ORIGINAL ARTICLE

High sensitivity to carcinogens in the brain of a mouse model of Alzheimer's disease

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Cancer and Alzheimer's disease (AD) are commonly found among elderly patients. Chronic inflammation is the characteristic of both diseases. Amyloid-β peptide is the main inducer of inflammation in AD. Moreover, chronic inflammation promotes cancer, suggesting that AD patients may be more prone to develop cancer than non-demented people. To test this hypothesis, we injected the carcinogen 20-methylcholanthrene in the brain of transgenic mice overexpressing the mutant forms of amyloid precursor protein (APP) and presenilin 1 (PS1), as a model of AD, and their wild-type (WT) littermates. Mutant mice developed tumors faster and with higher incidence than their WT counterparts. Expression of the inflammatory markers interleukin (IL)-1α, IL-1β, IL-6, IP-10 and tumor necrosis factor-α (TNF-α) was measured in AD and WT mice of 3 and 12 months of age that had not been exposed to the carcinogen. These cytokines were elevated in older AD mice, indicating the existence of a highly inflammatory milieu in these animals. We also found elevated expression of a mutated form of p53 in older AD mice, suggesting an alternative mechanism for the predisposition of AD brains to develop brain tumors. Clinical studies reporting comorbidity of AD and brain cancer are needed to understand whether our observations hold true for humans.

Introduction

Both cancer and Alzheimer's disease (AD) are common clinical findings in elderly people. The probability of developing either one of them increases with age (Anisimov, 2007), although for cancer this probability may experience a slight reduction after the eighth decade (Driver et al., 2008). Chronic inflammation is a common trait in the pathogenesis of both diseases (Leonard, 2007; Paugh et al. 2009). Nowadays, it is widely accepted that aging is accompanied by a low-grade chronic upregulation of proinflammatory responses and, even if in many individuals this inflammation remains subclinical, in some people it may promote a number of age-associated diseases such as cancer or AD (Giunta et al., 2008).

More than 20 epidemiologic surveys have consistently showed that common non-steroidal anti-inflammatory drugs may protect against the development of AD (Rogers, 2008) and, although mono-targeted therapies have proven so far ineffective in preventing AD, new agents that block several cellular pathways simultaneously are being analyzed (Harikumar and Aggarwal, 2008). It seems that amyloid-β peptide, which is derived from the longer amyloid precursor protein (APP), is the main stimulator of the inflammatory response found in the brain of AD patients (Sastre et al., 2008), resulting in the presence of activated microglia and astrocytes around neuritic plaques (Rodrigo et al., 2004) and increased levels of inflammatory mediators (Hoozemans et al., 2008). Some current hypotheses suggest that even peripheral chronic infections, such as periodontal episodes, may affect the onset and progression of AD (Kamer et al., 2008).

In contrast, the link between inflammation and cancer is widely accepted (Coussens and Werb, 2002; Hussain et al., 2003). During tumor progression a proinflammatory crosstalk gets established among the different components of the tumor and the stroma (Mbeunkui and Johann, 2009). Cancer cells and neighboring epithelial cells activate inflammatory pathways such as the arachidonic acid-cyclooxygenase cascade that secrete prostaglandins to the extracellular milieu. These molecules exert an effect as chemoattractants for macrophages and other infiltrating cells that would secrete proinflammatory interleukins, especially interleukin-6 (IL-6), in the proximity of the tumor. These interleukins promote tumor cell proliferation and clonal expansion (Hong et al., 2000), thus establishing a difficult-to-break positive feedback loop that may even stimulate metastasis production (Kim et al., 2009). In addition, several studies have found elevated expression of key oncogenes, such as p53 or epidermal

growth factor receptor, and loss of tumor suppressor genes, such as *PTEN* (phosphatase and tensin homolog deleted on chromosome 10), in AD patients when compared with age-matched controls (Griffin *et al.*, 2005; Lanni *et al.*, 2008; Uberti *et al.*, 2008; Di *et al.*, 2009).

Given this scenario, we would suspect that a high degree of comorbidity must exist for these two diseases. Clinical data on this issue are scarce and somehow contradictory. In most studies, cancer seems to be a prevalent comorbidity for patients with AD, and for males in particular (Gambassi *et al.*, 1999; Gasper *et al.*, 2005), although specific proportions vary from 8% (Formiga *et al.*, 2007, 2008) to almost 23% in patients with reported exposure to carcinogens (Yamada *et al.*, 1999). Other studies find that the risk of developing cancer, in organs distant from the central nervous system, is lower among AD patients than in non-demented patients (Beard *et al.*, 1996; Roe *et al.*, 2005). In a post-mortem histopathological study, 42% of patients diagnosed with a brain tumor also had unreported signs of AD (Nelson, 2002), indicating that the coexistence of both diseases may be grossly under-reported in the clinical literature.

