

Ammonia Induced Microglial Activation Modulates Connexin 43 and Aquaporin 4 Expression in Astrocyte-microglia Co-culture Model

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Abstract

Background Hepatic encephalopathy (HE) is a neurological complication resulting from acute or chronic liver disease. Hyperammonemia leading to astrocyte swelling and cerebral edema in combination with neuroinflammation including microglia activation, mainly contribute to the pathogenesis of HE. However, little is known about microglia and their inflammatory response, as well as their influence on astrocytic channels and astrocyte swelling under hyperammonemia.

Objective To investigate the effects of ammonia on the microglial activation, glial viability, connexin 43 (Cx43) and aquaporin 4 (AQP4) expression in astrocytes in *in vitro* astrocyte-microglia co-culture model.

Methods Primary rat glial cocultures of astrocytes containing 5% (M5, representing "physiological" conditions) or 30% (M30, representing "pathological" conditions) of microglia were incubated with 3 mM, 5 mM, 10 mM and 20 mM ammonium chloride (NH₄Cl) for 6 h and 24 h in order to mimic the conditions of HE. An MTT assay was performed to measure the viability, proliferation and cytotoxicity of cells. The microglial phenotypes were analysed by immunocytochemistry. The expression of Cx43 and AQP4 were quantified by immunoblot analysis.

Results A significant reduction of glial viability was observed in M30 co-cultures after incubation with 20 mM NH₄Cl for 6 h, whereas in M5 co-cultures the viability remained unchanged. Microglial activation was detected by immunocytochemistry after incubation with 3 mM, 5 mM and 10 mM NH₄Cl for 6 h and 24 h in M5 as well as in M30 co-cultures. The Cx43 expression was increased significantly in M30 co-cultures after 6 h incubation with 5 mM NH₄Cl. The AQP4 expression was increased significantly in M5 co-cultures treated with 10 mM NH₄Cl for 6 h.

Conclusions Our findings showed a significant microglial activation, decrease of viability and increase in Cx43 and AQP4 expression after NH₄Cl incubation in astrocyte-microglia co-culture model. Based on previous *in vitro* studies suggesting that microglia activation influences astrocytic networks, it can be assumed that the microglial activation under hyperammonemia can modulate the Cx43 and AQP4 expression in astrocytes in a dynamic way and this can contribute to astrocytic dysfunction in HE.

Background

Hepatic encephalopathy (HE) is a neurological complication resulting from acute or chronic liver disease. High ammonia level resulting from impaired liver function can cross the blood-brain-barrier and lead to morphological changes of astrocytes [1–2]. Further, astrocytes are the main cells in the central nervous system (CNS) which can metabolize ammonia into glutamine using glutamine synthetase [3]. Elevated glutamine level in astrocytes results in increased water accumulation, cytotoxic astrocyte swelling and increased osmotic pressure contributing to cerebral edema [4]. Studies have also shown that the transmembrane water channel protein Aquaporin 4 (AQP4), which is localized on astrocyte end-feet and regulates maintaining brain water homeostasis, is up-regulated during acute liver failure and HE [5–10].

Microglia, the main immune cells of the CNS, are found in a resting ramified form in the healthy brain and range from 5 to 20% of the glial cell population [11]. Under pathological conditions, microglial activation includes proliferation of microglia, change of the morphological phenotype from the resting ramified type (RRT) to the activated, rounded phagocytic type (RPT), expression of immune molecules and release of inflammatory mediators [12]. Studies have demonstrated that hyperammonemia in HE leads to microglia activation in terms of stimulation of microglial cell migration, morphological changes, oxidative stress and up-regulation of the microglial activation marker ionized calcium-binding adaptor molecule-1 (Iba-1) [13]. Media from ammonia-treated microglia cell culture added to cultured astrocytes contributes to astrocyte swelling suggesting a link between astrocytes and microglia under hyperammonemia conditions [14].

In summary, hyperammonemia leading to astrocyte swelling and cerebral edema in combination with neuroinflammation including microglia activation, mainly contribute to the pathogenesis of HE.

Connexin 43 (Cx43) is the predominant protein in astrocytes that contributes to formation of gap junctions (GJs) and functional astrocytic network allowing the exchange of small molecules, ions and second messengers, and ensuring the homeostasis [15]. Connexin 43 is further involved in spatial buffering of potassium, cell proliferation, regulation of transmitter uptake and dissipation, support of neurons, and volume regulation [11, 15]. No data about effects of ammonia on Cx43 expression in astrocytes are available to date. Also, little is known about microglia and their influence on astrocytic channels and astrocyte swelling under hyperammonemia. In this study, we aimed to investigate the effects of ammonia on the microglial activation, glial viability, Cx43 and AQP4 expression in astrocytes in *in vitro* astrocyte-microglia co-culture model.

