Role of 11β-HSD type 1 in abnormal HPA axis activity during immune-mediated arthritis

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Abstract

Patients with chronic immune-mediated arthritis exhibit abnormal hypothalomo-pituitary-adrenal (HPA) axis activity. The basis for this abnormality is not known. Immune-mediated arthritis is associated with increased extra-adrenal synthesis of active glucocorticoids by the 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) enzyme. 11β-HSD1 is expressed in the central nervous system, including regions involved in HPA axis regulation. We examined whether altered 11β-HSD1 expression within these regions contributes to HPA axis dysregulation during arthritis. The expression of 11β-HSD1, and other components of glucocorticoid signaling, were examined in various brain regions and the pituitary gland of mice with experimentally induced arthritis. Two arthritis protocols were employed: The K/BxN spontaneous arthritis model for chronic arthritis and the K/BxN serum transfer arthritis model for acute arthritis. 11β-HSD1 mRNA (Hsd11b1) was expressed in the hippocampus, hypothalamus, cortex, cerebellum and pituitary gland. Hypothalamic Hsd11b1 expression did not change in response to arthritis in either model. Pituitary Hsd11b1 expression was however significantly increased in both chronic and acute arthritis models. Hippocampal Hsd11b1 was decreased in acute but not chronic arthritis. Chronic, but not acute, arthritis was associated with a reduction in hypothalamic corticotropin-releasing hormone and arginine vasopressin expression. In both models, serum adrenocorticotropic hormone and cortisol levels were no different from non-inflammatory controls. These findings demonstrate inflammation-dependent regulation of Hsd11b1 expression in the pituitary gland and hippocampus. The upregulation of 11β-HSD1 expression in the pituitary during both chronic and acute arthritis, and thus, an increase in glucocorticoid negative feedback, could contribute to the abnormalities in HPA axis activity seen in immune-mediated arthritis.

Background

Acute inflammation is typically associated with an activation of the hypothalamic-pituitary-adrenal (HPA) axis, which results in an increase in the level of glucocorticoids within the circulation (1). In situations where this response is insufficient, inflammation can be excessive and often life threatening. Abnormal HPA axis
responses have been implicated in the persistence of various inflammatory conditions but most prominently rheumatoid arthritis (RA) (2, 3). In several studies in patients with RA, a failure of the HPA axis to increase in activity in response to high levels of inflammation has been observed (4, 5). The basis for these abnormal HPA axis responses is still not clear, but suppression of the HPA axis by pro-inflammatory cytokines has been proposed (6).

The function of the HPA axis is to maintain an appropriate level of glucocorticoids within the circulation; however, it is now known that the level of active glucocorticoids varies within tissues due to the local expression of glucocorticoid-metabolizing enzymes. The most extensively studied of these are the 11β-hydroxysteroid dehydrogenases (11β-HSDs) that interconvert hormonally inactive cortisone with hormonally active cortisol (and their rodent equivalents dehydrocorticosterone and corticosterone) (7, 8). The 11β-HSD type 1 enzyme is bidirectional but in vivo appears to act primarily as an activator of cortisol from cortisone (7). The 11β-HSD type 2 enzyme by contrast is a powerful inactivator of glucocorticoids. Both 11β-HSD enzymes have been reported to be expressed within the inflamed joint. 11β-HSD2 is expressed in a subset of macrophages (9, 10), whereas 11β-HSD1 expression is much more widespread, being observed in synovial fibroblasts, macrophages and lymphocytes (9, 11). The activity of 11β-HSD1, and thus, the ability to generate active glucocorticoids, increases in synovial tissue in proportion to the degree of systemic inflammation (11). Additionally, total body production of active glucocorticoids from inactive precursors, a measure of systemic 11β-HSD1 activity, is increased in patients with immune-mediated arthritis but can be reduced by antitumor necrosis factor α therapy (11, 12).

11β-HSD1 is also expressed within the central nervous system (CNS) where it appears to act as an exclusive activator of glucocorticoids (13) and has been reported to be important in the regulation of basal activity of the HPA axis (14, 15, 16). For instance, mice with transgenic deletion of Hsd11b1, at least on some genetic backgrounds, show an increase in the circulating level of corticosterone (17, 18). It is thus possible that the failure of the HPA axis to be activated in chronic arthritis could be due to an increased expression of 11β-HSD1 within the CNS, which would increase the degree of negative feedback from glucocorticoids.