To analyze whether there is a causal connection between AD and brain cancer, we designed a study in which genetically engineered mice that mimic AD and their wild-type (WT) counterparts were exposed to a carcinogen in the brain and the time until they developed brain tumors was recorded. Mice suffering from AD symptoms developed tumors much earlier and with higher incidence than control mice, indicating that AD may be a predisposing condition for brain cancer.

Results

Tumor generation in the brain of mutant and WT animals
Mice carrying the double transgene for mutated APP and presenilin 1 (PS1) and their WT littermates were exposed to equal intracranial amounts of the carcinogen 20-methylcholanthrene. The first symptoms of the presence of a brain tumor were lethargy, insecure gait and hair erection. In some cases, the tumor grew through the skull burr hole and under the scalp. The final phase of tumor progression was exponential and mice had to be killed at <48 h after the onset of symptoms for welfare reasons. Histologically, tumors were malignant gliomas (either astrocytomas or glioblastomas) as indicated by their invasiveness, the presence of nuclear atypia and specific histogenetic markers, including glial fibrillary acidic protein, Sox9 and vimentin (Figure 1). A case of sarcoma was detected surrounding the carcinogen that was incorrectly located in contact with the meninges in a double-mutant mouse (data not shown). This animal was excluded from the analysis. No histological differences were found between tumors produced in mutant or WT mice. Interestingly, double-mutant mice began to develop brain tumors much earlier than their WT counterparts and with higher incidence (88% for the mutants vs 54% in the WT). A Kaplan–Meier tumor-free survival curve shows

the striking differences between genotypes ($P<0.001$) with a mean tumor-free survival of 275 days for the WT animals and of only 80 days for the double transgenic mice (Figure 2).

Quantification of inflammatory mediators
Numerous studies point out the presence of inflammatory mediators in the AD brain (Sastre *et al.*, 2008; Salminen *et al.*, 2009). To analyze whether this is also true in our mouse model and whether these inflammatory markers increase with age and disease progression, we performed quantitative real-time PCR for several inflammatory mediators (Figure 3). Expression of IL-1α, IL-1β and IP-10 increased in older mutant mice when compared with younger animals (Figures 3a, b and d). IL-6 and tumor necrosis factor-α (TNF-α) had a similar expression pattern, in which older mutant mice had significantly higher expression than younger mice of the same genotype and than WT animals of the same age (Figures 3c and e). These data indicate that mice suffering from AD symptoms present a brain parenchyma that is rich in inflammatory cytokines.

Oncogene expression quantification
Accumulation of altered forms of p53 has been reported in AD patients (Uberti *et al.*, 2008). In our animal model we have observed a significant increase in the expression of mutated p53 in older AD animals when compared with younger mutants and with WT counterparts of the same age (Figure 3f), thus confirming the previously reported accumulation of these p53 variants.

Discussion

In this study we have shown that mice carrying mutations in the AD-specific genes, APP and PS1, are much more sensitive to the carcinogen 20-methylcholanthrene than their WT counterparts. In addition, aged AD mice that had not been exposed to carcinogens presented higher levels of the proinflammatory mediators IL-1α, IL-1β, IL-6, IP-10 and TNF-α than younger or WT mice. The tumor marker p53 was also elevated in untreated AD mice when compared with their WT littermates.

Tumors developed in our WT mice with a timeframe (mean = 275 days) perfectly in agreement with Zimmerman's initial manuscript (mean = 279 days for gliomas; Zimmerman and Arnold, 1941). In our experimental set, we obtained a 54% tumor incidence rate among the WT animals in comparison with 47% observed in Zimmerman's paper (Zimmerman and Arnold, 1941). This small discrepancy may be due to the use of different mouse strains. On the other hand, double transgenic mice generated tumors faster (mean = 80 days) and had a higher tumor incidence rate (88%), indicating that AD may predispose to develop brain tumors in the presence of a carcinogen.