Methods

Cell culture

Primary astrocyte-microglia co-cultures were derived from brain of postnatal Wistar rats (postnatal day 0–2, P0–P2) according to Faustmann et al., 2003 [11]. All experiments were performed according to the German animal welfare act and the ethical standards of Ruhr University Bochum, and were approved by the local authorities in Bochum, Germany. All animals were kept under standard laboratory conditions with access to food and water.

The P0–P2 rats were decapitated without sedation, according to the German animal welfare act. After removing of cerebellum, meninges and choroid plexus, the brains were kept in ice-cold phosphatebuffered saline (PBS), then treated with 0.1% trypsin for 30 min at 37 °C and centrifuged at 500 × g for 12 min to remove the supernatant. Following this, the pellet was resuspended in 5 ml of DNase I solution (100 µl/ml with Dulbecco's minimal essential medium, DMEM) for 5 min at room temperature, centrifuged at 200 × g for 5 min and after washing steps filtered through a 60-µm nylon mesh. Cells were kept at a density of one brain per plastic tissue-culture flask in 7% CO₂ at 37 °C. After 5 days, the cultures were about 100%

confluent. Adherent microglial cells and oligodendroglia on the astroglial surface were separated from the culture by shaking the flasks manually. The amount of microglial cells in the co-cultures varied between 5% and 30% depending on the extent of shaking and was determined by counting after fixation and staining.

Treatment of cultures

The primary rat glial co-cultures of astrocytes containing 5% (M5, representing "physiological" conditions) or 30% (M30, representing "pathological" conditions) of microglia were incubated with 3 mM, 5 mM, 10 mM and 20 mM ammonium chloride (NH₄Cl) (Sigma-Aldrich, Germany) for 6 h and 24 h in 7% CO₂ at 37 °C in order to mimic the conditions of HE.

MTT assay

An MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Roche) was performed to measure the viability, proliferation and cytotoxicity of cells. The co-cultures from tissue-culture flask were placed on poly-L-lysine-coated glass cover slips at 12,000 cells per well in 96-well plates in 7% CO₂ at 37 °C until they were confluent. Cells were incubated with ammonium chloride as described above. In the next step, incubation with 10 µl MTT reagent for 4 h in 7% CO₂ at 37 °C was performed. Following this, 100 µl of solubilization solution were applied to the co-cultures and the samples were incubated overnight. The next day, the Bio-rad microplate reader was used to measure the cell viability in the wells at a wavelength of 550 nm.

Immunocytochemistry

The microglial phenotypes as well as Cx43 and AQP4 expression were analysed by immunocytochemistry. The astrocyte-microglia co-cultures were placed on poly-L-lysine-coated glass cover slips at 70,000 cells per well in 24-well plates and incubated with ammonium chloride as described above. Cover slips with the cell cultures were fixed with 70% ethanol for 10 min and incubated in PBS-blocking solution containing 1% bovine serum albumin. The cover slips were treated with rabbit anti-Cx43 (1:2000) and rabbit anti-AQP4 (1:200) in combination with mouse anti-ED1 (1:250) (Serotec, Germany) and incubated at 4 °C for 2 h. In the next step, the wells were incubated with secondary antibodies (1:500) (Invitrogen, Germany) including goat anti-mouse IgG conjugates (Alexa fluor® 568) and goat anti-rabbit IgG conjugates (Alexa fluor® 488) for 1 h. Immunocytochemically labeled cells were counterstained with DAPI (4,6-diamidino-2-phenyl-indol, 1:2500) for quantification of cell numbers. The ratio of microglia to astrocytes was identified by comparison of the number of ED1-stained microglia with the total number of DAPI-labeled cells. The microglia morphology was evaluated in a minimum of three different visual fields on each cover slip at a primary magnification of 630×. ED1 staining allowed the classification of microglia as ramified, intermediate and activated rounded phagocytic phenotype [11].

