Gemst: a taylor-made combination that reverts neuroanatomical changes in stroke

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Abstract

In a single transient middle cerebral artery occlusion model of stroke and using immunohistochemical techniques, the effects of a new therapeutic approach named Gemst (a member of the Poly-L-Lysine innovative therapies) have been studied in the rat brain. The expression of inflammatory (CD45, CD11b), oxidative (NO-tryptophan, NO_2-tyrosine) and indoleamine 2, 3-dioxygenase pathway (kynurenic acid, 3-hydroxy anthranilic acid) markers has been evaluated in early and late phases of stroke. For this purpose, we have developed eight highly specific monoclonal antibodies directed against some of these markers. In the early phase (3 and 5 days of the stroke, we observed no effect of Gemst treatment (7.5 mg/day, subcutaneously for 3, 5 days). In the late phase (21 days) of stroke and exclusively in the ipsilateral side of non-treated animals an overexpression of kynurenic acid, 3-hydroxy anthranilic acid, CD45, CD11b, GFAP and ionized calcium-binding adapter molecule 1 (IBA-1) was found. In treated animals, the overexpression of the four former markers was completely abolished whereas the overexpression of the two latter ones was decreased down to normal levels. Gemst reversed the pathological conditions of stroke to normal situations. Gemst exerts a multifunctional action: down-regulates the indoleamine 2, 3-dioxygenase pathway and abolishes brain infiltration, microglial activation and gliosis. Moreover, Gemst has no effect on the expression of doublecortin, a protein involved in neuronal migration. Gemst could be a new drug for the treatment of stroke since it reverses the pathological findings of stroke and normalizes brain tissue conditions following the ischemic insult.

Introduction

Stroke is a multifactorial disease that imposes a major health problem. Stroke mortality will double worldwide by 2020 and it will have an increasing incidence in developing countries. Around 20% of population aged from 55 to 75 years will suffer at least one stroke episode during their lives. To date, unfortunately, there is no cure for stroke. Currently, the only effective drug approved by the FDA and EMA for the treatment of this disease (ischemic stroke) is the tissue plasminogen activator (TPA), a well-known thrombolytic agent. However, TPA has a short therapeutic window because its clinical use is limited (*e.g.*, it is contraindicated in hemorrhagic stroke). About 80%-85% of stroke cases are ischemic, but 15%-20% are haemorrhagic.

There are 3 phases in stroke: i) The excitotoxicity phase: it is characterized by an increase in both glutamate release and reactive oxygen species (ROS) production and by a breakdown of the blood-brain barrier.[1] It is known that the onset of ischemia leads to augmented intracellular calcium ion concentrations mediated by increased levels of catecholamines. Excessive ROS, accumulated in the tissue environment, activates signal transduction pathways that will produce secondary ROS, causing cellular injury in form of lipid peroxidation, DNA damage and protein oxidation and modifications. Moreover, disruption of both cell function (*e.g.*, cell transport, energy production, ion balance) and cell structural integrity also occurs.[2] ii) The second phase is characterized by an inflammatory response, apoptotic processes and by activation of cytokines, caspases and both indoleamine 2, 3-dioxygenase (IDO) and nitric oxide (NO) pathways.[1,3] The activation of nitric oxide synthase (NOS) accelerates superoxide production[4] which rapidly reacts with NO, producing peroxynitrite that with other ROS cause an oxidative damage to proteins, lipids and DNA, disrupting mitochondrial respiratory chain and ATP production.[5,6] iii) Finally, the third phase of stroke is the regeneration period that extends for days or weeks after the ischemic insult and is variable depending of the volume infarcted; that is, it depends on the damaged tissue and the number of apoptotic cells.[1] Paradoxically, after the regeneration phase, the ischemic insult triggers mechanisms that provoke the worst effects. In stroke, the importance played by ROS and several neurotransmitters is widely known.[2,7-9] In this sense, it is known that the disruption of the blood-brain barrier can be counteracted by inhibiting the formation of ROS.[9] When ROS and NO production is reduced, inflammatory processes will be decreased and lipid and protein oxidation will be also reduced. It has been also suggested that neurons located in the ischemic peri-infarcted zone can potentially be rescued after stroke.[10] To be successful, stroke therapies should be targeted against multiple targets and must protect neuronal, glial and endothelial cells. Thus, due to its multifactorial nature, treatment of stroke needs new multifactorial strategies.

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During the last ten years, a new generation of drug candidates (*e.g.*, Gemsp, Gemals) has been developed[11-13] by GEMAC's group. In general, these drugs exert anti-oxidative and anti-inflammatory effects (*e.g.*, Gemsp blocked brain leukocyte infiltration).[12] Gemsp and Gemals drug compounds consist of amino acids and their derivatives, vitamins and fatty acids linked to Poly-L-Lysine (PLL) that makes them more accessible to the brain tissue.[13,14] Because these PLL-linked compounds have been previously demonstrated to exert both anti-oxidative and anti-inflammatory effects,[13,14] these compounds may also have beneficial effects in stroke as well. Here, we investigate the effects of Gemst, a new member of the PLL-compounds family, on stroke. Chemical constituents of Gemst were selected in order to fight adequately against the oxidative/inflammatory milieu conditions occurring during the different phases of stroke. The composition of Gemst is very close to Gemsp and Gemals.[12,13,15] The chemical composition of Gemst is slightly different than Gemsp and Gemals and it has a higher concentration of PLL-linked compounds (*e.g.*, vitamins, amino acids), in order to act more effectively against the deleterious changes occurring abruptly after stroke. For one side, this is due to the fact that both multiple sclerosis and amyotrophic lateral sclerosis are chronic conditions while stroke changes appear suddenly; for other side, the Gemst composition is adapted to the specific deleterious processes engaged after the reperfusion of the tissue during the excitotoxicity phase.

In light of the above, the main aim of this study was to know the effects of the new therapeutic approach named Gemst in stroke. Thus, in an experimental model of stroke (single transient middle cerebral artery occlusion)[16,17] and using immunohistochemical techniques, we studied the expression of inflammatory (*e.g.*, CD45, CD11b), oxidative (*e.g.*, NO-tryptophan, NO_2-tyrosine) and IDO pathway (*e.g.*, kynurenic acid, 3-hydroxy anthranilic acid) markers in the rat brain after treatment with Gemst. Moreover, for this purpose, we have developed for the first time several highly specific monoclonal antibodies directed against some of these markers.

