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its neuroprotective/neuroregenerative properties in EAE and MS, which share an immunological etiology, are still subject to speculation and may involve anti-inflammatory, antiapoptotic, antioxidative and neurotrophic effects (reviewed in [17] and [20]).

We wondered whether EPO would also show neuroprotection in an etiologically different situation of even more pronounced demyelination. Hence, we used the copper chelator cuprizone, which leads to almost complete demyelination of the corpus callosum. This model permits focusing on demyelination without interfering T cell–mediated immune-inflammatory components. Considering the seemingly beneficial effects of EPO in chronic progressive MS where acute immune-inflammatory processes are replaced by smoldering progression, we hypothesized that EPO treatment would also alleviate the cuprizone-induced changes of neurological, imaging and histological readouts.

MATERIALS AND METHODS

Animals

All experiments were approved by and conducted in accordance with the regulations of the local animal care and use committee (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittel sicherheit; AZ 33.11.42502-04-040/08). For all experiments, male C57BL/6 mice were used. They were housed in groups of five in standard plastic cages and maintained in a temperature-controlled environment (21 ± 2°C) on a 12-h light/dark cycle with food and water available ad libitum. During experiments, body weight was monitored twice weekly.

Experimental Design

In a double-blind (for food and injections), placebo-controlled longitudinal four-arm design, 8-wk old male C57BL/6 mice (n = 26 per group) were started on cuprizone-containing (0.2%; Sigma-Aldrich, Taufkirchen, Germany) or regular food (standard ground chow) for a total of 6 wks. After 3 wks, mice were injected every other day (11 injections in total) with placebo (solvent control) or EPO (5,000 IU/kg body weight intraperitoneally; NeoRecormon, Roche, Basel, Switzerland) up to the end of cuprizone feeding (compare Figure 1A). Half of the mice were exposed to behavioral testing, MRI and histology immediately after cessation of treatment, whereas the other half were allowed a 3-wk treatment-free recovery period before behavioral testing and MRI.

It should be noted that preliminary experiments performed with cuprizone-containing pellets instead of ground chow failed to result in any demyelination, although toxicological analysis of the pellets had proven that cuprizone was intact and active. This result may be due to the necessity of a parallel intake of cuprizone as fine powder via skin, airways and gut for producing demyelination.

Behavioral Testing

Group size in all behavioral experiments amounted to n = 13–26.

Rotarod. Rotarod is a relatively crude test for motor function, gross coordination and motor learning and consists of a rotating drum (Med Associates Inc., St Albans, VT, USA) that is accelerated from 4 to 40 revolutions per minute over the course of 5 min. Each mouse is placed individually on the drum and the latency of falling down from the drum is recorded. To assess motor learning, the rotarod test is repeated 24 and 48 h later.

Beam balance. Beam balance is a sensitive test for (fine) motor coordination (balance) and vestibulomotor function. On the first day, two habituation phases are conducted where mice are placed on an elevated horizontal beam (25 mm in diameter, 59 cm in length), illuminated at the start side and with a dark little cage with bedding at the other end. With the goal to enter the little cage, mice are first placed directly in front of the cage (phase 1) and then in the middle of the beam (phase 2). On the second day, all mice are first habituated again on the 25-mm beam, this time being positioned on the illuminated start. This forces them to cross the whole beam for entering the little cage. For the following test, mice are placed on the start side of a 10-mm beam, and the time needed to pass the beam is recorded. On the third day, mice are again first put on the 25-mm beam and then switched onto an 8-mm beam for crossing-time measurement. If a mouse falls down, the test is repeated (maximally three trials/mouse). If all trials fail, a cutoff time of 60 s is used for calculations (average time in a healthy mouse amounts to <8 s). The results are calculated as duration on the beam in percentage of placebo control.

MRI Analysis

Upon completion of the behavioral analyses, six mice per group at both week 6 and 9 were studied by MRI. Animals were anesthetized by 1–1.5% isoflurane in a mixture of oxygen and ambient air and positive ventilated via endotracheal tube. A 3D Fast Low-Angle Shot (FLASH) MRI (repetition time [TR]/echo time [TE] = 14.9/3.9 ms, 45 min each) was performed at 9.4T (Bruker Biospin, Ettlingen, Germany) with 110-μm isotropic spatial resolution using a four-element phased-array surface coil (Bruker Biospin) for signal reception. Maps of T1 relaxation time were obtained by off-resonance irradiation (frequency offset 3 kHz, Gaussian pulse, duration 3.5 ms, flip angle 135°) to calculate the percent magnetization transfer ratio (%MTR). Brain volume (total brain, brain matter, hippocampi, ventricles) was determined by manual segmentation using Amira software (Visage Imaging GmbH, Berlin, Germany). In addition, multislice T2-weighted images were acquired with use of a fast-spin echo MRI sequence (TR/TE = 5,300/64 ms, 8 echoes, 26 sections) at an in-plane resolution of 80 μm and a section thickness of 300 μm (21).