Immunoblot (western blot) analysis

The expression of Cx43 and AQP4 were quantified by immunoblot analysis according to the protocol. A total number of 300.000 cells were seeded in each culture dish. After reaching confluence, the cells were

treated with ammonium chloride as described above. In the next step, the cells were washed with PBS and lysed with 200 µl Laemmli 1 x buffer and 4 µl protease inhibitor cocktail. The cells were detached from the culture dishes using a silicone scraper and the lysates were kept on ice. The protein concentrations were measured by Bradford assay (Bio-rad Bradford Protein Assay) based on the protocol.¹⁶ Next, loading of 10 µg solution onto 10% or 15% sodium dodecyl sulfate (SDS) gel and electrophoresis at 100 V for 20 min and following 150 V was performed. After transfer of the gels to nitrocellulose membrane for Cx43 or polyvinylidene fluoride (PVDF) membrane for AQP4 and blocking with Odyssey blocking buffer (LI-COR Bioscience, Germany) for 1 h, the membranes were incubated with anti-β-actin (1:10000) (Sigma, USA), anti-Cx43 (1:5000) (Invitrogen, Germany) or anti-AQP4 (1:2000) (Invitrogen, Germany) antibodies at 4 °C overnight. After washing with 0.1% Tween®20 (Applichem) in PBS (PBST) for 3 × 15 min, the membranes were treated with secondary anti-mouse (1:20000) and anti-rabbit (1:10000) fluorescent antibodies (Sigma, USA) for 1 h. Washing step with PBST was performed. An Odyssey Infrared Imaging System (LI-COR Bioscience, Germany) was used to visualize the bands. The expression of Cx43 and AQP4 could be quantified with the ratio to beta-actin bands.

Data analyses and statistics

All statistical analyses and graphs were performed with GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego, USA) using One-way analysis of variance (One-way ANOVA) and Bonferroni post hoc test for parametric data and Kruskal-Wallis with Dunn's post-hoc test for non-parametric data. The significance was set at $p < 0.05$ and the results were reported as mean ± standard error of the mean.

Results

Glial viability after incubation with ammonia

A significant reduction of glial viability was observed after incubation with 20 mM NH₄Cl for 6 h in M30 co-cultures, representing „pathological“ conditions ($p < 0.001$). This effect could not be observed after incubation for 24h. In M5 co-cultures, representing „physiological“ conditions, the glial viability was not altered by hyperammonemia (Figure 1).

Hyperammonemia-induced microglial activation

We detected a significant and dose-dependent increase of amount of activated microglia in M5 and M30 co-cultures by immunocytochemistry after incubation with 3 mM, 5 mM and 10 mM NH₄Cl for 6 and 24 h. In parallel, the amount of resting microglia decreased significantly and dose-dependent under the same conditions (Figure 2). Further the amount of intermediate type of microglia was increased significantly in M5 co-cultures after incubation with 5 mM and 10 mM NH₄Cl for 6 h ($p < 0.05$) and in M30 co-cultures after incubation with 3 mM and 5 mM NH₄Cl for 6 h ($p < 0.01$) (data not shown).

Influence of ammonia on Cx43 expression

The Cx43 expression in M5 co-cultures was not changed significantly in immunoblot analysis after dose-dependent incubation with NH₄Cl for 6 or 24 h. Under „pathological“ conditions in M30 co-cultures, the Cx43 expression was significantly increased after 6 h incubation with 5 mM NH₄Cl ($p < 0.05$) and weakly, but not significantly increased after incubation with 10 mM NH₄Cl (Figure 3). Longer incubation for 24 h with NH₄Cl did not alter the Cx43 expression in the astrocyte-microglia co-cultures ($p = ns$).

Influence of ammonia on AQP4 expression

The AQP4 expression was increased significantly in M5 astrocyte-microglia co-cultures treated with 10 mM NH₄Cl for 6 h ($p < 0.05$). This effect could not be determined after incubation with NH₄Cl for 24 h. In M30 co-cultures, AQP4 expression was not changed after incubation with ammonia (Figure 4).

Discussion

In this study, we demonstrated a significant, dose-dependent increase of microglial activation after incubation with ammonia both in physiological and pathological co-culture model. In parallel, increase in Cx43 and AQP4 expression was observed suggesting a link between microglial activation and astrocytic function under hyperammonemia conditions.

