BENEFICIAL NUTRACEUTICAL MODULATION OF CEREBRAL ERYTHROPOIETIN EXPRESSION AND OXIDATIVE STRESS: AN EXPERIMENTAL STUDY

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The main object of this study is to examine the effect of Klamin®, a nutraceutical containing phenylethylamine, phycocyanins, mycosporine-like aminoacids and aphanizomenon flos aquae-phytochrome on the learning and memory ability, the oxidative status and cerebral erythropoietin and its receptor EPO/EPOR system in prematurely senescent (PS) mice. A total of 28 PS mice, selected according to a prior T-maze test, and 26 non-prematurely senescent mice (NPS) mice were chosen. PS animals were divided into 3 groups and followed for 4 weeks: A) normal chow diet; B) added with Klamin® at 20 mg/kg/day (low dose); C) added with Klamin® at 100mg/kg/day (high dose). A further group of NPS mice given either normal food (group D) or high dose Klamin® (group E) was also considered. The behavioral procedures of spatial learning ability (Morris test) showed that PS mice had significantly longer learning time as compared to their NPS counterpart ($p<0.01$), but this effect was prevented especially in mice supplemented with high-dose Klamin® ($p<0.05$) which improved performances in NPS mice ($p<0.05$). High-dose Klamin® supplementation restored the depleted total thiol concentration in the brain observed in PS mice while normalizing their increased malonildialdehyde level ($p<0.05$). Moreover, the high-dosage only caused a significant upregulation of EPO/EPOR system both in PS and in NPS animals ($p<0.05$). Taken together, these data suggest that this specific alga Klamath extract has considerable antioxidant and adaptogenic properties, also through a stimulatory effect of cerebral EPO/EPO system.

Erythropoietin is a glycoprotein and together with its receptor (EPO-EPOR system) was initially documented to be produced by interstitial fibroblasts in the kidneys of the adult and in hepatocytes in the fetus. EPO is circulated towards the bone marrow where it regulates red cell production by preventing apoptosis (1). Recently it was reported that EPO-EPOR system is expressed also in the brain (2-3) where EPO exerts its biological activity through binding to its cognate receptor EPOR, possibly activating the

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Janus kinase-signal transducer. It affects neurons and astrocytes (4-5) to possibly exert protective effect during neurodegenerative diseases (6-8) although long-term cognitive outcomes and optimal dosing regimens have not been clarified. This has also been shown during acute illnesses such as traumatic brain injury, ischemia and stroke (9-11). In particular, it targets the vascular endothelium by stimulating neurovascular protection and angiogenesis (12), without enhancing vessel permeability (13), unlike VEGF which may bring about bleeding phenomena and haemodynamic derangement (14-15). It is known that oxidative stress is implicated in brain aging processes (16), thus, antioxidants play an intriguing role in preventing brain degeneration and may inhibit the age-related deficits in motor learning and memory ability (17). It has been shown that the age-dependent oxidative processes can be moderated by the application of various antioxidants, such to improve the brain morphology and the motor learning in aged rats. (18-19). De la Fuente et al. (20), by using the T-maze test as a clear-cut parameter, have shown that some mice express overt features of premature aging with immunologic impairment and a shorter life span when compared to their age-matched fast-performing counterparts and this may represent a useful model to test pharmacological and nutraceutical intervention. Among the latter, the microalgae of lake Klamath has always been appreciated not only for its peculiar nutritional properties, but also for its significant positive effects on mental attention, mood and anxiety. Such compounds contains phenylethylamine (PEA) which is an endogenous neurotransmitter synthesized by the decarboxylation of the aminoacid phenylalanine in the dopaminergic neurons of the nigrostriatal system (21), mycosporine-like aminoacids (MAAs), AFA-phycocyanins and AFA- phytochrome which, to different extents, are endowed by powerful antioxidants and anti-inflammatory properties (22-23). Recently, a patented nutraceutical made of specific extracts of Klamath alga has been devised (24) (Klamin®: PEA -β-phenylethylamine- 12 mg./gr. phycocyanins -AFA-PCs 10%, mycosporine-like aminoacids –MAAs- 2% and AFA-phytochrome 1% produced by Nutritherapy Research Centre, Urbino, Italy) and successfully used in clinics (25). Therefore, the main purpose of this study is to examine the effect of Klamin® on the learning and memory ability, the oxidative status and cerebral EPO/EPOr system in prematurely senescent (PS) mice.

