

“Shikonin inhibits microglia activation and reduces CFA-induced mechanical hyperalgesia in an animal model of pain”[☆]

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ARTICLE INFO

Keywords:

Inflammation

Mechanical thresholds

Microglia

Shikonin

ABSTRACT

Shikonin is an ointment produced from *Lithospermum erythrorhizon* which has been used in traditional medicine both in Europe and Asia for wound healing and is associated with anti-inflammatory properties. The goal of this work is to assess the analgesic properties of Shikonin in the CFA-induced inflammation model of pain. Rats were subjected to inflammation of the hind paw by CFA injection with a preventive injection of Shikonin and compared to either a control group or to a CFA-inflamed group with the vehicle drug solution. Inflammation of the hind paw by CFA was assessed by measurement of the dorsal to plantar diameter. Mechanical thresholds were established by means of the Von Frey filaments which are calibrated filaments that exert a defined force. Finally, the spinal cord of the studied animals was extracted to analyse the microglia population through immunohistochemistry using the specific marker Iba-1. Our results show that Shikonin reduces the paw oedema caused by CFA inflammation. Subsequently, there is a concomitant restoration of the mechanical thresholds reduced by CFA hind paw injection. Additionally, spinal microglia is activated after CFA-induced inflammation. Our results show that microglia is inhibited by Shikonin and has concomitant restoration of the mechanical thresholds. Our findings demonstrate for the first time that Shikonin inhibits microglia morphological changes and thereby ameliorates pain-like behaviour elicited by mechanical stimulation.

1. Introduction

Pain is a physiological process that informs us of real or potential harm. Higher organisms have developed the nociceptive pathway by which we can perceive pain attributes (e.g. location, type, intensity) which is part of the normal functioning of the body [1]. When the perception of pain persists over time and loses its capacity for sensory functional information is defined as pathological pain [2]. Indeed, pain pathologies with a prevalence of 20% (NIH reports 40 million patients suffering severe pain) are characterized by a long duration (e.g. chronic), have a deterioration in the quality of life and represents a significant economic effort on society [3–6].

One of the most widespread forms of chronic pain is chronic pain secondary to inflammatory processes (e.g. rheumatoid arthritis) [7]. The inflammatory response normally arises in response to destroy possible

pathogens, tissue or organ repair and is mediated by the release of proinflammatory cytokines [8]. The counterpoint of cytokine production during inflammation is that, in addition, they promote sensitization of neurons. As a consequence, sensitized neurons promote hyperalgesia and allodynia [9]. Although the acute and chronic inflammatory processes are at the base of the pain development and maintenance have been widely studied in human and experimental models [10], an effective treatment has not been determined yet.

In addition to increased neuronal activity, other cell types are activated during inflammatory processes. Particularly, the release of proinflammatory induces the activation of microglia which passes from a resting to a reactive state (also known as M1 microglia) [11]. This reactive M1 microglia is well known to trigger pain behaviour both in inflammation and neuropathies [12,13]. Thus, the effectiveness of microglia inhibition by compounds such as minocycline has been

[☆] Sponsor: Universidad Europea de Madrid (Grant number: 2020/UEM38).

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<https://doi.org/10.1016/j.bioph.2022.112961>

Received 3 February 2022; Received in revised form 30 March 2022; Accepted 11 April 2022

Available online 19 April 2022

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demonstrated to reduce pain behaviours in inflammatory processes [14]. However, there is not an effective treatment to block microglia reactivity which makes the necessity to research new therapeutic targets that are effective in the treatment of chronic pain.

Lithospermum erythrorhizon and *Alkanna tinctoria* are traditional plants which belong to the *Boraginaceae* family. They have been used in tinctures for colouring cloths, cosmetics and in traditional medicine both in Europe and Asia [15]. These root extracts have been largely used as a medical remedy for the treatment of ulcers and described for the first time by Hippocrates in the IV-V centuries BC [16]. The ointments produced from their roots contain the chiral isomers of Shikonin (R-configuration, *Lithospermum erythrorhizon*) and Alkannin (S-configuration, *Alkanna tinctoria*) which derive from the red dye naphthoquinone have been successfully isolated [17].

