# Hemin Induces the Activation of NLRP3 Inflammasome in N9 Microglial Cells

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## ABSTRACT

**Background:** Hemin is an important sterile component that induces a neuroinflammatory response after intracerebral hemorrhage, in which NLRP3 inflammasome activation has also proved to be involved. Although microglial activation acts as a key contributor in the neuroinflammatory response, the relationship between hemin and NLRP3 in microglia remains poorly understood. **Objective:** To investigate whether or not hemin regulates microglia-mediated secondary injury through activating the NLRP3/caspase-1 signaling pathway in microglia. **Methods:** In this study, N9 microglial cells were treated with hemin, and subsequently used to detect the production of caspase-1 p10 and NLRP3 inflammasome assembly. An ELISA was subsequently performed to measure the secretion of IL-1β. **Results:** It was found that the production of activated caspase-1 was dose- and time-dependent with regards to hemin. Moreover, hemin was observed to be capable of inducing the assembly of the NLRP3 inflammasome without any increase in IL-1β. Similarly, the supernatant of hemin-treated primary microglial cells did not increase in IL-1β secretion. Furthermore, hemin-induced NLRP3 inflammasome activation did not significantly affect pyroptosis. **Conclusion:** Hemin is a potential sterile danger signal molecule that can induce inflammasome activation without directly mediating inflammation damage on microglia.

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**Keywords: Caspase-1, Hemin, Microglia, NLRP3 Inflammasome**

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## INTRODUCTION

Intracerebral hemorrhage (ICH) is a common clinical disease entailing high mortality, morbidity and disability. One leading cause of the disease progression is secondary injury, which includes inflammation, excitotoxicity, edema and cell death (1-3). Recently, neuroinflammation has been found to play a major role during the secondary damage following intracerebral hemorrhage, as increased levels of inflammatory cytokines often induce neuronal apoptosis (4-6). Certain studies posit that hemin has a crucial role in neuroinflammation following ICH (7-9). Hemin is derived from the hemoglobin of red blood cells which becomes unstable at physiological temperatures after being released from red blood cells. Its ferrous heme groups are spontaneously oxidized into ferric hemin, which is detached from the globin part as a free hemin (10). Following intracerebral hemorrhage, the level of hemin can augment up to 10mM (8). Because the intracellular level of hemin rapidly increase, it can be considered as a major damage signal after ICH (8,11,12). Like macrophages, activated microglia act as key contributors to the neuroinflammatory response after ICH (13,14). Specifically, neuroinflammatory cascade is initiated via the recognition of damage-associated molecular patterns (DAMPs) by pattern recognition receptors (PPRs) on microglia. Myriad studies have proposed that NLRP3, a specific NOD-like receptor, belong to a major class of receptors that can recognize sterile hazard molecules (15-17) which may be inorganic (silica, asbestos) (18,19) or organic (monosodium urate, amyloid-β, cholesterol and ATP) (15,20-22) in nature. After NLRP3 protein recognizes the appropriate danger signal, it is activated to form an NLRP3 inflammasome comprised of the NLRP3, ASC and pro-caspase-1 molecules (23). Upon activation, the NLRP3 inflammasome mediates the autocatalytic cleavage and activation of caspase-1, forming a protease-activated subunit caspase-1 p10 / p20. Active caspase-1 p10 / p20 recognizes pro-IL-1β and other cytokine precursors for substrate hydrolysis so as to produce active inflammatory factors (24-26). In addition to the hydrolysis of inflammatory cytokines, caspase-1 p10 / p20 induces cell pyroptosis, a specific caspase-1 dependent type of programmed cell necrosis. Pyroptosis involves cell swelling until the cell membrane ruptures, releasing intracellular contents and inducing a strong inflammatory response with both apoptotic and necrotic features(27).That said, the specific mechanism by which hemin induces neuroinflammation after ICH is yet to be fully fathomed. Accumulating evidence suggests that NLRP3 exerts a critical role when DAMPs evoke an inflammatory insult. With most studies focusing on the influence of the oxidative stress mediated by hemin to microglia (9,28,29), less attention is paid to the effects of hemin on NLRP3 in hemin-mediated microglia activation.

