1. Introduction

Spinal cord injury (SCI) stands as a profoundly devastating neurologic condition, marked by severe dysfunction, paralysis, and even death [1-3]. The aftermath of trauma triggers the activation of microglia in situ, initiating a cascading neuroinflammatory response. This response, in turn, sets off the innate immune system, recognizing exogenous neurotoxic substances and pro-inflammatory stimuli [3,4]. The consequence is an accumulation of excessively activated microglia in the vicinity of the injury focus, releasing massive pro-inflammatory cytokines that induce further neuronal damage [4]. Furthermore, the mediators produced by these activated microglia exert additional stimulation on astrocytes, leading to their accumulation around the injury epicenter [5].

Despite the partial protective effect of the glial barrier against necrocytotoxins in the injury focus, neurogenesis and neurostructural remodeling are significantly impeded during the neurologic repair stage [6]. Clinically, methylprednisolone has emerged as a primary treatment against neuroinflammation, offering a crucial means to limit secondary damage, alongside surgical interventions [7]. However, its effectiveness in rescuing neurologic loss remains limited. Consequently, there is an urgent need to explore novel and effective therapeutic strategies to address the pervasive issue of SCI-induced neuroinflammation.

Dihydrotestosterone (DHT), a metabolite of testosterone, has shown notable neuroprotective effects in preclinical settings, including conditions such as Alzheimer's disease (AD) [8], Parkinson’s disease (PD) [9], and multiple sclerosis (MS) [10]. These effects are attributed to its anti-apoptotic and antioxidative stress properties. Despite these promising findings, few studies have delved into the specific role of DHT in neuroinflammation. While the anti-inflammatory effects of androgens in peripheral inflammatory diseases have been documented in both animal and clinical studies [11], the impact of DHT on neuroinflammation in the context of SCI and the central nervous system remains elusive.

In light of these considerations, there is a pressing need to investigate the effects and underlying mechanisms of DHT on neural tissue post-SCI. Previous studies have indicated the protective role of gonadal hormones, such as estradiol and DHT, in safeguarding spinal motoneurons following SCI [12,13]. Notably, androgens have been reported to rescue avian embryonic lumbar spinal motoneurons from injury-induced cell death [14]. However, whether DHT administration can effectively reduce neuron...
Dihydrotestosterone reduces neuroinflammation in SCI

2. Materials and Methods

2.1. Cell culture and treatment

The BV2 microglia line and was obtained from the Hubei University of Medicine. Cells were cultured in 5×5 cm² flasks containing 5 mL dulbecco's modified eagle medium (DMEM, Gbico, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS, Gbico, Rockville, MD, USA) and 100 U/mL penicillin-streptomycin (Gbico, Rockville, MD, USA). For cell treatment, DHT (MedChemExpress, Shanghai, China) was dissolved in 0.1% dimethyl sulfoxide (DMSO, sigma, St. Louis, MO, USA). Then the microglia medium was added to 10 nM DHT for 30 min. Lipopolysaccharide (LPS, 1 μg/mL Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 0.1% dimethyl sulfoxide (DMSO, sigma, St. Louis, MO, USA) and 100 U/mL penicillin-streptomycin (Gbico, Rockville, MD, USA). For cell treatment, DHT (MedChemExpress, Shanghai, China) was dissolved in 0.1% dimethyl sulfoxide (DMSO, sigma, St. Louis, MO, USA) and added to microglia medium for 18 h.

2.2. Animals and grouping

Adult male C57/B6J mice, aged 6-8 weeks (20-22 g), were housed in Hubei University of Medicine. Standard laboratory conditions, such as 12 h/12 h light/dark cycle, available food and water, 50% humidity as well as 22 ± 1°C temperature, were provided. Mice were randomly assigned to three different experimental groups. The Sham group (Sham), SCI group, and SCI+DHT group (n=9) were established in the study. The study was approved by the animal ethical committee of Hubei University of Medicine.