Recent studies have shown that Shikonin has antimicrobial properties [18], antiapoptotic activity [19], antitumoral effects [20] and is also used as a general ointment for wound repair [16]. Interestingly, Shikonin has a dose dependent anti-inflammatory capacity that reduces the hind paw oedema caused by exposure to the Complete Freund's adjuvant (CFA) inflammatory reagent [21]. Moreover, a recent study by Gupta et al. [22] shows the potential role for Shikonin in analgesia through the blockade of the NaV1.7 sodium channel in an experimental model of CFA-induced mechanical hyperalgesia [22].

The antecedents described above, the wide distribution of both *Lithospermum erythrorhizon* and *Alkanna tinctoria* plants, the ease of obtaining its isolated active principles and their analgesic and anti-inflammatory effects, led us to consider the Shikonin as a promising drug for the treatment of inflammatory pain. The main goal of this work is to further investigate the mechanisms of action responsible for the effect of Shikonin in the experimental model of CFA-induced mechanical hyperalgesia. Our findings demonstrate for the first time that Shikonin inhibits microglia morphological changes and thereby reduces mechanical hyperalgesia.

2. Materials and methods

2.1. Animals

Animals, 6 months old 400–500 g male Sprague Dawley rats, were breed and maintained on a 12-h-light/dark cycle at constant ambient temperature with free access to food and water until the experiment procedures at the animal facility of the *Hospital Universitario de Getafe*. Experiments took place during the light phase. Behavioural tests and animal care were conducted in accordance with the standard ethical guidelines (European Communities Directive 86/609 EEC; National Institutes of Health 1995) and approved by the local ethical committee (*Comunidad de Madrid, Spain*).

2.2. Experimental procedure

Animals were taken into the behavioural laboratory, left to accommodate for 30 min in a mesh grid behavioural box, tested for baseline behaviour and randomly assigned to the following experimental groups: (1) Control group (n = 6), (2) hind paw CFA injection and vehicle administration (CFA+veh) group (n = 6) and (3) hind paw CFA injection and preventive Shikonin administration (CFA+Shik) (n = 6) group. Shikonin (Sigma-Aldrich, Madrid, Spain) was administered intraperitoneally at a concentration of 150 µmol/Kg of body weight in phosphate buffered saline (PBS) with a Hamilton syringe and a 25-G needle. Intraperitoneal injection of PBS alone was performed as vehicle. After 20 min of resting to allow the maximum pharmacological benefit of the Shikonin, peripheral inflammation was induced by unilateral intraplantar injection at the left hind paw with 50 µL of CFA (Sigma-Aldrich, Madrid, Spain) with a 1-mL syringe and a 25-G needle [14]. To avoid animal distress at the time of the inflammation induction, the CFA inflammatory agent was administered under 4% isoflurane anaesthesia.

After CFA intraplantar injection, animals were returned to their behavioural box until further experimentation 1 h later. Control animals remained untreated.

2.3. Hind paw inflammation testing

The oedema caused by inflammation was estimated by measuring the dorsal to plantar diameter of the hind paw at the metatarsus area as previously reported [14]. Measurements (in millimetres) were taken at baseline (referred as PRE groups) and then 1 h post hind paw inflammation induction (referred as POST groups). The oedema was also calculated as the ratio (in percentage) between the PRE and POST groups in order to compare the reduction of paw oedema after Shikonin treatment.

