pyruvate (EP, 50 mg/kg body weight, MCE,

125   USA) was injected intraperitoneally (i.p.) to inhibit HMGB1 release, or the same volume

126   phosphate buffered solution (PBS) were injected as a negative control before rats subjected to

127   heat stress. A neutralizing antibody specific for rats against HMGB1 (3 mg/kg body weight,

128   Santa Cruz Biotechnology, Santa Cruz, CA, USA) was immediately injected i.p. into the rats

129   before heat stress. In some rats, TLR4 neutralizing antibody specific for rats (8 μg/kg body

130   weight, Abcam, Cambridge, MA, USA), RAGE neutralizing antibody (80 μg/kg body weight,

131   R&D, USA), or both were injected i.p. before rats subjected to heat stress. Other rats were

132   injected i.p. with Ac-Tyr-Val-Ala-Asp-chloromethylketone (ac-YVAD-cmk, 0.3 mg/kg body

133   weight, Enzo Biochem Inc, New York, USA) which is the caspase-1 inhibitor or PBS containing

134   2.8% dimethyl sulfoxide (DMSO) as a control before rats subjected to heat stress. Some rats

135   were given MCC950 (20 mg/kg body weight, Selleckchem, Houston, TX, USA), a specific

136   blocker of NLRP3, 1 h before heat stress. Some rats were injected i.p. with the antioxidant N-

137   acetylcysteine (NAC, 300 mg/kg body weight, MCE) immediately before heat stress. At the end

138   of the experiment, rats were anesthetized by inhalation of isoflurane, and sacrificed to collect

139   blood from abdominal aorta.

140   *Platelet count*

141   50 μL of whole blood was collected into a sodium citrate anticoagulation tube to measure
142   the platelet count using a blood cell analyzer (BC-3000, Mairui, Shenzhen, China).

143 *Platelet isolation*

144   Platelets were isolated as described previously (*Qiao et al., 2018*). Briefly, 3 mL of
145 anticoagulant blood samples were centrifuged at 150 g for 15 min to obtain platelet-rich plasma
146 (PRP), and about 3/4 of the PRP was carefully pipetted into 2 volumes of modified Tyrode
147 buffer (12 mM $NaHCO_3$, 138 mM NaCl, 5.5 mM glucose, 2.9 mM KCl, 2 mM $MgCl_2$, 0.42 mM
148 NaH_2PO_4, 10 mM HEPES, pH 7.4). The mixture was centrifuged at 37℃ at 500 g for 8 min in
149 the presence of PGE1 (0.1 μg/mL, Cayman Chemical) and apyrase (1 U/mL, Sigma-Aldrich),
150 washed twice with CGS buffer (120 mM sodium chloride, 12.9 mM trisodium citrate, 30 mM D-
151 glucose, pH 6.5), and resuspended in modified Tyrode buffer.

152 *Transmission electron microscopy examination*

153   One volume of PRP prepared above was added into 45 volumes of 3% glutaraldehyde and
154 to stand for 4 h. The samples were centrifuged at 800g for 10 minutes and the supernatant was
155 discarded. The pellet was washed with 0.1 M phosphate buffer for twice, postfixed with 1%
156 osmium tetroxide at 4°C for 1 h, and dehydrated in a graded ethanol series and embedded in
157 Epon. Epon-embedded platelets were cut into sections (70-80 nm) with an Ultracut UCT
158 ultramicrotome, transferred to copper grids, and stained with uranyl acetate and lead citrate. The
159 sections were then observed with transmission electron microscope (TEM) (H-7560, Hitachi,
160 Japan).

161 *Flow cytometric analyses*

162   Freshly isolated platelets (10^6 to 10^7) were resuspended in 1 mL of modified Tyrode buffer,
163 and treated with 0.2% triton before to measure intracellular cytokines. A minimum of 10,000
164 events per gate was acquired using a flow cytometer (BD LSR Fortessa). P-selectin (CD62P)
165 was determined using the anti-CD62P monoclonal antibody (1:1000, Biolegend, USA), and
166 TLR2 (toll-like receptor 2), TLR4, and RAGE were assessed with antibodies targeted to TLR2
167 (1:500, Abcam), TLR4 (1:1000, Abcam), and RAGE (1:1000, Abcam). NLRP3 expression was
168 evaluated by an NLRP3 antibody (1:1000, Abcam), and activation of caspase-1 was assessed by
169 the fluorescent probe green fluorescent-labeled inhibitor of caspase-1 (FLICA, 1:100,
170 Immunochemistry Technologies, Bloomington, MN), which irreversibly binds to activated
171 caspase-1. IL-1β was evaluated by the anti–IL-1β antibody (1:1000, Biorbyt). Mitochondrial-
172 derived reactive oxygen species (ROS) were detected using the molecular probes Mito-SOX (for
173 mitochondria O_2•-, 1:1000, Invitrogen), DHE (for cytoplasmic O_2•-, 1:1000, Invitrogen), and
174 DCF-DA (for H_2O_2, 1:1000, Invitrogen). All indices were doubly labeled with a phycoerythrin-
175 or fluorescein isothiocyanate–conjugated CD61 monoclonal antibody (1:1000, Biolegend) to
176 label platelets. The results were analyzed using FlowJo software 7.6.