Glial cell viability under hyperammonemia

Cytotoxic effects of ammonia on primary astrocyte cultures were previously demonstrated, but data about effects on astrocyte-microglia co-culture models were not available [17–18]. In our study, we examined the effects of ammonia on glial cell viability in astrocyte-microglia co-culture models under physiological and pathological conditions. A significant reduction of glial viability was observed after incubation with high-dose ammonia for 6 h in M30 astrocyte-microglia co-cultures, representing „pathological“ conditions, but not after incubation for 24 h. This could refer to a regulatory effect within the culture after a prolonged time of incubation and could further be explained by a very short half-life of ammonia (1-6.5 sec). In M5 co-cultures, representing „physiological“ conditions, the glial viability was not affected by hyperammonemia. This effect could not be modulated by changing the concentration of ammonia which underlines how stable and compensatory a physiological culture reacts to stress via ammonia even if the used concentrations was higher than described in previous studies of hepatic encephalopathy [17]. In another study, treatment with “low” concentrations of ammonia (5 mM) for 18 h had also no effects on the cell viability in astroglial-enriched cultures containing up to 10% microglia and in microglia-enriched cultures [19].

Microglial activation and modulation of Cx43 and AQP4 expression under hyperammonemia

Our findings showing a significant and dose-dependent increase of activated microglia in M5 and M30 co-cultures after incubation with ammonia, are consistent with previous study results from primary microglia cultures [13–14]. Incubation of primary microglia cultures with ammonia caused an increase in synthesis and release of IL-6 and TNF α compared to untreated microglia [20]. However, in another study ammonium chloride did not influence LPS-induced up-regulation of transcription of microglia activation markers such as IL-6 and TNF α in microglia mono-cultures, but reduced it in co-cultured astrocytes and microglia [20]. These results indicated that astrocytes reduced the up-regulation of microglia activation markers induced by LPS [20]. Further studies are required to evaluate the exact effects of ammonia on microglia activation in the different contexts of HE.

On the other hand, there is evidence for interactions between astrocytes and microglia under hyperammonemia conditions contributing to astrocyte swelling [14]. Previous *in vitro* studies by Faustmann et al., 2003 demonstrated that microglia activation in M30 astrocyte-microglia co-culture model, representing "pathological" conditions, was associated with reduced astroglial Cx43 expression [11]. However, microglial activation under hyperammonemia was related to increase in Cx43 expression in our study. These findings indicate that the degree of intercellular coupling in the astroglial network may be modulated by the activation of microglia under pathological conditions in different ways.

The AQP4 expression in our study was increased after incubation with ammonia similar to Cx43. This is consistent with results of previous studies [21]. Further, AQP4 up-regulation after incubation with ammonia correlated with astrocyte swelling [21]. The exact mechanisms leading to increase in AQP4 expression are not yet known. Protein tyrosine nitration was detected in ammonia-treated astrocyte cultures, thus ammonia-induced nitrosative stress may contribute to the up-regulation of AQP4 [22]. It is also conceivable that microglial activation following exposure to pathophysiological concentrations of ammonia can modulate AQP4 expression similar to Cx43 expression.

Conclusions

Our findings showed a significant microglial activation, decrease of viability and increase in Cx43 and AQP4 expression after NH₄Cl incubation in astrocyte-microglia co-culture model. Based on previous *in vitro* studies suggesting that microglia activation influences astrocytic networks, it can be assumed that the microglial activation under hyperammonemia can modulate the Cx43 and AQP4 expression in astrocytes in a dynamic way and this can contribute to astrocytic dysfunction in HE.

Abbreviations

AQP4: Aquaporin 4

CNS: Central nervous system

Cx43: Connexin 43

DMEM: Dulbecco's minimal essential medium

DAPI: 4',6-Diamidino-2-phenylindole

GJs: Gap junctions

HE: Hepatic encephalopathy

Iba1: Ionized calcium-binding adaptor molecule 1

IL-6: Interleukin 6

LPS: Lipopolysaccharide

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NH₄Cl: Ammonium chloride

PBS: Phosphatebuffered saline

PVDF: Polyvinylidene fluoride

RRT: Resting ramified type

RPT: Rounded phagocytic type

SDS: Sodium dodecyl sulfate

TNF α : Tumor necrosis factor α

Declarations

Ethics approval and consent to participate

All experiments were performed according to the German animal welfare act and the ethical standards of the Ruhr University Bochum, and were approved by the local authorities in Bochum, Germany.

Consent for publication

Not applicable.

Data availability statement

The datasets used or analysed during this study are available from the corresponding author on reasonable request.

Competing interests

FSI, TJF, AT, ZM, PMF declare that they have no competing interests.

Study funding

No targeted funding reported.

Authors' contributions

PMF conceived and designed the experiments. AT performed the experiments. All authors analysed and interpreted the experimental data. FSI and TJF wrote the manuscript. All authors read and approved the final manuscript. All authors have agreed to be personally accountable for own contributions and to ensure that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and the resolution documented in the literature.