To examine whether 11β-HSD1 expression within the CNS is regulated by inflammation, we utilized two murine models of immune-mediated arthritis. In the K/BxN spontaneous arthritis model, KRN mice transgenic for a T cell receptor recognizing an epitope of bovine RNase are bred onto a NOD background leading to the development of severe arthritis (19). In the K/BxN serum transfer model of arthritis, serum from the K/BxN mice is transferred to normal mice causing induction of arthritis (20). Although both models share some features with human immune-mediated arthritis, they differ in their chronicity of arthritis with the K/BxN model representing an exposure dependent on age (mice spontaneously develop joint inflammation at day 25–35 postnatal, which is subsequently sustained) and the serum transfer model of arthritis with 14 days of joint inflammation after serum transfer representing a comparatively short period of exposure. We have examined expression of 11β-HSD1 in the pituitary and various regions of the brain implicated in HPA axis activity or feedback regulation in these two models of immune-mediated arthritis and their non-inflammatory controls.

Methods

Mouse models

Mice were housed under standard laboratory conditions on a 12:12-h light–darkness cycle with free access to standard chow and water. Animals were kept at the animal facility of the ANZAC Research Institute, in accordance with Institutional Animal Welfare guidelines. All studies were approved by the Sydney Local Health District Animal Welfare Committee.

K/BxN spontaneous arthritis model

Male KRN transgenic mice were crossed with female mice from the NOD strain. To differentiate the offspring between T cell receptor transgene-positive K/BxN mice and their transgene-negative control littermates, the genotype was determined by conventional PCR from toe clip samples. At the age of 60 days, mice were killed and serum was collected and pooled for induction of serum transfer arthritis. Tissue samples including the brain and pituitary glands were harvested. Groups of mice were assigned to overlapping experimental techniques (clinical scoring, hormone assays, gene expression studies and immunohistochemistry). As such, the experimental group numbers varied between studies. The number of male and female mice in each experiment was matched between controls and arthritis mice.

K/BxN serum transfer arthritis model

In the serum transfer model of arthritis, 150 µL of serum from 60-day-old K/BxN transgenic mice was injected
intraperitoneally to healthy 8-week-old C57BL/6 male mice. Control mice (also male) received 150 µL of normal saline vehicle instead. The injection was repeated once after two days. Mice underwent clinical scoring until day 7, at the peak of inflammation, when mice were killed and serum and tissue samples were collected for hormone assays and gene expression studies.

**Clinical assessment of arthritis**

Severity of joint inflammation was clinically assessed as described previously (21, 22, 23). Each limb was examined and scored according to the following categories: 0 = normal joint; 1 = mild-to-moderate swelling of the ankle and/or one swollen digit; 2 = swollen ankle or swelling in two or more digits; 3 = severe swelling along all aspects of paw or all five digits swollen. Summing up the scores of each limb, the mice ranked on a joint inflammation intensity scale from 0 to 12 points.

**Serum assays for corticosterone and adrenocorticotropic hormone**

Serum samples were collected at 12:00h, before animals were killed and stored at −80°C until further examination. Blood was collected by cardiac puncture and approximately 0.8 mL blood was collected from each mouse. After collection, blood was transferred to autoclaved 1.5 mL microcentrifuge tubes, followed by centrifugation at 4°C (13,400 g, 20 min). The transparent supernatant was then transferred to a fresh microcentrifuge tube as serum. Levels of corticosterone and adrenocorticotropic hormone (ACTH) were determined with commercial enzyme immunoassay kits (Mouse ACTH ELISA, Sigma Aldrich; Corticosterone EIA Kit, Arbor Assays, Ann Arbor, MI, USA).

**Tissue harvesting and brain dissection**

Pituitary glands were dissected and instantly frozen in liquid nitrogen and then stored at −80°C until processing. The brain of each mouse was collected as a whole and stored in RNAlater (Life Technologies) at 4°C overnight. Brain samples were then further dissected on ice into distinct regions. Hippocampus, cerebellum and cortex were dissected according to a previously reported protocol (24). The hypothalamus was then dissected out from the remaining brain tissue by a method adapted from Baker and coworkers (25). The macroscopic landmarks used as borders were as follows: Rostral; optical chiasma and anterior commissure, caudal; interpeduncular fossa, lateral; choroidal fissure. The tissue samples were stored at −80°C until processing.