Materials and Methods

Single transient middle cerebral artery occlusion (tMCAO)

In this work, we followed the guidelines of the legal and ethics recommendations of French, Spanish and European laws. Moreover, the research commission of the University of Salamanca (Spain) approved this study. All efforts were made to minimize the number of animals used and their suffering. Forty-two adult male Wistar rats (negative control (non-operated): 6 animals; tMCAO procedure: 36 animals) weighing 350 g were used. Animals were deeply anesthetized (induction, 4% isoflurane; maintenance, 2.5%) by means of a facial mask. As previously described,[16-20] the tMCAO chirurgical procedure was carried out. A transient middle artery cerebral occlusion (50-55 min) was carried out by using a rounded tip monofilament.[16,17]

Behavioral studies were carried out before and after the surgical procedure and the day of the perfusion of the animal. Two tests were performed: Open Field test (general motor abilities) and Cylinder test, used to detect unilateral forelimb impairments.[21]

Experimental groups

Animals were divided into three groups: 3days (D), 5D and 21D. Animals belonging to the 3D group were perfused three days after the tMCAO; the 5D group animals was perfused five days after the occlusion, and the 21D animal group was perfused twenty-one days after the surgical procedure. In each experimental group, 14 animals were used: 2 negative control rats (non-operated) and 12 tMCAO animals. Moreover, animals with surgery were divided into two subgroups: a) rats treated with NaCl solution (positive control group) and b) animals treated with Gemst. In groups 3D and 5D (early stroke phase) and in group 21D (late phase of stroke), animals received Gemst (7.5 mg/day, subcutaneously) or vehicle (NaCL 9g/L) immediately before the surgical procedure and every day until the perfusion of the animals.

Gemst

Gemst is a functional mixture of PLL-compounds belonging to a new generation of drug candidates (Gemsp, Gemals) for the treatment of inflammatory, neurodegenerative and chronic diseases.[12,13,15] The chemical composition of Gemst derived from the previous published chemical compositions of Gemsp and Gemals.[12,13] Both latter compounds have been patented [6114388 (USA) and 792167 (EU)]. The patent application of Gemst (INPI, Institut National de la Propriété Intellectuelle) is in course (DSO2017000421). In the three drugs above mentioned, several compounds (amino acids and their derivatives, fatty acids, vitamins) were linked to PLL by different linkages (*e.g.*, *via* glutaraldehyde).[14]

Development of monoclonal antibodies

In this study, we have developed eight monoclonal antibodies directed against kynurenic acid (KYNA), 3-hydroxy anthranilic acid (3-HAA), anthranilic acid (Anthra), kynurenine, quinolinic acid (Quino), quinaldic acid (Quina), NO-tryptophan (NO-W) or NO_2-tyrosine (NO_2-Tyr). The latter two antibodies were generated to show oxidative processes in the tMCAO model and the other six antibodies to observe metabolites of the IDO pathway. Each primary antiserum was developed in BALB/c mice after immunization with the corresponding antigen [*e.g.*, kynurenine-bovine serum albumin (BSA)].

As previously reported,[16,17,22] the following protocol was conducted: 10 mg of the corresponding small molecule (*e.g.*, kynurenine) were dissolved in methanol or in sodium acetate solution (solution 1); later, 20 mg of BSA were dissolved in water or in sodium acetate solution (solution 2). Then, solution 1 containing the small molecule (*e.g.,* kynurenine) was activated with ethyl-chloroformiate or glutaraldehyde. After the activation process, solutions 1 and 2 were mixed. After that, the linkage of the small molecule (*e.g.*, kynurenine) to BSA was over and the immunogenic compounds (*e.g.*, kynurenine-BSA) were purified using dialysis membranes (cut-off limits 12-16 KDa).[16,17,22]

After the synthesis of the immunogenic compounds, mice were immunized (one injection every 2-3 weeks) with the corresponding immunogen [50 µL of antigen (1 mg/mL) and 50 µL of complete or incomplete Freund adjuvant]. After the second immunization, serum samples were collected and antisera were pre-purified and tested by ELISA.[16,17,22,23] Once the polyclonal antibodies were characterized, a fusion of mice splenocytes and SP2/O/Ag myeloma cells was conducted. After the spleen dissection, the following steps were performed *in vitro*. In order to select specific clones producing antibodies directed against the targets, a screening was carried out. Once the monoclonal antibodies were obtained, cells were expanded. Supernatants were collected, pre-purified and dialyzed as previously reported.[16,17] Finally, monoclonal antibodies were purified in HiTrap protein G HP columns (17-0404-01, GE Healthcare, Marlborough, MA, USA)[16,17] and isotypes were determined using an Isotyping kit (26179, ThermoScientific, Waltham, MA, USA).

According to previous described protocols,[16,17,23] the specificity of each antibody was carried out by ELISA with close-relat-

ed chemical structure competitors. Thus, the specificity of the anti-KYNA antibody was evaluated by competition experiments with the following antigens: KYNA, kynurenine, Quino, picolinic acid, xanthurenic acid, 3-HAA, phenylalanine or tryptophan;[17] anti-L-kynurenine antibody specificity was evaluated by competition experiments with the following antigens: L-kynurenine, D-kynurenine, picolinic acid, xanthurenic acid, Quina, L-tryptophan, 3OH-kynurenine, Anthra, Quino, KYNA or L-kynurenine (free); anti-Quino antibody specificity was evaluated by competition experiments with the following antigens: Quino, Quina, KYNA, picolinic acid, Anthra, 3-HAA, nicotinic acid, 3-OH-kynurenine, L-kynurenine, xanthurenic acid or Quino (free); anti-3-HAA antibody specificity was evaluated by competition experiments with the following antigens: 3-HAA, Anthra, xanthurenic acid or picolinic acid;[16] anti-Anthra antibody specificity was evaluated by competition experiments with the following antigens: Anthra, 3-HAA, xanthurenic acid or picolinic acid; anti-Quina antibody specificity was evaluated by competition experiments with the following antigens: Quina, Quino, xanthurenic acid, Anthra, picolinic acid or kynurenine; anti-NO-W antibody specificity was evaluated by competition experiments with the following antigens: NO-W, NO-L-tyrosine, L-tryptophan, NO-L-cysteine or NO-L-histidine,[24] and finally, anti-NO_2-Tyr antibody specificity was evaluated by competition experiments with the following antigens: NO_2-Tyr or L-tyrosine.