The concept of 'seed and soil', initially coined by Stephen Paget, postulates the important role that the
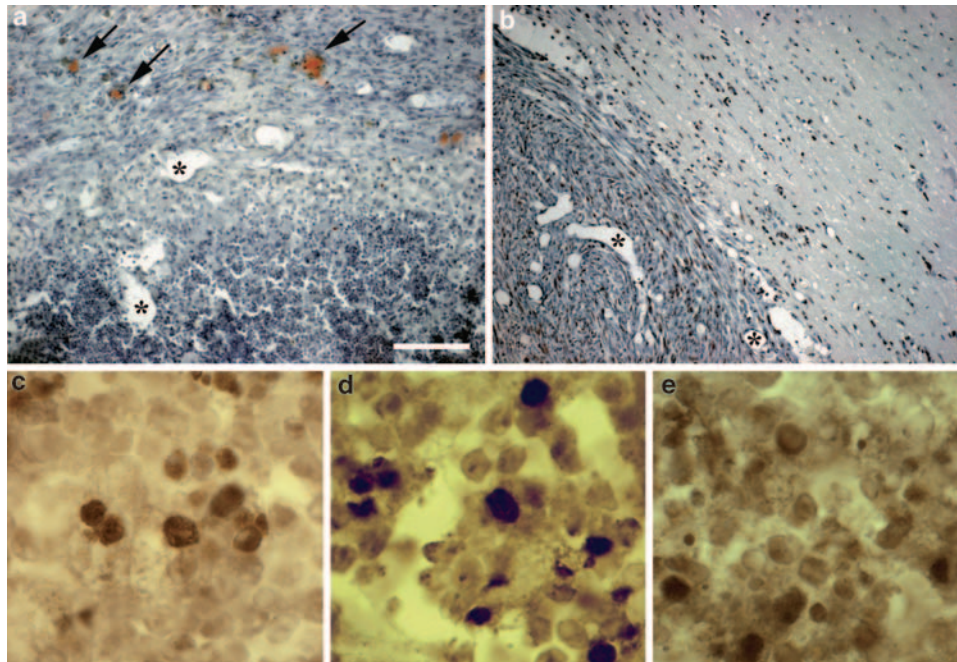
Figure 1 Histological sections of the brain of double transgenic (**a**) and wild type (WT) (**b**) mice, which had been injected with the carcinogen and developed tumors, stained with Congo red and counterstained with hematoxylin. Neuritic plaques containing β-amyloid stain positive for the Congo red dye in the APP-PS1 mice (arrows in **a**) but they are absent in the parenchyma of the WT animals (**b**). Tumors and their periphery contain a large number of blood vessels (asterisks in **a, b**). Immunohistochemical staining for glial fibrillary acidic protein (GFAP) (**c**), Sox9 (**d**) and vimentin (**e**) in a tumor that developed in a mutant mouse. Bar = 200 μm for **a** and **b**, and 20 μm for **c–e**.
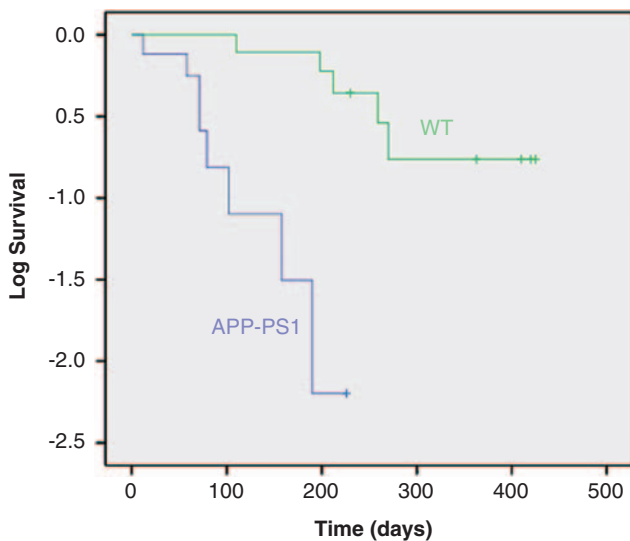

Figure 2 Kaplan–Meier tumor-free survival curves comparing the double mutant mice (APP-PS1) ($n = 9$) with their wild-type (WT) littermates ($n = 10$) after carcinogen exposure at day 0. Little crosses represent censored data. Analysis of the curves shows a 50% tumor-free survival for APP-PS1 animals of 80 days whereas for WT mice it goes to 275 days. An important statistically significant difference was observed between genotypes ($P < 0.001$).

microenvironment has in the development of primary and metastatic tumor growth, and proinflammatory stimuli have primary roles in the conditioning of the tumor niche (Ribatti et al., 2006; Psaila and Lyden,

