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An effective novel delivery strategy of rasagiline for Parkinson's disease

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ABSTRACT

This is the first report on the efficacy of a new controlled release system developed for rasagiline mesylate (RM) in a rotenone-induced rat model of Parkinson's disease (PD). PLGA microspheres in vitro released RM at a constant rate of 62.3 μg/day for two weeks. Intraperitoneal injection of rotenone (2 mg/kg/day) to Wistar rats produced typical PD symptoms. Catalepsy, akinesia and swim tests outcomes in animals receiving RM either in solution or within microspheres showed a reversal in descent latency when compared to rotenone-treated animals, being this reversal specially pronounced in animals receiving RM microspheres (dose equivalent to 1 mg/kg/day RM injected i.p. every 15 days). Nissl-staining of brain sections showed selective degeneration of the substantia nigra (SNc) dopaminergic neurons in rotenone-treated animals which was markedly reverted by RM microspheres. PET/CT with ^{18}F-DG resulted in mean increases of accumulation of radiotracer in striatum and SNc of around 40% in animals treated with RM microspheres which also had significant beneficial effects on Bcl-2, Bax, TNF-α mRNA and SOD2 levels as detected by real-time RT-PCR. Our results confirm the robust effect achieved by the new controlled release system developed for RM which exhibited better in vivo efficacy than RM given in solution.

1. Introduction

Parkinson's disease (PD) is one of the most prevalent neurodegenerative diseases which affects around 2% of adults over the age of 60 years (Nussbaum and Ellis, 2003). The main symptoms of PD are related to movement abnormalities, such as rigidity, tremor, akinesia, bradykinesia, masked face and gait and, posture alterations. PD patients are also often characterized by weight loss which usually begins before the process is diagnosed and continues with disease progression. The core pathological feature of PD is the accelerated loss of dopaminergic neurons in the substantia nigra (SNc), associated with abnormal protein aggregation (Taylor et al., 2002). The pathogenesis of PD remains still undetermined; however, factors contributing to its development are aging, mitochondrial dysfunction, oxidative stress and apoptosis (Mounsey and Teismann, 2011). Current therapies for PD ameliorate symptoms in the early phases of disease but become less effective over time (Gárdián and Vécsei, 2010).

Rasagiline (N-propargyl-1-R-aminoindan) mesylate (RM), a selective and irreversible second generation inhibitor of monoamine oxidase type B (MAO-B) (Mandel et al., 2005; Weinreb et al., 2010), is used for symptomatic treatment as monotherapy in early PD (Parkinson Study Group, 2002), or as adjuvant therapy for levodopa in advanced stages of the disease (Parkinson Study Group, 2005; Rascol et al., 2005). RM has been introduced in therapeutics in 2005 under the brand name Azilect® (0.5 mg and 1.0 mg, RM) which is given as oral tablets. The maximum oral dose recommended in monotherapy as well as in adjunct therapy is 1.0 mg once daily due to the risks associated with non-selective inhibition of MAO if daily doses exceed the maximum recommended dose.

Several studies have reported that RM can prevent cell death induced in vitro by several dopaminergic toxins including 1-methyl-4-phenyl pyridinium (MPP^+), the nitric oxide donor 3-morpholinosydnoniminehydrochloride (SIN-1), 6-hydroxydopamine (6-OHDA), glutamate, N-methyl(R)salsolinol, β-amyloid, peroxynitrite, tetrahydroisoquinoline, and serum and growth factor deprivation (Maruyama et al., 2002; Mandel et al., 2005). Neuroprotection by RM has been confirmed in vivo in several animal models including neurodegeneration induced by the neurotoxins MPTP (*N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) and 6-hydroxydopamine (6-OHDA) (Blandini et al., 2004a; Sagi et al., 2007). However, these animal models cannot show direct relevance to human PD, that is; motor deficits, formation of Lewy bodies and gradual loss of dopamine neurons in adults, based on a single mutation and short time course (Betarbet et al., 2000).

Several studies have used rotenone as an experimental model of PD (Dong et al., 2003; Sherer et al., 2003; Alam and Schmidt, 2002). Rotenone is a natural compound obtained from the roots of certain plant species which is frequently used as an alternative to synthetic pesticides. Rotenone is highly lipophilic being able to

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easily and rapidly cross the blood–brain barrier. Rotenone exhibits high-affinity for specific inhibition of mitochondrial complex I, which exerts its inhibitory action by blocking electron transfer in nicotinamide adenine dinucleotide diaphorase-quinone (NADH-Q) reductase, thus preventing the utilization of nicotinamide adenine dinucleotide diaphorase (NADH) as an enzyme in oxidative phosphorylation (Betarbet et al., 2000). Taking all these facts into consideration, the rotenone model appears to be a more accurate model in that systemic complex I inhibition results in specific, progressive and chronic degeneration of the nigrostriatal pathway similar to that observed in human PD (Betarbet and Sherer, 2002).

RM is rapidly absorbed and achieves the peak plasma concentration within 30 min; however is has a low oral bioavailability (36%) with a very short elimination half-life (0.6–2 h) (Chen et al., 2007). These biopharmaceutic and pharmacokinetic characteristics and its efficacy in a chronic disease such as PD make RM a suitable candidate for the development of a controlled release system. The use of biodegradable and biocompatible microspheres for the controlled release of RM could represent an attractive alternative to its oral administration. The system would not require its removal once the treatment is finished and controlled drug release from the microparticles will allow decreasing the number of administrations thereby leading to a better patient compliance and possibly a reduction of adverse side-effects.

This is the first report on the efficacy of a new controlled release system developed for rasagiline mesylate (RM). In this study, we explored the neuroprotective potential of chronic administration of RM-loaded PLGA microspheres in a rodent model of rotenone-induced PD. We have selected poly-D,L-lactide-coglycolide (PLGA) microspheres as RM carriers due to its excellent tissue compatibility, biodegradable nature, safety profile and ability to control the release of drugs (Gander et al., 2001).

2. Materials and methods

2.1. Chemicals

Rasagiline mesylate (RM) was obtained from Hangzhou Onion Chemical Co. Ltd. (China). Resomer® RG 502 (poly D,L-lactide-co-glycolide, 50:50), Mw 15,000 Da, inherent viscosity 0.2 dL g^{-1} was obtained from Boehringer Ingelheim Chemicals Division (Germany). Polyvinyl alcohol (PVA) Mw 72,000 Da was purchased from Merck (Germany). Water was purified by Milli-Q filtration system (Millipore, USA) and used in the preparation of buffers/solutions. Rotenone was purchased from Sigma–Aldrich (USA). Sunflower oil was obtained from Sigma–Aldrich (Spain). All other reagents/solvents used were of analytical grade and purchased from Panreac (Spain).

