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Spinal glial activation and oxidative stress are alleviated by treatment with curcumin or coenzyme Q in sickle mice

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Sickle cell anaemia (SCA) is accompanied by unpredictable episodes of recurrent acute pain during vasoocclusive crises (VOC), superimposed on chronic pain.\textsuperscript{1} Pain in SCA can start in infancy and may continue throughout life, leading to sustained activation of the nociceptive mechanisms resulting in poor therapeutic outcomes. Pain is an outcome of nociceptive processing in the central nervous system (CNS), triggered by peripheral nervous system response to exogenous and endogenous stimuli. Activation of transient receptor potential vanilloid 1 (TRPV1) channels on C-fibers, neurogenic inflammation, mast cell activation, systemic inflammation, and oxidative stress in the periphery have been demonstrated in sickle SCA.\textsuperscript{2, 3} However, the extent and mechanisms of CNS involvement remain unknown in SCA. Activation of inflammatory and neuronal cells in the CNS plays a pivotal role in nociception.\textsuperscript{4} We recently observed that spinal nociceptive neurons are sensitized in sickle mice suggestive of central sensitization.\textsuperscript{5} Bidirectional signaling occurs between neurons and immunocompetent cells present in the CNS, including microglia, astrocytes and oligodendrocytes.\textsuperscript{4} Activated microglia release reactive oxidative species (ROS), inflammatory cytokines, neurotrophic factors, and prostaglandins that excite nociceptive neurons and contribute to the persistence of chronic pain.\textsuperscript{4} It is therefore likely that activation of central nociceptive mechanisms contributes to chronic pain in SCA.

Remarkable decrease in inflammation, thiobarbituric acid reactive substances (TBARS, an indicator of oxidative stress), and VOC have been observed in SCA patients receiving coenzyme Q10 (CoQ10).\textsuperscript{6} Additionally, curcumin reduced markers of oxidative stress in thalassemia patients and also ameliorates pain hypersensitivity in rats with monoarthritis by decreasing spinal neuroinflammation.\textsuperscript{7, 8} Since excess free iron due to hemolysis contributes to oxidative stress and inflammation in SCA, we examined glial activation, inflammation and oxidative stress in the spinal cords of sickle mice and tested the possibility of a synergistic effect of CoQ10 and/or curcumin to ameliorate spinal oxidative stress, glial activation and hyperalgesia.

To examine our hypotheses, we used female transgenic HbSS-BERK sickle mice with murine $\alpha$ and $\beta$ globin knockouts and expressing human sickle, or normal haemoglobin A (designated sickle or control mice, henceforth, respectively). We bred and characterized these mice by pheno- and geno-typing (Please see Online Supplementary Appendix for details).\textsuperscript{3, 9} These
sickle mice have severe haematologic disease, organ damage and tonic hyperalgesia similar to that observed in human SCA.9-11

Sickle mice received either vehicle (olive oil), curcumin (15 mg/kg), CoQ10 (45 mg/kg), or both CoQ10 and curcumin (cotreatment) daily for 4 weeks by gavage. Female mice were used because BERK female mice show more hyperalgesia as compared to males.11 Pain behaviors were evaluated during the proestrous/estrous cycle before treatment and weekly. Mice were tested for, mechanical hyperalgesia using paw withdrawal frequency (PWF) in response to von Frey filaments, paw withdrawal latency (PWL) in response to a heat stimulus using a Hargreave’s apparatus and sensitivity to cold was determined by PWL and PWF per 2 min on a cold plate (please see detailed procedures in the Online Supplementary Appendix).11 Spinal cords were harvested after 4 weeks of treatment. Sections were examined by laser scanning confocal microscopy (LSCM) for Iba1, a microglial marker (Wako, Richmond VA), glial fibrillary acidic protein (GFAP), an astrocyte marker (Abcam, Cambridge, MA), neuropeptide substance P (SP, Abcam) and detection of ROS with dihydroethidium (DHE, Life Technologies, Grand Island, NY).