**Gene expression studies**

Due to the small volume of pituitary tissue in each mouse, two pituitaries were pooled together in order to yield sufficient amounts of RNA. Whenever possible, these two samples were matched according to litter and gender. In the figure legends, the two pooled samples are referred to as one sample number. Samples from other tissues were processed individually.

RNA was isolated from the tissue samples using a TRIzol RNA extraction protocol and further purified with a commercial RNA extraction kit (Nucleospin RNA by Macherey Nagel, Düren, Germany). Briefly, samples were immersed in bead tubes (Lysis Matrix D, MP Biomedicals, Santa Ana, CA, USA) filled with 1 mL TRIzol (Invitrogen) and then homogenized using a Powerlyzer machine (MO BIO Laboratories, Carlsbad, CA, USA). After that, the homogenates were centrifuged and the supernatant was added to 200 µL of chloroform. The tubes were then vigorously shaken before spun down, and the aqueous phase was added to 500 µL 2-propanol followed by 2-h incubation on ice. Subsequent steps followed the protocol of the company’s RNA extraction kit. The quality of the yielded RNA was determined by gel electrophoresis and spectrophotometry. RNA was quantified using a NanoPhotometer (Implen, München, Germany) with an a260/a280 ratio greater than 2.0 was considered of sufficient purity.

Complementary DNA was generated from RNA using a commercial reverse transcription kit (SuperScript III First-Strand Synthesis for qRT-PCR, Life Technologies). Complementary DNA was synthesized from 600 µg RNA, and 200 µL Milli Q water was added to each cDNA sample for dilution before qPCR. Relative mRNA expression levels were determined using a real-time PCR machine (CXCF Connected, Bio-Rad) in combination with Sybr Green Supermix solution (Bio-Rad). The primer sequences are shown in Supplementary Fig. 1 (see section on supplementary data given at the end of this article). Glyceraldehyde 3-phosphate dehydrogenase (Gapdh) was used as a reference gene on the basis of published work (26) and pilot experiments demonstrating that the expression of this gene in the brain was stable during glucocorticoid treatment (Supplementary Fig. 2). Post hoc analysis of Gapdh expression in all brain regions studied demonstrated no differences in expression between controls and arthritis animals (Supplementary Fig. 3).
The protocol for the real-time PCR analysis was: 95°C for 3 min, 40 cycles 95°C for 10 s + 60°C for 15 s + 72°C for 30 s. The abundance of specific mRNAs in a sample was normalized to that of Gapdh RNA. Data were obtained as Ct values and used to determine ΔCt values (Ct target – Ct Gapdh). Gene expression in arbitrary units (AU) was calculated from ΔCt values with the equation 2^ΔΔCt.

**Immunohistochemistry**

Samples for histological examination were fixed in 4% paraformaldehyde at 4°C for 24 h before embedding in paraffin wax (Paraplast Tissue Embedding Medium, Tyco Healthcare Group, Mansfield, MA, USA). A manual microtome (Leica Microsystems, Wetzlar, Germany) was used to cut 6 µm thick coronal sections of whole brain samples. After identifying the level of the paraventricular nucleus (PVN), sections were collected for immunostaining.

After dewaxing the samples, antigens were retrieved in 10 mM citric acid just below boiling temperature for 2 h, followed by endogenous peroxidase inactivation with 3% hydrogen peroxide for 20 min. Samples were then blocked with 5% goat serum for 30 min and incubated overnight with the primary antibody for 11β-HSD1 (1:100, Cayman Chemicals) and glucocorticoid receptor (GR) (1:400, sc-1004, Santa Cruz Biotechnology). A biotinylated secondary antibody was applied together with the corresponding Avidin–Biotin-Complex and diaminobenzidine solution following the company’s protocol (all Vector Laboratories, Burlingame, CA, USA). Stained and unstained cells in the PVN were counted and the percentage of stained cells was determined.

**Statistical analysis**

To compare normally distributed values between two groups, the Student’s t-test was used. A P value of less than 0.05 was considered statistically significant. Results are shown as the mean and the standard error of the mean.