Accordingly, the objective of the present research was to investigate whether hemin regulates microglia-mediated secondary injury by activating the NLRP3/caspase-1 signaling pathway in microglia. This study may further our understanding on neuroinflammation by providing an experimental basis for the prevention and treatment of secondary inflammation following cerebral hemorrhage.

## MATERIALS AND METHODS

**Reagents.** Hemin, ATP and LPS were purchased from Sigma-Aldrich, Dulbecco's Modified Eagle's Medium was from Gibco and Fetal bovine serum (FBS) was obtained

from Biological industries. IL-1β ELISA kits was purchased from E-Bioscience. NLRP3 antibody was purchased from Cell Signaling Technology. Caspase-1 antibody, IL-1β and β-actin were obtained from Abcam. Cytotoxicity LDH Assay Kit was from DojinDo, Annexin V-FITC-PI Apoptosis detection kit was bought from Bioworld, and Protein G PLUS-Agarose was purchased from Santa Cruz Biotechnology.

**Animals.** All experiments were conducted in accordance with the National Institute of Health guidelines for the humane use of laboratory animals and approved by local ethical review for the Animal center of Daping Hospital.

**Primary Microglial Cells and N9 Microglial Cell Culture.** Primary microglial cells were isolated from P1-P3 C57BL/6J mouse pups and cultured in Dulbecco's Modified Eagle Medium/F12 media supplemented with 10% FBS (30). N9 microglial cells, generously provided by Professor Yun Bai (Department of Medical Genetics, Army Medical University) were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS. N9 microglial cells were treated with hemin at final concentrations of 10 μM ~ 50 μM, respectively. The positive control group which had induced inflammasome activation was treated with LPS (1 μg/mL) for 3 hours followed by ATP (3 mM) for 30 minutes. The supernatants were collected to detect the secretion of IL-1β and the release of LDH, the former detected by an E-bioscience IL-1β ELISA and the latter assayed by a Cytotoxicity LDH Assay, and analyzed by a microplate reader at 450 nm.

**Western Blotting and Immunoprecipitation.** Cell total protein lysates (100 μg) were immunoprecipitated overnight with specific primary antibody at 4 ℃. The next day, agarose beads were added into the mixture and incubated on a rotator for 4 hours at 4 ℃. Finally, 2× SDS sample buffer was added to the agarose beads, with the mixture boiled for 5 minutes. Total cell lysates and immunoprecipitation samples were mixed with SDS sample buffer, separated by 15% SDS-PAGE, and transferred onto polyvinylidene fluoride (PVDF) membranes. Blots were probed with rabbit polyclonal antibody of mouse caspase-1 (1:1000), NLRP3 (1:1000), and IL-1β (1:1000) overnight at 4℃. The following day, the membranes were washed via TBS with 0.1% Tween-20 (TBST) and incubated with a horseradish peroxidase (HRP) conjugated goat anti-rabbit secondary antibody. The OD density values of bands were analyzed using Quantity One Software.

**Annexin V and Propidium Iodine Staining.** N9 microglial cells were seeded on coverslips in a 12-well plate at a density of $0.5 \times 10^6$ cell/mL and cultured overnight. After replacing them with a new medium, the cells were treated with hemin, LPS and ATP. After discarding the supernatant, cells were washed with cold PBS. Added to these cells was 400 μl of binding solution containing 5 μL of Annexin V-FITC and 10 μl of Propidium Iodine incubated at 4°C in the dark. After two washes with PBS, the nuclei were stained with DAPI, and the cells were observed and analyzed by fluorescence microscopy.

**MTT Assay.** N9 microglial cell viability was determined through a 3- [4,5-dimethyl-thiazol-2-yl]-2,5- diphenyltetrazolium bromide (MTT) assay. The N9 microglial cells were incubated with MTT reagent (Beyotime) for 4 hours in 5% CO2 at 37 ℃. Following MTT treatment, the supernatants of N9 cell was discarded and DMSO was added to dissolve formazan crystals. Cell viability was quantified in a plate reader by measuring the absorbance at 570 nm.