*Spinal glial activation, neuroinflammation and oxidative stress in sickle mice:* LSCM revealed increased immunoreactivity (ir) for GFAP, Iba1, SP and DHE/ROS staining in the dorsal horn of the spinal cord of sickle mice as compared to control mice, suggestive of activation of astrocytic and microglial cells accompanied by neuroinflammation and oxidative stress (Figure 1 A-D). Control spinal cords exhibited a significantly larger proportion of “resting” microglia with small cellular body (somata) and long ramified processes that act as sensors of change in the microenvironment (Figure 1B, lower panel). However, in sickle spinal cords microglial cells show shorter and fewer processes suggestive of activated microglia (Figure 1B, lower panel). We also observed increased levels of neuropeptide SP-ir and ROS (Figure 1 C, D) in superficial dorsal horn of spinal cords from vehicle-treated sickle mice Vs control mice (p<0.0001; Figure 1 G, H).

*Curcumin and/or CoQ10 treatment reduces spinal glial activation, neuroinflammation and oxidative stress:* Treatment with curcumin and/or CoQ10 significantly reduced activated astroglial (p<0.01) and microglial cells (p<0.001), SP-ir (p<0.05), and ROS (p<0.05) compared to vehicle-treatment in sickle mice (Figure 1 A-H). The reduction in activated astroglia was
similar to that found in control mice while activated microglia remained significantly elevated after treatment as compared to control mice. Thus, curcumin and CoQ10 are both able to reduce SP and ROS expression in sickle mice, and together reduce ROS to levels seen in control mice. Microglial activation is dependent upon MAPK signalling. Previously, we observed increased phosphorylation of MAPK/ERK and p38MAPK simultaneously with nociceptor sensitization in the spinal cords of these sickle mice as compared to control mice. Therefore, it is likely that glial activation contributes to spinal MAPK activation, nociceptor sensitization and chronic hyperalgesia observed in sickle mice.

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Astrocyte activation is a result of calcium influx into the astrocytes, which is caused by increased blood flow. Previously we observed increased blood flow in the skin of sickle mice as compared to control mice. Therefore, it is likely that blood flow may be higher in the spinal cord of sickle mice, contributing to astrocytic activation observed in our studies. Astrocytes are activated by glutamate, SP and many other neurotransmitters released upon stimulation of hyperexcitable nociceptive neurons in nerve injury. In turn, activated astrocytes release these same neurotransmitters, further augmenting neuronal excitability. In the setting of nerve injury or chronic neuropathic pain conditions, astrocyte activation is maintained by microglia-derived inflammatory factors. The increase in concurrent expression of ROS and SP strongly suggests a global constitutive inflammatory state and oxidative stress in the CNS, which may underlie central sensitization leading to chronic pain observed in patients with SCA and sickle mice. Of note, increased circulating levels of SP have been reported in patients with SCA as compared to normal healthy subjects and in the skin and blood of sickle mice as compared to control mice. It is plausible that high amounts of spinal SP are released into the periphery due to antidromic release that may occur in central sensitization due to continued neuronal excitation. It is therefore likely that reduced inflammation and oxidative stress orchestrated by ‘nutraceuticals’ and supplements already used widely by consumers may reduce central sensitization and chronic pain.

Curcumin and/or CoQ10 treatment ameliorates mechanical and thermal hyperalgesia in sickle mice: Similar to our previous observations, sickle mice showed increased sensitivity to mechanical and thermal stimuli, suggestive of increased mechanical and thermal hyperalgesia in sickle mice as compared to control mice (Figure 2). Chronic treatment with curcumin and
CoQ10, alone or in combination, significantly reduced mechanical and heat hyperalgesia as compared to baseline or vehicle-treated sickle mice (P<0.0001; Figure 2 A-B). This decrease started at week 1 irrespective of treatment, but at week 4 cotreatment was slightly more effective than either treatment alone (Figure 2 A-B). Sickle mice exhibited cold hyperalgesia with higher PWF and shorter PWL compared to control mice (p<0.001), which was significantly attenuated after 2 weeks of treatment with curcumin and/or CoQ10 (Figure 2 C-D). All treatments of sickle mice presented increased PWL on a cold plate at week 4 in comparison to sickle vehicle group, but it was significantly lower than control mice (p<0.0001), suggesting that cold hyperalgesia is more challenging to treat. Together, these data suggest that curcumin and CoQ10 independently as well as in combination ameliorate chronic hyperalgesia in sickle mice, consistent with their antinociceptive effects observed in other rodent models of hyperalgesia and/or pain in SCA patients.6,8 Therefore, one or the other may be equally effective perhaps because of their similar mechanism of action of ameliorating oxidative stress, glial activation and SP modulation.