177 *Confocal fluorescence microscopy*

178   Immunofluorescence for NLRP3 and apoptosis-associated speck-like protein containing a
179   caspase-recruitment domain (ASC) was performed according to previous research (*Feng et al.,*
180   *2018*). Platelets attached on a poly-L-lysine-coated coverslip were fixed with pre-cooled
181   methanol for 20 min, washed twice with PBS (135 mM NaCl, 4.7 mM KCl, 10 mM Na_2HPO_4, 2
182   mM NaH_2PO_4, pH 7.4), and blocked with 10% fetal calf serum (FCS) in PBS. Then, platelets
183   were incubated with goat anti-rat NLRP3 antibody (1:1000, Abcam) or rabbit anti-rat ASC
184   antibody (1:500, Santa Cruz) for 30 min, washed twice with PBS, and labeled with
185   corresponding Dlight 488 and Dlight 594 conjugated secondary antibodies (1:1000, Invitrogen)
186   for 30 min respectively. Preparations were analyzed under a laser scanning confocal microscope
187   (Fluo View FV1000, Olympus), and FV1000 operations software was used for recording.

188   *Plasma HMGB1 and IL-1β levels measurement*

189   According to the manufacturer's instructions, plasma HMGB1 levels were measured by a
190   commercially available HMGB1 enzyme-linked immunosorbent assay kit (Shino-Test
191   Corporation, Sagamihara, Japan), and IL-1β levels were measured by a commercially available
192   IL-1β enzyme-linked immunosorbent assay kit (Invitrogen, America).

193   *Statistical analysis*

194   All data were presented as the mean ± SD unless stated otherwise. All data followed
195   a normal distribution except for one group, which was analyzed with Kruskall-Wallis. For the
196   data followed a normal distribution, statistical significance was determined with the least
197   significant difference t-test or one-way analysis of variance (ANOVA). IBM SPSS Statistics 26
198   software was used for data. $P < 0.05$ was considered statistically significant.

199   **Results**

200   *HS induces platelet activation and thrombocytopenia in HS rats*

201   The platelet count began to decline at 3 h after HS onset (Fig. 1A), whereas the expressions
202   of IL-1β (Fig. 1D) and CD62P (Fig. 1E) in platelets increased progressively during the same
203   period after HS onset. All changes were peaked at 9 h after HS onset. The platelet parameters
204   such as mean platelet volume (MPV) and platelet distribution width (PDW) were assessed. MPV
205   (Fig. 1B) and PDW (Fig. 1C) at 3 h and 6 h after HS maintained in normal ranges, with no
206   statistical differences comparing to the sham group. However, both MPV and PDW at 9 h after
207   HS increased significantly compared to those in the control group after 9 h of HS ($P < 0.01$).
208   Therefore, for the remaining experiments, we observed heat-stressed animals only at the time

209   point of 9 h after HS onset.

NLRP3 inflammasome activation in platelets in HS rats

211   The association of NLRP3 and ASC as well as caspase-1 represents activation of the
212   inflammasome. The colocalization of NLRP3 (green) and ASC (red) in platelets was observed
213   under a confocal microscopy (Fig. 2C). In addition, flow cytometric analyses showed that
214   NLRP3 (Fig. 2A) and cleaved caspase-1 (Fig. 2B) increased at 3 h and reached the peak levels at
215   9 h after HS onset. All results indicate the HS-inducing NLRP3 inflammasome activation in
216   platelets.

NLRP3 inflammasome mediates platelet activation and thrombocytopenia in HS rats

218   To investigate whether HS-activated NLRP3 inflammasome mediates platelet activation
219   and thrombocytopenia, we injected rats i.p. with MCC950, a specific inhibitor of the NLRP3
220   inflammasome, before the rats subjected to heat stress. The results showed that inhibited NLRP3
221   inflammasome significantly down-regulated the levels of cleaved caspase-1 (Fig. 3A), IL-1β
222   secretion (Fig. 3B) and CD62P expression (Fig. 3C) and reduced the drop of platelet count (Fig.
223   3D) at 9 h after HS onset. Moreover, pharmacological inhibition of caspase-1 activity with ac-
224   YVAD-cmk also markedly down-regulated the IL-1β secretion (Fig. 3E) and CD62P expression
225   (Fig. 3F) as well as the drop of platelet count (Fig. 3G) at 9 h after HS onset.

HMGB1 activates platelet NLRP3 inflammasome in HS rats

227   Previous studies found that early increased extracellular HMGB1 levels in HS were
228   negatively correlated with its prognosis (*Tong et al., 2013*). HMGB1 activates the NLRP3
229   inflammasome in liver cells, which in turn promotes hepatocyte pyroptosis (*Geng et al., 2015*).
230   Consistent with previous studies (*Tong et al., 2011*), we found that plasma HMGB1
231   concentrations in HS rats were gradually increased (Fig. 4A). We also unraveled the role of
232   HMGB1 in inducing NLRP3 inflammasome activation, platelet activation, and
233   thrombocytopenia in HS. We injected rats i.p. with EP to inhibit HMGB1 release and used anti-
234   HMGB1 neutralizing antibody to block the effect of HMGB1 before submitting rats to HS.

235   EP inhibiting or neutralizing antibody against HMGB1 significantly reduced the expression
236   of NLRP3 (Fig. 4B) and cleaved caspase-1 (Fig. 4C) at 9 h after HS onset. Notably, levels of
237   secreted IL-1β (Fig. 4D), expression of CD62P (Fig. 4E), and thrombocytopenia (Fig. 4F) at 9 h
238   after HS onset were significantly alleviated after pretreatment with EP and anti-HMGB1
239   antibody. These results indicate that HMGB1 plays an important role in activating NLRP3

240   inflammasome and possibly participates in platelet activation and thrombocytopenia in HS.