2.3. Spinal cord injury

The modeling method was described as follows: mice were anesthetized intraperitoneally using xylazine (5 mg/kg) and ketamine (95 mg/kg) by normal saline, then we conducted laminectomy and impacted the 10th spinal cord (100 kg) and ketamine (95 mg/kg) by normal saline, then we were anesthetized intraperitoneally using xylazine (5 mg/kg) and ketamine (95 mg/kg). Mice received a 2.5 mm transverse laminectomy at the T10 level. A 1 mm-wide, 10 mm-deep defect was created in the T10 spinal cord. The injured area was repaired using a one-way or two-way ANOVA. Data were collected and analyzed using Statistical Product and Service Solutions (SPSS) 18.0 software (SPSS Inc., Chicago, IL, USA). P<0.05 means statistical significance.

2.4. Enzyme-linked immunosorbent assay (ELISA)

Microglia medium and tissue homogenate were collected. We centrifuged them for 5 min and isolated the supernatant. ELISA of pro-inflammatory factors was conducted using an ELISA Kit (KeyGen, Nanjing, China) according to manufacturer’s instructions. The absorbance (OD value) of each well was measured at 450 nm using a spectrophotometer.

2.5. Western Blotting

Protein was extracted from cells using a Total Protein Extraction Kit (KeyGen, Nanjing, China) directed by the manufacturer’s protocols. Concentration determination was performed with a Bicinchoninic Acid (BCA) Assay Kit (Thermo Scientific, Waltham, MA, USA). Then equivalent protein was performed electrophoresis using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer and immuno-blocking. Incubation of primary and secondary antibodies (iNOS (1:250, Abcam, Cambridge, MA, USA), COX-2 (1:1000, Abcam, Cambridge, MA, USA), p-p65 (1:1000, Abcam, Cambridge, MA, USA), p-p65 (1:1000, Abcam, Cambridge, MA, USA), p-p65 (1:1000, Abcam, Cambridge, MA, USA), and Goat Anti-Rabbit IgG H&L (HRP) (1:2000, Abcam, Cambridge, MA, USA)), protein was exposed using an enhanced chemiluminescence (ECL) system (Tanon, China) and quantified using ImageJ software (USA).

2.6. Immunofluorescence (IF) and immunohistochemical staining (IHC)

Cord tissues were collected in the study. The study was approved by the animal ethical committee of Hubei University of Medicine. Standard laboratory conditions, such as 12 h/12 h light/dark cycle, available food and water, 50% humidity as well as 22 ± 1°C temperature, were provided. Mice were randomly assigned to three different experimental groups. The Sham group (Sham), SCI group, and SCI+DHT group (n=9) were established in the study. The study was approved by the animal ethical committee of Hubei University of Medicine.

2.6. Immunofluorescence (IF) and immunohistochemical staining (IHC)

Cord tissues were collected after mouse sacrifice. Cells and tissues were fixated with 4% paraformaldehyde (PFA) for 24 h. Following dehydration by ethanol and permeation by xylene, tissue was embedded into paraffin and cut into 6 μm sections using a rotary microtome. Roasted for 48 h, sections were deparaffinized, hydrated and antigen repaired. For IF, Sections and cells were incubated with iNOS (1:100, Abcam, Cambridge, MA, USA), IBA-1 (1:300, Abcam, Cambridge, MA, USA), TNF-α (1:200, Abcam, Cambridge, MA, USA), IL-1β (1:200, Abcam, Cambridge, MA, USA), GFAP (1:200, Abcam, Cambridge, MA, USA) and fibronectin (1:200, Abcam, Cambridge, MA, USA) overnight at 4°C. For IF, sections were incubated with Alexa Fluor® 488 or 594 (1:200, Abcam, Cambridge, MA, USA) for 1 h. The nucleus was stained with diamidine phenylindole (DAPI, Mounting Medium With DAPI-Aqueous, Fluoroshield, Abcam, Cambridge, MA, USA), protein was exposed using an enhanced chemiluminescence (ECL) system (Tanon, China) and quantified using ImageJ software (USA).