In clinical trials, curcumin was efficacious in reducing inflammation and oxidative stress and improving the outcomes in several conditions including both rheumatoid- and osteo-arthritis.14 Short-term use of Theracurcumin with relatively higher bioavailability than curcumin was effective in reducing pain and symptoms of osteoarthritis in a randomized, double-blind, placebo-controlled prospective study for treating knee osteoarthritis.15 In this study, 8 weeks of Theracurcumin treatment significantly reduced knee pain and lowered Celecoxib dependence as compared to placebo. Curcumin reduced oxidative stress in thalassaemia patients and decreased iron overload and oxidative stress in the liver and spleen of chronic iron-overloaded rats.7,16 Thus, curcumin may influence the sickle microenvironment that includes large quantities of cell-free iron due to haemolysis and oxidative stress. Treatment with curcumin and/or CoQ10 could be a promising alternative strategy to reduce pain in SCA. Considering the challenges with treating chronic sickle pain and requirements of high doses of opioids to treat pain, this approach may reduce the requirement of opioid analgesics and/or improve the outcomes of opioid analgesia.
Footnotes

For definitions, please see Table 1 in the Online Supplementary Appendix

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References


Figure legends

**Figure 1. Glial activation, neuroinflammation and oxidative stress in spinal cords of sickle mice is ameliorated with curcumin and/or CoQ10.** Analysis of activation in glial cells in L4-L5 superficial dorsal horn segments of female sickle mice after daily treatment with curcumin (HbSS Curc), CoQ10 (HbSS CoQ), or both (HbSS Cotreatment) for 4 weeks. Sickle mice (HbSS Vehicle) show increased levels of activated cells as compared to control mice (HbAA Vehicle) which is reduced by all treatments. Representative images of (A) astrocytes (GFAP, upper panel; lower panel showing a magnified field of activated astroglial cells), (B) activated microglia (Iba1 positive cells, upper panel; lower panel shows magnified fields of activated microglial cells), (C) neuropeptide SP, and (D) ROS (green) with DAPI counterstained nuclei (blue). Quantitative analysis of (E) GFAP immunoreactivity, (F) numbers of resting and activated microglial cells, (G) SP immunoreactivity, and (H) ROS levels expressed as percentage DHE positive pixel. Each image representative of images from 8 mice per treatment. Scale bars as indicated. Quantitation of fluorescence expressed as the mean value of the pixel density ± SEM of 3 nonadjacent fields per mouse, except (H) activated microglial expressed as number of immunoreactive cells per field. Significance was determined by one-way ANOVA with Bonferroni’s multiple comparison.

**Figure 2. Curcumin and coenzyme Q10 reduce chronic hyperalgesia in sickle mice.** Sickle mice (HbSS) were treated with Vehicle, Curcumin, coenzyme Q10 (CoQ10) or both in combination for 4 weeks, while control mice (HbAA) received vehicle. Pain measures were obtained before starting treatments, Baseline (BL), and weekly after initial treatment. Measures of (A) mechanical hyperalgesia and (B-D) thermal sensitivity to heat and cold are shown. *p<0.05, **p<0.0001 for BL vs weeks of treatment; #p<0.05, ##p <0.0001 HbSS Vehicle vs treatments; $ p<0.05 HbSS Cotreatment vs HbSS CoQ10 or HbSS Curcumin. Each value is the mean ± SEM from 6-12 female mice (mean age = 5.6 month old) with 3 observations per mouse. Significance was determined by two-way ANOVA with Bonferroni’s and Students t-test.
Supplementary Appendix for the Letter to the Editor:

Oxidative stress in a mouse model of sickle cell disease is associated glial activation that is alleviated by treatment with curcumin or coenzyme Q

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Methods

Mice

Transgenic female sickle mice (HbSS-BERK) and control mice (HbAA-BERK) were used. HbSS-BERK are homozygous for knockout of both murine alpha and beta globins and carry linked transgenes for human alpha and beta-S globins. Thus, HbSS-BERK mice produce only human alpha and beta S globin chains (human hemoglobin S) and have severe disease that simulates human sickle cell anemia.\textsuperscript{1-3} Control HbAA-BERK have the same mixed genetic background as HbSS-BERK, but exclusively express normal human alpha and beta globins (hemoglobin A). Mice were bred and phenotyped for sickle and normal human hemoglobin by IEF.\textsuperscript{2} Genotyping for the mouse knockout and human hemoglobin transgenes was done by Transnetyx (Cordova, TN). All experiments were performed following approved protocols from the University of Minnesota’s Institutional Animal Care and Use Committee and conform to the statutes of the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals.\textsuperscript{4}

Drug treatment of mice

Mice were treated daily for 4 weeks with Coenzyme Q10 (CoQ10) and curcumin (both from Sigma, St Louis, MO) at 15 mg/kg and 45 mg/kg, respectively, in olive oil by gavage of 0.1 ml/25 g body weight.\textsuperscript{5, 6} Sickle and control mice received either vehicle, curcumin, CoQ10, or both CoQ10 and curcumin (cotreatment).
Pain Behavior Analysis

All behavioural tests were performed in a quiet room maintained at a constant temperature of 23-25°C during the proestrous/estrous cycle once a week for a total of four weeks before that day's treatment. Mice were habituated to each test protocol and environment for three consecutive days before collecting baseline measurements as described earlier. Three measurements were recorded for each test, or as described, for the following behaviors.

Mechanical hyperalgesia. The paw withdrawal frequency (PWF) evoked by 10 consecutive applications of a 1.0 g (4.08 mN) von Frey (Semmes-Weinstein) monofilament (Stoelting Co., Wood Dale, IL) were used to assess mechanical sensitivity. Mice were placed on a wire-mesh under a glass container (10x6.5x6.5 cm) and were allowed to acclimate for 60 min. The von Frey filament was applied to the plantar surface of each hind paw, avoiding the toes, heel and pads, for 1-2 seconds with a force sufficient to bend the filament. An inter-stimulus interval of at least 5 seconds was observed. Only vigorous withdrawal responses were counted.

Thermal hyperalgesia. To test for heat sensitivity, mice were placed on the glass floor of Hargreave's apparatus and a radiant heat stimulus was applied to the plantar surface of the hind paw from below with a projector lamp bulb (CXL/CXR, 8 V, 50 W). Paw withdrawal latency (PWL) to the nearest 0.1 second was automatically recorded when the mouse withdrew its paw from the stimulus. A 20 second stimulus cutoff was used to prevent damage to the paw. To test for cold sensitivity, mice were placed on an aluminum plate set in an ice bath (3°C) and the latency to initial lifting of either forepaw from cold plate (PWL) and the number of times mice lifted or rubbed the forepaws together (PWF) over a period of 2 minutes were determined.