MATERIALS AND METHODS

All studies were conducted in agreement with the policy and procedures as detailed in the "Guide for the Care and Use of Laboratory Animals" (NIH Publication, no. 86-23, 1985). Fifty Balb-c mice (25–30 g) were bred under conventional conditions, housed in a pathogen-free environment at 23±1°C with an alternating 12-h light/dark cycle and supplied with food and water *ad libitum*. At 70 weeks of age, the T-maze test was performed once a week for 4 weeks, and prematurely senescent (PS) mice were regarded as those animals that failed at all times to complete the test within the maximum allotted time (60 s). Animals with intermediate performances were excluded so as to obtain a "fast" and a "slow" group, containing 100% and 0%, respectively. Altogether, 28 PS mice and 26 non-prematurely senescent mice (NPS) mice were chosen. Afterwards, PS animals were divided into 3 groups and followed-up for 4 weeks: A) given normal chow diet; B) added with Klamin® at 20 mg/kg/day (low dose); C) added with Klamin® at 100mg/kg/day (high dose). A further group of NPS mice given either normal food (group D) or high dose Klamin® was also considered (group E).

Morris-test

The behavioral procedures of spatial learning ability was performed according to McClure (26) in both PS and NPS mice. Briefly, the maze consisted of a pool (120 cm diameter, 40 cm deep) and platform, modified for use with mice, with the interior of the pool being painted white and the pool filled to a height of 30 cm with water at 26±1°C, rendered opaque by adding 30 ml of skimmed milk; a circular transparent escape platform located 1 cm beneath the surface of the water near the center of one of the four quadrants of the maze acted as the 'exit' from the maze, and overtly visible cues, consisting of differing geometric symbols, were placed 1 cm above the water at each of four locations between quadrants. Spatial learning ability was determined by measuring the time required to escape by reaching the platform on 6 consecutive days. During tests, each mouse was placed in the water at a fixed starting point facing the pool wall. If a mouse failed to reach the platform within 120 s, the trial was terminated and the time recorded as the maximum of 120 s. Each mouse was tested four times per day on all 6 test days. The pool was located in a test room containing clear cues external to the maze (consisting of black symbols) that could be used by the mouse for overall spatial orientation. The swim

paths, distances and latencies to swim to the platform were monitored with a CCD video camera connected to a computer system. At the end of the tests on the tenth day, the platform was removed and the mice placed in the water at a random point, while the time which the rats took to reach the original platform within 120 s was recorded.

Measurement of plasma EPO levels

The animals were anesthetized and blood was obtained by cardiac puncture for measurement of EPO levels. Plasma EPO levels were measured by ELISA (R&D Systems) according to the manufacturer's instructions. A standard curve was generated with recombinant human EPO. The results are presented as relative values that have been normalized to the mean EPO level in untreated mice.

Measurement of malonildialdehyde (MDA) and total thiols in brain tissues

The mice were anesthetized and sacrificed after the learning and memory examination. MDA was measured using the LPO-586 kit which contains a chromogenic reagent that reacts with MDA at 45°C, yielding a stable chromophore with a maximum absorbance at a wavelength of 586 nm. Dissected brain tissue sections of each treated group were homogenized in 10 volumes of 20 mM Tris-HCl buffer (pH 7.4) and measured directly for MDA. The levels of MDA were measured in all prepared samples as follows. Immediately after centrifugation of brain homogenate at 2,500 x g for 10 min at 45°C, the pellet was resuspended in 2 or 3 volumes of 20 mM Tris-HCl buffer and 200 µl was used for the assay. In the next step, 650 µ1 of freshly prepared chromogenic reagent, in 11.4 mM of acetonitrile, were added to each test tube and then mixed with 200 µl of the sample. After the addition of 150 RI of 10.4 M methanesulfonic acid, the reaction mixture was thoroughly mixed and then incubated for 40 min at 4°C. The test tube was placed on ice after incubation to stop the reaction prior to measurement of absorbance at 586 nm. The measurement of Total Thiols (TT) was carried out as follows. Briefly, the assay mixture contained 0.2 ml of homogenate (10% w/v), 0.1 M Tris-EDTA (pH 8.2) and 1.0 mM DTNB in total volume of 2.7 ml and was mixed well. Thereafter, 8.3 ml methanol was added and centrifuged at 3,500 x *g* for 5 min. The yellow color developed and was read immediately at 412 nm in a spectrophotometer (UV-1800, Shimadzu, Japan). The total thiol content was calculated by using a molar extinction co-efficient of $13.6x10^3$ M^{-1} cm^{-1}.