2.7. Behavioral assessment

Mice in each group were allowed to free movement in an open field for 4 min at 1 days, 3 days, 7 days, 14 days, and 28 days post SCI. Two researchers scored sports situations according to the Basso Mouse Scale (BMS) in a blind way. Then data were collected and analyzed in statistics.

2.8. Statistical analysis

Data were displayed as the means ± SD. The difference in statistics between the two groups was assessed using Student’s t-test. Difference among more than two groups was evaluated via one-way or two-way ANOVA. Data were collected and analyzed using Statistical Product and Service Solutions (SPSS) 18.0 software (SPSS Inc., Chicago, IL, USA). P<0.05 means statistical significance.
3. Results

3.1. DHT reduces LPS-induced inflammatory activation in BV2 microglia

To provoke an inflammatory response in microglia, we performed LPS administration for stimulation. Then the released pro-inflammatory cytokines in medium, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 were detected using ELISA, showing that the expressions of TNF-α, IL-1β and IL-6 were significantly increased following LPS treatment, whereas DHT reduced the levels of the above pro-inflammatory cytokines in LPS treated microglia medium (Figure 1A-1C). To verify inflammatory activation in BV2 microglia, the representative mediator inducible nitric oxide synthase (iNOS) and the microglial marker ionized calcium-binding adaptor molecule-1 (IBA-1) were visualized using IF, the Figure 1D showed that LPS administration significantly increased iNOS (Red) expressions in microglia (Green), however, DHT administration reduced iNOS expression in microglia. It was indicated that DHT decreased inflammatory microglia activation after LPS stimuli. Furthermore, the pro-inflammatory cytokines in cells including TNF-α and IL-1β were examined using IF, exhibiting that the levels of TNF-α (Red) and IL-1β (Green) elevated markedly post LPS utilization while synthetic inflammatory cytokines reduced in microglia with DHT treatment (Figure 1E). Hence, it is proved that DHT could reduce LPS-induced inflammatory microglia activation.

![Fig. 1. DHT reduces LPS-induced inflammatory activation in BV2 microglia. (A-C) Representative ELISA of TNF-α, IL-1β and IL-6 in control, LPS and LPS+DHT treated microglia medium. (D) Representative IF of iNOS (red) and COX-2 (green) in control and LPS-treated microglia, (magnification: 100×). (E) Representative IF of TNF-α (red) and IL-1β (green) in control, LPS and LPS+DHT treated microglia, (magnification: 200×). “***” P<0.001, “**” P<0.01 and “*” P<0.05 vs. control group and “###” P<0.001, “##” P<0.01 and “#” P<0.05 means vs. LPS group with statistical significance.](image)

3.2. DHT treatment inhibits TLR4-mediated p65 and MAPK-p38 pathways in inflammatory microglia

Further, we investigated whether DHT inhibited toll-like receptor 4 relative signaling pathways to exert an anti-inflammation effect. Western blot exhibited increased expressions of phosphor-NF-κB p65 (p-p65) and phosphor-MAPK p38 (p-p38) in LPS-induced inflammatory microglia, whereas DHT treatment decreased p-p65 and p-p38 protein levels (Figure 2A-2C). We also detected the expressions of iNOS and cyclooxygenase-2 (COX-2), the protein levels of iNOS and COX-2 exhibited consistent expressions with p-p65 and p-p38 (Figure 2D-2F). The above results indicate that DHT attenuates inflammatory response in microglia via down-regulation of NF-κB and MAPK p38 pathways.