2.2. Animals

Male Wistar rats (Harlan, France, 180–220 g, 2-month old at the beginning of the experiment, $n = 43$) were housed and maintained in a room at $22 \pm 2\,^{\circ}C$ with automatic light cycles (12 h light/dark). Food and water were offered *ad libitum* throughout the study. All procedures involving animals and their care were approved by the ethics committee of the Universidad Complutense de Madrid, and were conducted in accordance with the European Community Council Directive (86/609/EEC). All experiments conformed to international guidelines on the ethical use of animals. All efforts were made to minimize the number of animals used and their suffering.

2.3. Preparation and characterization of RM-loaded PLGA microsphere: in vitro release tests

Microspheres were prepared by oil–water (O/W) solvent evaporation method. Briefly, the organic phase was prepared by dissolving PLGA (400 mg) in 1 ml of CH_2Cl_2 using a vortex mixer and then dispersing 40 mg of pulverized RM in the PLGA solution by means of agitation with vortex mixer and further sonication (Heat Systems-Ultrasonics Inc., USA). The aqueous phase consisted of 5 ml PVA (1%, w/v, pH 10). Both phases were emulsified with a Polytron® system (RECO Kinematica GmbH PT 3000, Germany) for 60 s at 2500 rpm. The resulting O/W emulsion was immediately poured into 100 ml of PVA solution (0.1%, w/v, pH 10) with 5% NaCl and continuously stirred for 3 h at room temperature with a magnetic stirred (400 rpm) to allow complete evaporation of the organic solvent. The microspheres were vacuum-filtered through 5-μm membrane filters, washed three times with deionised water and freeze-dried (Flexy-Dry™, FTS Systems, USA) for at least 12 h. The lyophilized microspheres were kept in a desiccator until use. Blank PLGA microspheres were also prepared. The morphology of the microspheres was investigated by scanning electron microscopy (SEM, Jeol, JSM-6400, Japan).

To determine loading efficiency RM-loaded PLGA microspheres (30 mg) were dissolved in 5 ml of CH_2Cl_2 and extracted three times with 5 ml PBS (pH 7.4). The mixture was centrifuged (Universal 32, Hettich, Germany) at $6000 \times g$ for 5 min, the supernatant extracted, transferred to a 20 ml volumetric flask and diluted with PBS to complete the volume. All samples were filtered through 0.45 μm filters. Quantification of RM was performed by the HPLC method previously developed and validated by the authors (Fernández et al., 2009).

In vitro release of RM from the microspheres was investigated in PBS at pH 7.4. For this, microspheres (20 mg) were suspended in 3 ml of PBS (sink conditions) in a water shaker bath (NE-5, Clifton, UK) at 37 °C with constant agitation (100 strokes per minute). At predetermined time intervals samples were centrifuged and then the supernatant was completely extracted with a syringe, filtered through 0.45-μm filters and replaced with the same volume of fresh medium. Quantification of RM was performed by HPLC (Fernández et al., 2009). In vitro release tests were performed in triplicate.

2.4. Drugs

The rotenone was dissolved in sunflower oil and animals received 2 mg/kg rotenone per day. Control animals received vehicles; oil or saline at 2 ml/kg. RM, blank PLGA microspheres and RM-loaded PLGA microspheres were suspended in saline before administration. RM encapsulated in PLGA microspheres was assayed at two dose levels (0.5 mg/kg/day and 1 mg/kg/day). Doses were selected on the basis of previous studies conducted in our laboratory and reported in the literature (Parkinson Study Group, 2005). For this, appropriate amounts of RM-loaded PLGA microspheres were injected intraperitoneally in order to maintain RM levels for 15 days; therefore, 3 administrations were required (0, 15 and 30 days). The amount of microspheres injected was adapted to variations in animal weight throughout the study (32.5–50 mg for 0.5 mg/kg/day and 75–100 mg for 1 mg/kg/day). The amount of blank PLGA microspheres injected i.p. corresponded to that of the highest dose level of RM assayed (1 mg/kg/day). The administration of RM-loaded PLGA microspheres was performed every 15 days according to the zero-order release kinetics of the drug obtained in vitro.

2.5. Experimental design

Animals were divided into 6 groups each containing seven animals, except for group G1 which was composed of 8 animals. The first group (G1) received the vehicle of rotenone (sunflower oil) ($n=4$) or saline ($n=4$). Group 2 (G2) received rotenone (2 mg/kg/day) for 45 days. Groups 3–6 received rotenone (2 mg/kg/day) for 45 days and: blank PLGA microspheres (Group 3, G3); RM-loaded PLGA microspheres (high dose; amount of microspheres equivalent to 15 mg/kg RM injected every 15 days) (Group 4, G4); RM-loaded PLGA microspheres (low dose; amount of microspheres equivalent to 7.5 mg/kg RM injected every 15 days) (Group 5, G5); and RM in saline (1 mg/kg/day) (Group 6, G6). All treatments were given intraperitoneally.

The experimental groups can be summarized as follows:

Group 1 (G1): vehicle (sunflower oil or saline).
Group 2 (G2): rotenone.
Group 3 (G3): rotenone + blank PLGA microspheres.
Group 4 (G4): rotenone + RM-loaded PLGA microspheres (high dose).
Group 5 (G5): rotenone + RM-loaded PLGA (low dose).
Group 6 (G6): rotenone + RM in saline.

At the end of the study animals were killed by decapitation with the use of a guillotine. The brains were removed rapidly within 25–50 s and immediately frozen by slow immersion in isopentane cooled on dry ice. Frozen tissues were stored at −80 °C until analysis.

2.6. Body weight assessment

During the experimental study period, animals from each group were weighed on days 1, 5, 10, 15, 20, 25, 30, 35 and 45.

2.7. Behavioural testing

2.7.1. Catalepsy test

The term implies the inability of an animal to correct an externally imposed posture. In our study catalepsy tests were performed on days 15, 30 and 45. For this, each rat was hung by all four paws on a vertical grid (25.5 cm wide and 44 cm high with a space of 1 cm between each wire) and a stopwatch was started as soon as the rat held onto the grid. As the animals started to move their paws or showed first movement the stopwatch was stopped and the time noted as descent latency.

The second part of the catalepsy study was the bar test. The rats were placed with both forepaws on a bar which was 10 cm above and parallel from the base. The rats were placed with both forepaws on the bar in a half-rearing position. Latency with removal of the paw was noted. The maximum descent latency was fixed at 180 s for both tests. Catalepsy tests were repeated three times for each animal.

2.7.2. Akinesia test

Akinesia tests (difficulty in initiating movement) were conducted on days 15, 30 and 45 of the experiment. Measurement of akinesia was performed by noting the latency in seconds (s) of the animals to move all four limbs with the test finished when latency exceeded 180 s (Haobam et al., 2005). Before carrying out each akinesia test animals were acclimatized for 5 min on a wooden elevated (100 cm) platform (100 cm × 150 cm) used for measuring akinesia in rats. Using a stopwatch, the time taken by the animal to move all the four limbs was recorded. The test was performed in triplicate for each animal.