**Results**

**Arthritis generation and changes in circulating levels of ACTH and corticosterone**

Arthritis developed in both models of immune-mediated arthritis. Mice on a K/BxN genetic background develop arthritis at around 30 days postnatal and were harvested at 60 days of age. Serum transfer mice were harvested 7 days after induction of arthritis. The indices of joint inflammation for the arthritic mice at the time point of tissue harvest are shown in Fig. 1. The severity of arthritis (assessed by mean clinical score) was significantly greater (P<0.01) in the K/BxN spontaneous arthritis model compared to the serum transfer model of arthritis. Mice in either of the two groups of control mice did not show clinical signs of inflammation.

Serum ACTH and corticosterone were measured at 12:00 h, which corresponds to the nadir of the rodent circadian glucocorticoid production rhythm. Levels of ACTH and corticosterone were not found to be significantly different between mice with either type of arthritis and their non-inflammatory controls as shown in Fig. 2. These results suggest that these models of immune-mediated arthritis do not cause a major change in circulating corticosterone levels, at least at the time point selected.

**Expression levels of Hsd11b1 mRNA in murine brain and pituitary in the absence of inflammation**

The expression of Hsd11b1 mRNA in various parts of the brain and the pituitary was examined in K/BxN transgene-negative littermates. These mice do not develop arthritis and were thus used as a non-inflammatory control. In the brain, expression was detectable in all examined areas, but the greatest expression was in the hippocampus, which parallels the results of earlier studies on rodent brains (27, 28). Interestingly, there were even higher Hsd11b1 mRNA levels in the pituitary. The relative expression of Hsd11b1 mRNA in various parts of the brain and the pituitary is illustrated in Fig. 3.
Changes in expression of genes involved in HPA axis regulation in K/BxN spontaneous arthritis mice

The expression of Hsd11b1 mRNA in various parts of the CNS was compared between 12 K/BxN mice and 11 non-inflammatory control mice (13 and 7 sample pairs respectively for the pituitaries). The level of expression was unchanged in the hippocampus, hypothalamus, cerebral cortex or cerebellum. However, the level of expression within the pituitary was significantly increased by 29% \( (P<0.013) \) in the arthritis group (Fig. 4A).

The expression levels of various other genes involved in HPA axis activity and feedback were examined in the hypothalamus and pituitary. In the hypothalamus, there was a 9% \( (P<0.01) \) increase of the mRNA expression level for GR, but no change in expression of the mineralocorticoid receptor (MR). There was however a 19% \( (P=0.015) \) decrease in the level of corticotropin-releasing hormone (CRH) and a 29% \( (P<0.01) \) decrease in the level of arginine vasopressin (AVP) expression (Fig. 4B). In the pituitary gland, arthritis was not associated with a change in the level of GR, but an increase in the mRNA expression of MR (29%), corticotropin-releasing hormone receptor 1 (CRHR1) (24%) and proopiomelanocortin (POMC) (59%) (Fig. 4C); all changes significant with \( P<0.05 \).

Changes in expression of genes involved in HPA axis regulation in the serum transfer model of arthritis

To determine whether the changes seen in immune-mediated arthritis are consistent across different strains of mice and durations of inflammation, the expression of genes involved in HPA axis regulation was examined in 7 mice with serum transfer arthritis and 7 of their controls (4 and 3 sample pairs respectively for the pituitaries). As noted earlier, no difference in the plasma level of corticosterone or ACTH was seen between animals with immune-mediated arthritis and their non-inflammatory controls. The level of Hsd11b1 expression in the hippocampus was significantly decreased by 24% \( (P=0.016) \) in the serum transfer model of arthritis group (Fig. 5A). This is in contrast to the K/BxN model where the level was unchanged. In keeping with the K/BxN model, the expression of 11\( \beta \)-HSD1 was unchanged in the hypothalamus and significantly increased in the pituitary by 50% \( (P<0.01) \) (Fig. 5A). With regards to other genes involved in HPA axis regulation, the level of GR in the pituitary was increased by 48% \( (P<0.01) \) in the serum transfer model of arthritis but POMC expression was unchanged as was the expression of the other genes examined (Fig. 5B and C).
Immunohistochemical analysis of 11β-HSD1 expression in the PVN of K/BxN spontaneous arthritis mice

The experiments earlier examined mRNA expression in relatively large areas of the brain. This approach was unable to rule out the possibility that there are changes in mRNA expression within smaller regions in response to inflammation. In particular, it was impossible to determine whether the expression of Hsd11B1 might be selectively regulated in the PVN of the hypothalamus, a region critical to the production of CRH and communication with the anterior pituitary. Due to the small size of the mouse hypothalamus, it was impossible to selectively dissect this nucleus. Instead, we used semi-quantitative immunohistochemistry to examine for changes in 11β-HSD1 expression specifically within the PVN in K/BxN mice and their control littermates.