**Statistical Analysis.** Data and figures are illustrated as mean ± Standard Error of the Mean (SEM). One-way ANOVA and pairwise comparison LSD among groups was carried out by use of IBM SPSS 21 statistical package. $P<0.05$ was considered statistically significant.

## RESULTS

### Hemin-Induced Activation of Caspase-1 in N9 Microglial Cell.

Upon activation, the NLRP3 inflammasome mediates the autocatalytic cleavage and activation of caspase-1which form the protease-active subunit caspase-1 p10/p20. In order to investigate whether hemin is involved in the NLRP3 inflammasome activation in microglia, N9 microglial cells treated with hemin were used as a cell model. Western blotting results showed that the expression of caspase-1 p10 significantly increased at 40 and 50μM ($p<0.05$; Figures 1A, B). To further characterize the effect of hemin on N9 microglial cells, different time points (0, 0.5, 1, 2, 4, 6, and 24 hours) were examined following hemin (40μM) treatment. Experimental results indicated that the production of caspase-1 p10 peaked at 6 hours after treatment and was significantly diminished at 24 hours ($P<0.05$; Figures 1C, D).

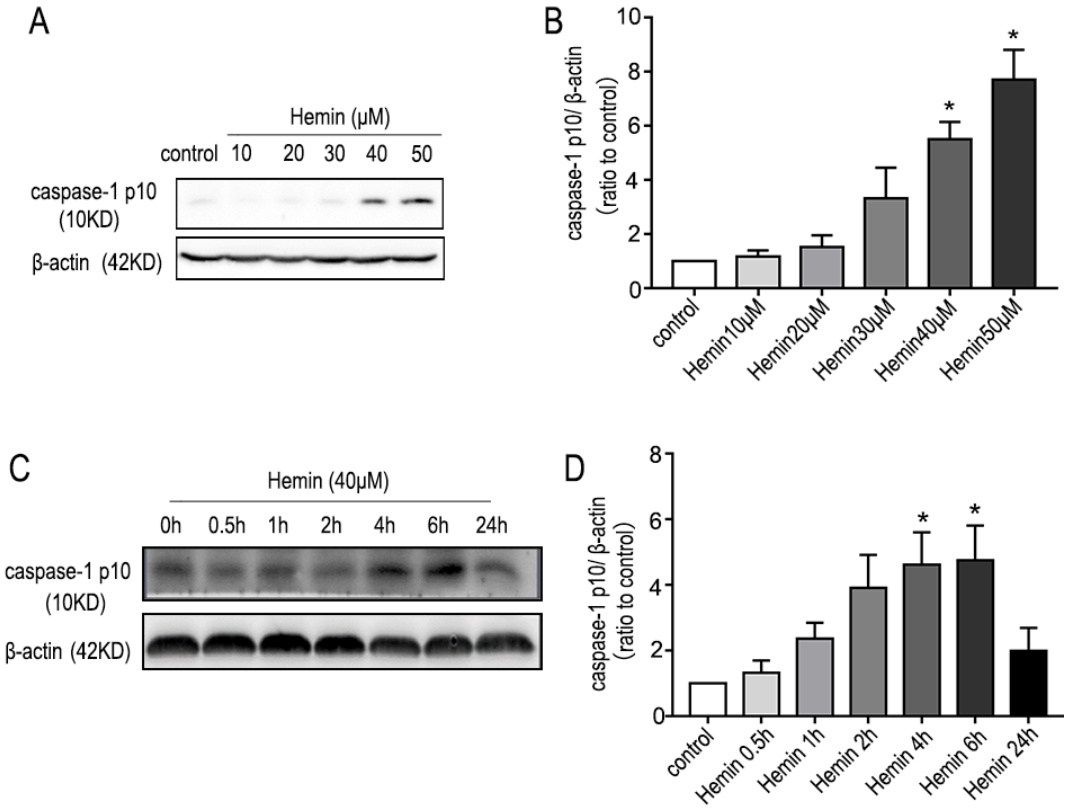


**Figure 1. Hemin induces the production of caspase-1 p10 in N9 microglial cells. (A)** Western blot analysis of caspase-1 p10 levels in N9 microglial cells, which were treated without or with hemin at the indicated concentrations for 6 hours. **(B)** The caspase-1 p10 levels of A are quantified and normalized to the β-actin levels. The data are presented as means ± SEM, and each experiment was repeated three times. *, $P<0.05$ when compared with control. **(C)** Western blot analysis of caspase-1 p10 levels in N9 microglial cells, which were treated without or with hemin (40 μM) for 30 min-24 hours **(D)** The caspase-1 p10 levels of C are quantified and normalized to the β-actin levels. The data are presented as means ± SEM, and each experiment was repeated five times. *, $P<0.05$ when compared with control.