2.4. Behavioural testing

Rats were tested for cutaneous mechanical sensitivity before and after 1 h post hind paw inflammation by a blind observer. The calibrated von Frey (VF) filaments (Stoelting, Woodvale, IL, USA) were used to analyse flexion withdrawal reflexes (i.e. mechanical thresholds) in response to punctuate mechanical stimulation of the dorsal surface of the hind paw [14]. Measurements were taken at baseline (referred as PRE groups) and then 1 h post hind paw inflammation induction (referred as POST groups). The mechanical thresholds were also calculated as the ratio (in percentage) between the PRE and POST groups in order to compare the changes in mechanical thresholds after Shikonin treatment.

2.5. Immunostaining

The tissues from the rats used in the inflammation and behavioural tests were collected for immunohistochemistry analyses. Thus, rats were overdosed with 1 mL of Euthanal (0.3 g/mL). The lumbar spinal cord was removed and fixed in 4% paraformaldehyde for 24 h at 4 °C and preserved in 30% sucrose in PBS at 4 °C. Microglia was detected using the specific marker ionized calcium binding adaptor molecule 1 (Iba-1) [14]. Briefly 50-µm-thick cryostat sections were cut and stained using a primary antibody anti-Iba-1 (1/1000; Wako Chemicals USA, Inc., Richmond, VA, USA) coupled with a fluorescent secondary antibody (1/250, Alexa-593, Molecular Probes, Invitrogen, Madrid, Spain). Images were acquired with Leica system with the 10X magnification lens at constant exposure.

The images generated by immunohistochemistry were analysed with ImageJ 1.46j (NHS) software [23]. Two parameters were analysed: (1) Spinal microglia Iba-1 positive cells were counted in the dorsal horn spinal cord ipsilateral to the injection site and (2) the area of microglia cell bodies (n = 6 rats with 4–6 tissue sections per animal).

2.6. Statistical analysis

Data was analysed by using a ONE-WAY ANOVA. All pair wise comparison post-hoc comparisons (Student-Newman-Keuls Method, SNK) were performed in case of significant interaction between factors. The percentage of oedema reduction and the percentages of mechanical thresholds changes produced between the CFA+veh and CFA+Shik group were analysed by t-test. Statistical analyses were performed by the SPSS 19.0 software package (SPSS Inc., Chicago, IL, USA). All results are expressed as the mean average ± the standard error of the mean (SEM).

3. Results

3.1. Effect of Shikonin on paw oedema after CFA treatment

Intraplantar injection of CFA showed an increase of paw oedema 1 h after treatment in the CFA+veh POST group (8.4 mm ± 0.3) compared

to both the Control group ($6.2 \text{ mm} \pm 0.2$) and the CFA+veh PRE group ($6.5 \text{ mm} \pm 0.1$, ANOVA $p < 0.001$, post hoc SNK $p < 0.05$, $F = 14.636$, $DF = 4$, $n = 6$ rats per experimental group). Shikonin partially but significantly reduced the hind paw oedema effect produced by the CFA inflammation and the paw oedema was reduced to values of $7.3 \text{ mm} \pm 0.3$ in the CFA+Shik POST group (Fig. 1A, ANOVA $p < 0.001$, post hoc SNK $p < 0.05$, $F = 14.636$, $DF = 4$, $n = 6$ rats per experimental group). Compared to the control group, the CFA+Shik POST group it only shows an increase of the hind paw oedema by $16.4\% \pm 4.3$ while the CFA+veh POST group has an oedema increase by $30.6\% \pm 4.6$. Thus, Shikonin provokes a significant inhibition (approximately a 53% reduction) of the hind paw oedema (t-test $p < 0.05$, $t = 2.192$, $DF = 20$, $n = 6$ rats per experimental group, Fig. 1B).

Interestingly, no differences were found when comparing the Shikonin treatment with the control group (CFA+Shik POST versus control)

or its baseline (CFA+Shik POST versus CFA+Shik PRE).