241   In the early stages of sterile inflammation, HMGB1 activates the NLRP3 inflammasome
242   through TLR4 or RAGE receptors (*Xu et al., 2013*). As shown in Figures 4G-I, expressions of
243   TLR2, TLR4, and RAGE in platelets were gradually increased after HS onset. To define the
244   receptors that mediate HMGB1-induced NLRP3 inflammasome activation, we applied anti-
245   TLR4 and anti-RAGE neutralizing antibodies before heat exposure. Either anti-TLR4 or anti-
246   RAGE neutralizing antibody significantly inhibited HS-induced NLRP3 (Fig. 4J) and cleaved
247   caspase-1 (Fig. 4K) expression in platelets at 9 h after HS onset. In addition, the combination of
248   anti-TLR4 and anti-RAGE neutralizing antibody induced greater inhibition of NLRP3 and
249   cleavage of caspase-1 expression. Synchronous changes were also observed in HMGB1-induced
250   upregulated expression of IL-1β (Fig. 4L), and thrombocytopenia (Fig. 4N) at 9 h after HS. Anti-
251   TLR4 or anti-RAGE neutralizing antibody inhibited HS-induced CD62P expression, while
252   combination of anti-TLR4 and anti-RAGE neutralizing antibody made no significantly effect
253   compared with single neutralizing antibody (Fig. 4M)..These data indicate that HMGB1 activates
254   NLRP3 inflammasome via both TLR4 and RAGE receptors, which is possibly associated with
255   platelet activation and thrombocytopenia in HS.

256   *HMGB1 activates platelet NLRP3 inflammasome by upregulating ROS in HS rats*

257   ROS mainly includes the superoxide anion (O2–), hydrogen peroxide (H2O2), and the
258   hydroxyl radical (HO–) (Leytin et al., 2012). Many studies confirmed HMGB1 could up-regulate
259   ROS (Tsung et al., 2007; Pietraforte et al., 2014), which was the key to activate NLRP3
260   inflammasome (Dostert et al., 2008; Zhou et al., 2011). To study the changes of ROS in platelets
261   and the effects of ROS on the HMGB1-induced NLRP3 inflammasome in HS, we used a
262   fluorescence probe to detect the expression of mitochondrial O2•-, cytoplasmic O2•-, and H_2O_2
263   in platelets in HS rats. We found that mitochondrial O2•- (Fig. 5A), cytoplasmic O2•- (Fig. 5B),
264   and H_2O_2 (Fig. 5C) increased at 3 h after HS onset. Inhibiting HMGB1 significantly reduced the
265   level of ROS, which indicated that HMGB1 was involved in high-level of ROS in HS (Fig. 5D-
266   F).

267   To explore the relationship between ROS and the NLRP3 inflammasome activation, we
268   pretreated rats with the antioxidant NAC. In platelets, NAC significantly inhibited the expression
269   of NLRP3 (Fig. 5G) and cleaved caspase-1 (Fig. 5H) at 9 h after HS onset. Similar results were
270   observed for secreted IL-1β (Fig. 5I), CD62P expression (Fig. 5J), and thrombocytopenia (Fig.
271   5K). These results indicate that ROS is responsible for platelet NLRP3 inflammasome-induced
272   activation and thrombocytopenia in HS.

273 **Discussion**

274   In the current study, we investigated the NOD-like receptor signaling pathway of platelet in

275 HS rats, and explored the mechanisms involved in platelet activation and thrombocytopenia. The

276 main results of our investigation were as following: (1) HS activatied the NOD-like receptor

277 signaling pathway, which induced thrombocytopenia and platelet activation in HS rats. (2) High

278 levels of extracellular HMGB1, through TLR4 and RAGE pathway, induced NLRP3

279 inflammasome activation via ROS possibly in HS rats.

280   MPV and PDW are indices of average platelet volume and variation in platelet size

281 respectively, both of which are markers of platelet activation and thrombocytopenia

282 (*Pogorzelska et al., 2020*). In this experiment, we found that MPV and PDW did not change at 3

283 h but were increased significantly at 9 h after HS onset. Previous study showed no evidence of

284 megakaryocyte damage (*Gader et al., 1992*), We found platelet aggregation gradually decreased

285 after HS onset (Fig.SA), which consists with the results that hyperthermia inhibit platelet

286 aggregation (*Etulain et al., 2011*), indicating that peripheral platelet destruction may be a

287 possible explanation of the thrombocytopenia of HS.

288   Zhuang et al. found that heat stress facilitated the procession of Px-caspase-1 (a caspase

289 gene) expression, an important effector molecule of the NLRP3 inflammasome (*Zhuang et al.,*

290 *2011*). Murthy et al. found that NLRP3 inflammasome affects platelet activation, aggregation,

291 and thrombosis (*Murthy et al., 2017*). Additionally, one team in our institute previously found

292 that HMGB1-activatied NLRP3 inflammasome in liver cells during HS was associated with liver

293 damage (*Geng et al., 2015*). Therefore, it is speculated that the HMGB1-activatied NLRP3

294 inflammasome possibly mediates platelet activation and thrombocytopenia in HS. We observed

295 that the expressions of NLRP3, ASC, and cleaved caspase-1 in platelets of HS rats were

296 increased at early stage after HS onset and remained increased thereafter (Fig. 2A, B, C).   All

297 these results reflect the assembly and activation of NLRP3 inflammasome in HS. Consistent with

298 the results of a previous study (*Murthy et al., 2017*), we found that inhibiti   NLRP3 and

299 cleavage of caspase-1 alleviated platelet activation and thrombocytopenia (Fig. 3), indicating that

300 the NLRP3 inflammasome played an important role in platelet activation and thrombocytopenia

301 possibly.