Laser Scanning Confocal Microscopy (LSCM)

Sample preparation. Spinal cords were fixed in Zamboni’s solution (0.03% w/v picric acid and 2% w/v paraformaldehyde) and transferred into 20% sucrose with 0.05% sodium azide after 24-48 hr at 4°C (Kohli 2010). Fixed tissues were embedded in OCT and cryosections (15 µm) were stained with rabbit anti-Iba1, a microglial cell marker (Wako, Richmond VA), goat anti-GFAP, an astrocyte marker (Abcam, Cambridge, MA), or guinea pig anti-Substance P (SP; Abcam). Cy2- or Cy3-conjugated species-specific donkey secondary antibodies (Jackson ImmunoResearch, West Grove, PA) were used to detect immunoreactive proteins and samples were mounted with Vectashield (Vector Labs, Burlingame, CA).
Quantitative analysis. Spinal cord sections from L4 and L5 were imaged using LSCM (Olympus FluoView 1000 BX2, Olympus Corporation, Center Valley, PA). SP immunoreactivity in the superficial dorsal horn was quantitated in optical sections of 1.68-μ (30 steps) captured with a 20X objective (UPLAPO Olympus BX61). GFAP-immunoreactivity was quantitated in optical sections 0.48-μ (38 steps) captured with a 40X objective (UPLFLN Laser 40XO Olympus BX61). For each dorsal horn, a threshold intensity corresponding to the average intensity of labeled regions within the superficial dorsal horn was measured using Image J (NIH). Data was collected from three sections, averaged and expressed as percentage of ir pixels as described by Malmberg. Lba-1 immunoreactivity was quantitated by counting number of microglial cells exhibiting a clear somata and discernible processes per single field of view (FOV) from optical sections of 0.48-μ (38 steps) captured at 40X with a 2.6X electronic zoom following the protocol by Hains et al. Activated microglia were classified based on the following criteria: somata exhibiting hypertrophia and retracted processes. Three representative non-adjacent FOV from the dorsal horn were selected. For uniformity the FOV were collected from the superficial dorsal horn, superficial lateral dorsal horn and in proximity to the central canal. Each segment and the total number of glial cells in the three FOV were calculated for each group. The average of the three sections/mouse for 8 mice was averaged for each group.

Reactive oxygen species

Lumbar spinal cords sections (30 μm) were incubated with the superoxide indicator dihydroethidium (DHE; Life Technology, Grand Island, NY) at 5μM and imaged using LSCM (Olympus FluoView 1000 BX2). The percentage of fluorescence pixels divided by the total area was quantitated.

Statistical analysis

All data were analyzed using Prism software (v 5.0a, GraphPad Prism Inc., San Diego, CA). A one-way ANOVA with Bonferroni’s multiple comparison correction and t-test was used to compare treatments. A two-way ANOVA with Bonferroni’s multiple comparison correction was used to compare the pain behaviour responses between treatments. A p-value of < 0.05 was considered significant. All data are presented as mean ± SEM.
Supplementary References


**Supplementary Table S1:** Definitions of pain related terminology

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<thead>
<tr>
<th>Term</th>
<th>Definition</th>
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<tr>
<td>Analgesia</td>
<td>absence of pain in response to stimulation which would normally be painful</td>
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<tr>
<td>Gliosis</td>
<td>a nonspecific reactive change of glial cells (astrocytes, microglia, and oligodendrocytes) in response to damage to the central nervous system</td>
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<tr>
<td>Hyperalgesia</td>
<td>increased sensitivity to pain or enhanced intensity of pain sensation and a raised threshold to painful stimuli</td>
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<tr>
<td>Mechanical hyperalgesia</td>
<td>increased sensitivity in response to touch or pressure</td>
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<tr>
<td>Neurogenic inflammation</td>
<td>inflammation arising from the local release from neurons of inflammatory mediators such as Substance P, Calcitonin Gene-Related Peptide (CGRP), neurokinin A (NKA), and endothelin-3 (ET-3).</td>
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<tr>
<td>Nociception</td>
<td>processing of harmful stimuli by the nervous system to sense potential harm or pain; and a feeling of distress, suffering, or agony, caused by stimulation of specialized nerve endings</td>
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<tr>
<td>Nociceptor sensitization</td>
<td>a reduction in threshold and an increase in responsiveness of specialized nerves</td>
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<tr>
<td>Thermal hyperalgesia</td>
<td>increased sensitivity in response to heat or cold</td>
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