2009). Inflammation has been shown to be critical for promoting apoptosis resistance, proliferation, invasion, metastasis and secretion of proangiogenic and immunosuppressive factors in tumors of different origins, including lung (Peebles et al., 2007), prostate (Stock et al., 2008), breast (Hu and Polyak, 2008), stomach (Correa and Houghton, 2007), pancreas (Chu et al., 2007), gut (Quante and Wang, 2008) and, of course, the brain (Murat et al., 2009; Paugh et al., 2009). Thus, it is easy to imagine how the brain parenchyma of an AD patient, which is rich in proinflammatory mediators, would be a good environment for the development and progression of brain tumors.

To show that the brain of AD mice had higher levels of proinflammatory markers, the expression of several interleukins was analyzed using real-time PCR in the brain of untreated mutant and WT mice. IL-1α, IL-1β, IL-6, TNF-α and IP-10 were all upregulated in older AD mice, suggesting that their brain parenchyma was specially prepared to host a developing tumor. IL-1 is a cytokine that promotes tumorigenesis, tumor invasiveness, metastasis and tumor–host interactions (Apte et al., 2006). IL-6 is also involved in tumor progression and it has been shown clinically that its levels correlate negatively with patient survival (Chang et al., 2005). TNF-α induces tumor cell death but it can also exert an effect as a mitogen and migration agent (Chicoine and Silbergeld, 1997), representing a dual component of the inflammatory cascade that can either drive or repress tumor growth depending on the environmental context. IP-10, also known as CXCL10, is an antiangiogenic
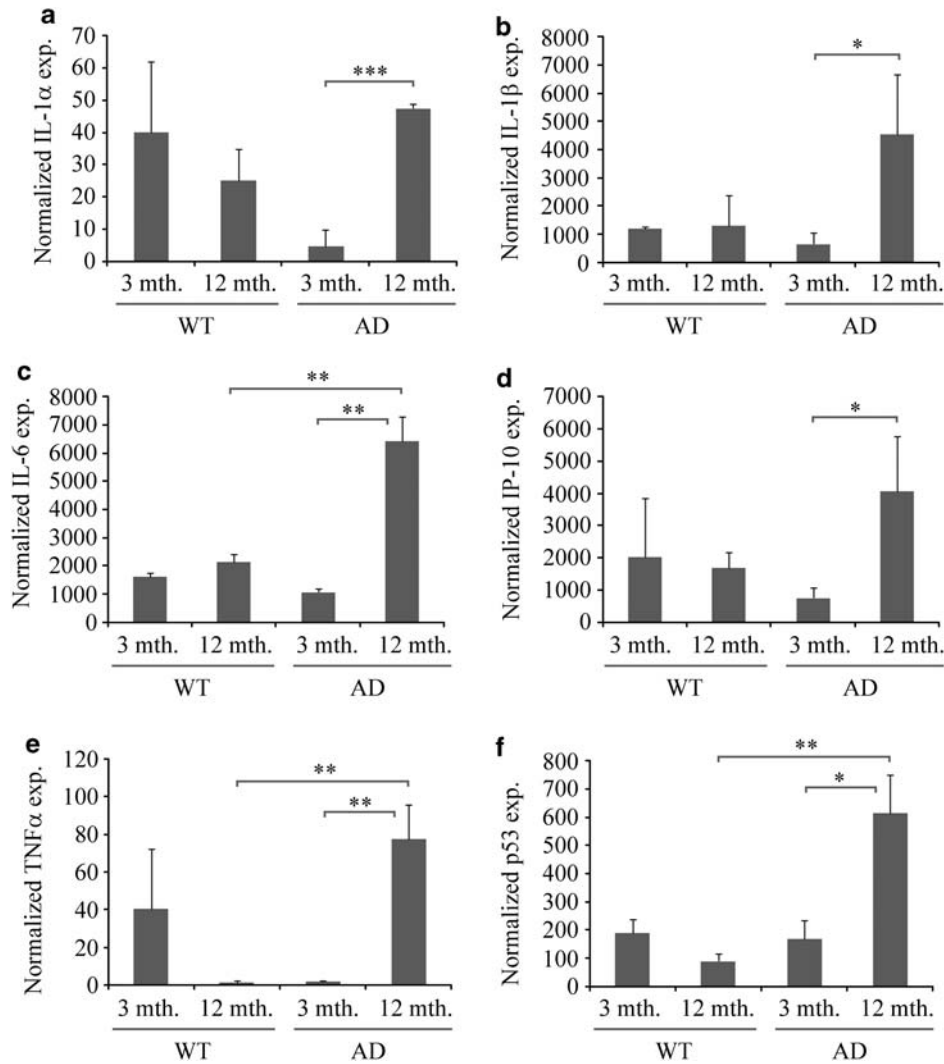
Figure 3 Expression of inflammatory markers and oncogenes as revealed using quantitative real-time PCR. RNA was extracted from the brain of double-mutant (AD) and wild-type (WT) mice at the ages of 3 and 12 months (mth.). After reverse transcription, the complementary (c)DNAs were tested for IL-1α (**a**), IL-1β (**b**), IL-6 (**c**), IP-10 (**d**), TNF-α (**e**) and p53 (**f**). All data were normalized by the 18S ribosomal (r)RNA value in each sample. Bars represent mean ± s.d. of three independent samples. Statistically significant differences are indicated by asterisks. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