2.7.3. Swim-test

For the swim-test we have adapted the method described by Haobam et al. (2005). This test was used to assess motor impairment and it was performed three times throughout the study; each one carried out the day after catalepsy/akinesia tests were done. The test was performed in water tubs (40 cm length × 40 cm width × 30 cm height). The depth of water was kept at 30 cm and the temperature was maintained at 27 ± 2 °C. The animals were wiped dry right after the experiment using a dry towel. Swim-scores were recorded on a performance intensity scale of 0–3: 0, hind part sinks with head floating; 1, occasional swimming using hind limbs while floating on one side; 2, occasional floating/swimming only; 3, continuous swimming. Behavioural performance in the swim-test was videotaped for 5 min and analyzed by using video replay. The test was repeated three times for each animal.

All behavioural tests were performed during the animal's light cycle.

2.8. Histology

In order to perform the histological analyses on brain sections Nissl staining was carried out. For this, forty-micrometer brain coronal sections were taken serially through of substantia nigra according to the rat brain atlas of Paxinos and Watson (1998) using a cryostat (Leica CM1850, Germany) at −20 °C. The slices were thaw-mounted onto gelatine-coated and slides were stored at −80 °C until analysis.

The Nissl staining of brain sections was performed as described by Sindhu et al. (2005) but with some modifications. Briefly, brain sections were fixed with 4% formaldehyde in buffer Tris–HCl 50 mM, pH 7.4. Then the brain sections were stained for 30 min with 0.5% cresyl violet (Sigma–Aldrich Chemical Co., USA) in 0.34 M acetic acid. After rinsing in water, the sections were submerged in ethanol 70% 6-times; then submerged in ethanol 95% and 100%, 5 min each. Finally, the sections were dehydrated with xilene for 10 min. The sections were mounted and sealed under coverslips, and then viewed under microscope (Leica DC300F, Germany) and photographed.

2.9. Positron emission tomography/computed tomography (PET/CT)

To perform PET/CT 12 h before the beginning of the studies the animals were left without food, in order to avoid interference of plasma glucose with positron-emitting radiotracer ^{18}F-deoxyglucose (^{18}F-DG). All the animals (control ant treated) were studied with the marker ^{18}F-DG (18.5 MBq/rat), administered via a tail vein. To ensure immobilisation during all the microPET studies, the rats were anaesthetised by inhalation of isofluorane (2% isofluorane in 100% oxygen). Depth of anaesthesia was monitored by measuring respiration frequency. Animals were fixed with adhesive tape and positioned in the camera such that the head was placed symmetrically in the center of the field of view. Body temperature was controlled by rectal probe.

^{18}F-DG imaging was performed in groups G1, G2, G4 and G6. For each group 4 rats with a similar weight were used. The time point of tracer injection into the first animal was set as $t=0$ and represented the reference time point for all decay corrections. Immediately after radioligand injections, animals were anesthetised. The images were obtained by using a microPET Albira ARS (Oncovisión, Spain), with 1.5 mm resolution, 4.0 cm axial field and 8.0 cm transaxial field. The PET images obtained for the animal were confluenced with CT images to obtain transverse, sagital and coronal PET images, CT images and PET/CT images. The PET/CT scan was started immediately after 30 min since ^{18}F-DG injection and continued for 30 min

(20 min for PET scanner and 10 min for CT images). All PET images were reconstructed using an OSEM cross algorithm and, death time, decay and dispersion corrections were applied. The CT images were reconstructed with FBP (filtered back projection) algorithm. Images of functional (PET) and structural (CT) were finally adjusted in an automatic spatial manner.

The studies were quantified through the definition of the volumes of interest (VOIs) from the activity images obtained with the Albira ARS system. Image analysis was performed using PMOD software 3.0 on reconstructed images with and without scatter correction for each study.

2.10. Real time quantitative PCR (RT-PCR)

RT-PCR was performed in animal groups G1, G2, G4 and G6. For this, the striatum of the rats were used for total RNA isolation using the method described by the manufacturer (RNeasy Kit, Qiagen, Spain). RNA was eluted in RNAse free water, aliquoted and stored at −70 °C. RNA concentration was measured at 260 nm, and sample quality was checked using RNA Nano LabChips in a 2100B Bioanalyzer (Agilent Technologies, USA).

The transcriptional profile was analyzed with the use of the GeneChip® Affymetrix platform and the Mouse Genome 430 2.0 microarray. This array contains 45,000 mouse probe sets representing more than 39,000 transcripts and variants. Sequences for probe sets were selected from Gen-Bank®, dbEST and RefSeq databases (http://www.affymetrix.com). Five micrograms of total RNA from each biological sample were used as starting material for all array hybridization protocols. These protocols were performed as described by the manufacturer (Affymetrix, USA). For each treatment, four samples for independent assays were tested.

Changes in the expression of selected genes were assessed by quantitative PCR with the use of the same RNA sample extracted for microarrays. Reverse transcription of 1 μg total RNA (20 μl final volume) for cDNA synthesis was performed using the High Capacity cDNA Archive Kit (Applied Biosystems, USA) and random primers following the recommendations of the manufacturer.

Amplification was performed using a total reaction volume of 20 μl in a MicroAmp Optical 96-well reaction plate covered by optical adhesive covers. Real-time reactions were carried out using the "TaqMan® Fast Universal PCR Master Mix" (Applied Biosystems, USA) and LNA probes from the "Universal ProbeLibrary" (Roche Applied Science, USA), or the "Power SYBR Green PCR Master Mix" (Applied Biosystems, USA). Primers and probes for each gene were designed at the "Universal Probe Library Assay Design Center" (http://www.roche-applied-science.com). The forward and reverse primers were supplied by Isogen Life Science (Spain) and the final concentration of primers and probes on the assay was 200 and 100 nM, respectively. All primers and probes for quantitative PCR are described in Table 1. For the normalization of cDNA loading in the PCR reaction, the amplification of 18S ribosomal RNA (rRNA) for every sample was used. Relative changes in gene expression were calculated using the $2^{-\Delta\Delta CT}$ method (Livat and Schmittgen, 2001).

Quantitative PCRs were run on a 7900HT Fast Real Time PCR System (Applied Biosystems, USA) with a denaturation step at 95 °C for 20 s followed by 40 PCR cycles of denaturation at 95 °C for 1 s and annealing/extension at 60 °C for 20 s. All PCR assays for a particular gene were done at the same time under identical conditions and carried out in duplicate.

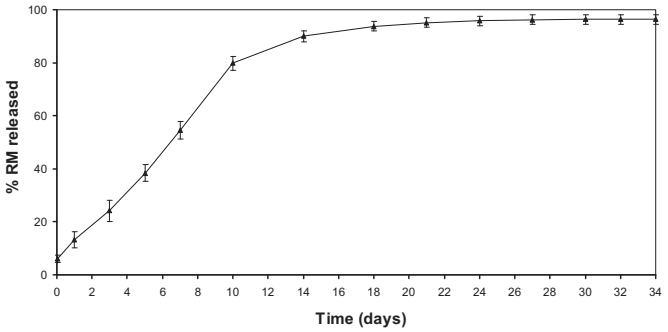

Fig. 1. Mean release profiles (± S.E.M., $n = 3$) of RM from PLGA microspheres. RM: rasagiline mesylate.