Immunohistochemical study

Animals belonging to 3D, 5D, and 21D tMCAO experimental groups were anaesthetized and perfused as previously described.[16,17,22,23] Brains were post-fixed and cryoprotected; 40-50 µm-thick brain sections were obtained using a freezing microtome and processed for immunohistochemistry. Free-floating sections were treated with mixed hydrogen peroxide and methanol solution in order to prevent possible interference with endogenous peroxidases. Then, sections were washed in phosphate-buffered saline (PBS) and pre-incubated in the mix solution [PBS/normal horse serum (1%)/Triton X-100 (0.3%)]. Later, sections were incubated overnight at 4ºC in the mix solution containing the following polyclonal antibodies, rabbit anti-GFAP (diluted 1/100, Z0334, Dako, Glostrup, Denmark), goat anti-ionized calcium-binding adapter molecule 1 (IBA-1) (diluted 1/1,500, ab5076, Abcam, Cambridge, UK), anti-doublecortin (C-18) (diluted 1/300, SC-8066, Santa Cruz Biotechnologies, Dallas, TX, USA) or the following monoclonal antibodies, anti-KYNA (diluted 1/1,000, AM011, Gemacbio, Saint-Jean-d'Illac, France), anti-kynurenine (diluted 1/1,000, AM009, Gemacbio), anti-Quina (diluted 1/1,000, AM016, Gemacbio), anti-Quino (diluted 1/1,000, AM008, Gemacbio), anti-NO-W (diluted 1/1,000, AM012, Gemacbio), anti-NO_2-Tyr (diluted 1/500, AM014, Gemacbio), anti-3-HAA (diluted 1/1,000, AM013, Gemacbio), anti-Anthra (diluted 1/1,000, AM015, Gemacbio), anti-glial fibrillary acidic protein (GFAP) (diluted 1/400, ab10062, Abcam), anti-CD11b (diluted 1/50, ab1211, Abcam), anti-CD45 (diluted 1/50, ab33923, Abcam). After washing in PBS, sections were incubated in the mix solution containing the corresponding biotinylated anti-mouse/rabbit/goat immunogammaglobulin (1/200) (BA-9200/BA-1,000/BA-5000, Vector). Sections were washed in PBS, incubated with the avidin-biotin-peroxidase complex (ABC) (1/100) (Vectastain PK-6100, Vector Laboratories, Burlingame, CA; USA) and rinsed firstly with PBS and later with Tris-HCl buffer. Finally, using 3, 3' diaminobenzidine (DAB) as chromogen, the tissue-bound peroxidase was developed with hydrogen peroxide as previously described.[16,17,24]

In order to control the specificity of the immunoreactivity observed, the following histological controls were performed: i) pre-absorption of the first antibody with an excess (0.1 mg/mL) of the corresponding antigen; and ii) omission of the primary and/or secondary antibodies. In both cases, we observed no residual immunolabelling. Moreover, the immunological characteristics of the anti-KYNA, anti-3HAA and anti-NO-W have been previously published.[16,17,24] Furthermore, in order to control the specificity of the eight monoclonal antibodies developed in this study, ELISA tests were carried out. In all cases, the monoclonal antibodies specifically recognized the corresponding target.

According to a previous published protocol,[25] sections in which KYNA was detected by the DAB developing procedure, were prepared for double-labelling immunohistochemistry. To reveal the second immunohistochemical reaction, the chromogen 4-chloro-1-naphol was used. This substance provides a blue precipitate (3-HAA) easily distinguishable from the brown product (KYNA) of DAB.[25]

The stereotaxic atlas of Paxinos and Watson[26] was followed for nomenclature and mapping. Using an Olympus DP50 digital camera attached to a Kyowa Unilux-12 microscope, photomicrographs were taken. Adobe Photoshop CS software was used: to improve the visualization of results, only the contrast and brightness of the images were adjusted. Photographs at low-power magnification were also obtained using a Leica DMRB photomicroscope/Neurolucida system (8.0 Microbrightfield-bioscience, USA) (Figure 1).

Results

Monoclonal antibodies

Using ELISA tests, the eight monoclonal antibodies developed here were fully characterized as previously reported.[16,17,22-24] These antibodies showed a very high affinity (the estimated IC_{50} was between 10^{-11} M and 10^{-10} M) (Table 1) and an excellent discrimination from close chemical analogue structures (high specificity). The quantity (titration) and quality (affinity and specificity) of the monoclonal antibodies were studied by ELISA.[16,17,22-24] The antibodies developed here recognized specifically their respective targets, at a very low concentration, and hence the affinities were very high.[15,23,27] Using ELISA, the monoclonal

Table 1. Affinity of the developed monoclonal antibodies.

Monoclonal antibodies	Affinity	Specificity
NO-Tryptophan (NO-W)	10^{-11} M	Very high
NO_2-Tyrosine	10^{-11} M	Very high
Kynurenic acid (KYNA)	10^{-10} M	Very high
L-Kynurenine	10^{-11} M	Very high
3OH-Anthranilic acid (3-HAA)	10^{-11} M	Very high
Anthranilic acid (Anthra)	10^{-11} M	Very high
Quinolinic acid (Quino)	10^{-10} M	Very high
Quinaldic acid (Quina)	10^{-10} M	Very high

antibodies showed a very low cross-reactivity with close chemical analogue structures. The data show that the monoclonal antibodies developed here are highly specific against their respective targets. Furthermore, immunohistochemical controls (omission of the first antibody and preabsorption of the antibodies with their respective targets) showed an absence of immunoreactivity: this demonstrates the specificity of the immunoreactivity observed in our study. Moreover, monoclonal antibodies were purified on HiTrap columns and isotyped. Thus, the anti-KYNA antibody was characterized as an Ig G_1 isotype and λ chain; anti-3-HAA, anti-Anthra, anti-kynurenine and anti-Quino antibodies were characterized as an Ig G_1 isotype and κ chain; anti-NO-W antibody was characterized as an Ig G_{2b} isotype and κ chain and the anti-NO_2-Tyr antibody was characterized as an Ig G_{2b} isotype and λ chain.

Immunohistochemical study

In the contralateral side of treated (Gemst) and non-treated (positive control) animals (3D, 5D, 21D), the distribution patterns of the immunoreactivity for all markers studied were identical to those found in negative control animals (non-operated) (Table 2). In the latter animals and after the application of the immunohistochemical technique, no immunoreactivity was found for the markers studied, except for IBA-1, GFAP, doublecortin and NO-W (Table 2). As expected, in these animals and throughout the whole brain, IBA-1 was observed in microglia and GFAP in astrocytes. Moreover, immunopositive cells containing doublecortin (*e.g.*, in the hippocampus, cerebral cortex, hypothalamus) or NO-W (exclusively found in the septum) were also observed. In negative control animals, the degree of the immunoreactivity and the distribution pattern of both NO-W and doublecortin were similar to those found in treated (Gemst) and non-treated (positive control) rats (Table 2).