![Fig. 2. DHT treatment inhibits TLR4-mediated p65 and MAPK-p38 pathways in inflammatory microglia. (A) Representative western blotting including p-p65, p65, p-p38 and p38 in control, LPS and LPS+DHT treated microglia. (B-C) Quantification of the ratios of p-p65/p65 and p-p38/p38. (D) Representative western blotting including COX-2 and iNOS in control, LPS and LPS+DHT treated microglia. (E-F) Quantification of the protein levels of COX-2 and iNOS, “***” P<0.001, “**” P<0.01 and “*” P<0.05 vs. control group and “###” P<0.001, “##” P<0.01 and “#” P<0.05 means vs. LPS group with statistical significance.](image)

3.3. Administration of DHT attenuates systemic neuroinflammation after SCI

We next evaluated the possible therapeutic effect of DHT in SCI mice. Firstly, we measured the microglia activation via IF, founding massive inflammatory microglia neighboring the injured centre following SCI, but DHT treatment reduced activated microglia surrounding injured focus (Figure 3A). IHC staining showed increased p-p65 and p-p38 in injured site after SCI, however, DHT administration reduced p-p65 and p-p38 positive area in injured site at 3 days post-trauma (Figure 3B). ELISA showed that the expressions of TNF-α, IL-1β and IL-6 dramatically increased in injured tissue while treatment with DHT decreased the levels of the pro-inflammatory cytokines (Figure 3C-3E). Hence, we demonstrate that DHT reduces neuroinflammation in injured tissue via inhibition of NF-κB and MAPK p38 pathways.

3.4. Effect of DHT mitigates glial accumulation and fibrosis focus in injured site

Furthermore, we investigated the effect of DHT treatment on protection of neural repair and locomotor func-
neural injury [17]. Previous studies [18,19] have shown that androgens inhibited the progression of inflammatory responses by inhibition of macrophage activation. Therefore, DHT potentially plays an anti-inflammatory role in systemic diseases associated with neuroinflammation. Inflammatory microglia respond rapidly to LPS stimulation and thereby release inflammatory mediators such as NO, PGE2, TNF-α and IL-1β [8,20]. Androgen has been found to reduce the expression of inflammatory factors including TNF-α, IL-1β, and IL-6 in peripheral neuroinflammatory diseases as an alternative therapy. Here, we found that DHT inhibited the activation of pro-inflammatory microglia and reduced proinflammatory cytokines including iNOS, COX-2, TNF-α, IL-1β and IL-6, and the reduction of these inflammatory mediators is also regulated via DHT in injured spinal cord. These findings are consistent with previous results that androgen therapy reduces TNF-α and IL-1β [8,21]. As adverse effects after neuroinflammation, glial chemotaxis and long-term stimulation of chronic inflammation mediate the formation of glial scar and the occurrence of fibrosis in lesion after SCI. Previous studies have exhibited that glial hyperplasia and alternative fiber repair could hinder the recovery of neurogenic function, while the effective reduction of glial scar area and the pathogenesis of fibrosis promoted the recovery of neural tissue [22-24]. We discovered that DHT also reduced aggregation of glial cells and fibronectin levels at the last phase of SCI, which may be related to the negative regulation of neuroinflammation in the early phase. In terms of the mechanism by which DHT downregulates inflammation, previous studies have demonstrated that TLR4 expression is significantly reduced in macrophages and endothelial cells treated with androgens [25]. Moreover, androgen supplementation inhibited increased expression of TLR4 in casparted animals [26]. Therefore, we hypothesized that DHT might inhibit the pathogenesis of neuroinflammation in microglia cells through TLR4-related signaling pathways. TLR4 mediates inflammation through downstream signa-

4. Discussion

Neuroinflammation is one of the important physiologi- nal recovery. IF staining showed that SCI resulted in accumulation of excessive microglia (IBA-1 positive) and astrocytes (GFAP positive) around the epicenter at 28 days post-trauma, whereas administration of DHT reduced the area of glial scar in neurologic tissue (Figure 4A). Then we further measured the fibrosis degree in injured site using IF, showing that the positive region of fibronectin in injured focus significantly decreased in injured site after DHT treatment at 28 days post SCI (Figure 4B). Moreover, we evaluated BMS scores in each group at 1 day, 3 days, 7 days, 14 days and 28 days, respectively. The BMS scores in the Sham group were at 9 points during 4 weeks, scores in other groups were at 0 points on 1 day after trauma, the scores in the SCI+DHT group were significantly higher than those in the SCI group and SCI+Mel+EX527 group beginning at 7 days post-SCI and continued to 28 days (Figure 4C). Hence, the results proved that administration of DHT protects neural tissue from gliosis and fibrosis, improving locomotor functional recovery after SCI.