2.11. Statistical analysis

Results are expressed as mean ± standard error of the mean (mean ± S.E.M). Statistical analyses were carried out using the Statgraphics® *Plus* v. 5.1 software (John Wiley and Sons, USA). The catalepsy and akinesia data were analyzed for statistical significance employing non-parametric analyses (multifactorial Kruskal–Wallis one-way ANOVA followed by Mann–Whitney *U*-test). $P < 0.05$ and $P < 0.01$ were taken as levels of statistical significance.

3. Results and discussion

This is the first report on the efficacy of a new controlled release system developed for parenteral administration of rasagiline mesylate (RM). We investigate a novel delivery strategy for RM in a rotenone-induced animal model of Parkinson's disease. The biodegradable RM microspheres were prepared by solvent evaporation technique from an oil–water emulsion using PLGA as encapsulating biomaterial. RM-loaded PLGA microspheres showed entrapment efficiency of 52.8 ± 2% and appropriate yield (70.5 ± 1.8%). SEM observation showed that microspheres were spherical with smooth surfaces and with an average size of 103.1 ± 20.2 μm, which can be considered suitable (10–200 μm) for parenteral administration. In vitro release of RM from PLGA microspheres resulted in a zero-order release constant of 62.3 μg/day/20 mg microspheres, which was maintained for two weeks (Fig. 1).

Parkinson's disease is characterized by a progressive and selective degeneration of dopaminergic neurons of the substantia nigra pars compacta (SNc), leading to disorders in movements. There are a variety of animal models available for the study of the pathogenesis of Parkinson's disease (PD), such as 1-methyl-4-phenylpyridinium ion (MPP+), 6-hydroxydopamine (6-OHDA), paraquat, and rotenone, (Mazzio et al., 2004; Saravanan et al., 2005). Systemic administration of rotenone to animals

Table 1
Forward and reverse primers used for RT-PCR.

Gene	GenBank access	Forward primer (5′–3′)	Reverse primer (5′–3′)
Bcl-2	L14680	GCAACCGAACGCCCGCTGTG	GTGATGCAGGCCCCCACCAG
Bax	U49729	GCGAATTGGAGATGAACTGG	GTGAGCGAGGCGGTGAGGAC
TNF-α	X66539	TCGTAGCAAACCACCAAGCA	CCCTTGAAGAGAACCTGGGAGTA
SOD1	Y00404	GCGGTCCAGCGGATGA	GTCCTTTCCAGCAGCCACAT
SOD2	Y00497	CACGACCCACTGCAAGGAA	GCGTGCTCCCACACATCA
18 S	X01117	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG

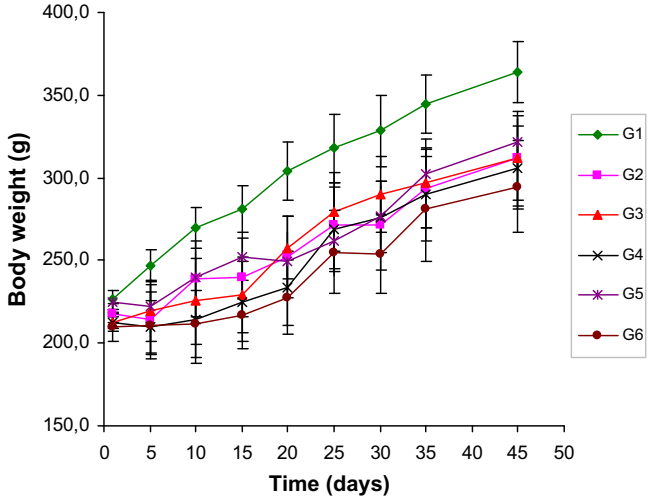

Fig. 2. Evolution of rat-body weight throughout the study (45 days). G1: control group; G2: rotenone-treated control group; G3 to G6 rotenone-treated animals also receiving; G3: blank PLGA microspheres; G4: RM-loaded PLGA microspheres (amount of microspheres equivalent to 15 mg/kg RM injected every 15 days); G5: RM-loaded PLGA microspheres (amount of microspheres equivalent to 7.5 mg/kg RM injected every 15 days) and; G6: RM in saline (1 mg/kg/day for 45 days). Values are mean ± S.E.M. ($n = 7$). RM: rasagiline mesylate.

resembles most of the features of PD including Lewy body formation in the SNc and loss of dopaminergic neurons, unlike other models. Therefore, the rotenone model can be very useful to study the performance of novel therapeutic approaches at different clinical stages of PD.Weight loss is a common feature of PD patients with gastrointestinal dysfunction being directly related to degeneration of the enteric nervous systems (Pfeiffer, 2003). All rotenone-treated groups (G2–G6) showed statistically significant differences ($P < 0.05$) in body weight when compared to control group G1 (Fig. 2).

We have observed that animals treated with rotenone required a minimum 10–15 days to exhibit hypokinetic movements and less intake of food as compared to control animals. All the surviving animals till the last administration of rotenone lost around 15% body weight as compared to control animals. In the first 5 days the average weight loss was approximately 13% in all rotenone-treated rats. A study performed by Sánchez-Reus et al. (2007) indicated that rats treated with rotenone (2 mg/kg/day) for 45 days showed minimum treatment-related effects in their body weights as compared to control animals. Their results also revealed non-statistically significant differences for weight loss between animal groups at the end of the experiment.

Around 10–15 days after initiating rotenone several side-effects were observed such as sialorrhea (increased salivation) and hyperpnea (increased respiration) as well as changes in hair colour and texture when compared to control animals. No convulsions were observed. Mortality occurred only in one case each in groups G2 and G3.

Reduced DA activity causes parkinsonism symptoms in humans such as rigidity (specific muscle tension), bradykinesia (slower movement) and akinesia (inability to move). This state is shown as catalepsy in the rat. To determine the behavioural consequences of chronic rotenone administration to rats, and the effects of RM given either in solution or encapsulated within PLGA microspheres, we conducted behavioural tests that had been previously used to assess motor performance in rodent models of PD.