The following results make always reference to the ipsilateral side (infarcted region) of treated and non-treated animals. In comparison with negative control animals, in the early phase of stroke (3D, 5D), we observed in the infarcted regions (ipsilateral cerebral cortex and/or ipsilateral striatum) of non-treated and treated animals an overexpression of IBA-1, GFAP, KYNA and 3-HAA (Table 2). The two latter markers, belonging to the IDO pathway, were observed in astrocytes, since cells containing KYNA or 3-HAA showed the same morphological characteristics than those

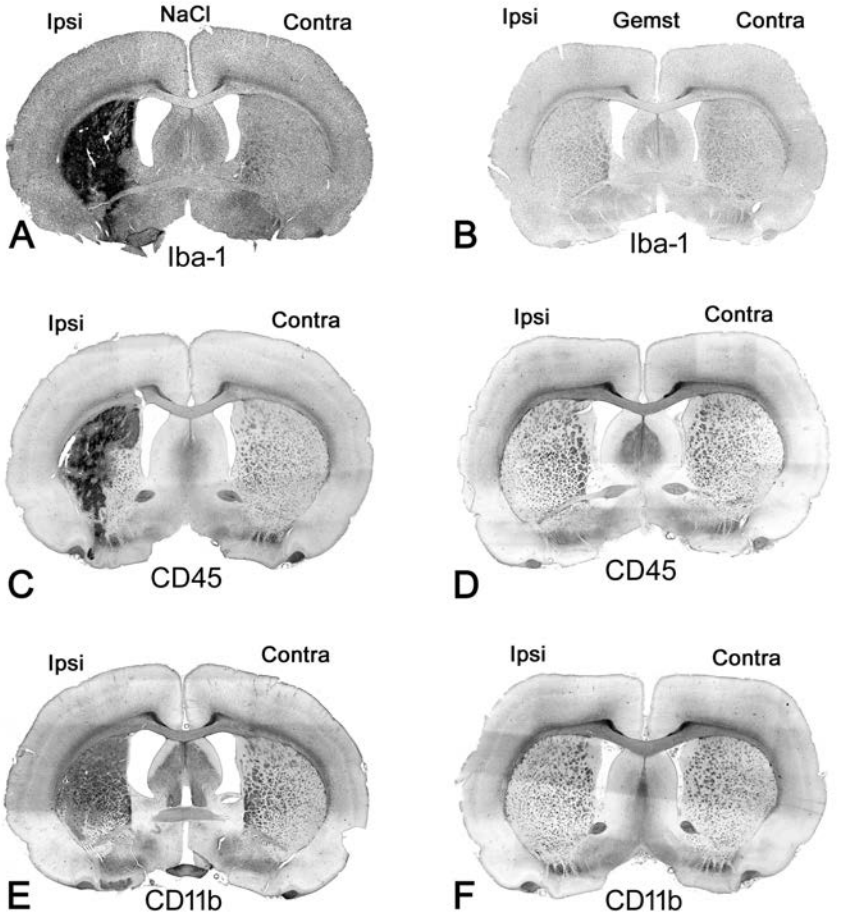

Figure 1. 21D group. Immunoreactivity for IBA-1 (A, B), CD45 (C, D) and CD11b (E, F) in non-treated (NaCl, positive control) (A, C, E) and treated (Gemst) (B, D, F) animals. Contra: contralateral side; Ipsi: ipsilateral side. IBA-1 is overexpressed in the infarcted region (A), whereas the immunoreactivity observed in this region for CD45 (C) or CD11b (E) was completely abolished in the ipsilateral side of treated animals (D, F). Photographs were taken using a Leica microscope/Neurolucida software.

Table 2. Immunoreactivity found in the infarcted region in treated (Gemst) and non-treated (positive control) groups. Data observed in the negative control group are also indicated.

Group	Positive control				Gemst				Negative control
Marker	3D/5D		21D		3D/5D		21D		
	I/C		I/C		I/C		I/C		
IBA1	+++	+	+++	+	+++	+	+	+	+
GFAP	+++	+	+++	+	+++	+	+	+	+
DCX	+	+	+	+	+	+	+	+	+
CD45	+++	-	+++	-	+++	-	-	-	-
CD11b	+++	-	+++	-	+++	-	-	-	-
NO-W	-	-	-	-	-	-	-	-	-
NO2-Tyr	-	-	-	-	-	-	-	-	-
KYNA	+++	-	+++	-	+++	-	-	-	-
Kynurenine	-	-	-	-	-	-	-	-	-
3-HAA	+++	-	+++	-	+++	-	-	-	-
Anthra	-	-	-	-	-	-	-	-	-
Quina	-	-	-	-	-	-	-	-	-
Quino	-	-	-	-	-	-	-	-	-

I/C, ipsilateral side/contralateral side; +++, high density (see Figure 1A); +, low density (see Figure 1B); -, absence of immunoreactivity.

containing GFAP.[16,17] Moreover, immunoreactivity for CD45 (marker for leukocytes) and CD11b (activated microglia) was exclusively observed in the infarcted region in treated and non-treated animals, but no immunoreactivity for both markers was observed in negative control animals (Table 2). Although the degree of the immunoreactivity was higher for both CD45 (marker for infiltration) and CD11b (Table 2), it is important to note that the immunoreactivity for CD45 was much stronger than for CD11b. In the early phase of stroke (3D and 5D), in both treated and non-treated animals, no immunoreactivity was found for NO_2-Tyr, kynurenine, Anthra, Quina and Quino (Table 2).

In the late phase of stroke (21D) (positive control, non-treated animals) the immunoreactivity distribution patterns of the markers studied were identical to those described in the early phase of stroke (3D, 5D) (Table 2). By contrast, in the late phase of stroke (21D) (animals treated with Gemst), we observed significant neuroanatomical changes in the distribution patterns of the immunoreactivity studied (Table 2; Figures 1-6). Thus, in comparison with non-treated animals, in animals treated with Gemst, the degree of immunoreactivity for IBA-1 (Figure 1 A,B and Figure 2 A-F) and GFAP decreased considerably; the immunoreactivity for CD45 and CD11b was absent (Figures 1 C-F, 3 A-D, 4 A-D) and the immunoreactivity for KYNA and 3-HAA disappeared completely (Figures 5 A-D, 6 A-F). That is, we observed the same results in animals treated with Gemst (21D) than in negative control animals (non-operated) (Table 2). Finally, by double immunohistochemistry, the coexistence of 3-HAA and KYNA in astrocytes has been also demonstrated (Figure 7 A-E).

Behavioral studies confirmed that the tMCAO model was correctly carried out because, after the surgical procedure, animals showed a unilateral forelimb impairment. Those animals in which this impairment was not observed were discarded.

Discussion

The main finding of our study is that Gemst exerts a beneficial effect in the late phase of stroke, counteracting the neuroanatomical changes observed in tMCAO non-treated animals. At an early phase of stroke (3D, 5D), we observed no effect on these changes. In the case of the NO pathway and in all experimental groups (early and late phases), we did not observe immunoreactivity for NO-W (except in the septum) or NO_2-Tyr. However, in the late phase of stroke, Gemst modulated the IDO pathway since the overexpression of both KYNA and 3-HAA (observed in tMCAO non-treated animals) was completely abolished. Moreover, in the late phase of stroke, Gemst completely abolished the immunoreactivity for CD45 (infiltration) and CD11b (activated microglia) and decreased considerably the expression of IBA-1 and GFAP until the normal expression observed in non-operated animals; that is, Gemst abolishes gliosis. In sum, Gemst reversed the pathological conditions of stroke to a normal situation since, in stroke animals treated with Gemst, the distribution patterns of the immunoreactivity for the markers studied were similar to those found in non-operated animals.