protein whose expression is regulated by IL-1β, interferon-γ and TNF-α (Yeruva et al., 2008); thus, in view of the overexpression of these markers in the AD brains, the activation of this downstream protein is expected. Although IP-10 can be considered an 'anti-tumoral' cytokine, its upregulation in AD mice shows that there is a marked elevation of all members of the inflammatory cascade. In summary, all these data suggest that the cerebral parenchyma of mice suffering from AD symptoms contains a dangerous cocktail of proinflammatory mediators that would promote tumor growth once the initial malignant mutations occur. An important experiment to be performed in the future will imply the chronic treatment of AD mice with anti-inflammatory drugs to analyze whether there is a delay in brain cancer onset.

But inflammation is not restricted to AD among neurodegenerative disorders and other common diseases of the CNS (Steinman, 2008). On the contrary, inflammatory mediators are commonly produced in Parkinson's disease (Tansey et al., 2008), multiple sclerosis (Sanders and De, 2007), schizophrenia (Saetre et al., 2007) or AIDS dementia complex (Vesce et al., 2007), to cite just some examples. Therefore, we could expect that the brain microenvironment in all these conditions will be especially conducive for cancer growth. Clinical studies correlating inflammatory diseases of the brain and tumor development and progression are warranted.

An interesting observation is the accumulation of mutated p53 in older AD mice. This has been previously reported in patients (Uberti et al., 2008) and has been shown as a link between neural stem cells and tumors of the brain (Wang et al., 2009). The researchers of these studies postulate that the AD brain is characterized by high levels of reactive oxygen/nitrogen species and a concomitant decrease in the levels of antioxidant enzymes (Di et al., 2009). Under these conditions, the mutant-like form of p53 becomes elevated, glutathiony-

lated and suffers conformation changes in a way that favors monomers or dimers while preventing formation of the tetramers, which is the aggregate form needed for effective action of p53 (Lanni et al., 2008; Di et al., 2009). As p53 is the main guardian of the cell division checkpoint, blocking this molecule will result in progressive accumulation of mutational events and will eventually lead to cancer development. Obviously, this oncogene hypothesis is complementary rather than mutually excluding with the involvement of inflammation in the onset of the brain tumors.

Clinical studies reporting the comorbidity of brain tumors and other diseases of the central nervous system are needed to confirm whether these observations in mouse models correlate with the human paradigm.

Materials and methods

Animals
Two transgenic mice strains, carrying the human mutated alleles for APP (Hsiao et al., 1996) or PS1 (Duff et al., 1996), were a generous gift from Professor Ignacio Torres (Instituto Cajal, Madrid, Spain). Both strains were crossed to obtain heterozygotes for both alleles, which were used as the parental generation for the experiment. The offspring of these double heterozygotes were genotyped and the WT and double-mutant male littermates were chosen for carcinogen exposure. Double mutants for APP and PS1 (APPswe/PS1-A246E) have been shown to produce AD symptoms much earlier than either one of the single mutants (Borchelt et al., 1997).