### Hemin Stimulates NLRP3 Inflammasome Assembly in N9 Microglial Cells.

Further analyzed was the assembly of the inflammasome complex by immunoprecipitation since different studies have shown that caspase-1 activation is

induced following NLRP3 inflammasome assembly. In our initial studies, the coupling of NLRP3 with pro-caspase-1 in N9 microglial cells was detected 6 hours after hemin (40μM) treatment. As reported in several studies, a combination of LPS and ATP treatment can induce the assembly of the NLRP3 inflammasome and proinflammatory cytokine secretion (31,32). In this regard,LPS and ATP treatment groups were employed as a positive control for the activation of NLRP3 inflammasome. Immunoprecipitation results revealed that pro-caspase-1 could bind to NLRP3 after being treated with 40μM hemin for 6 hours, in a similar manner as LPS + ATP treatment group($p<0.05$; Figures 2A, 2B).

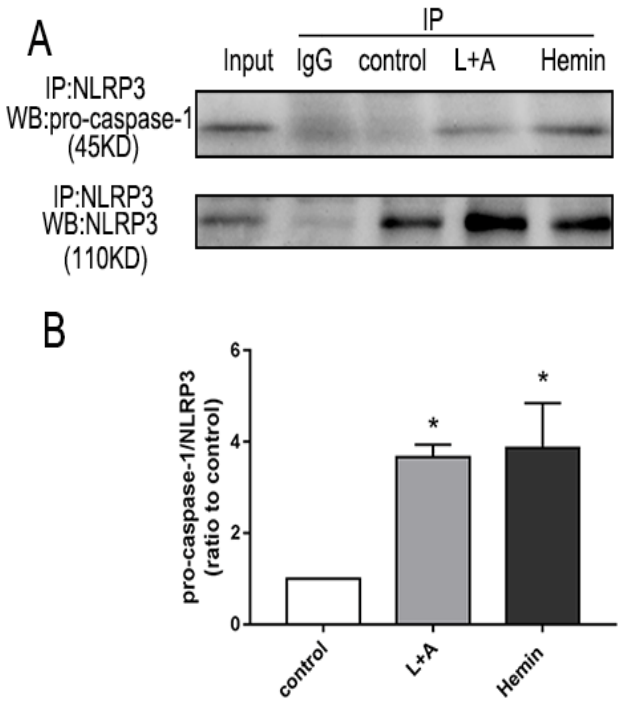


**Figure 2. Hemin induces the assembly of the NLRP3 inflammasome in N9 microglial cells.(A)** Co-IP of pro-caspase-1 and NLRP3 in N9 microglial cells using anti-NLRP3 antibody as the immunoprecipitating antibody. IP, immunoprecipitation. WB, Western blot. Rabbit IgG served as a negative control. The experimental control was the N9 microglial cells lysate without hemin treatment. L+A, LPS (1 μg/mL) pretreatment cell for 3 hours, then ATP (3 mM) was added for 30 minutes, this group is used as the positive control of NLRP3 inflammasome assembly. Input is the total cell lysis of control without NLRP3 antibody immunoprecipitation. **(B)** The pro-caspase-1 are quantified and normalized to the NLRP3 levels. The data are presented as means ± SEM, and each experiment was repeated three times. *, $P<0.05$ when compared with control.