Monoclonal antibodies

In this study, we have developed eight monoclonal antibodies against KYNA, kynurenine, 3-HAA, anthranilic acid, quinaldic acid, quinolinic acid, NO-W or NO_2-Tyr. The immunological characteristics of the monoclonal anti-KYNA, anti-3HAA and anti-NO-W have been recently published.[16,17,24] In all cases, antibodies showed a very high affinity and specificity for their respective targets, since they did not recognise close analogue chemical structures used as competitors in order to evaluate a possible cross-linking (*e.g.*, the monoclonal antibody directed against KYNA did not recognise L-kynurenine, Quino, picolinic acid, xanthurenic acid, 3-HAA, L-phenylalanine or L-tryptophan).

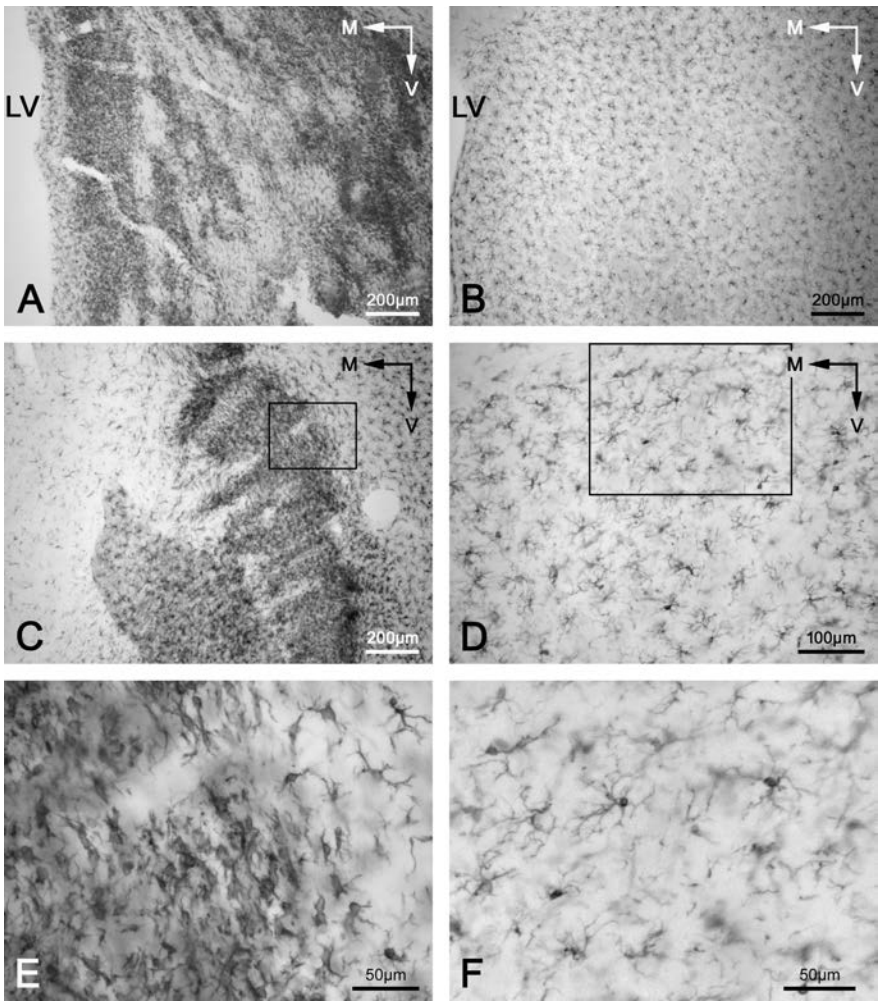

Figure 2. 21D group. Ipsilateral side. Immunoreactivity for IBA-1 (A-F) in non-treated (NaCl, positive control) (A, C, E) and treated (Gemst) (B, D, F) animals. Compared with Gemst treated animals, note the overexpression of IBA-1 in non-treated animals. E) High-power magnification of the region delimited by the rectangle in C. F) High-power magnification of the region delimited by the rectangle in D. LV, lateral ventricle; M, medial; V, ventral.

Moreover, ELISA tests and histological controls showed the specificity of the immunoreactive signal observed for the eight above mentioned monoclonal antibodies. In the case of IBA-1, CD45, CD11b, GFAP and doublecortin (purchased from commercial sources), we carried out histological controls confirming the specificity of the immunolabeling.

Single transient middle cerebral artery occlusion model

A considerable variability (extension of the infarcted region) in the tMCAO model has been previously reported.[28] This is in agreement with our observations, since infarcted regions were observed in the striatum and/or cerebral cortex. In addition, the results found with the IBA-1 marker confirmed that the tMCAO experimental model was adequately carried out, since IBA-1 was exclusively overexpressed in the ipsilateral infarcted region in comparison with the immunoreactivity found through the whole brain.[16,17,29,30] Moreover, the presence of cells expressing CD45 or CD11b exclusively in the infarcted region confirmed that the tMCAO model was well conducted.

Neuroanatomical study and possible physiological actions

Regarding the early phase of stroke, animals treated and non-treated with Gemst showed in the infarcted region an overexpression of IBA-1, GFAP, CD45, CD11b, KYNA and 3-HAA in comparison with that found in non-operated animals. This is in agreement with previous studies in which non-treated animals were used.[16,17] Thus, for these markers, a perfect match occurs in the infarcted region (CD45, CD11b, KYNA and 3-HAA were exclusively found in the infarcted region). As previously reported,[16,17] GFAP was clearly observed in our study in astrocytes and the cells, expressing both KYNA and 3-HAA, showed similar morphological characteristics that those containing GFAP. In order to confirm this morphological observation, we have demonstrated by double immunohistochemistry the coexistence of 3-HAA and KYNA in astrocytes. This is in agreement with previous studies since the coexistence of GFAP/3-HAA or GFAP/KYNA has been recently reported in these cells.[16,17]

In stroke, and according to our morphological data, it seems that a balance between neuroprotective (KYNA) and neurotoxic (3-HAA) occurs. It is important to note that in the tMCAO model the expression of both 3-HAA and KYNA was observed from early stages and this was exclusively found in the infarcted regions. This is in agree-