Fig. 3. Administration of DHT attenuates systemic neuroinflammation after SCI. (A) Representative IF of IBA-1 (green) in Sham, SCI and SCI+DHT at 3 days post-SCI, (magnification: 200×). (B) Representative IHC of p-p65 and p-p38 in Sham, SCI and SCI+DHT at 3 days post-SCI, (magnification: 400×). (C-E) Representative ELISA of TNF-α, IL-1β and IL-6 in Sham, SCI and SCI+DHT group at 3 days post-SCI. (E) Quantification of Sirt-1 protein level. ‘###’ P<0.001, ‘##’ P<0.01 and ‘*’ P<0.05 vs. control group and ‘##’ P<0.01 and ‘*’ P<0.05 means vs. LPS group with statistical significance.

Fig. 4. Effect of DHT mitigates glial accumulation and fibrosis focus in injured site. (A) Representative IF of IBA-1 (green) and GFAP (red) in Sham, SCI, SCI+DHT group at 28 days post-SCI, (magnification: 200×). (B) Representative IF of fibronectin in Sham, SCI, SCI+DHT group at 28 days post SCI, (magnification: 200×). (C) Representative BMS scores in Sham, SCI, SCI+DHT group at 1, 3, 7, 14, and 28 days post-SCI. ‘###’ P<0.001, ‘##’ P<0.01 and ‘*’ P<0.05 means vs. LPS group with statistical significance.
ling pathways NF-κB and MAPKs [27]. Consistent with previous findings, DHT inhibited phosphorylation of NF-κB pathway and subsequent inflammatory responses. However, we further found that DHT inhibited phosphorylation of the TLR4-independent NF-κB p65 and MAPK p38 pathways in LPS-treated microglia and mouse spinal cord after SCI. Meanwhile, multiple studies have shown that DHT inhibits the expression of several MAPKs pathways, such as JNK, p38 and ERK signaling, in vitro and in vivo. In the current study, we verified the poor locomotor function in mice following trauma, however, treatment with DHT improved the recovery of locomotor function in SCI mice. Earlier studies likewise showed the neuroprotective role of DHT in LPS-induced acute encephalitis, in which cognitive impairment was ameliorated via DHT [28]. However, the mechanism concerning DHT reducing glial scar and fibrosis is still unknown and needs to be investigated in further studies. In summary, DHT treatment reduces post-SCI neuroinflammation through TLR4-mediated NF-κB and MAPK-P38 signaling pathways. The results implicate that the neuroprotection of DHT in SCI model improves behavioral function and DHT administration may be a selection of adjuvant therapy or drug combination to acute neuroinflammation in early phase of SCI.

5. Conclusion
The present research certifies that DHT reduces inflammation response in LPS-induced microglia and SCI mice, which inhibits NF-κB and MAPK-P38 signaling pathways. The neurohistology and behavioral recovery are thereby improved following DHT administration in SCI.

Conflict of Interests
The author has no conflicts with any step of the article preparation.

Consent for publications
The author read and approved the final manuscript for publication.

Ethics approval and consent to participate
The study was approved by the animal ethical committee of Hubei University of Medicine Animal Center.

Informed Consent
The authors declare not used any patients in this research.

Availability of data and material
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions
Jiarui Wei and Tao Li designed the study and performed the experiments, Shengyuan Lin and Bin Zhang collected the data, Shengyuan Lin, Bin Zhang and Xing Li analyzed the data, Jiarui Wei and Tao Li prepared the manuscript. All authors read and approved the final manuscript.

Funding
Non.

References

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