302   HMGB1, a class of non-histone chromosomal binding protein presenting in eukaryotic cells,
303   has been extensively studied as an important molecule activating NLRP3 inflammasome(*Park et*
304   *al., 2006*). Our previous studies showed that plasma HMGB1, significantly elevated at an early
305   stage in HS patients, was associated with more critical disease and poor prognosis (*Tong et al.,*
306   *2011*). Inhibiting HMGB1 release or blocking HMGB1 alleviated liver sinusoidal endothelial
307   damage (*Tong et al., 2013*) and reduced thrombosis in HS (*Murthy et al., 2017*). In this
308   experiment, we also found that inhibiting HMGB1 by EP and anti-HMGB1 neutralizing antibody
309   down-regulated the activation of the NLRP3 inflammasome, platelet activation, and
310   thrombocytopenia. Previous studies have showed that HMGB1 promotes inflammation via the
311   main receptors including TLR2,TLR4 and RAGE receptors (*Park et al., 2006*), TLR4-dependent
312   upregulation of the platelet NLRP3 inflammasome promotes platelet aggregation (*Vogel et al.,*
313   *2019*). Because there was no anti-TLR2 neutralizing antibody for rats, we only tested the effects
314   of TLR4 and RAGE receptors. Different to the results of a prior study that visfatin-induced
315   disruption on junction proteins of mouse vascular endothelial cells was conducted through the
316   HMGB1-RAGE rather than the HMGB1-TLR4 pathway (*Chen et al., 2015*), our results indicate
317   that both TLR4 and RAGE receptor-specific antibodies can inhibit NLRP3 inflammasome
318   activation, platelet activation and thrombocytopenia. Also, anti-TLR4 antibody pretreatment
319   significantly shortened the heat exposure time of rats (Fig.SB). These results suggest that
320   HMGB1 activates the NLRP3 inflammasome via TLR4 and RAGE receptors and participates in
321   platelet activation and thrombocytopenia possibly.

322   ROS, taking an important role in the homeostasis of aging and death of platelets
323   (*Pietraforte et al., 2014*), are involved in platelet activation, adhesion, and aggregation and
324   apoptosis (*Leytin et al., 2012*). Consist with previous studiess, 3 kinds of ROS in the platelets of
325   rats with HS increased progressively with time (Fig. 5A, B, C) in our experiments. HMGB1 can
326   upregulate intracellular levels of ROS in many diseases including HS (*Tang et al., 2011*).
327   Similarly, we found inhibition or blockade of HMGB1 could partially inhibit the production of
328   all the 3 kinds of ROS (Fig. 5D, E) in HS rats. It was found that HMGB1-induced elevated
329   intracellular ROS (*Abais et al., 2011; Han et al., 2015*) could promote NLRP3 inflammasome
330   activation. We found that NAC pretreatment could inhibit the expression of platelet NLRP3 and
331   cleaved caspase-1 as well as platelet activation and thrombocytopenia in HS rats, indicating that
332   ROS could activate NLRP3 inflammasome in platelets and may be invovled in
333   thrombocytopenia.

334   Pyroptosis is a caspase-1 dependent programmed cell death (*Geng et al., 2015*). In the
335   current study, thrombocytopenia and active caspase-1 showed opposite trends, suggesting that
336   there must be a link between them. Recent studies have shown that pyroptosis is a major cell

337   death pathway of platelets treated with dengue virus and envelope protein domain III (*Lien et al.,*
338   *2021*). Using different methods to validate the pyroptotic results, they they examined cleaved
339   caspase-1, cleaved GSDMD and cell-surface GSDMD levels, IL-1β and lactic dehydrogenase
340   release (*Lien et al., 2021*). However, we did not conduct experiments to verify pyroptosis in
341   platelet of HS. whether pyroptosis is a kind of cell death pathway of platelets in the HS rat model
342   need further research. In addition, we did not exclude the effect of direct thermal injury to the
343   platelet. Furthermore, other signaling pathways in platelet may also be activated and involved in
344   the inflammation process. Finally, our results did not fully reflect the effect of NLRP3
345   inflammasome on HS platelets for using rat models not patients.

346   **Conclusions**

347   In conclusion,   this study preliminarily demonstrates that elevated extracelluar HMGB1
348   induced high levels of ROS via both TLR4 and RAGE receptors of platelets, which in turn
349   participates NLRP3 inflammasome activation leading to thrombocytopenia in HS rats. The
350   recognition of this study may aid to develop novel therapeutic agents in future.

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475

Figure 1

Fig. 1. *HS induces platelet activation and thrombocytopenia in HS rats (n=6).*

(A) Thrombocytopenia in HS rats. *$p < 0.001$ vs sham. (B) Changes of MPV in HS rats. *$p < 0.001$ vs sham. (C) Changes of PDW in HS rats. *$p = 0.001$ vs sham. (D) Increased expression of IL-1β in HS rats. *$p < 0.001$ vs sham. (E) Increased expression of CD62P in HS rats. $p = 0.003$ between HS-3 h vs sham, $p < 0.001$ between HS-9 h vs sham, $p = 0.01$ between HS-3 h vs HS-6 h.