**Hemin Does Not Affect the Secretion of IL-1β in N9 Microglial Cells and Primary Microglial Cells.**

The supernatants of N9 microglial cells were collected with different treatment groups. The ELISA assays demonstrated that after 6 hours of treatment with 40μM hemin, IL-1β secretion in N9 microglial cells and primary microglial cells did not undergo any statistically significant increase ($p>0.05$, Fig 3A, C). However, in the positive control group (LPS+ATP) and LPS pretreatment group, the IL-1β secretion levels were robustly increased ($p<0.05$, Figures 3A, 3C). Moreover, the hemin treatment group did not augment IL-1β precursor in N9 microglial cell (Figure 3B).

In conclusion, without LPS pretreatment, hemindoes not affect the secretion of IL-1β in N9 microglial cells and primary microglial cells.

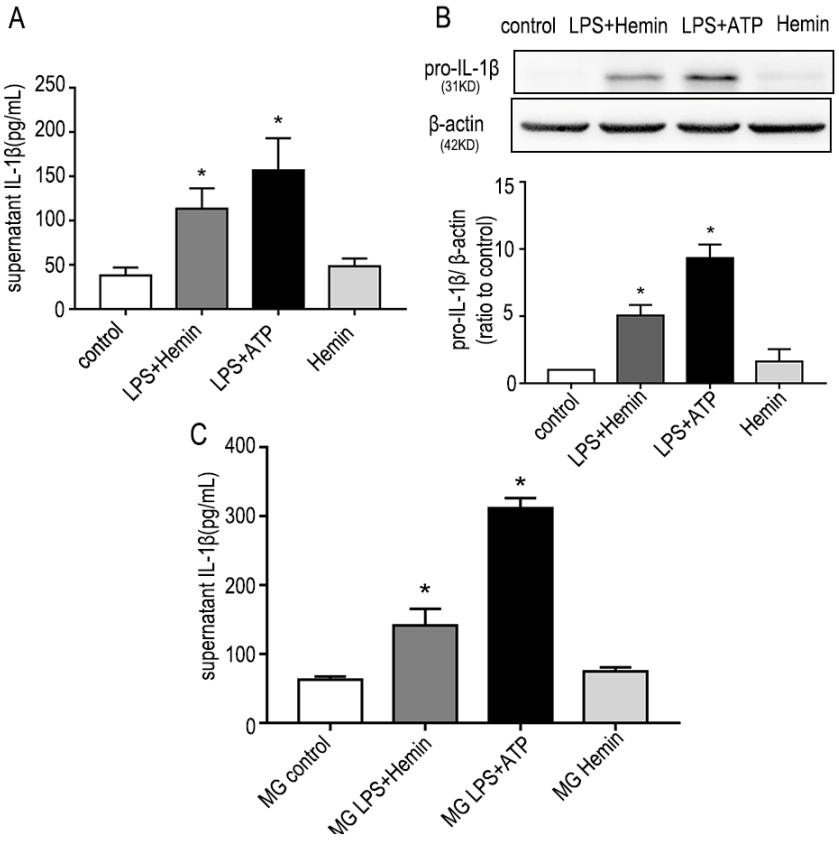


**Figure 3. Hemin has no effect on IL-1β secretion in N9 microglial cells and primary microglial cells supernatants.(A)** N9 microglial cells were treated with or without 40 μM hemin for 6 hours. Concentrations of IL-1β in the supernatants were measured by ELISA. LPS + Hemin, N9 microglial cells were pretreated with LPS for 3 hours, then treated with Hemin 40 μM for 6 hours. The data are presented as means ± SEM, and each experiment was repeated three times. *, P<0.05 when compared with control. **(B)** N9 microglial cells were treated with or without hemin at the concentration of 10 μM to 50 mM for 6 hours. The pro-IL-1β are quantified and normalized to the β-actin levels. The data are presented as means ± SEM, and each experiment was repeated three times. *, P<0.05 when compared with control. **(C)** Primary microglial cells (MG) were treated without or with hemin at the concentration of 40 μM for 6 hours. Concentrations of IL-1β in the supernatants were measured by ELISA. The data are presented as means ± SEM, and each experiment was repeated three times. *, P<0.05 when compared with control.

## Hemin Does Not Affect Pyroptosis in N9 Microglial Cells.

In addition to IL-1β secretion, a pyroptotic phenomenon was detected in N9 microglial cells. As a newly characterized type of cell death, pyroptosis possesses the characteristics of both apotosis and necrosis (4). Subsequently,the Annexin-V and Propidium Iodine double-labeling were utilizedto detect apotosis in N9 microglial cells. An LDH release experiment was furtherperformedto verify necrosis in these cells.