Preparation of Brain Tissue

Animals were anesthetized with 0.25% tribromoethanol (Avertin; 0.125 mg/g

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intraperitoneally) and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde. Brains were removed, fixed overnight at 4°C with 4% paraformaldehyde and placed in 30% sucrose/phosphate-buffered saline (PBS) for cryoprotection. They were frozen on liquid nitrogen. Whole mouse brains were cut into 30-μm thick coronal sections on a cryostat (Leica, Wetzlar, Germany) and kept in a storage solution (25% ethylene glycol and 25% glycerol in PBS).

**Immunohistochemistry**

For immunohistochemistry, serial coronal sections, spaced at regular intervals for each specific marker under investigation, were taken through the whole brain. Free floating sections were washed with PBS three times, mounted on Super Frost microscopic slides, microwaved three times in citrate buffer for 4 min and incubated with 0.5% hydrogen peroxide for 30 min to quench endogenous peroxidases. They were permeabilized and blocked for 1 h at room temperature with 5% normal serum of host species from which respective secondary antibodies were derived. Sections were incubated with mouse anti-APC (CC-1; 1:200; Merck, Darmstadt, Germany), rabbit anti-IBA1 (1:1,000; Wako, Neuss, Germany) and mouse anti-amyloid precursor protein (APP; 1:850; Millipore, Schwabach, Germany) antibodies diluted in 3% normal serum and 0.5% Triton-X in PBS for 48 h at 4°C. After three washes with PBS, sections were incubated with biotinylated secondary antibodies for 1.5 h. The staining was visualized by a peroxidase-labeled avidin-biotin kit (Vector Laboratories, Burlingame, CA, USA) and diaminobenzidine (Sigma-Aldrich, Taufkirchen, Germany). Sections stained for APP were counterstained with hematoxylin for 30 s. Slides were washed in PBS, air-dried overnight, put into xylol and cover-slipped using DePeX (Serva, Heidelberg, Germany).

**Quantification of Cell Numbers**

Digitized overlapping light microscopic images (400x oil; Zeiss, Oberkochen, Germany) from the rostral (bregma 1.10 to –0.10) and caudal (bregma –0.94 to –2.46) region of the corpus callosum (compare Figure 3A) were fused to continuous images using Photoshop CS. Cell counts were performed using ImageJ software. Cell numbers were calculated per millimeter squared. Intra- and inter-rater reliability of cell counts was conducted for all parameters and revealed highly significant correlations (r > 0.98; P < 0.001).

**Isolation of Total RNA and Quantitative Real-Time Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR; qPCR)**

For isolation of total RNA, two coronal brain sections per mouse were used, using the RNasy FFPE kit (Qiagen, Hilden, Germany). First-strand cDNA was generated from total RNA using N6 random primers. The relative concentrations of mRNAs of interest in different cDNA samples were measured out of
three replicates using the threshold cycle method (delta Ct) for each dilution and were normalized to a normalization factor derived from levels of “housekeepers,” glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin and hypoxanthine phosphoribosyltransferase 1 (HPRT1) mRNA, calculated with the geNorm_win_3.5 (22). Reactions were performed using the SYBR green PCR master mix (ABgene, Foster City, CA, USA) according to the protocol of the manufacturer. Cycling was done for 2 min at 50°C, followed by denaturation at 95°C for 10 min. The amplification was carried out with 45 cycles of 95°C for 10 s and 60°C for 60 s. The specificity for each primer pair was controlled with a melting curve analysis. For qPCR, the specificity of each primer pair was controlled with a melting curve analysis. Volume balancing was done for 15 s and 60°C for 60 s. The specificity of each primer pair was controlled with a melting curve analysis. Volumetric analyses of total brain volume (C) and of ventricles (D–F) show a clear effect of EPO after 6 wks, but no longer after 9 wks. For all panels: n = 4–6 (respective n number is always given in the bar); mean ± SEM is presented.