A

B

C

D

E
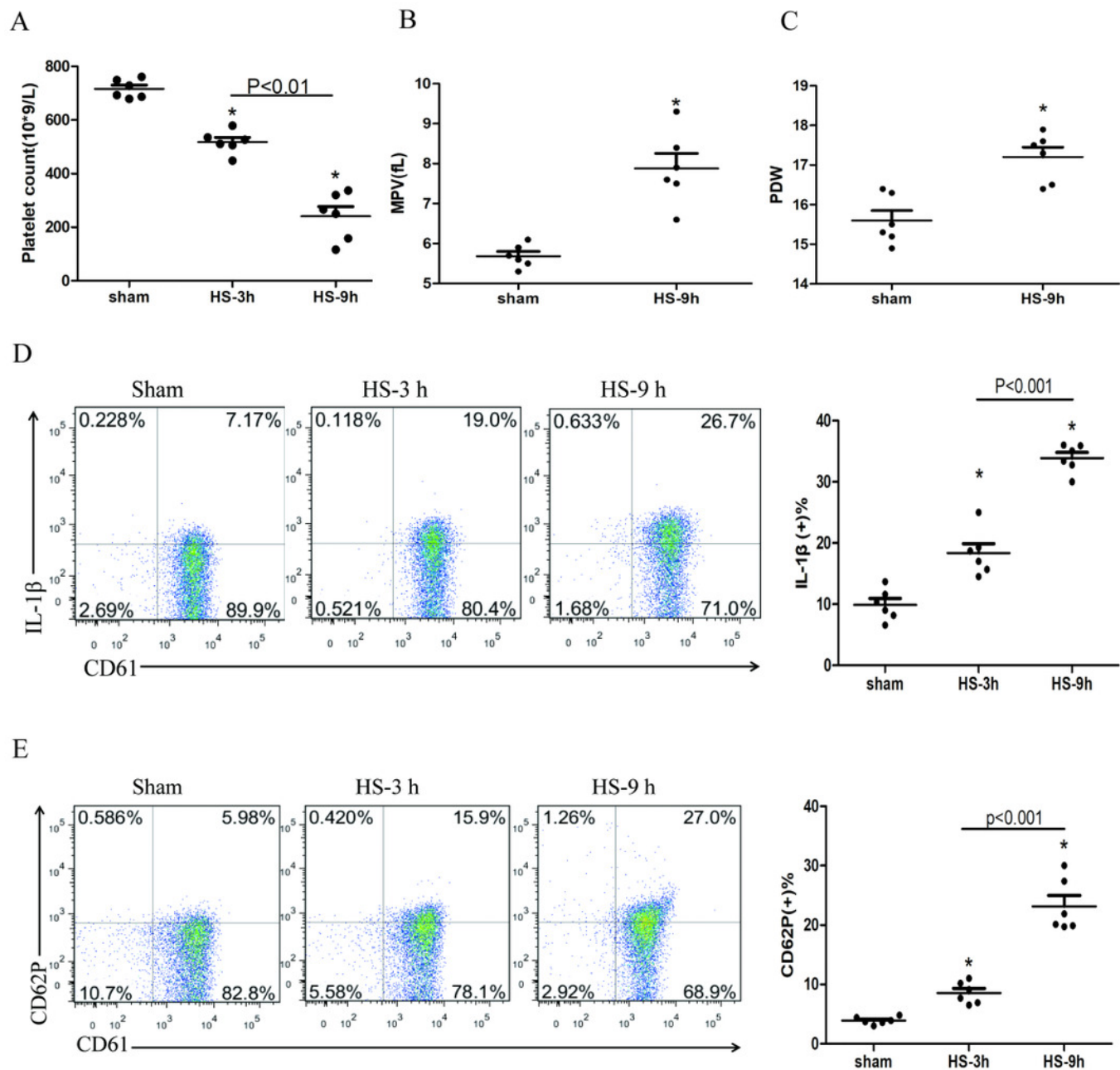
Figure 2

Fig. 2. *NLRP3 inflammasome activation in platelets in HS rats (n=6).*

(A) NLRP3 expression in HS rats. *p < 0.001 vs sham. (B) caspase-1 activation in HS rats. *p < 0.001 vs sham. (C) NLRP3 Inflammasome assembly in platelets of rat at 9 h after HS onset. NLRP3 (green) and ASC (red) and Merge (yellow) were observed under a laser confocal microscope. The white triangular arrows point to the NLRP3 inflammasome, suggesting the presence of NLRP3 and ASC in platelets from rats with HS. Bars represent 10,000 nm.
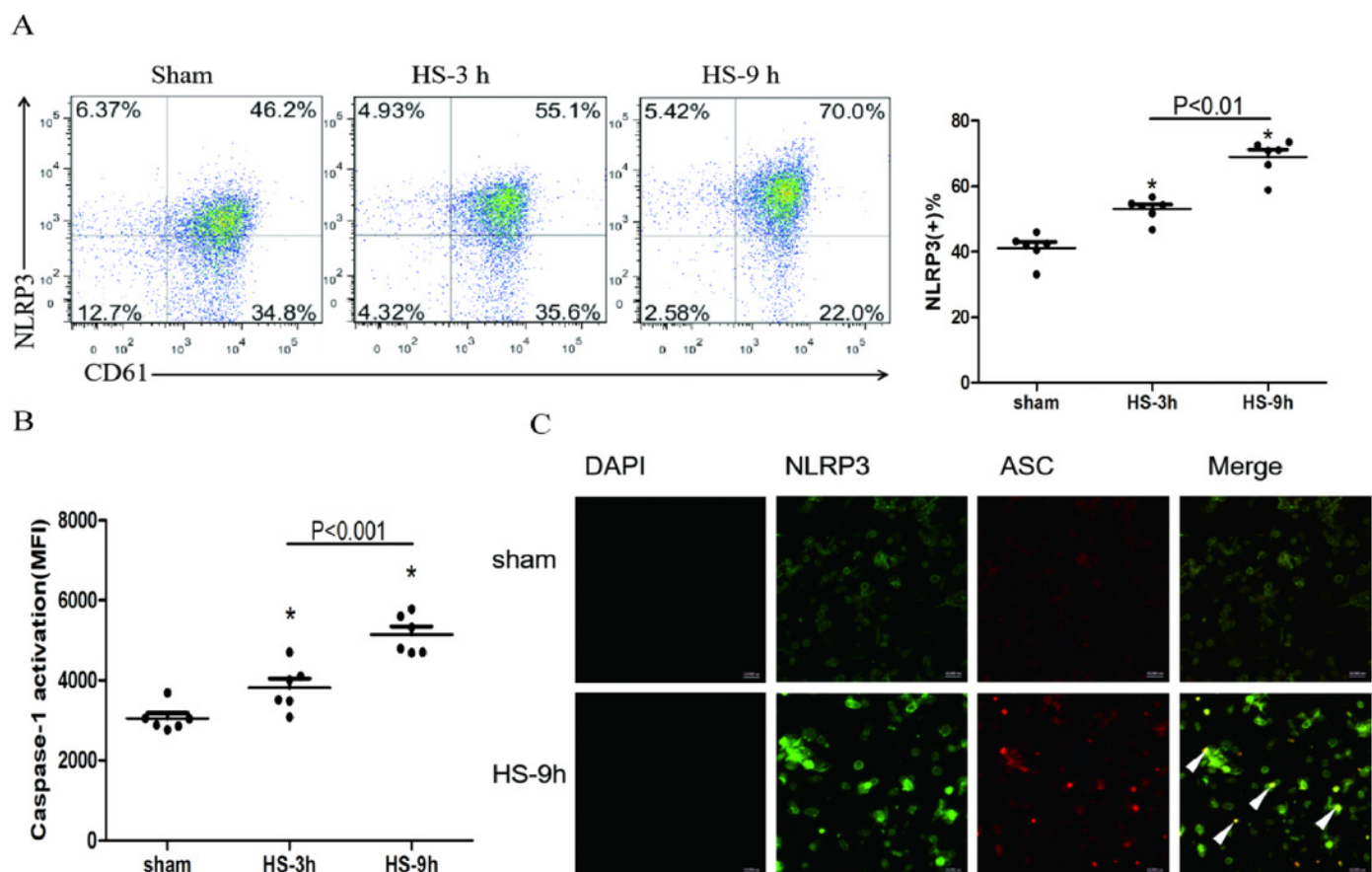
Figure 3

Fig. 3. *NLRP3 inflammasome mediates platelet activation and thrombocytopenia in HS rats (n=6).*