These experiments demonstrated that N9 microglial cells treated with 40μM hemin did not induce apoptosis. On the other hand, apoptosis was detected in cells treated with both LPS and ATP (Figure 4).

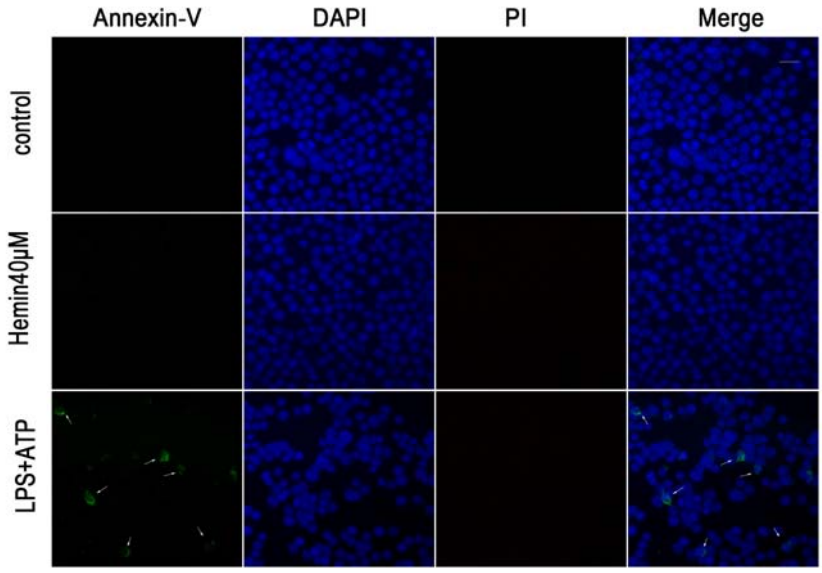


**Figure 4. Hemin has no effect on the apoptosis of N9 microglial cells.** N9 microglial cells were treated with or without 40 μM hemin for 6 hours. LPS+ATP, N9 microglial cells were pre-treated with LPS (1 μg/mL) for 3 hours, then treated with 3 mM ATP for 6 hours. Annexin V/Propidium Iodine staining on N9 microglial cells was used to verify apoptotic status. Arrows (white) indicate the Annexin V positive cells. Bar, 20 μm.

Additionally, the LDH release assays showed no significant increase in N9 microglial cells treated with hemin ($P>0.05$, Figure 5A). Moreover, based on the MTT viability assays, no obvious cell death was observed in hemin-treated N9 microglial cells ($P>0.05$, Figure 5B). In conclusion, these results suggest that hemin does not directly induce pyroptosis in N9 microglial cells.

## DISCUSSION

There is increasing evidence that NLRP3 is an important PPR in cytoplasm, playing a major role in neurological injury, especially in the case of non-infectious inflammation (17). Following its release from hemoglobin, hemin accumulates rapidly in the extracellular space of intracerebral hemorrhage (8). It may, therefore, be recognized as a sterile danger signal for brain injury (8,11,33). Though there are reports suggesting that ICH models show a significant increase in NLRP3 activation(34,35), the role of NLRP3 as a sterile sensor in hemin-mediated microglial injury is a quite untrodden path.

For the first time, we conceptually demonstrated that hemin can induce the production of caspase-1 p10 in a dose- and time-dependent manner. More specifically, a concentration of 40μM hemin generated NLRP3 inflammasome assembly. However, it is surprising that the secretion of IL-1β and pyroptosis was not affected by hemin. The reason behind the lack of pyroptosis after hemin treatment warrants further investigation. In our study, the production of caspase-1 p10 was augmented via hemin in microglia. As several reports have suggested, active caspase-1 can be released to the extracellular space so as to exert such afunction (36).