3.2. Effect of Shikonin on mechanical withdrawal thresholds after CFA inflammatory treatment

Intraplantar injection of CFA significantly decreases the mechanical thresholds 1 h after treatment in the CFA+veh POST group ($34.1 \text{ g} \pm 9.6$) compared to both the control group ($140 \text{ g} \pm 15.1$) and the CFA+veh PRE group ($133.8 \text{ g} \pm 12.7$, ANOVA $p < 0.001$, post hoc SNK $p < 0.05$, $F = 16.445$, $DF = 4$, $n = 6$ rats per experimental group). Shikonin treatment restores the mechanical thresholds by CFA injection and the mechanical threshold of the CFA-Shik POST group was only of $86.2 \text{ g} \pm 16.6$ which was significant higher than that of the CFA+veh POST group but lower compared to the control group and the CFA-Shik PRE group ($153.3 \text{ g} \pm 13.3$, ANOVA $p < 0.001$, post hoc SNK $p < 0.05$,

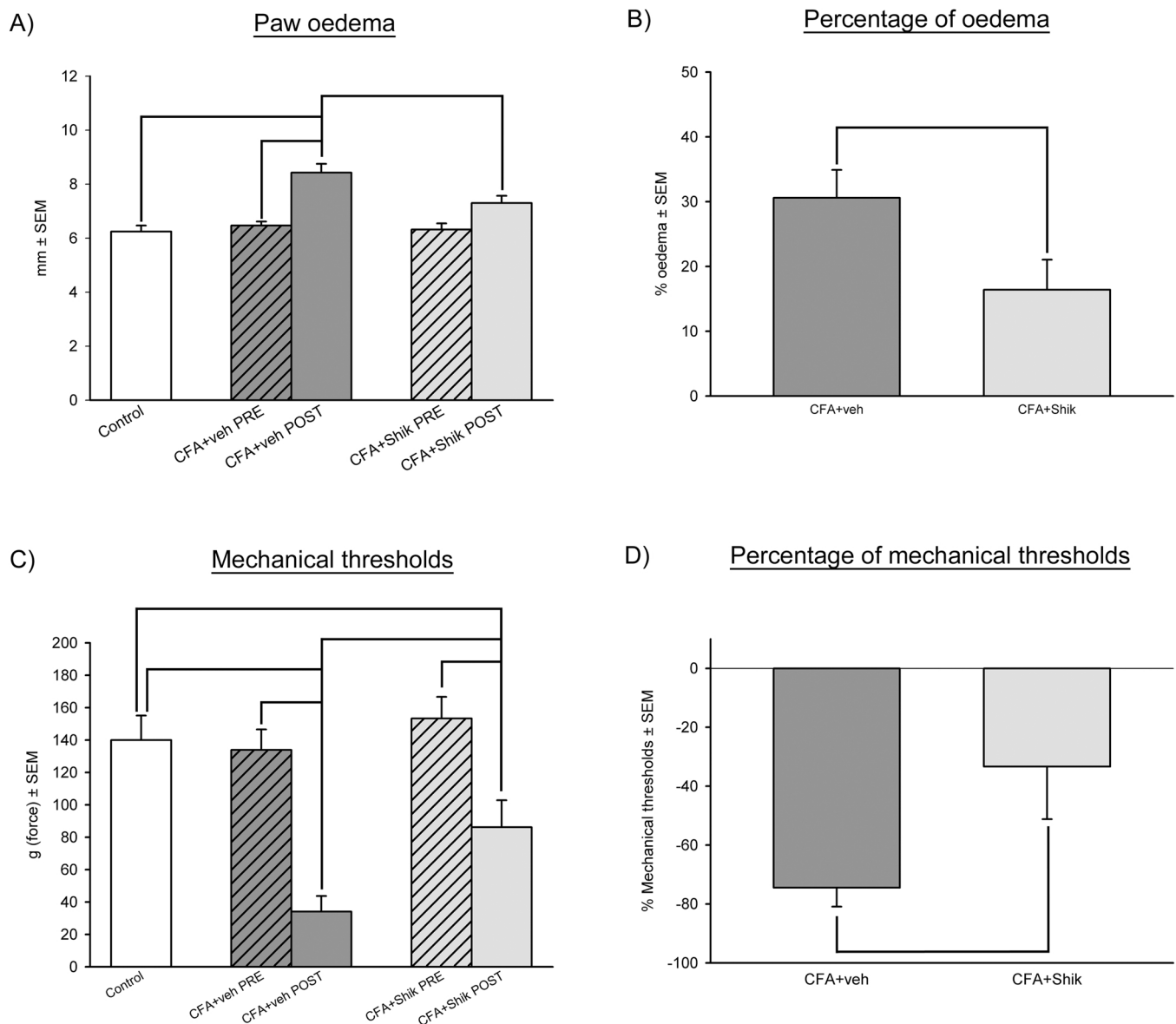


Fig. 1. Effect of CFA inflammation and preventive Shikonin treatment on hind paw oedema and mechanical thresholds. A) Intraplantar injection of CFA induces hind paw inflammation (CFA+Veh Post) which is reduced by preventive treatment with Shikonin (CFA+Shik Post). B) The percentage of paw oedema increase is observed in both CFA+Veh and CFA+Shik groups but significantly reduced in the CFA+Shik group. The 0% indicates the normal size of the hind paw. C) Intraplantar injection CFA caused a significant decrease of the mechanical thresholds compared to the Control group and baseline while preventive Shikonin administration partially reversed the paw oedema. D) The massive reduction in mechanical thresholds in the CFA+veh group is partially restored after Shikonin preventive treatment (CFA+Shik group). The 0% indicates the normal mechanical thresholds. The lines indicate a significant difference ANOVA $p < 0.001$, post hoc SNK test $p < 0.05$ (A and C) or t-test $p < 0.05$ (B and D) among the experimental groups ($n = 6$ rats per experimental group).