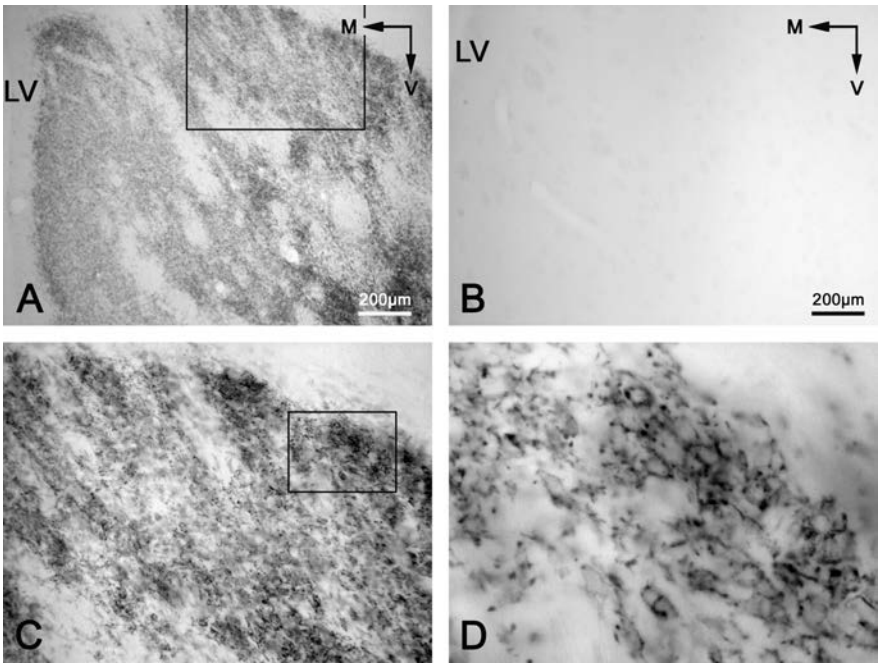

Figure 3. 21D group. Ipsilateral side. Immunoreactivity for CD45 (A-D) in non-treated (NaCl, positive control) (A, C, D) and treated (Gemst) (B) animals. Note the absence of immunoreactivity in animals treated with Gemst (B). C) High-power magnification of the region delimited by the rectangle in A. D) High-power magnification of the region delimited by the rectangle in C. LV, lateral ventricle; M, medial; V, ventral.

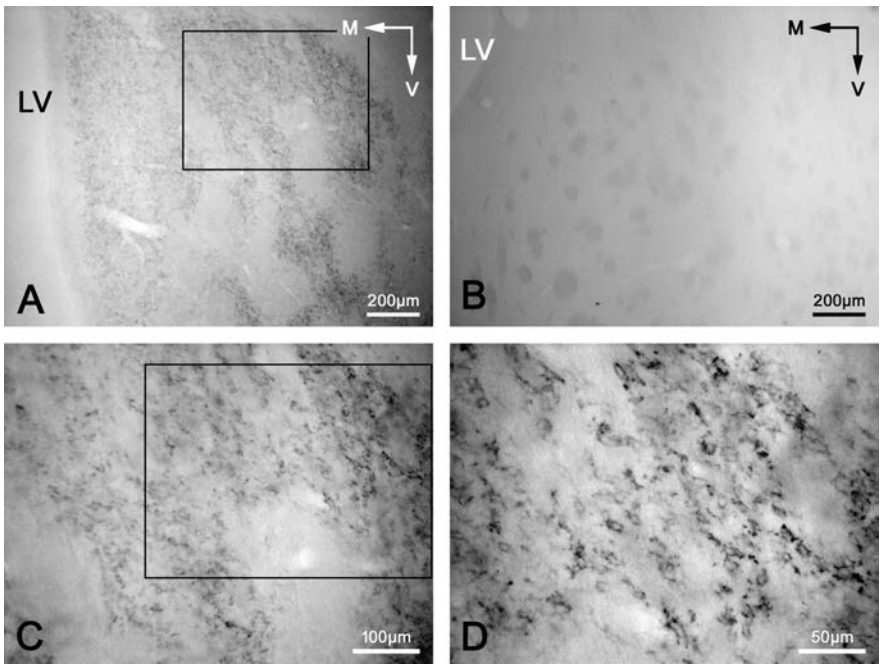

Figure 4. 21D group. Ipsilateral side. Immunoreactivity for CD11b (A-D) in non-treated (NaCl, positive control) (A, C, D) and treated (Gemst) (B) animals. Note the absence of immunoreactivity in animals treated with Gemst (B). C) High-power magnification of the region delimited by the rectangle in A. D) High-power magnification of the region delimited by the rectangle in C. LV, lateral ventricle; M, medial; V, ventral.

ment with previous studies.[16,17] It is known that KYNA is an intermediate molecule of the catabolism of tryptophan; it is mainly synthesized by astrocytes; acts as a scavenger; exerts an antioxidant action and a neuroprotective effect against the neuronal loss induced by ischemia, and it is a broad-spectrum glutamate receptor antagonist.[31-35] The overexpression of KYNA could be explained as an endogenous mechanism to block the deleterious effects mediated by glutamate. By contrast, it has been demonstrated that 3-HAA is involved in the initiation, development and amplification of neurodegenerative processes, exerting a cytotoxic effect.[3,36-38] It is known that a concentration of 3-HAA (>100 nM) is neurotoxic, but the level of NAD^+ was increased at a lower concentration, exerting a beneficial effect on cultured human astrocytes and neurons.[37,39] It has been also reported that the IDO pathway is altered in several pathologies (*e.g.*, stroke, multiple sclerosis, schizophrenia, depression, Parkinson, Huntington, Alzheimer, amyotrophic lateral sclerosis) and that regulates the immune response.[3,38,40-42] In stroke, a relationship between the kynurenine/tryptophan balance (IDO pathway) and the post-stroke cognitive impairment has been also reported.[41]

One aim of this study was to evaluate oxidative processes in stroke. For this reason, we have developed two new monoclonal antibodies directed against NO-W or NO_2-Tyr. In both cases and in all animals studied, we did not observed immunoreactivity by the whole brain (including the infarcted region), except the presence of perikarya containing NO-W in the lateral septum. As previously described,[24] the latter was observed in treated, non-treated and non-operated animals. This means that the immunohistochemical technique was correctly carried out and that these two markers are not expressed in the stroke model used here. However, it is possible that the absence of immunoreactivity for NO-W/NO_2-Tyr was due to several reasons: i) other markers not studied here could be involved in stroke (*e.g.*, NO-cysteine) and/or ii) these molecules have a short half-life and they cannot be detected at the days studied here by immunohistochemical techniques, except when NO-W is endogenous (located in the lateral septum).[24] Thus, to show oxidative processes in stroke, it seems that NO-W/NO_2-Tyr must be studied immediately 1-2 h after the induction of stroke.