EPO and EPOR expression with Western blotting

Mice were sacrificed 1 h after the last treatment, and the brains were excised and tissue samples were homogenized in an ice-cold lysis buffer containing 50 mm Tris-HCl (pH 8.2), 0.5 M saccharose, 10 mM□pH 7.9□HEPES, 1.5 mM MgCl2, 10 mM KCl, 1 mM EDTA,10%□V/V□glycerine, 1 mM DTT, 1 mM PMSF, 10µg/ml Aprotinin, 5µg/ml Leupeptin. Crude homogenates were incubated on ice for 30 min, then centrifuged at 12,000 r.p.m for 30 min at 4°C. An aliquot of the supernatant was taken for protein assay, while the remainder was stored at -80°C until use. Samples (40 µg protein per lane) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis in a 12% gel and electrophoretically transferred to the polyvinyl diflouride membranes (Amersham Pharmacia, Piscataway, NJ, USA). The membranes were blocked in the blocking solution (5% skimmed milk, 10 mM Tris-HCl, 150 mM NaCl, 0.01% Tween-20) for 2 h at room temperature to block non-specific binding, and immersed with the rabbit anti-EPO and mouse anti-EPOR antibody overnight at 4°C. Membranes were washed three times for 10 min each in TBST (10mM Tris-HCl, 100mM NaCl, 0.01% Tween-20) and incubated for 2 h with horseradish peroxidase-conjugated secondary antibody, goat anti-rabbit or anti-mouse IgG). After washing three times for 10 min each in TBST, the PVDF membrane was put into DAB fluid for 5□10 min. Termination of color reaction after rinsing with water, natural dried image scanning and acquisition were used for analysis. A western blotting of β-actin (used as a control) was performed in the same manner, using a monoclonal anti-β-actin antibody as the first antibody and a goat anti-mouse IgG as the second antibody. Western blot bands were scanned by ScanBiotools system. The intensities of bands were analyzed by a Bio-Rad software. Each EPO or EPOR band was normalized with its corresponding β-actin control band and the values were expressed as an intensity ratio by arbitrary units.

Statistical analysis

Significance was established by analysis of variance and the level of significance was determined by employing a Duncan's multiple-range test. Data were expressed in the text as means (SD) and a probability value of <0.05 was set as indicating that a statistically significant difference existed between experimental groups.

RESULTS

Body weight

The body weight and food intake showed no differences between the control and the Klamin®-treated groups in either PS or NPS mice (data not shown).