(A) Effect of MCC950 on caspase-1 activation in platelet of HS-9 h rats. *P<0.001 vs PBS+HS-9 h. (B) Effect of MCC950 on IL-1β secretion in HS-9 h rats. *P=0.001 vs PBS+HS-9 h. (C) Effect of MCC950 on CD62P in platelet of HS-9 h rats. *P<0.001 vs PBS+HS-9 h. (D) Effect of MCC950 on platelet count of HS-9 h rats. *P<0.001 vs PBS+HS-9 h. (E) Effect of ac-YVAD-cmk on IL-1β secretion in HS-9 h rats. *P<0.001 vs PBS+HS-9 h. (F) Effect of ac-YVAD-cmk on CD62P in platelet of HS-9 h rats. *P<0.001 vs PBS+HS-9 h. (G) Effect of ac-YVAD-cmk on platelet count of HS-9 h rats. *P=0.002 vs PBS+HS-9 h.
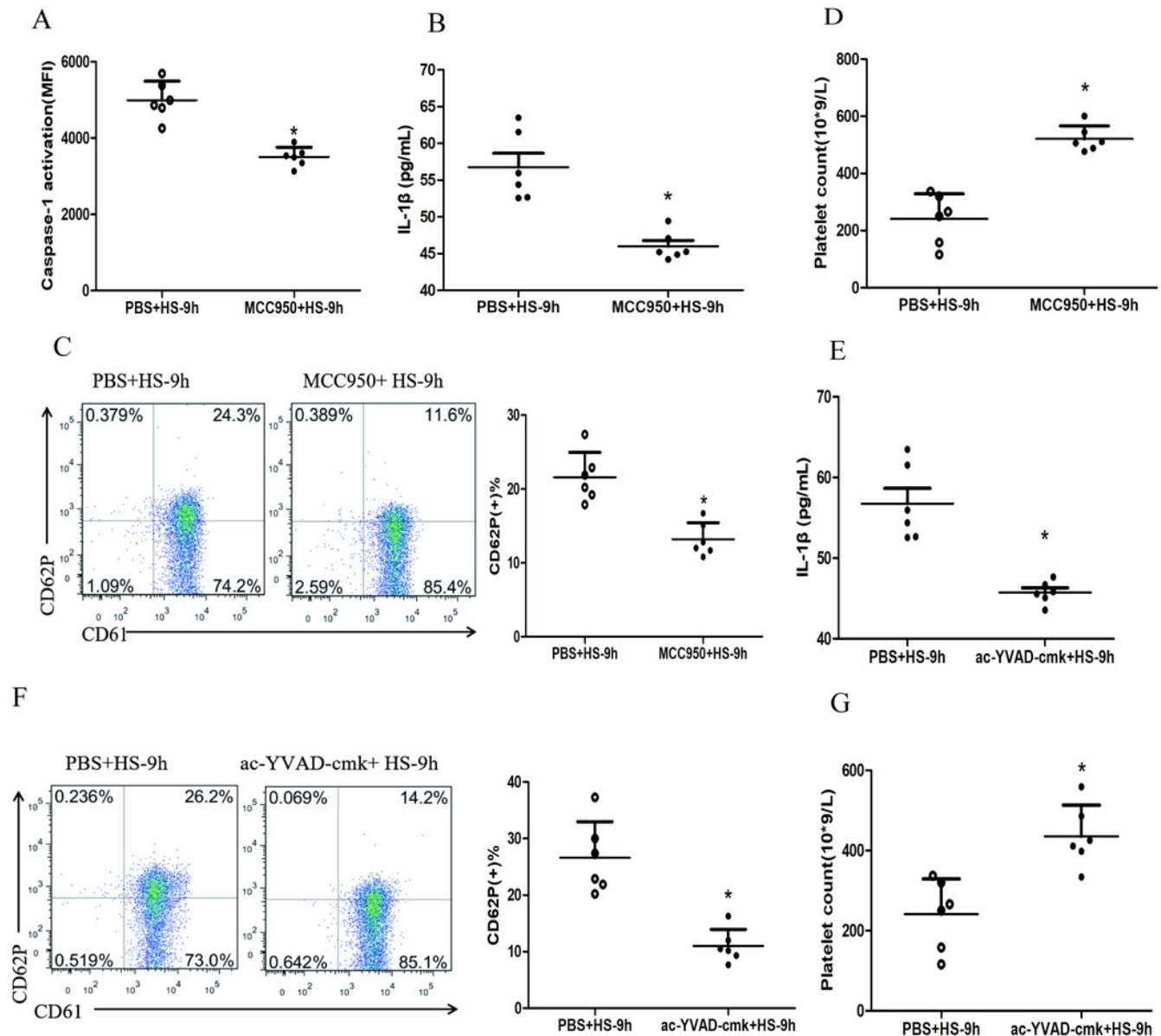
Figure 4