Regarding the late phase of stroke and in comparison with non-treated animals, in the Gemst treated animals we observed that the overexpression of IBA-1 and GFAP (gliosis) was decreased to normal values (as observed in non-operated animals) and that the expression of CD45, CD11b, KYNA and 3-HAA (exclusively found in the infarcted region of non-treated animals) was completely abolished. Moreover, a similar expression/distribution of doublecortin (a protein involved in neuronal migration) was observed by the brain of all animals. This suggests that Gemst has no effect on the expression of this marker for neurogenesis. We have demonstrated here that stroke induced changes in the IDO pathway and that Gemst reversed them. Only two of the six markers studied of the IDO pathway showed immunoreactivity in the infarcted region (KYNA and 3-HAA). As indicated above, KYNA is considered as a neuroprotector and 3-HAA is considered as neurotoxic[3] and it is known that in the IDO pathway, both KYNA and 3-HAA are generated from kynurenine. In stroke, and according to our morphological data, it seems that a balance between neuroprotective (KYNA) and neurotoxic (3-HAA) occurs. The absence of immunoreactivity for kynurenine and the other markers studied here could be due to the low concentration of these small molecules and for this reason immunohistochemical techniques cannot detect them. In the late phase of stroke, Gemst has a double effect: i) abolishes the neurotoxic action of 3-HAA and ii) abolishes the expression of a neuroprotective factor/glutamate receptor antagonist (KYNA) because Gemst exerts a general beneficial effect in stroke (abolishing infiltration, gliosis, microglial activation and the expression of neurotoxic factors), and then, endogenous neuroprotective factors are not necessary to be expressed. One of our goals in this study was to evaluate the action of the Gemst in early and late phases of stroke. According to the morphological data observed here, it seems that Gemst did not exert an effect on the early phase of stroke. This is in agreement with previous *in vitro* unpublished data carried out by Bioalternatives (France). In this study, PLL-compounds did not exert a neuroprotective role after the administration of glutamate and this means that the PLL-compounds tested did not counteract the excitotoxic effects mediated by glutamate. However, in order to confirm the no effect of Gemst in the early phase of stroke, other markers not studied here must be tested. Our data clearly demonstrated that Gemst exerts a beneficial effect in the late phase reversing stroke conditions to normal conditions (infiltration, gliosis, activation of microglia and IDO pathway signals disappeared).

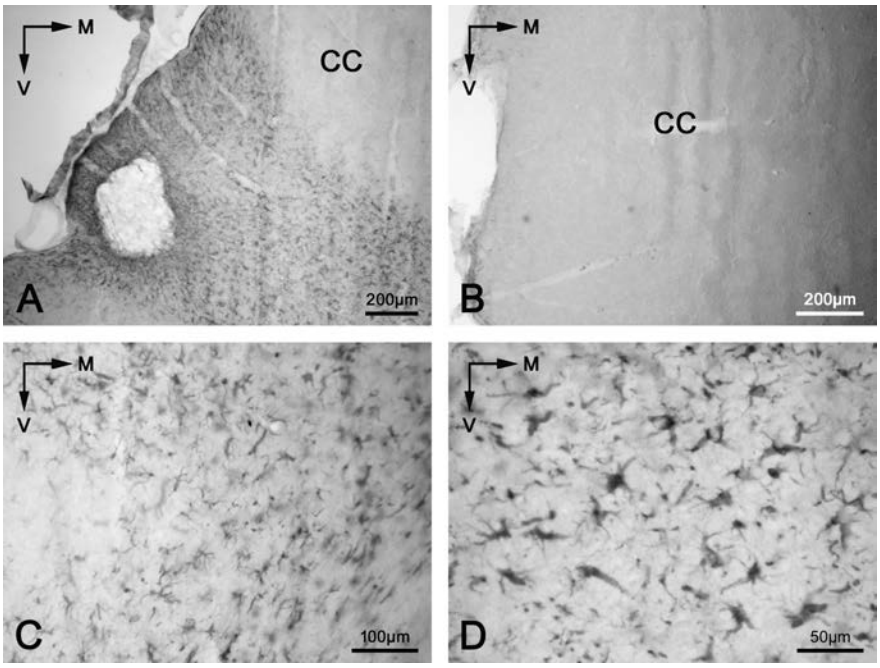

Figure 5. 21D group. Ipsilateral side. Immunoreactivity for KYNA (A-D) in non-treated (NaCl, positive control) (A, C-D) and treated (Gemst) (B) animals. Note the absence of immunoreactivity in animals treated with Gemst (B). CC, cerebral cortex; M, medial; V, ventral.

PLL-compounds

In addition to Gemsp and Gemals, Gemst belongs to a family of drugs constituted by innovative PLL-compounds for the treatment of inflammatory, autoimmune, neurodegenerative and chronic diseases (*e.g.*, multiple sclerosis).[43] This family of drugs, including Gemst, are a tailor made combination in which fatty acids, vitamins, amino acids and their derivatives are included.[12,13] It has been demonstrated that vitamins, amino acids and their derivatives included in Gemst exert an important cellular protector role (*e.g.,* increase neuronal survival), exert antioxidant effects, act as scavengers of free radicals, prevent cell membranes (exerting a fatty acid protective action), prevent apoptotic processes and the excitotoxicity caused by glutamate, diminish the oedema formation and the damage produced in ischemia. They play an important role in neuronal survival and neurite outgrowth, modulate calcium signalling, the elimination of heavy metals, the osmoregulation and the level of pro-inflammatory cytokines, control the immune response and the redox state of cells, downregulate several stress associated proteins, exert a beneficial effect concerning the growth of stem cells, and finally, exert a protective effect against hypoxia in neural stem cells increasing the expression of survival proteins (*e.g.*, phosphorylated Akt) and decreasing the expression of death-related proteins (*e.g.,* caspase 3).[44-51] In sum, PLL-compounds exert anti-inflammatory, anti-oxidative and neuroprotective effects and also abolish brain leukocyte infiltration.[12,52] Thus, these drug candidates exert a multimodal action as we have demonstrated here for Gemst in an experimental model of stroke. Furthermore, Gemst was conceived in order to fight against oxidative and inflammatory conditions occurring in stroke. Compared with Gemsp and Gemals, the concentration of PLL-compounds was slightly higher in Gemst in order to act specifically against the deleterious conditions appearing in stroke. According to previous studies[12,13] regarding the dose of PLL-compounds, we have administered here 7.5 mg/day. This dose was chosen because it was the most efficient in experimental models of amyotrophic lateral sclerosis and multiple sclerosis.[12,13] By using a Fourier Transform Infra-Red Spectroscopy, the stability of Gemst was confirmed for at least one year (unpublished data). The toxicity of PLL-compounds (Gemsp, Gemals and Gemst) has been previously tested: after a single intravenous dose of 10 mg/kg, no mortality of animals was observed (LD0 and LD50 > 10 mg/kg).[11,52] Moreover, it is known that