RESULTS

Study Design and Basic Readouts Under Cuprizone and EPO

In a double-blind (for food and injections) placebo-controlled longitudinal four-arm design, 8-wk-old C57BL/6 mice (n = 26 per group) were started on cuprizone-containing (0.2%) or regular food (ground chow) for a total of 6 wks. After 3 wks, mice were injected every other day with placebo or EPO (5,000 IU/kg body weight intraperitoneally) up to the end of cuprizone feeding (study design shown in Figure 1A). Already within the first week, cuprizone led to reduced body weight of mice (monitored twice weekly), which then (independent of EPO) stayed at this lower level compared with control mice until cessation of cuprizone treatment and returned to control values thereafter (Figure 1B). Expectedly, the hematocrit increased upon EPO treatment and returned to the control range at 3 wks after termination of EPO injections (Figure 1C).

Alleviation by EPO of the Detrimental Effect of Toxic Demyelination on Vestibulomotor Coordination and Balance

Three-day rotarod tests immediately upon cessation and 3 wks after cuprizone feeding with or without EPO did not reveal any significant changes in gross motor performance and motor learning (Figures 1D, E). A more sensitive test of motor coordination and vestibulomotor function requiring interhemispheric communication is the beam balance task where mice have to cross two elevated
beams of 10 and 8 mm diameter, respectively. After 6 wks of cuprizone, mice needed significantly longer to cross the 10-mm as well as the 8-mm beam compared with untreated control mice \( (P < 0.001) \). EPO treatment of cuprizone mice improved performance compared with placebo (Figure 1F). After 3 wks without any treatment, all mice had reached a similar performance, underlining the pronounced spontaneous recovery after cuprizone (Figure 1F).

**Mitigation of Ventricular Enlargement and Degree of Demyelination by EPO**

After wk 6, the %MTR as a magnetic resonance imaging (MRI) readout correlating with myelination in the corpus callosum was lower in cuprizone-treated mice, pointing to demyelination. EPO tended to counteract this decrease. After the 3-wk recovery period, there was still a decrease of %MTR compared with controls, but no measurable further benefit of EPO treatment (Figure 2A). Figure 2B illustrates typical T2-weighted brain images of a control and a cuprizone-treated animal after 6 wks. Quantification of total brain volume on the basis of high-resolution T1 maps showed an increase in cuprizone-fed mice \( (P = 0.005) \), an effect mitigated by EPO treatment at wk 6 (Figure 2C). This increase is explained by the known cuprizone-induced enlargement of the whole ventricular system (left and right lateral ventricle as well as third ventricle) \( (24) \), which is attenuated by EPO (Figures 2D–F). Compared to the 6-wk time point, all cuprizone-treated mice displayed a pronounced spontaneous recovery at wk 9, with no further benefit of EPO.

**Attenuation by EPO of a Cuprizone-Induced Increase in Microglial Numbers and APP Accumulations as Readout of Neuroinflammation and Axonal Degeneration**

To better understand the observed effects of EPO on behavioral and imaging parameters after wk 6, we chose this time point to investigate the number of oligodendrocytes and microglia as well as the degree of axonal degeneration within the corpus callosum (anatomical overview of screened corpus callosum regions). The number of adult oligodendrocytes as stained by CC-1 (anti-APC) was remarkably reduced upon cuprizone feeding \( (P < 0.001) \) without any measurable benefit of EPO (Figures 3B–D). Moreover, cuprizone resulted in a significant increase in microglial numbers in the corpus callosum \( (P < 0.001) \), an effect diminished by EPO treatment (Figures 3E–G). In addition, axonal degeneration induced by cuprizone within the corpus callosum, as quantified by APP-positive axonal swellings, was attenuated upon EPO (Figures 3H–J). Quantitative mRNA analysis of cytokines (IL-1\( \alpha \), IL-1\( \beta \), TGF-\( \beta \)) and microglia activity markers (C1qA, Trem-2) yielded a significant increase under cuprizone of the inflammation mediators IL-1\( \alpha \), IL-1\( \beta \), C1qA and Trem-2 and a significant decrease of the protective growth factor TGF-\( \beta \) \( (P < 0.05) \). EPO showed in several cases a tendency in the respective other direction, consistent with its counteraction of the detrimental cuprizone effects. These EPO