Klamin® effect on space-memory performance as measured by Morris test

With the increasing training times, the escape

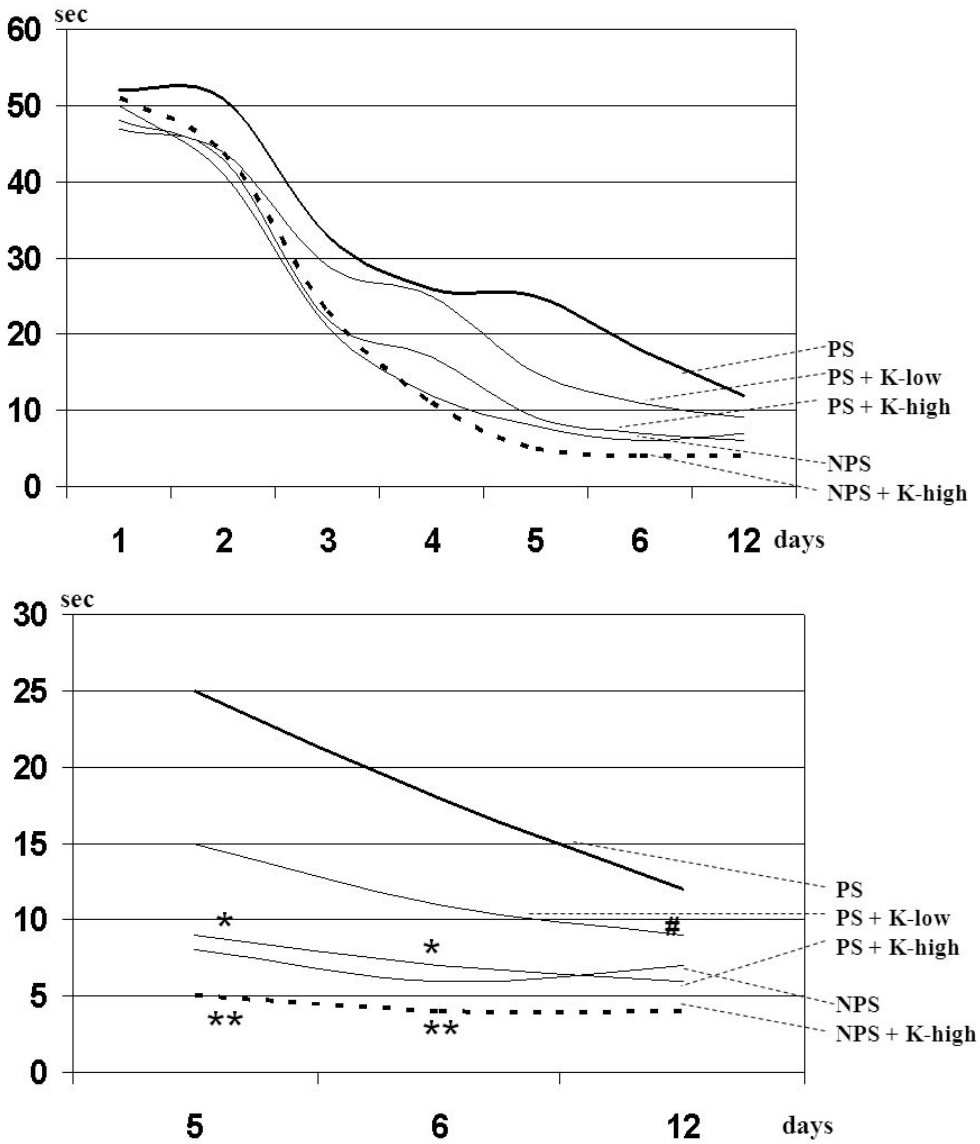

Fig. 1. *Effect of Klamin® on space-memory performance test. Upper case: PS, NPS see text. K-low and K-high: low dosage and high dosage of* Klamin® *respectively. Significance level is detailed in the text. Lower case: close up view of the last 3 observation times of the upper graph. * PS + K-high vs PS + K-low: p<0.05; ** NPS + K-high vs NPS fed normal food: p<0.05; # PS + K-low vs PS fed normal food: p<0.05.*

latency in all groups significantly decreased. PS mice showed a significantly longer learning time as compared to their NPS counterparts ($p<0.01$, Fig. 1). In particular, from the fourth day, the escape latency of both PS and low-dosage Klamin® were significantly longer than NPS mice. Low-dosage Klamin® exerted a significant improvement only on the last observation day ($p<0.05$). On the other hand, on the fourth observation day, supplementation with high-dosage Klamin® to PS mice brought about a significantly shortened escape latency ($p \square 0.05$) which was comparable to NPS mice. Moreover, high-dose Klamin® supplemented to NPS enabled a significant improvement from the fifth day ($p<0.05$).