It has been demonstrated that rotenone causes an increase in catalepsy probably due to a decrease of striatal dopamine resulting from mitochondrial complex I deficiency (Alam and Schmidt, 2002). In our study catalepsy tests on grid and bar were performed on days 15, 30 and 45 (end of the experiment). Chronic administration of rotenone resulted in a significantly prolonged descent latency as compared to control animals (Fig. 3a and b, G2 vs. G1). Our results also revealed highly significant differences ($P < 0.01$) between control animals (G1) and rotenone-treated animals receiving blank PLGA microspheres (G3) after analysis by Kruskal–Wallis ANOVA followed by Mann–Whitney *U*-test. When comparing descent latency in animal groups G2 and G3 similar results were obtained. All animals receiving RM either in solution or encapsulated within PLGA microspheres (G4, G5 and G6) showed a marked reversal in the descent latency when compared to rotenone-treated animals (G2) ($P < 0.01$). This reversal was specially marked in group G4 in which the descent latency was reduced by approximately 80% at the end of the study when compared with group G2. Group G4 corresponds to animals receiving the high-dose of RM-loaded PLGA microspheres assayed in this study (amount of microspheres equivalent to 15 mg/kg RM injected i.p. every 15 days). Moreover, the latency value obtained in group G4 was non-statistically significant from that of control animals (G1), thereby demonstrating the efficacy of the new controlled release system developed for RM.

For the other two groups; one receiving the low-dose of RM-loaded PLGA microspheres (RM 0.5 mg/kg/day given i.p. every 15 days) (G5), or RM in solution (1 mg/kg/day given i.p. for 45 days) (G6) differences found with respect to control group (G1) were statistically significant ($P < 0.05$). Moreover, descent latency obtained in G6 (60% at the end of the study when compared to G2) was lower than that achieved in group G5. It can be therefore stated that administration of RM-microspheres at the highest dose level assayed (RM 1 mg/kg/day every 15 days) resulted in a robust reversal of catalepsy which was significantly better than that achieved with the same dose of RM when given in solution.

When analyzing the evolution of catalepsy on grid and bar throughout the study (Fig. 3d and e) it can be noted that only for group G4 catalepsy was non-statistically different from control animals (G1) with a practically constant behaviour recorded on days 15, 30 and 45 of the study. The other two groups of RM-treated animals (G5 and G6), and as indicated before, showed a lower reversal of catalepsy than G4 being this reversal effect more pronounced at the end of the study period (45 days) for both animal groups. Regarding groups G2 and G3 descent latency steadily increased throughout the study period without statistically significant differences found between them.

Chronic rotenone administration also caused significant akinesia ($P < 0.05$) in 2.0 mg/kg/day rotenone-treated animal groups G2 and G3 when compared to control animals (G1) (Fig. 3c). At the end of the study (45 days) the latency was found to be 5 and 6-folds for G2 and G3 as compared to control group

Administration of RM either in solution or encapsulated within PLGA microspheres resulted in statistically significant reversal in the descent latency when compared to rotenone-treated animals (G2) ($P < 0.01$). This reduction was specially marked in group G4 in which the descent latency was reduced by approximately 75% after 45 days with respect to group G2. Moreover, the latency value obtained in group G4 was non-statistically significant with respect to that of control group (G1) thereby confirming the robust beneficial effect achieved with the high dose of the controlled release system developed for RM.

For the other two groups receiving RM-loaded PLGA microspheres (RM 0.5 mg/kg/day every 15 days) (G5), or RM in solution (1 mg/kg/day) (G6) differences found with respect to control group (G1) were also statistically significant ($P < 0.05$) but reversal in descent latency was lower, specially in G6 which resulted in 42% reduction of latency when compared to group G2.

The evolution of akinesia was also analyzed throughout the study (days 15, 30 and, 45) (Fig. 3f). It can be noted that only in

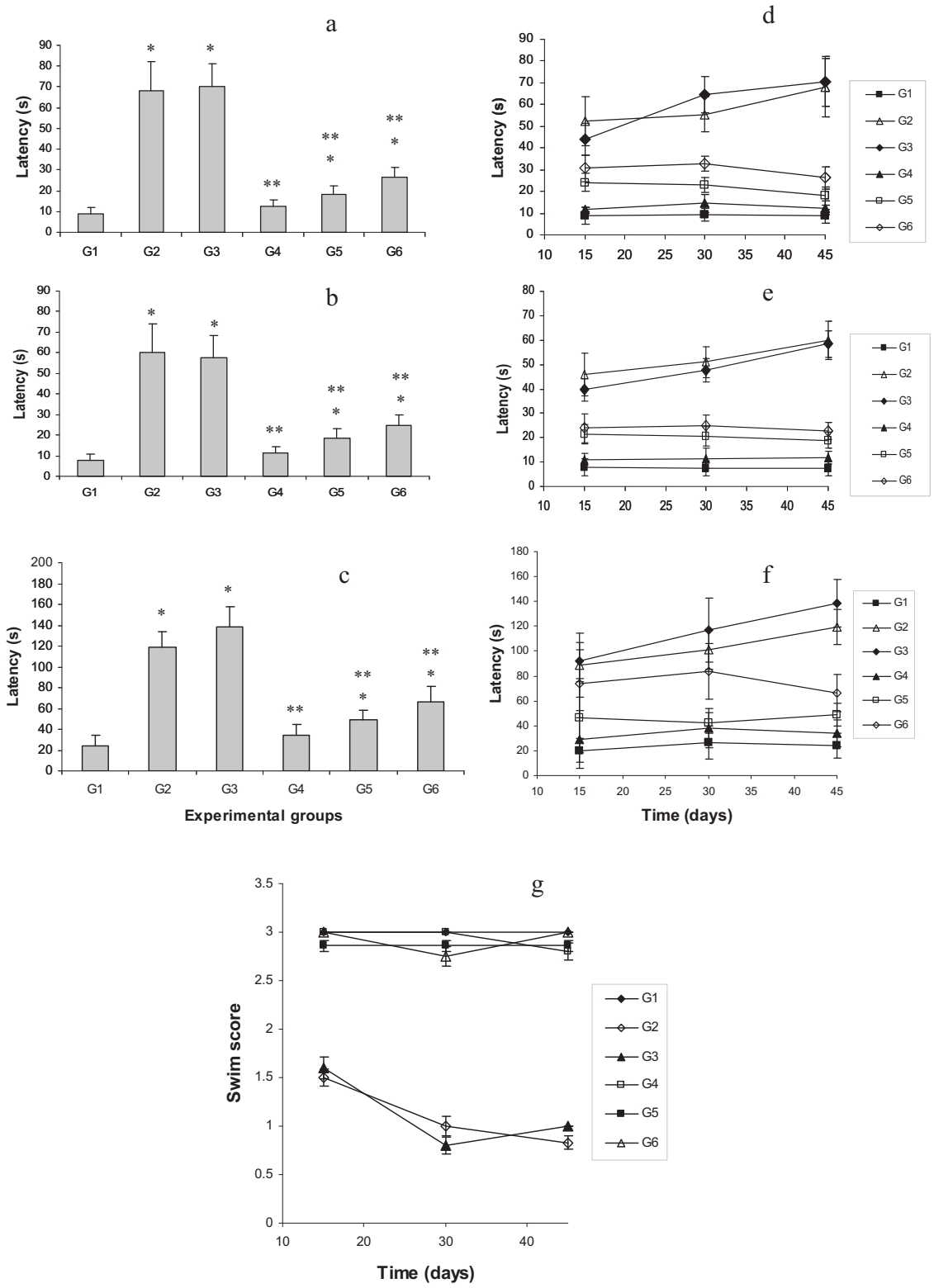

Fig. 3. Catalepsy test performed on grid (a) and bar (b) and, akinesia test (c) performed on all animal groups at the end of the study period (45 days). * Significant at $P < 0.05$ (comparison between control group G1 and all other animal groups), ** Significant at $P < 0.01$ (comparison between rotenone-treated groups G2/G3 and all other animal groups). Evolution of catalepsy test on grid (d) and bar (e), akinesia (f), and swim-test (g) performed on days 15, 30 and 45 to all animal groups. Values are mean ± S.E.M ($n = 7$).