Immunohistochemistry for 11β-HSD1 demonstrated expression in cells within the PVN (area highlighted in Fig. 6A) in K/BxN mice and their control littermates. The number of cells within the area of the PVN that stained positive for 11β-HSD1 and GR protein was counted and compared between mice with and without arthritis. No difference in the percentage of stained cells was detected suggesting that, in keeping with the mRNA findings, there was no selective change in 11β-HSD1 or GR expression within the PVN in response to K/BxN arthritis (Fig. 6B).

Discussion

We report here that in two murine models of immune-mediated arthritis, inflammation resulted in changes in expression of 11β-HSD1 in some CNS tissues involved in the regulation of the HPA axis. The changes were most marked and consistent at the level of the pituitary gland where Hsd11B1 expression was increased substantially in both models. Such an increase in 11β-HSD1 would be expected to result in increased levels of active glucocorticoids within the pituitary and thus would be likely to modify through negative feedback the functioning of the HPA axis. Rodent studies employing targeted deletion of the GR in the PVN or pituitary demonstrate that both sites are important for feedback regulation of the HPA axis (29).
GR deletion in the pituitary leads to elevated levels of corticosterone, particularly in young animals, and a reduced suppression of corticosterone production in response to dexamethasone treatment (30). As such, pituitary glucocorticoid signaling is an important part of the feedback of the HPA axis. Therefore, an increase

Figure 5
Comparison of expression levels between serum transfer model of arthritis (STA) and their control littermates. All data are mean ± s.e. (A) Relative expression of 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) in different parts of the central nervous system. (B) Relative expression of different genes in the hypothalamus. AVP, arginine vasopressin; CRH, corticotropin-releasing hormone; GR glucocorticoid receptor; MR, mineralocorticoid receptor. (C) Relative expression of different genes in the pituitary. CRHR1, corticotropin-releasing hormone receptor 1; POMC, proopiomelanocortin.

Figure 6
Immunohistochemical staining of the paraventricular nucleus (PVN) of the hypothalamus in K/BxN spontaneous arthritis mice (K/BxN) and their controls. (A) Representative examples of immunostaining for 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) protein expression. (B) Percentage of positive-stained cells in the PVN for 11β-HSD1 and the glucocorticoid receptor (GR) (mean ± s.e.).
in the expression of GR or an increase in the level of 11β-HSD1 would be predicted to suppress the activity of the HPA axis. Of note, in keeping with previous literature, these changes in 11β-HSD1 were associated with failure to upregulate the activity of the HPA axis in inflammatory arthritis (4, 5).

In our study, the expression of 11β-HSD1 in the pituitary is upregulated by inflammation, but the expression in most brain regions is not. The most likely explanation for this is that pro-inflammatory cytokines can more easily access the pituitary than the rest of the CNS. Pro-inflammatory cytokines are a major stimulator of 11β-HSD1 activity in a range of tissues of diverse origins (31, 32, 33), but these cytokines are largely unable to cross the blood–brain barrier.

This study demonstrates 11β-HSD1 expression in the mouse pituitary, which has been described for rats (27) and humans (15, 34), but to our knowledge not for adult mice previously. In contrast to the studies on rats, we found much higher relative expression levels of 11β-HSD1 in the pituitary compared to the other examined tissues from the CNS. This could, especially in the light of the aforementioned findings, indicate a more significant role for 11β-HSD1 in pituitary glucocorticoid feedback regulation than previously considered.

In the K/BxN spontaneous arthritis model, there was also a decrease in the expression of CRH and AVP. The basis for the decrease in these factors was unclear but appeared unrelated to any change in 11β-HSD1 expression within the CNS.