Fig. 1C). Interestingly, the CFA-Shik group has a reduction of the mechanical thresholds by $33.3\% \pm 17.8$ which is significantly lower compared to the CFA+veh group (reduction of $74.4\% \pm 6.4$) that indicates a significant reversal of the mechanical thresholds by approximately 47.8% (t-test $p < 0.05$, $t = -2.469$, $DF = 20$, $n = 6$ rats experimental groups, Fig. 1D).

3.3. Spinal microglia activation after CFA-induced peripheral inflammation

Morphometric analysis of microglia was performed at the spinal cord dorsal horn. Representative immunohistochemical staining using the microglia specific marker Iba-1 is shown in Fig. 2A. Quantitative analyses of microglia cells shows no significant increase in microglia cell numbers (Fig. 2B) after CFA intraplantar induced inflammation (CFA+veh group; $179.9 \text{ cells} \pm 9.71$) when compared to the control group ($192.2 \text{ cells} \pm 10.64$) or with the preventive Shikonin treatment (CFA+Shik group; $192.9 \text{ cells} \pm 5.3$; $p = 0.579$, $F = 0.552$, $DF = 2$ and

$n = 6$ rats with 4–6 tissue sections per animal). However, we have observed that the CFA+veh group produces a significant change in cell morphology. Thus, there is an increase of about 39% of the microglia cell area 1 h after CFA intraplantar induced inflammation (CFA+veh group; $458.1 \mu\text{m}^2 \pm 5.1$) compared to the control group ($318.5 \mu\text{m}^2 \pm 3.0$, ANOVA, $p < 0.001$, post hoc SNK test $p < 0.05$, $F = 359.7$, $DF = 2$ and $n = 6$ rats with 4–6 tissue sections per animal). Interestingly, the preventive treatment with Shikonin (CFA+Shik group) had a remarkable inhibitory effect over microglia cell area ($318.7 \mu\text{m}^2 \pm 2.65$) which was comparable to that of the control levels and no significant differences were found between them (Fig. 2C).