group G4 akinesia was non-statistically different from control animals (G1) with an almost constant evolution throughout the study. The other two groups of RM-treated animals (G5 and G6) and as indicated before resulted in a lower reversal of akinesia. As with catalepsy, akinesia was steadily increased throughout the study period in groups G2 and G3.

While akinesia is an acute behavioural manifestation that is visible, swimming is latent and manifested only when tested in a new

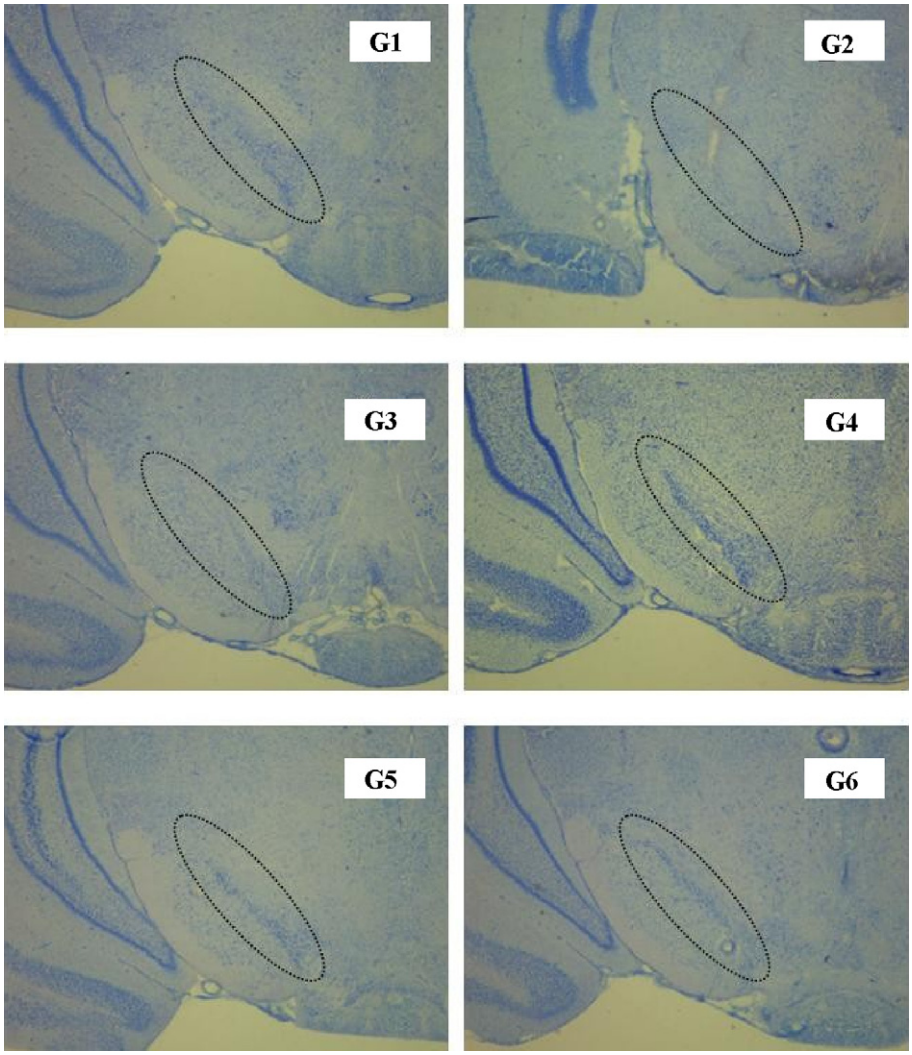

Fig. 4. Representative Nissl staining of nigral neurons of brain sections (substantia nigra pars compacta, 40 μm) corresponding to all animal groups (G1–G6). Nissl body-containing neurons defined by dashed ovals.

environment. Akinetic responses are short lasting, whereas swim deficit could be setting in slowly, along with depletion of striatal dopamine (Haobam et al., 2005). Swim-test is sensitive to various neuroprotective agents, such as salicylic acid and bromocriptine (Muralikrishnan and Mohanakumar, 1998; Mohanakumar et al., 2000), and can be used to evaluate the potential efficacy of treatments designed to restore dopaminergic function in animal models of PD (Sindhu et al., 2005).

Swimming in cold water may induce stress in the animals, and may interfere with the performance of the test. To overcome the cold-induced stress water temperature should be maintained at the ambient level. In our study we have maintained the temperature at $27 \pm 2\,^{\circ}C$. Due to the fact that in rotenone-treated animals swim ability was markedly decreased (Fig. 3g, G2 and G3) when compared to control animals (G1) ($P < 0.05$), it was possible to carry out the experiment only for 5 min. During this observational time swim scores obtained were 1.6 or less, when compared to that achieved by group G1 (swim-score 3). Animals receiving RM either in solution or encapsulated within PLGA microspheres showed a marked improvement in swim-scores throughout the study period. For instance on days 30 and 45 swim scores were ≥ 2.75 for all animals receiving RM.

Pathologically PD is defined by the intracytoplasmic accumulation of proteinaceous inclusions-Lewy bodies and the degeneration of dopamine neurons in the SNc. Mesencephalic dopaminergic neurons are the main source of dopamine in the mammalian CNS being located in the ventral tegmentum (VTA), the retrorubral field (RRF), and the SNc. Their main innervation targets are the basal ganglia, where they play a major role in controlling emotion, motivation, and motor behaviour (Girault and Greengard, 2004). Histologic evaluation of serially cut stained sections throughout the brains of the control group (G1) showed that vehicles (sunflower oil or saline) caused no loss of neurons in the ipsilateral SNc (Fig. 4).

By means of Nissl-staining it has been observed that rotenone-treatment induced a considerable decrease of dopaminergic cells in the SNc (group G2) as expressed by Nissl-staining deprivation; thereby confirming that dopaminergic neurons in the nigrostriatal pathway are sensitive to the action of rotenone (Alam and Schmidt, 2002; Sindhu et al., 2005; Cannon et al., 2009). Sherer et al. (2003) have demonstrated the implication of oxidative damage in rotenone toxicity. Moreover, Panov et al. (2005) showed that rotenone intoxication for 6 days resulted in multiple mitochondrial dysfunctions which included increased reactive oxygen species (ROS) production and inhibition of NAD-dependent substrate oxidation.