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Not applicable.

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Figures

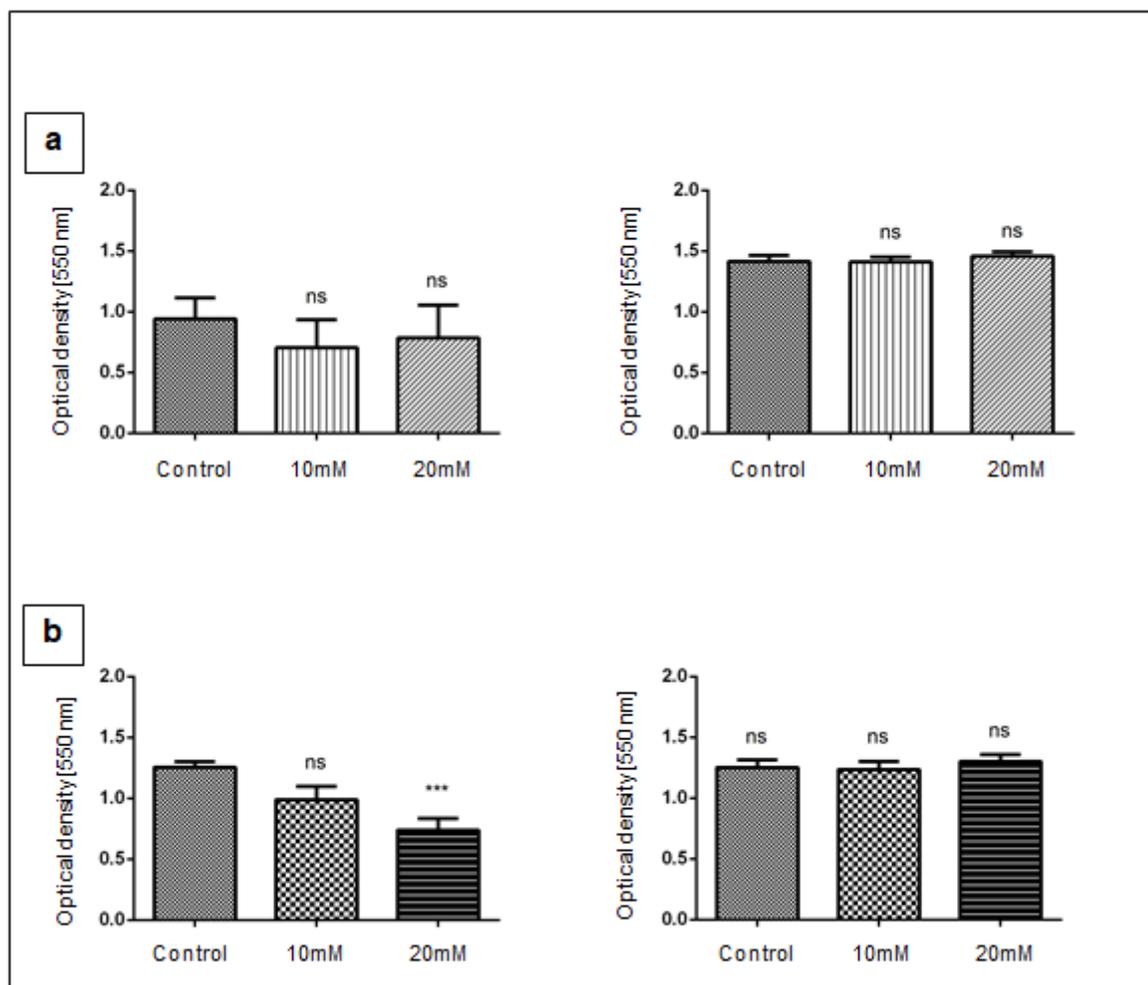


Figure 1

Glial viability detected by MTT assay a) The glial viability was significantly reduced after incubation with 20 mM NH₄Cl for 6 h in M30 co-cultures, representing „pathological“ conditions. This effect could not be detected following NH₄Cl incubation for 24 h. b) In M5 co-cultures, representing „physiological“ conditions, the glial viability was not altered by hyperammonemia. Data were collected by at least eight independent experiments and tested with One-way ANOVA and Kruskal-Wallis with Dunn’s post-hoc test, *p < 0.05, **p < 0.01, ***p < 0.001.