4. Discussion

In the present work we have shown the potential role of Shikonin as analgesic drug which inhibits the microglia morphological changes in an inflammatory pain animal model. Thus, the present work shows that preventive Shikonin administration reduces the mechanical

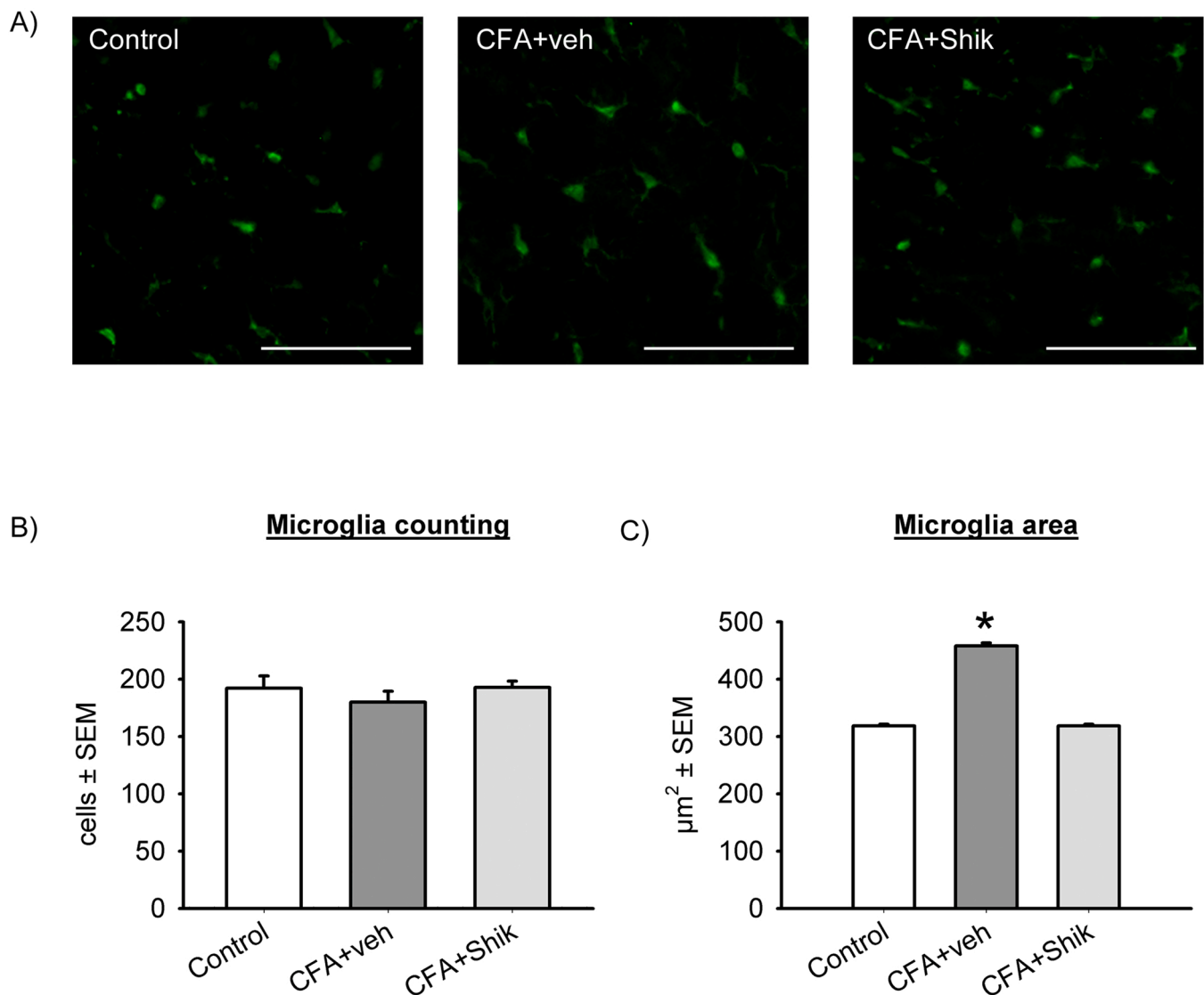


Fig. 2. Microglia reaction to CFA inflammation and Shikonin treatment. A) Microglia staining with microglia marker Iba-1 from Control, CFA+veh and CFA+Shik groups in L3–L5 dorsal horn of the spinal cord at 1 h post-CFA injection (scale bar: 100 μm). The microglia from both the control and the CFA+Shik groups are smaller in size and with less process compared to the CFA+Veh group. B) Microglia cell number did not experience significant changes with any treatment compared to the control group at 1 h post-inflammation ($p = 0.579$, $n = 6$ rats, and 4–6 tissue sections per animal). C) Microglia cell size (measured the area of the cell body and the processes) shows a significant increase in microglia cell area the CFA+Veh but not in CFA+Shik groups 1 h post-inflammation. *: Indicates a significant difference ANOVA $p < 0.001$, post hoc SNK test $p < 0.05$ ($n = 6$ rats, and 4–6 tissue sections per animal).

hyperalgesia caused by the unilateral intraplantar injection of CFA. Additionally, animals pre-treated with Shikonin showed smaller oedema formation and higher mechanical thresholds in the paw injected with CFA than the animals without Shikonin treatment. We have also observed that CFA treatment induced spinal microglia activation which was strongly reversed by the Shikonin preventive treatment.

4.1. Shikonin reduces oedema and ameliorates mechanical hyperalgesia

Shikonin has a general role to ameliorate inflammation and to reduce the production of proinflammatory cytokines (e.g. TNF- α and IL1- β) in several models of inflammatory models such as ulcerative colitis, autoimmune encephalomyelitis and osteoarthritis [19,24,25]. It is no wonder that all these experimental models have been associated with pain [26] but the role of Shikonin to reduce the pain behaviours such as mechanical hyperalgesia is yet to be explored.