Plasma EPO levels after treatment with Klamin®

Plasma EPO levels did not significantly change

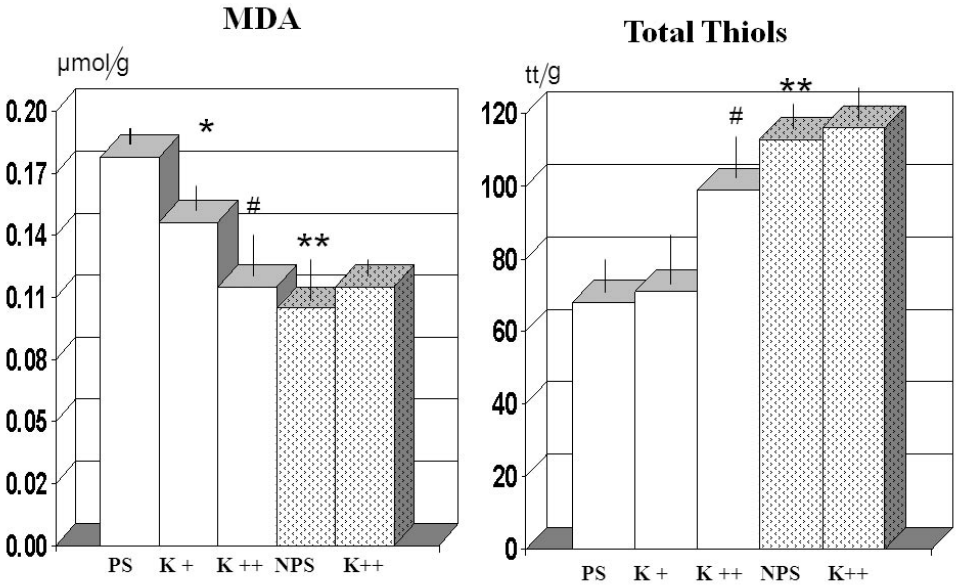

Fig. 2. *The MDA and total thiol concentrations of the brain tissues.*
*PS: Prematurely senescent; K+: low dose of Klamin®; K++: high dose of Klamin®; NPS: non-prematurely senescent. Tt: total thiols. * low and high dose of Klamin® vs PS mice fed normal diet; # high dose of Klamin® vs low dose of Klamin®: p<0.05; ** NPS vs PS: p<0.01.*

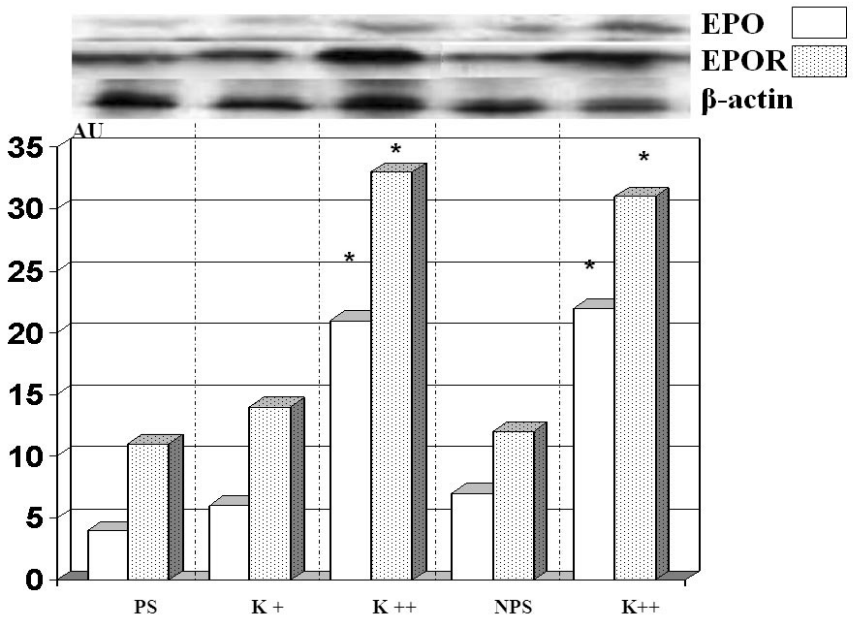

Fig. 3. *After treatment with high-dosage Klamin®.*
*PS: Prematurely senescent; K+: low dose of Klamin®; K++: high dose of Klamin®; NPS: non-prematurely senescent.. AU: arbitrary units (see text); * high dose of Klamin® vs PS and NPS mice fed normal diet: p<0.01.*

after treatment with both dosages of Klamin® nor was any difference observed between PS and NPS mice (data not shown).