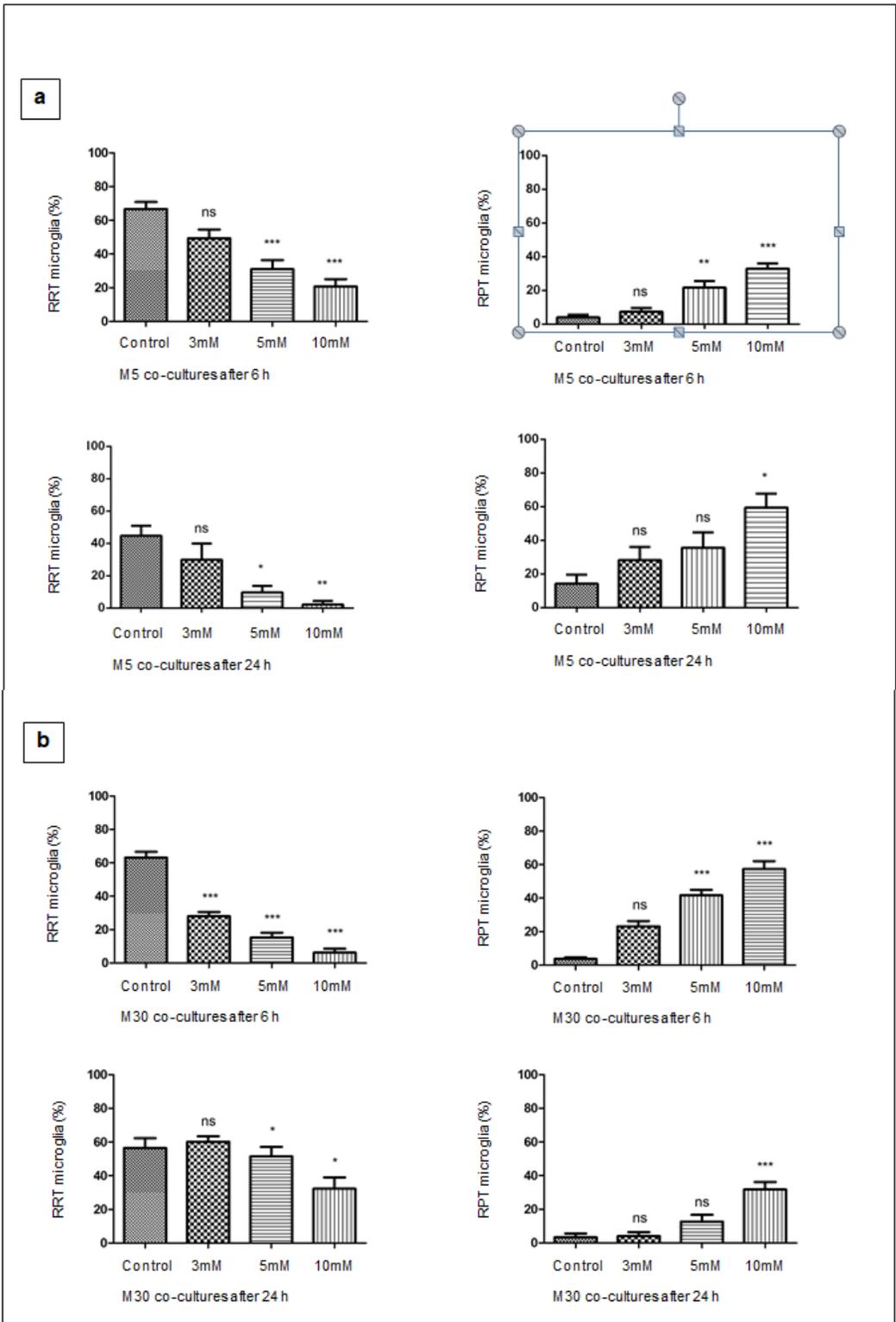


Figure 2

Immunocytochemical analyses of microglial phenotypes under hyperammonemia a) A significant and dose-dependent increase of activated, rounded phagocytic type of microglia (RPT) was observed in M5 co-cultures by immunocytochemistry after incubation with 3 mM, 5 mM and 10 mM NH₄Cl for 6 and 24 h. In parallel, the amount of resting ramified type of microglia (RRT) decreased significantly and dose-dependent. b) Similar results were obtained in M30 co-cultures. Data were collected by at least six

independent experiments and tested with One-way ANOVA and Kruskal-Wallis with Dunn's post-hoc test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