Carcinogen exposure
Following Zimmerman's methodology (Zimmerman and Arnold, 1941), 20-methylcholanthrene (MP Biomedicals, LLC, Solon, OH, USA) pellets were deeply implanted into the brains (right parietal subcortex) of 9-month old mice (9 double mutants and 10 WT littermates) by intracranial incision using stereotaxic guidance. Mice were deeply anesthetized with pentobarbital (10 mg/kg) and atropine (90 mg/kg) intraperitoneally and placed in a mouse stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). While under deep anesthesia, a sagittal incision was made through the skin to expose the skull, and a burr hole was drilled at 0.1 mm anterior and 2.25 mm lateral to bregma. A Hamilton syringe (Hamilton,

Reno, NV, USA) with a 27-gauge needle was inserted at the depth of 2.7 mm from brain surface to open an access to the CP, and the crystals were inserted there with the help of thin forceps. The burr hole was then filled with spongostan (Ferrosan A/S, Soeborg, Denmark) and the scalp sutured. The animals were periodically observed for signs of neurological alterations and/or tumor growth and were killed when these alterations compromised their comfort and quality of life. Some brains were fixed with 4% paraformaldehyde in phosphate buffer 0.1 M pH 7.4, stored in cryoprotectant (30% sucrose in phosphate buffer) overnight and cut into 40-μm-thick sections with the help of a cryostat. These sections were then stained. Stains included Congo red and hematoxylin to study morphology and the presence of neuritic plaques and immunohistochemistry with antibodies against glial fibrillary acidic protein (1:1000, rabbit polyclonal, DK 2600, Dako, Glostrup, Denmark), vimentin (1:1000, rabbit polyclonal, Abcam, Cambridge, MA, USA) and Sox9 (1:2000, rabbit polyclonal). The presence of transcription factor Sox9 was screened with antibodies that have been previously characterized (Stolt et al., 2003) and were a generous gift from Dr Michael Wegner (Institut fur Biochimie, Universitat Erlangen-Nuremberg, Germany). Immunohistochemistry was performed following standard protocols (Serrano et al., 2008). Kaplan–Meier tumor-free survival curves were plotted and statistically analyzed using SPSS software (SPSS, Inc., Chicago, IL, USA).

All procedures were carried out in accordance with the European Communities Council Directive (86/609/EEE) on animal experiments and with approval from the ethical committee of the Instituto Cajal.

Expression analysis of oncogenes and inflammatory mediators
Additional animals ($n = 3$ for each group) of different ages (3 and 12 months) and genotypes that had not been exposed to the carcinogen were used to obtain mRNA. After deep anesthesia, the brain was rapidly dissected out and frozen in liquid N_2. The RNA was extracted with Trizol (Invitrogen, Carlsbad, CA, USA) and reverse transcribed using SuperScript reverse transcriptase (Invitrogen). Real-time PCR was performed using the Chromo4 (MJ Research, Hercules, CA, USA) thermocycler and software. Amplification was performed in a final volume of 25 μl, containing 2 μl complementary DNA (diluted 1:10), 2 μl of primer mixture (at 10 nM) and 12.5 μl of $2 \times$ SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). Values were determined by interpolation within a standard curve. At the

Table 1 Primers used for quantitative real-time PCR

Gene	Primer sequence	Annealing temperature
IL-1α	Sense: 5′-GCACCTTACACCTACCAGAGT-3′ Antisense: 5′-AAACTTCTGCCTGACGAGCTT-3′	60 °C
IL-1β	Sense: 5′-GCAACTGTTCCTGAACTCAACT-3′ Antisense: 5′-ATCTTTTGGGGTCCGTCAACT-3′	60 °C
IL-6	Sense: 5′-GAAACCGCTATGAAGTTCCTCTCTG-3′ Antisense: 5′-TGTTGGGAGTGGTATCCTCTGTGA-3′	60 °C
IP-10	Sense: 5′-CAGTGAGAATGAGGGCCATAGG-3′ Antisense: 5′-CGGATTCAGACATCTCTGCTCAT-3′	60 °C
TNF-α	Sense: 5′-CCCTCACACTCAGATCATCTTCT-3′ Antisense: 5′-GCTACGACGTGGGCTACAG-3′	60 °C
p53	Sense: GTCACAGCACATGACGGAGG-3′ Antisense: 5′-TCTTCCAGATGCTCGGGATAC-3′	60 °C
18S rRNA	Sense: 5′-ATGCTCTTAGCTGAGTGTCCCG-3′ Antisense: 5′-ATTCCTAGCTGCGGTATCCAGG-3′	60 °C

Abbreviations: IL, interleukin; rRNA, ribosomal RNA; TNF-α, tumor necrosis factor-α.

end of the PCR, a melting curve was generated to ascertain amplicon quality. All gene expression values were normalized according to the 18S ribosomal RNA concentration of each sample. The primers are shown in Table 1.

Conflict of interest

The authors declare no conflict of interest.

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