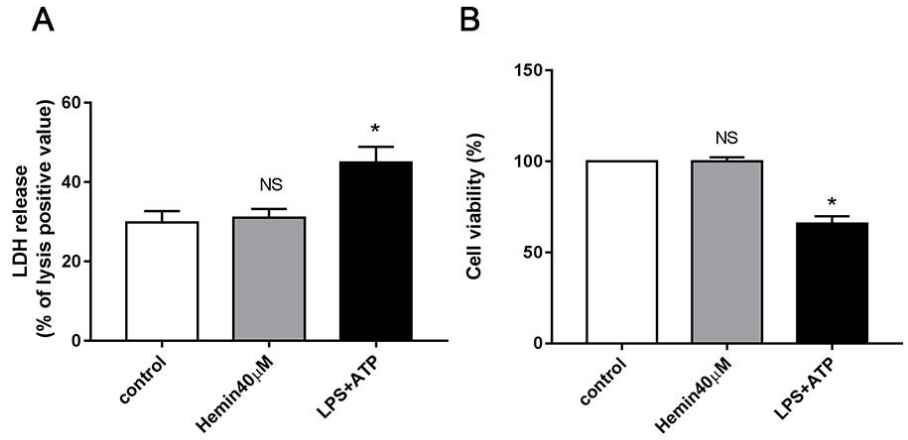


**Figure 5. Hemin has no effect on the necrosis of N9 microglial cells. (A)** N9 microglial cells were treated with or without 40 μM hemin for 6 hours. An LDH release assay was used to test necrotic status. The data are presented as means ± SEM, and each experiment was repeated five times. NS, P>0.05 when compared with control. *, P<0.05 when compared with control. **(B)** N9 microglial cells were treated with or without 40 μM hemin for 6 hours. MTT assays were used to test the cell viability. The data are presented as means ± SEM, and each experiment was repeated six times. NS, P>0.05 when compared with control. *, P<0.05 when compared with control.

Based on the present results, the production of caspase-1 p10 may function following extracellular secretion to affect another neighboring cellular type to propagate further neuronal injury.In addition to cleaving proinflammatory cytokines, caspase-7 can be hydrolyzed by activated caspase-1 in order to regulate the formation of phagosomes in macrophages,thereby regulating cellular function (37,38). Research has shown that macrophages specifically perform a regenerative role in vascular development and remodeling after microhemorrhage. As a macrophage subpopulation, microglial cells are indispensable concerning the repair of blood-brain barrier after injury in mice (39). In the present cellular model, hemin might not have been able to regulate the inflammatory response of cells through the activation of NLRP3; hemin was therefore suspected of regulating phagocytosis and repair function of microglia by activating caspase-1, an assumption yet to be corroborated.

Taken together, the present study posits that under non-infectious conditions, hemin plays a novel role as a sterile activator of NLRP3 inflammasome assembly. LPS pretreatment robustly increased the secretion of IL-1β in N9 and primary microglial cells. It is proposed that when intracerebral hemorrhage occurs, infection has to be avoided in order to control the expansion of the inflammatory damage caused by hemin.Our studies demonstrated that in the absence of LPS pretreatment, hemin was able to activate the NLRP3 inflammasome in microglia, even though inflammatory cytokine release and pyroptosis were not observed. This indicates that inflammatory injury caused by hemin, released during non-infective intracerebral hemorrhage,mainly does not function through the NLRP3/caspase-1 inflammasome pathway. Previous

studies have pointed out that hemin is absorbed by both microglia and neurons (11), hence the fact that our future studies will be focused on the direct neuronal cytotoxicity mediated by hemin. Such studies eventually conduce to the verification of the secondary injury induced by hemin after cerebral hemorrhage, providing the experimental basis for a novel treatment for cerebral hemorrhage. In conclusion, hemin induces NLRP3 inflammasome assembly to form the active enzyme caspase-1 p10 which does not significantly induce the secretion of IL-1β nor a pyroptoticphenomenon in N9 microglial cells.Moreover, hemin is a potential sterile danger signal molecule capable of activating NLRP3 inflammasome in microglia. In N9 microglial cells, hemin does not further mediate a pronounced inflammatory response through NLRP3 inflammasomes. This research enhances our understanding of neuroinflammation by providing an experimental basis for the prevention and treatment of the secondary inflammation following cerebral hemorrhage.

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