It is of particular interest the hind paw inflammatory pain model as it has been largely used to study inflammatory pain [9,14]. As a feature of the hind paw inflammatory pain model swollen hind paw have been observed elsewhere [26]. Thus, our results show a robust inflammation after CFA injection. The intraperitoneal injection of 150 μ mol/Kg of body weight of Shikonin prevents the formation of the oedema and we only observe an increase in volume of the hind paw of 16.4%. This is in agreement with the previous work of Kouronakis et al. [21], which shows a 20% volume of the hind paw at the same conditions [21].

Regarding the potential role of Shikonin as an analgesic agent, we have observed that CFA-induced inflammation of the hind paw produces a clear mechanical hyperalgesia demonstrated by the massive reduction on mechanical thresholds. Shikonin ameliorates mechanical hyperalgesia as the mechanical thresholds are partially restored. Thus, our results show that intraperitoneal Shikonin administration prevents the massive drop in mechanical thresholds observed in the CFA treated animals and reversed the mechanical thresholds by approximately 48%. Our results are in agreement with previous findings. Hence, intraperitoneal or oral administration of Shikonin showed a similar reversal of the mechanical thresholds of approximately 45% and 39% respectively at 1 h after CFA-inflammation induction [22].

Finally, it is also interesting to note that our results show a close correlation between the paw oedema inhibition by approximately 53% and the reversal of the mechanical thresholds by approximately 48% at 1 h post CFA inflammation induction. All together, these results suggest an important role of Shikonin in analgesia which should be further studied.

4.2. Shikonin inhibits microglia activation and ameliorates the pain behaviour in the hind paw in CFA-inflammatory pain model

Microglia is a resident immune cell in the central nervous system which has key role in inflammatory and neuropathic pain [14]. There is a resting microglia which has a physiological role to survey the environment and act as scavengers to remove debris. However, there is a reactive microglia associated with pain; acting as a trigger to provoke peripheral, central sensitisation and lower mechanical thresholds (mechanical hyperalgesia) [27]. This reactive microglia activates upon release of proinflammatory cytokines (e.g. IL-1 β , IL-6) and is termed as M1 microglia [11,13]. Compared to resting microglia, reactive M1 microglia is more abundant in total cell numbers and is also characterised by morphological changes such as increased cell body with longer and more abundant processes [14,28].