Effect on brain MDA and total thiols

The MDA and total thiol concentrations of the brain tissues were analyzed and the results are

shown in Fig. 2. MDA concentration in PS mice was significantly elevated when compared to NPS animals ($p<0.01$). However, the MDA concentrations in both Klamin® groups were significantly lower than that in the control PS group ($p<0.05$). High-dosage Klamin® supplementation caused a normalization of MDA concentration, irrespective of the mice group ($p<0.05$ *vs* low-dose Klamin®). PS mice showed significantly depleted cerebral thiol levels as compared to normal mice ($p<0.01$) and low-dosage supplementation did not show any significant effect. On the other hand, total thiol level was restored by high-dosage Klamin® supplementation ($p<0.05$). No further improvement of total thiols in NPS mice was observed when their diet was supplemented with high-dosage Klamin®. In prior in-house tests we had shown that MDA in PS mice was comparable to NPS ones and Klamin® did not bring about any significant change (data not shown).

Klamin® upregulation of EPO and EPOR expression in the cortex by Western blotting analysis

Treatment with low-dosage of Klamin® did not significantly affect EPO/EPOR gene expression. Moreover, there was no significant difference between PS and NPS mice. However, after treatment with high-dosage Klamin® both in PS and NPS mice the density of EPO and EPOR bands had a significant almost two-fold increase as compared with that of the control (Fig. 3, $p<0.05$).

DISCUSSION

The microalgae *Aphanizomenon flos aquae* (AFA) from Klamath Lake in USA is widely used as a nutrient-dense nutritional supplement. AFA algae is also rich in specific nutraceutical molecules, such as phenylethylamine (PEA) and unique AFA-phycocyanins, with strong antioxidant and anti-inflammatory properties on the one hand and powerful neuromodulating and neuroprotective activities on the other (22-23, 25). We found that PS mice had a significant depletion of antioxidant defense system at cerebral level with baseline higher MDA concentration while at plasma level there was no change (data not shown). However, we have no comparative data on the overall plasma antioxidant system in these different groups of mice. Even low dosages of Klamin® proved to significantly decrease MDA cerebral level but being unable to affect the depletion of total thiols noted in the brains of PS mice. Higher dosage of Klamath algae extract enabled a more significant antioxidant effect at a cerebral level. These data need further corroboration by analyzing the reduction of lysates for protein oxidation levels using DNP/HNE/NT antibody following treatment with Klamath extract. Interestingly, low dosages improved the Morris test performance but did not modify the EPO/EPOR status. It has to be noted that the EPO/EPOR system was comparably expressed in both mice groups, thus suggesting that the cognitive-learning impairment in this mouse model is likely to follow different pathways. Indeed, since the level of Epo receptor expression in brain tissue has been proposed to determine its cytoprotective effects (27), we can postulate that this mouse model is not associated with damage *per se*, although this was outside the scope of the present work. EPO is known to be a multifunctional growth factor playing a relevant role in the nervous system since it can cross blood brain barrier (28) and exert protecting properties for neurons from ischemic damage (29) and for endothelial cells by promoting neurovascular unit repair (30). However, whether Epo is indifferent, protects or even damages the brain tissue seems to be related to the amount of Epo reaching the brain (31) and the timing of the hypoxia or ischemia (32). In our experiment it was interesting to observe that even higher dosages of Klamin® did not enhance plasma level of EPO but upregulated EPO and EPOR expression in the brain in PS. Moreover, such dosage brought about the most significant improvement of the spatial memory test in PS mice and, interestingly, also enabled a significant test improvement in normal mice where Klamin® determined also a significant EPO/EPOR upregulation. It is worth noting that recent studies suggest that EPO could prevent cognitive impairment and spatial recognition also in non-hypoxic brain injury models (33-34). These data and our preliminary findings are somewhat in agreement with the observation that optimal neuroprotection by erythropoietin is associated with elevated expression of its receptor (35). Very recently, Hussein et al. (36) suggested that the neuroprotective effect of EPO might be at least

partly explained by its activity to enhance biological-antioxidant-potentials and we may postulate that this may be one of the mechanisms which have taken place with higher dosages of Klamin®. Further studies aimed to obtain more detailed information on EPO/EPOR system in different brain regions and cellular compartments are ongoing given that not all brain areas similarly express *EpoR* gene or respond similarly to rhEpo (37). Moreover, while we showed an elevated mRNA level of EPO/EPOR gene after Klamin® administration, it remains to be clarified whether this compound acted also by enhancing the inducer/upstream genes of EPO/EPOR gene as well as to ascertain the involvement of other post-translational regulation. Finally, given the reported anti-inflammatory properties of phycocyanins (23) the inflammatory cascade pathways occurring in this model warrants further investigation.

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