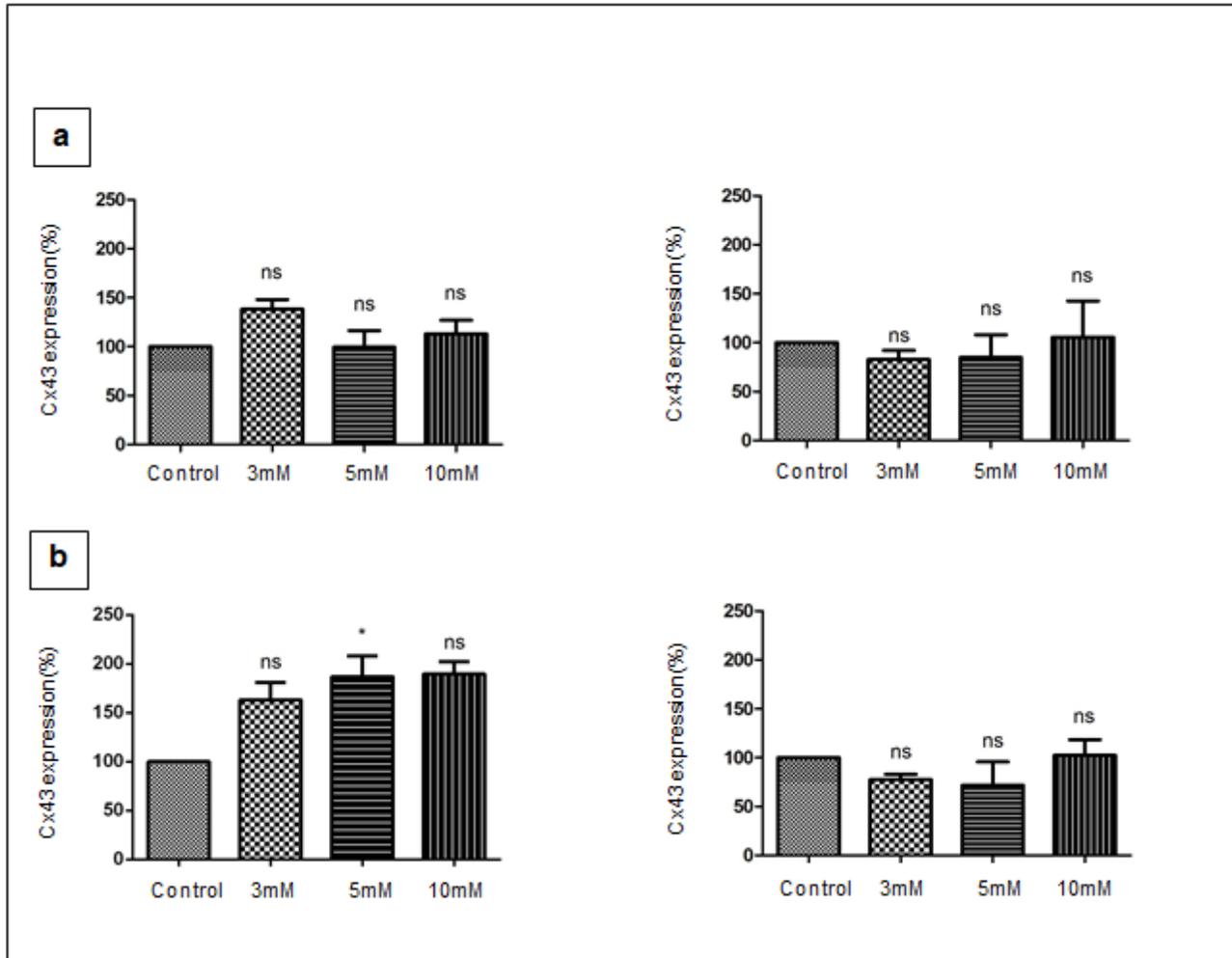


Figure 3

Cx43 expression under hyperammonemia detected by western blot analysis a) The Cx43 expression in M5 co-cultures was not altered significantly after dose-dependent incubation with NH₄Cl for 6 or 24 h. b) In M30 co-cultures, the Cx43 expression was significantly increased after 6 h incubation with 5 mM NH₄Cl and weakly, but not significantly increased after incubation with 10 mM NH₄Cl. Incubation for 24 h with NH₄Cl did not change the Cx43 expression in the astrocyte-microglia co-cultures. Data were collected by at least three independent experiments and tested with One-way ANOVA and Kruskal-Wallis with Dunn's post-hoc test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