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**Figure 3.** Cuprizone-induced changes of oligodendrocyte and microglia numbers as well as axonal degeneration in the corpus callosum: effect of EPO. All histological analyses presented have been performed after 6 wks of cuprizone \( \pm \) EPO. (A) Anatomical overview of screened corpus callosum regions. (B–D) Oligodendrocytes in the rostral and caudal corpus callosum are massively reduced upon cuprizone; there was no effect of EPO \( (D: \) histological sections with scale bar 50 \( \mu m \) ). (E–G) Microglial numbers in the rostral and caudal corpus callosum are highly increased, dampening effect of EPO \( (G: \) histological sections with scale bar 50 \( \mu m \) ). (H–J) APP-positive accumulations in the corpus callosum \( (J: \) histological sections with accumulations indicated by arrows; scale bar 20 \( \mu m \) ). For all panels: \( n = 7–11 \) (respective \( n \) number is always given in the bar); mean \( \pm \) SEM is presented.
may at least partly explain the beneficial
produced by EPO treatment. This reduction
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Attenuation by EPO treatment of mi-
croglial numbers and microglia-mediated
inflammation has been demonstrated
among others in models of cortical lesion
(29), EAE (5,11), epilepsy (30), closed head
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reduction of APP accumulations by EPO
has not been demonstrated yet. This latter
finding may be secondary to EPO-medi-
cated dampening of microglial activation
and inflammation or be due to direct pro-
tective EPO effects on neurons/axons, in-
terrupting the vicious cycle of demyelina-
tion, inflammation, compromised axonal
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Oligodendrocyte numbers were se-
verely diminished by cuprizone but, sur-
prisingly, no protective effects of EPO on
this cell type were found here. In other
models, a clear beneficial EPO effect on
oligodendrocyte numbers was demon-
strated (29,33). This discrepancy may be
explained by the time point or brain
region of cell counting selected in the
present study or (more likely) by a mech-
anism of cell death that cannot be coun-
teracted by EPO. Interestingly, a recent
article reporting an advantageous influ-
ence of vitamin D on demyelination in
the cuprizone model, strikingly similar
to the results obtained here, also failed to
detect effects on oligodendrocytes (34).
The fact that both vitamin D and EPO act
via at least one common pathway, MAPK
(35), as well as seem to share mech-
anism or interact regarding iron absorp-
tion or metabolism (36,37), may make fu-
ture studies on a potential interplay of
these agents attractive.

The beam balance test turned out to be
a sensitive clinical test for measuring dis-
ability and neuroprotection by EPO in the
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viously requires a more sophisticated
function of neuronal networks in the
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munication. In contrast, the rotarod was
used here as a crude motor control test,
indicating simpler and more elementary
functions. Not too surprising, it did not
show any evidence of compromised
function, an observation that had already
led other authors to develop more sensi-
tive tests for this model (38).

The cuprizone model has a number of
difficulties and limitations, some of
which have been mentioned in previous
reports (reviewed in [26] and [39]): (a) it
is variable, requiring relatively large
numbers of mice for solid conclusions;
(b) it is characterized by damage and re-
cuperation running in parallel, making
predictions of the right time point for
measurements difficult; (c) it shows a
rapid and remarkable spontaneous re-
covery after cessation of toxin feeding,
rendering it less suitable for extended
pharmacological neuroregeneration
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mechanisms of damage by the copper
chelator cuprizone are still incompletely
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mately explain why our search for
discussion
This study used the cuprizone model
to explore the protective potential of EPO in the
absence of T cell–mediated acute immune-
inflammatory processes. In this regard,
the model may simulate some aspects of
chronic progressive MS. Indeed, we
found neurological, imaging and histo-
logical evidence for a beneficial effect of
EPO in this condition.

The strong inflammatory component
in this model is reflected by the highly
increased numbers of microglia in the
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![Figure 4. Cuprizone-induced alterations in brain mRNA levels of cytokines and microglia activity markers: effect of EPO. All qPCR analyses presented have been performed after 6 wks of cuprizone ± EPO. Analyses of mRNA for cytokines (IL-1α, IL-1β, TGF-β) (A–C) and microglia activity markers (C1qA, Trem-2) (D, E) yielded significant shifts under cuprizone (all P < 0.05). EPO showed in several cases only a slight tendency in the respective other direction (all P > 0.05). Expression levels are normed to the normalization factor (NF; i.e. geometric mean of GAPDH, β-actin, and HPRT1 mRNA levels). For all panels: n = 5 -11; mean ± SEM is presented. PL, placebo.](image-url)
further mechanistic insight by quantification of cytokines, known to be potentially influenced by EPO and/or cuprizone in other models (41–46), or of microglial activity markers (46,47) yielded tendencies at best, but no clear-cut results.

CONCLUSION

To conclude, despite all the problems connected with the cuprizone model, there was a surprisingly clear protective effect of EPO detectable, with not all readouts reaching nominal significance but essentially all parameters adding to a mosaic: We suggest that EPO ameliorates neurological symptoms in the cuprizone model of demyelination by reduction of inflammation-associated axonal degeneration in white matter tracts. This effect of EPO may also partially explain its beneficial action in chronic progressive MS, and is worthwhile to pursue.

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DISCLOSURE

H Ehrenreich has submitted/holds user patents for EPO in stroke, schizophrenia and MS.

REFERENCES