Fig. 4. *HMGB1 activates platelet NLRP3 inflammasome in HS rats (n=6).*

(A) HMGB1 levels in plasma of HS rats. *P<0.001 vs sham, P=0.003 between HS-3 h vs HS-6 h. (B) Effects of EP and a-HMGB1 on NLRP3 expression in platelets of HS-9 h rats. *P<0.001 vs PBS+HS-9 h. (C) Effects of EP and a-HMGB1 on caspase-1 activation in platelets of HS-9 h rats. *P<0.001 vs PBS+HS-9 h. (D) Effects of EP and a-HMGB1 on IL-1βsecretion in HS-9 h rats. *P=0.001 vs PBS+HS-9 h. (E) Effects of EP and a-HMGB1 on CD62P expression in platelets of HS-9 h rats. *P<0.001 vs PBS+HS-9 h. (F) Effects of EP and a-HMGB1 on platelet counts in HS-9 h rats. *P<0.001 vs PBS+HS-9 h. (G) TLR2 expression in platelets of HS rats. *P<0.001 vs sham. (H) TLR4 expression in platelets of HS rats. P= 0.004 between HS-3 h vs sham, p = 0.001 between HS-9 h vs sham, p = 0.007 between HS-3 h vs HS-6 h. (I) RAGE expression in platelets of HS rats. *P=0.001 vs sham. (J) Effects of a-TLR4 and a-RAGE on NLRP3 expression in platelets of HS-9 h rats. *P<0.001 vs PBS+HS-9 h. (K) Effects of a-TLR4, a-RAGE on caspase-1 activation in platelets of HS-9 h rats. *P<0.001 vs PBS+HS-9 h. (L) Effects of a-TLR4, a-RAGE on IL-1β in platelets of HS-9 h rats. *P<0.001 vs PBS+HS-9 h. (M) Effects of a-TLR4, a-RAGE on CD62P in platelets of HS-9 h rats. P = 0.003 between a-TLR4+HS-9 h vs PBS+HS-9 h, P = 0.003 between a-RAGE+HS-9 h vs PBS+HS-9 h, p<0.001 between a-TLR4+a-RAGE+HS-9 h vs PBS+HS-9 h, p=0.269 between a-TLR4+a-RAGE+HS-9 h vs a-TLR4+HS-9 h, p=0.242 between a-TLR4+a-RAGE+HS-9 h vs a-RAGE+HS-9 h. (N) Effects of a-TLR4, a-RAGE on platelet count in HS-9 h rats. *P<0.001 vs PBS+HS-9 h.
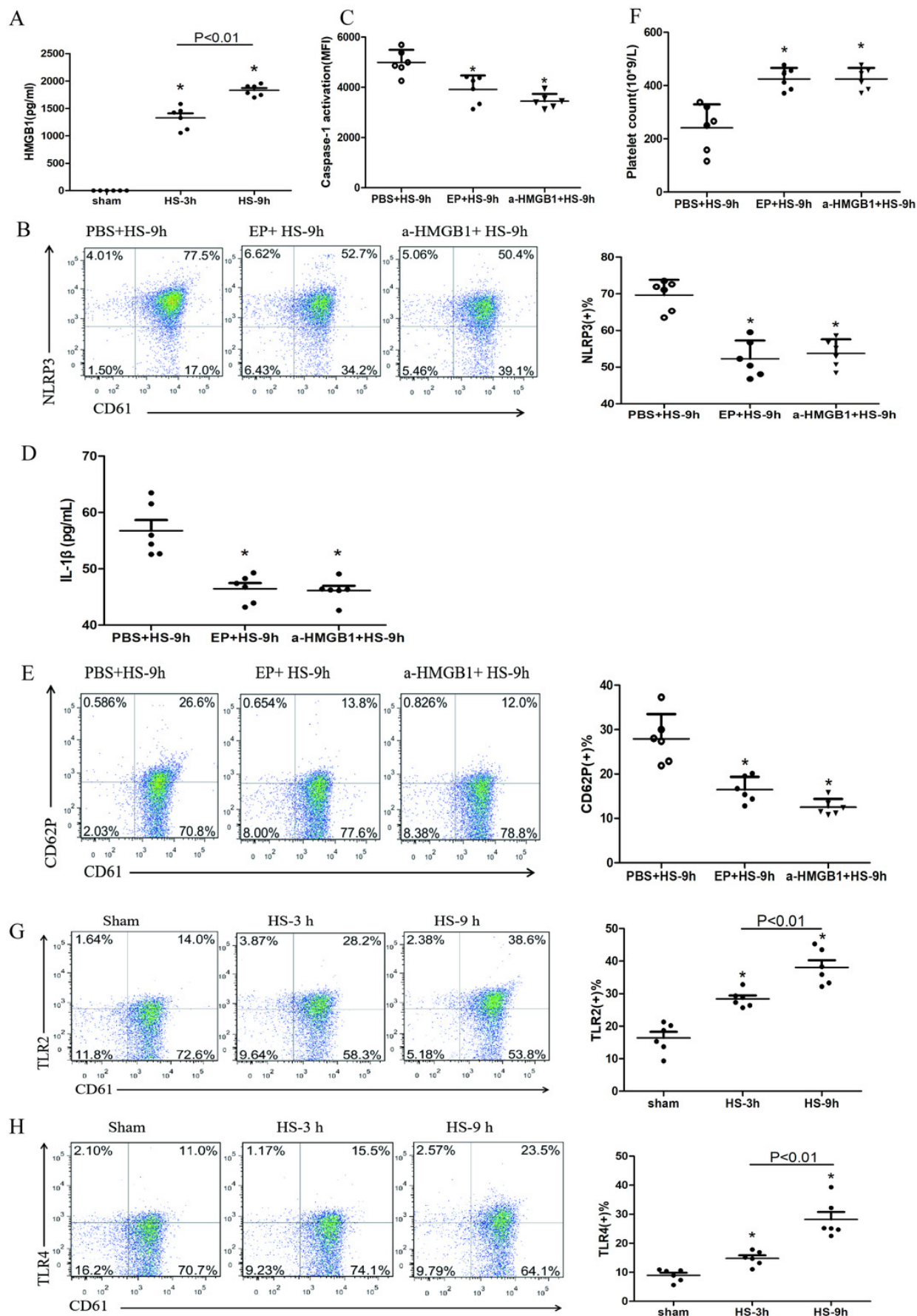
Figure 5

Fig.5. *HMGB1 activates platelet NLRP3 inflammasome by upregulating ROS in HS rats (n=6)*

(A) Mito-SOX levels in HS rats. *p < 0.001 vs sham. (B) DHE levels in HS rats. *p < 0.001 vs sham. (C) DCF-DA levels in HS rats. *p < 0.001 vs sham. (D) Effects of EP and a-HMGB1 on Mito-SOX in platelets of HS-9 h rats. *P=0.007 vs PBS+HS-9 h. (E) Effects of EP and a-HMGB1 on DHE in platelets of HS-9 h rats. *P=0.001 vs PBS+HS-9 h. (F) Effects of EP and a-HMGB1 on DCF-DA in platelets of HS-9 h rats. *P=0.003 vs PBS+HS-9 h. (G) Effects of NAC on NLRP3 in platelets of HS-9 h rats. *P<0.001 vs PBS+HS-9 h. (H) Effects of NAC on caspase-1 activation in platelets of HS-9 h rats. *P<0.001 vs PBS+HS-9 h. (I) Effects of NAC on IL-1βsecretion in HS-9 h rats. *P=0.005 vs PBS+HS-9 h. (J) Effects of NAC on CD62P in platelets of HS-9 h rats. *P=0.002 vs PBS+HS-9 h. (K) Effects of NAC on platelet count of HS-9 h rats. *P=0.001 vs PBS+HS-9 h.