Another notable finding was that in the serum transfer model of arthritis, the level of Hsd11b1 mRNA in the hippocampus was significantly decreased. The hippocampus is considered to be a major site for feedback regulation of glucocorticoids on the HPA axis (35) and thus even small changes in 11β-HSD1 activity might be expected to have an impact on glucocorticoid feedback. Interestingly, a decrease in 11β-HSD1 would be expected to reduce glucocorticoid feedback and thus activate the HPA axis. However, we did not see such an activation in terms of a difference in ACTH or corticosterone levels between mice with immune-mediated arthritis and control animals.

There are several limitations with the study. The exact pathways by which normal HPA axis activity is mediated, including those relating to negative feedback are not fully known. It is therefore possible that inflammation could lead to alterations in 11β-HSD1 activity elsewhere in the CNS that impact significantly on the HPA axis. However, this study examined the sites thought to be most important in the functioning of the HPA axis. We also did not directly examine the directionality of 11β-HSD1, but in the CNS, it appears to be exclusively in the direction of glucocorticoid activation (13). We also did not examine the expression of enzymes that might also metabolize glucocorticoids such as 11β-HSD2 or 5-reductases since these have not been associated with changes in the activity of the HPA axis. Furthermore, we did examine changes in 11β-HSD1 expression in non-CNS tissues in response to inflammation, which might indirectly regulate the activity of the HPA axis.

Another limitation is the reliance on one reference gene in gene expression analysis. In our preliminary studies (Supplementary Fig. 2) and previous work (26) examining the effects of glucocorticoids on the brain, Gapdh was the most stable gene. A recent publication highlighted that Gapdh is not a reliable reference gene when examining gene expression within the arthritic joints of mice with K/BxN serum transfer arthritis (36). However, post hoc analysis failed to show any effect of arthritis on Gapdh in any brain region examined (Supplementary Fig. 3). The mRNA analysis was also carried out on brain regions rather than isolated nuclei or even cells. It is thus possible that 11β-HSD1 expression in certain areas within specific brain regions could be changing dynamically in response to inflammation but that this signal is being lost when diluted by other nearby tissues. We attempted to overcome this by using immunohistochemistry to determine if there was a major difference in 11β-HSD1 protein expression in the PVN, a critical area of the hypothalamus involved in the integration of various signals involved in the HPA axis. Although no difference was seen, the limited sensitivity of this technique means we would not have been able to detect a small change in expression.

One finding that appeared paradoxical was an increase in Pomc expression in the pituitary glands of our K/BxN mice in the context of no apparent increase in circulating ACTH levels. The most likely explanation for this relates to the relatively well-developed pars intermedia in the mouse. This part of the pituitary gland is a major source of POMC in mice (but not humans), but this POMC is primarily used to make factors such as α-melanocyte-stimulating hormone, corticotropin-like intermediate peptide and β-endorphin. It is possible that the high POMC reflects increased activity of this part of the pituitary, which is regulated by pathways distinct from those of the anterior pituitary and HPA axis. Our analysis of ACTH and corticosterone levels were also limited by being restricted to one time point, with samples being obtained at the time of killing. As both hormones are
secreted in a pulsatile manner, it is possible that there were subtle differences in the activity of the HPA axis, which were not detectable at a single time point.

**Conclusion**

This study demonstrates upregulation of 11β-HSD1 activity within the pituitary glands of mice exposed to two different forms of immune-mediated arthritides. This increase would be expected to increase the degree of negative feedback of glucocorticoids at the level of the pituitary gland and thus could contribute to the impairment of the HPA axis seen in immune-mediated arthritides.

**Supplementary data**

This is linked to the online version of the paper at [https://doi.org/10.1530/EC-17-0361](https://doi.org/10.1530/EC-17-0361).

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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**Authors’ contribution statement**

J S carried out tissue collection, RNA, immunohistochemical and ELISA analysis and contributed to writing the manuscript. J T supervised all experiments, oversaw animal work, assisted in tissue collection and contributed to writing the manuscript. J L supervised RNA extraction and analysis and contributed to writing the manuscript. F B helped conceive the study, helped in data interpretation and contributed to writing the manuscript. M J S supervised the project and contributed to writing the manuscript. H Z assisted with study design, supervised all experiments and contributed to writing the manuscript. M S C conceived the study, supervised experiments and drafted the initial manuscript. All authors have reviewed and approved the final article.

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