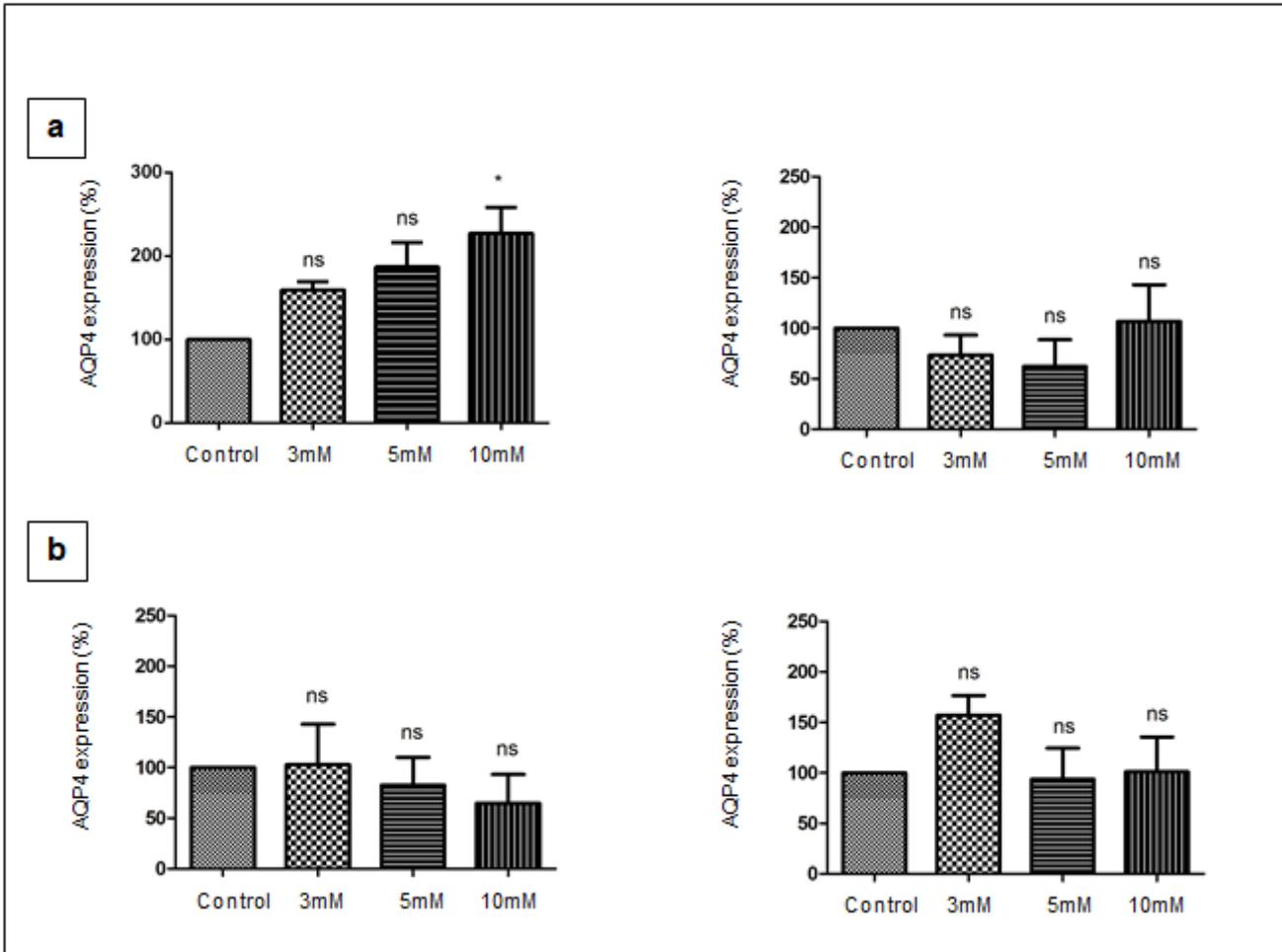


Figure 4

Western blot analysis of AQP4 expression under hyperammonemia a) The AQP4 expression was increased significantly in M5 astrocyte-microglia co-cultures treated with 10 mM NH₄Cl for 6 h. This effect was not shown after incubation with NH₄Cl for 24 h. b) In M30 co-cultures, AQP4 expression was not altered by ammonia. Data were collected by four independent experiments and tested with One-way ANOVA and Kruskal-Wallis with Dunn's post-hoc test, *p < 0.05, **p < 0.01, ***p < 0.001.

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