In our study the same results were obtained for rotenone-treated animals also receiving blank PLGA microspheres (group G3) when compared to group G1, thereby confirming the lack of effect

produced by the polymer used (PLGA). RM treatment (solution or microspheres) induced a marked decrease of dopaminergic cell loss in the SNc being this beneficial effect considerably higher in group G4. A qualitative analysis of Nissl-stained sections corresponding to groups G5 and G6 revealed comparable neuronal populations indicating that the performance of RM given in solution at a dose of 1 mg/kg/day was similar to that achieved with RM-loaded PLGA microspheres when given at the low-dose assayed in this study.

Our results also show that the high dose of RM-loaded PLGA microspheres significantly counteracts the SNc cell loss following injection of rotenone in rats.

PD is characterized by higher cerebral blood flow (CBF) and glucose consumption in the thalamus/capsula interna/lentiform intersection, the pons, cerebellum, and motor cortex/white matter (Ma et al., 2007). These pathophysiological changes can be easily monitored with the aid of techniques such as positron emission tomography (PET) which are also valuable tools to evaluate the effects of medical or surgical treatments. Among radiotracers, ^{18}F-DG is considered to be a marker of cerebral glucose consumption based on neuronal entrapment and accumulation of ^{18}FDG-6-PO$_4$, thereby indicating neuronal viability (Casteels and Lauwers, 2008). Several studies have demonstrated a marked reduction of ^{18}F-DG striatal uptake in PD, which reflects degeneration of the dopaminergic nigrostriatal pathways (Morrisch et al., 1998; Broussolle et al., 1999). More recently (Hou et al., 2010a) it has been indicated that changes occurring in PD are not only limited to the extrapyramidal system but are also accompanied by motor function changes of the frontal cerebral cortex in which a decrease in glucose metabolism has been detected in PD patients.

PET images obtained from control group (G1) (Fig. 5) revealed a normal heterogeneous distribution of radioactivity with higher activity detected in deep lying brain structures which probably represent SNc, globus pallidus and/or thalamic nuclei. Regarding group 2 (G2) PET images showed a marked modification of radioactivity distribution. ^{18}F-DG intensity of bilateral frontal cortex was reduced, but increased in the bilateral parietal cortex. Hou et al. (2010b) using ^{18}F-DG PET in a MPTP-induced cat model of PD also detected a continuous reduction of glucose metabolism in the bilateral frontal cortex with the aggravation of PD symptoms, whereas glucose metabolism was increased in the bilateral parietal cortex. The increasing glucose metabolism in the parietal cortex could be explained as a compensation mechanism for the functional disturbance occurred in the frontal cortex. In our study, quantification of the metabolic activities in SNc and striatum after termination of the PET scan revealed significant decreases with respect to the basal metabolic activity, which resulted in approximately 49.0% reductions (Fig. 5), indicative of neuronal hypometabolism and/or loss of cellular tissue.

PET images of brains corresponding to group 4 (G4) revealed a heterogeneous distribution of radioactivity (Fig. 5), which was increased in the frontal cortex and reduced in the parietal cortex when compared to rotenone-treated animals (G2). This increase in metabolic activity obtained in the frontal cortex of animals treated with the high dose of the new controlled release system developed for RM (G4) can be directly related to the behavioural motor improvements seen in catalepsy, akinesia and swim-tests. Finally, PET images of animal brains belonging to group 6 (G6) were similar to those obtained for group G4, with a slightly higher activity in the parietal cortex of group G6 with respect to G4.

Quantification of ^{18}F-DG radiotracer accumulation in SNc and striatum showed higher metabolic activity in groups G4 and G6 when compared to group G2 (Fig. 5). With respect to SNc the increase in cerebral metabolism was more pronounced in group G4 (mean increases of approximately 40%) than in group G6 (mean increases of approximately 25%) ($P < 0.05$), thereby confirming the better efficacy achieved by RM given in microspheres than in solution.

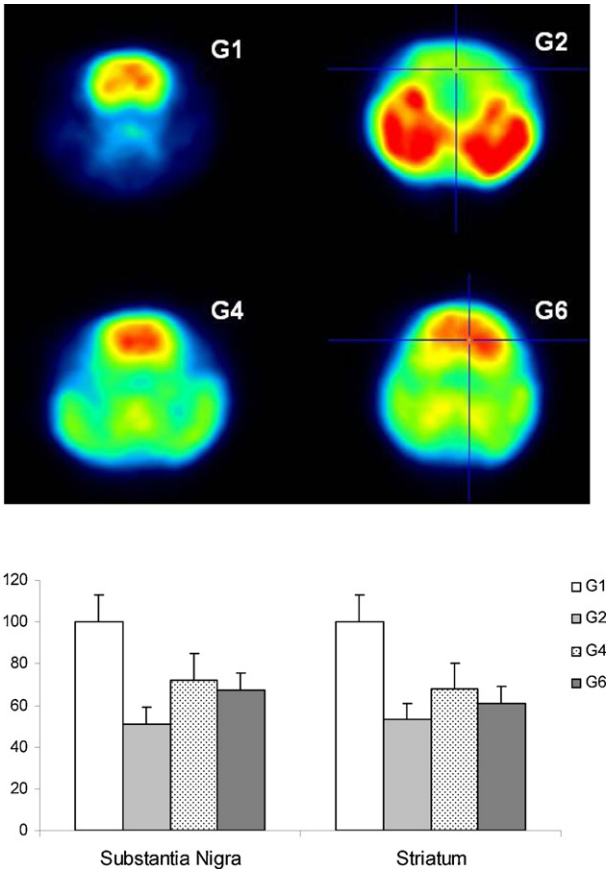

Fig. 5. Above: representative PET/CT ^{18}F-DG neuroimages from animal groups G1: control group; G2: rotenone-treated control group; G4 and G6: rotenone-treated animals also receiving; G4: RM-loaded PLGA microspheres (amount of microspheres equivalent to 15 mg/kg RM injected every 15 days) and, G6: RM in saline (1 mg/kg/day for 45 days). Below: metabolic activity (%) of ^{18}F-DG in substantia nigra and striatum of brains corresponding to animal groups G1, G2, G4 and G6. Values are mean ± S.E.M ($n = 4$).

Several studies have stated that RM can induce the expression of anti-apoptotic proteins and mRNAs coding for Bcl-2 and Bcl-x, with simultaneous effect decreasing the pro-apoptotic Bax and Bad protein (Bar-Am et al., 2005). Bcl-2 family proteins intervene regulating apoptosis being either death antagonists (Bcl-2, Bcl-XL, Bfl-1, A1 and Mcl-1) or agonists (Bax, Bak, Bad, Bid, Bik and Hrk) (Kroemr, 1997). Among them, Bcl-2 is mainly located in the mitochondrial inner membrane being able to promote survival of neurons and others cells undergoing apoptosis (Tsujimoto and Shimizu, 2000).