In the present work, we did not find an increase in microglia cell number. On the contrary, we describe a morphological change by an increase in the cell size in the CFA+veh group 1 h after treatment which can be interpreted as the microglia turning into the M1 reactive type. In support of our results, it has been shown the carrageenan inflammatory agent has the same effect (changes in morphology but not in cell numbers) when injected into the hind paw of young (P10) pups [14].

In the present work we show that Shikonin treatment inhibits the progression from resting microglia to the M1 reactive microglia. Thus, we hypothesised that the inhibition of microglia by Shikonin has a direct effect over the restoration of the mechanical thresholds. The reactive microglia plays such an important role in pain that its timed inhibition prevents sensitisation and pain behaviours [27,29]. In support to our hypothesis, Nam et al. [30] have demonstrated that the derivatives of Shikonin reduce microglia activation and reduce the levels of proinflammatory cytokines and other molecules such as COX-2 and PGE₂ [30]. All together, we could suggest that Shikonin prevents sensitisation by inhibiting the proinflammatory cytokines and microglia activation.

This M1 reactive microglia inhibition by Shikonin which we have observed is concomitant with a significant reversal of the mechanical thresholds by a approximately 48%. Microglia is part of a cascade to ultimately sensitise the neurons. However, it is known that microglia inhibition may not be sufficient to completely reverse hyperalgesia [14]. Thus, minocycline which is a potent microglia inhibitor [27] shows a comparable effect to Shikonin and it is only capable to partially reverse thermal hyperalgesia in the CFA-inflammation model of pain besides of completely block microglia activity [27]. The inflammatory process itself produces cytokines such as IL-1 β that can directly sensitise neurons [31]. Additionally, other glial cells such astrocytes are also known to mediate inflammatory pain [32]. Therefore, this direct effect of the inflammation over the neurons or the increased activity of other glial cells may explain the partial reversal of the mechanical thresholds observed in our work. Moreover, Shikonin can inhibit the nociceptor Sodium Channel Nav1.7 in an in vitro assay [22] which suggest a pleiotropic effect of the Shikonin in neurons and microglia. Thus, further studies will be needed to explore the role of the Shikonin on microglia at a molecular level.

5. Conclusion

In the present work we have demonstrated that, in the CFA inflammatory pain model, Shikonin administration reduces paw oedema, ameliorates mechanical hyperalgesia and partially reverses mechanical thresholds and inhibits the CFA-depending microglia activation. Our results highlight that Shikonin is a reliable microglia inhibitor which should be further studied to be included amongst the battery of analgesic compounds to ameliorate pain.

CRedit authorship contribution statement

Dr. Miguel Biscaia has performed the behavioural experiments, contributed to the immunohistochemistry assay and has significantly contributed to the overall study design and grant application, Dr. Ricardo Llorente: has performed the behavioural experiments, contributed to the immunohistochemistry assay and the data analysis. Dr. Jose Gomez: has performed the microglia analysis, Dr Daniela Grassi: Has contributed to the behavioural experiments and material collection (spinal cord extractions and further processing), Dr. David Vega-Avelaira is the principal investigator and supervisor of the project. Dr Vega-Avelaira has written the manuscript, performed the behavioural experiments, the immunohistochemistry assay and the data analysis and has written and submitted the grant application to support this manuscript, All authors have contributed to the data analyses and supervised and reviewed the manuscript. All data were generated in-house, and no paper mill was used. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by the Universidad Europea de Madrid (Grant number: 2020/UEM38). We would like to specially thank to Dr Arenillas, Hospital Universitario de Getafe (Spain) for his support and advice in animal welfare.

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