In this study, treatment with rotenone significantly decreased Bcl-2 levels when compared with control group G1 (Fig. 6). Administration of RM either in solution or encapsulated within PLGA microspheres resulted in statistically significant increases in Bcl-2 levels when compared to rotenone-treated animals (G2) ($P < 0.01$). This increase was specially marked in group G4 in which the mean value obtained (1.09 ± 0.07) was non-statistically significant with respect to control animals (1.16 ± 0.08). Other studies (Akao et al., 2002; Bar-Am et al., 2005) have found recovery of Bcl-2 levels in SH-SY5Y neuroblastoma cells after treatment with RM. In PD patients a negative correlation between Bcl-2 levels and both duration and severity of the disease has been demonstrated (Blandini et al., 2004b).

In cell cultures RM is able to reduce the levels of Bax protein (Bar-Am et al., 2005). In our case Bax levels were significantly increased in group G2 and markedly reduced in all animal groups treated

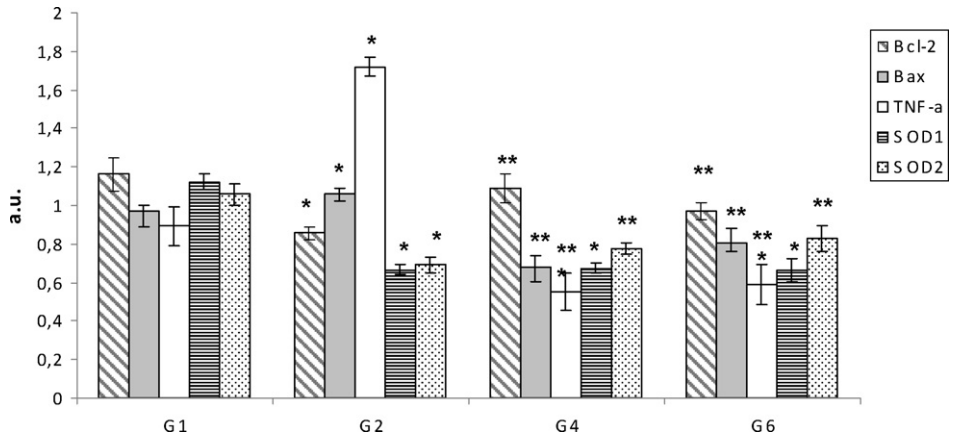

Fig. 6. Relative Bcl-2, Bax, TNF-α, SOD1 and SOD2 levels in brain homogenate of rats corresponding to animal groups G1: control group; G2: rotenone-treated control group; G4 and G6: rotenone-treated animals also receiving; G4: RM-loaded PLGA microspheres (amount of microspheres equivalent to 15 mg/kg RM injected every 15 days) and, G6: RM in saline (1 mg/kg/day for 45 days), assayed by RT-PCR after 45 days. * Significant at $P < 0.05$ (comparison between control group G1 and all other animal groups), ** Significant at $P < 0.01$ (comparison between rotenone-treated group G2 and all other animal groups). Values are mean ± S.E.M ($n = 4$).

with RM, being this reduction of approximately 33% in G4 when compared to rotenone-treated animals (G2) (Fig. 6).

In neurodegenerative disorders such as PD particular neurons deteriorate in a slow and continuous manner, where not only pro-survival but also apoptogenic factors should be activated. In PD, overexpression of TNF-α mRNA can play an important role in its pathogenesis being proposed TNF-α inhibition as a treatment strategy (Tweedie et al., 2007). TNF-α acts according to an inverted U-shaped dose-response curve in which at low concentrations TNF-α is neuroprotective whereas at high concentrations is neurotoxic (Maruyama et al., 2002). Treatment with rotenone almost doubled TNF-α mRNA levels when compared with control group (G1) (Fig. 6). Jin et al. (2008) also indicated a significant increase of TNF-α mRNA in 6-OHDA-induced PD in rats. In our study administration of RM either in solution (G6) or encapsulated within PLGA microspheres (G4) resulted in statistically significant reductions of TNF-α mRNA levels when compared to rotenone-treated animals (G2) ($P < 0.01$) being such reductions of approximately 66% for both groups.

Superoxide dismutases (SOD1 and SOD2) are enzymes that catalyze the dismutation of superoxide into less toxic oxygen and hydrogen peroxide being important antioxidant enzymes. SOD1 (Cu–Zn superoxide dismutase) is mainly found intracellularly in most tissues being one of the main antioxidants in the CNS together with glutathione peroxidase and catalase (Evans, 1993) and, SOD2 (Mn superoxide dismutase) is mainly located in the mitochondria. Although the activity of SOD1 has been described to be reduced in PD patients, especially with longer duration and/or progression of the disease (Tórsdóttir et al., 2006) there are conflicting data (Sudha et al., 2003). On the other hand, it has been postulated that mitochondrial SOD2, but not SOD1, can rapidly catalyze the conversion from more harmful mitochondrial superoxide to less harmful hydrogen peroxide with studies indicating that RM is able to increase SOD2 levels in the dopaminergic system of rotenone-induced PD in rats (Carrillo et al., 2000).

The results obtained in our study show that both SOD1 and SOD2 levels markedly decreased in rotenone-treated animals (G2) when compared to control group (G1) (Fig. 6). Administration of RM either in solution or encapsulated within PLGA microspheres did not have any effect on SOD1 levels but resulted in a slight but statistically significant increase of SOD2 levels when compared to G2.

These results are in agreement with those obtained in catalepsy, akinesia, swim-tests and PET thereby indicating that RM-loaded PLGA microspheres given at a dose of 1 mg/kg/day every 15 days is able to exert a marked beneficial effect on an animal model of rotenone-induced PD. The neuroprotective properties of rasagiline are attributed to its propargylamine structure (Bar-Am et al., 2005) and involve multiple survival pathways (Youdim et al., 2001) which may contribute to the neuroprotection observed in the present study. This study also demonstrates that RM microspheres provided dose-dependent neuroprotective effects against rotenone-induced PD in rats with better in vivo results than RM given in solution. The administration of our controlled release formulation during the induction phase of PD significantly ameliorated the ensuing neurological symptoms as well as delaying their onset.

4. Conclusions

Chronic treatment with a low dose of rotenone was able to induce neuronal degeneration and behavioural deficits that resemble PD very closely. The catalepsy, akinesia and swim tests as well as Nissl-staining of dopaminergic neurons showed clear evidence of selective degeneration of the SNc in rotenone-treated animals. Our results demonstrate a robust effect of RM when encapsulated within PLGA microspheres on all the analytical outcomes evaluated in the rotenone-induced rat model of PD which exhibited better in vivo efficacy than RM given in solution. These encouraging results make the new controlled release system developed a promising delivery strategy of RM for the treatment of Parkinson's disease.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpharm.2011.07.029.

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