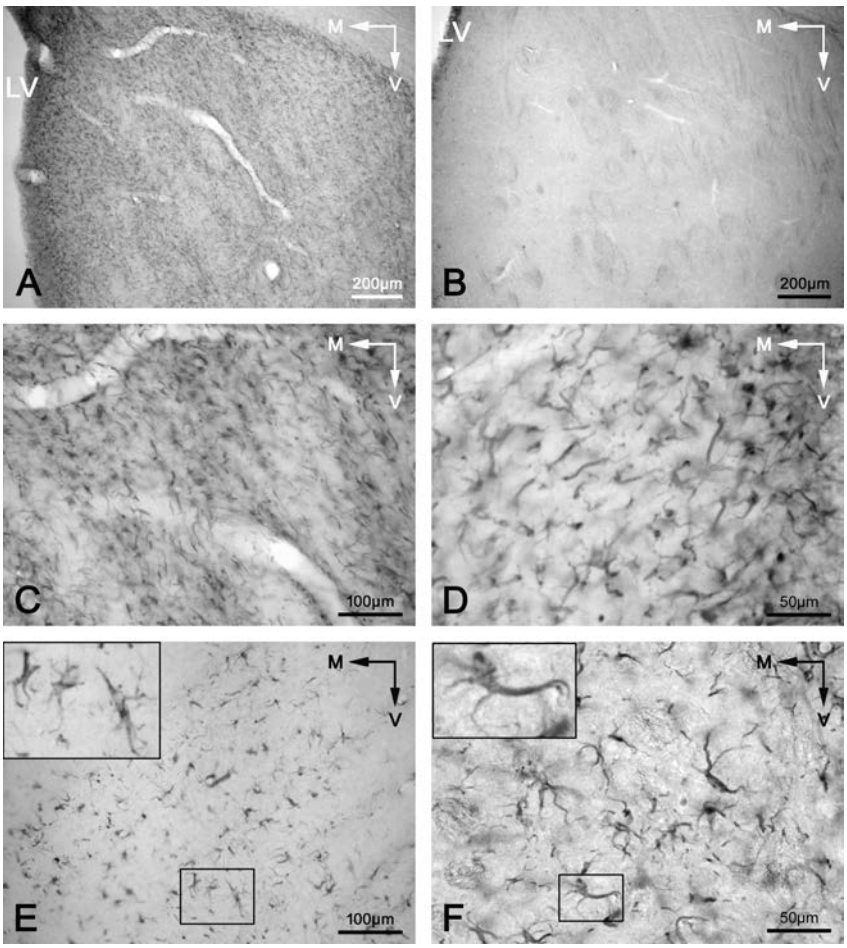

Figure 6. 21D group. Ipsilateral side. Immunoreactivity for 3-HAA (A-F) in non-treated (NaCl, positive control) (A, C-F) and treated (Gemst) (B) animals. Note the absence of immunoreactivity in animals treated with Gemst (B). E) Inset at top left: a higher magnification of the region delimited by the rectangle. F) Inset at top left: higher magnification of the region delimited by the rectangle. LV, lateral ventricle; M, medial; V, ventral.

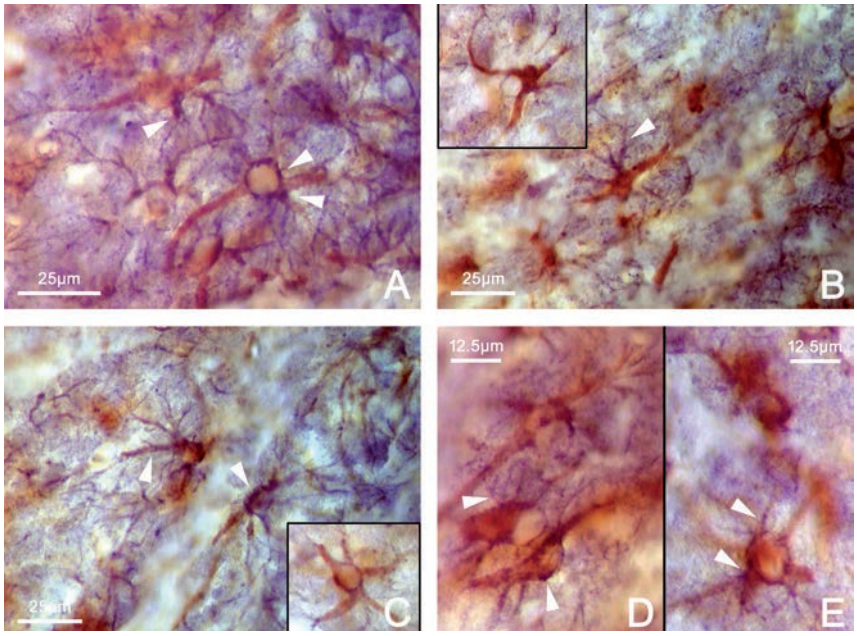

Figure 7. 21D group. Ipsilateral side. Double-labeling immunohistochemistry. The coexistence of KYNA (brown labelling) and 3-HAA (blue labelling, arrowheads) is shown in astrocytes.

PLL-compounds are not immunogenic, since during a chronic treatment (7.5 mg/day for one month) no immunological change was reported.[12]

This means that a long-period treatment is possible without side-effects because: i) PLL is not immunogenic; ii) PLL represents around 90% of the molecular weight and this means that the small molecules (*e.g.,* vitamins) coupled to PLL represent a low weight proportion of the whole PLL-compound molecular weight; iii) PLL compounds are not transporters of small molecules (they are not neither vectors nor liposomes); it should be noted that free constituents (small molecules not linked to PLL) have no effect since they are quickly incorporated into the metabolism and only PLL-compounds have a beneficial effect as previously reported;[12,52] in sum, PLL-compounds have an effect only when small molecules are linked to the PLL and neither free small molecules nor PLL alone have effect;[12,52] and iv) free small molecules are quickly degraded or incorporated to metabolism, but when they are linked to PLL the half-life is longer as well as their effects; for this reason the dose of PLL compounds is much lower than currently standard drugs.

Moreover, PLL compounds are not environmentally toxic since they are naturally recycled by microorganisms into the biotopes. In an open clinical trial, it has been also reported that PLL-compounds were safe and well-tolerated.[11] Thus, PLL-compounds did not show hepatic (e.g., gamma-glutamyl transpeptidase, glutamate oxaloacetate transaminase, glutamate pyruvate transaminase, alkaline phosphatase, bilirubin), biological (*e.g.*, glucose, apolipoproteins, triglycerides, cholesterol and proteins associated, creatinine) and hematological (*e.g.*, red and white blood cells count, hemoglobin, mean globular volume) side-effects.[11,12,52] All together, our data suggest that in humans Gemst could be a new drug candidate for stroke treatment, because Gemst restores the environmental conditions before the ischemic insult and because PLL-compounds are safe.

In sum, the morphological data described here in a tMCAO model of stroke suggests that Gemst exerts the following actions in the late phase of the disease: i) downregulates the IDO pathway; ii) abolishes infiltration; iii) abolishes the activation of microglia; and iv) abolishes gliosis. Moreover, Gemst has no effect on neurogenesis. Gemst exerts a multifunctional action which seems to be beneficial in stroke. In this disease, stem cells and progenitor cells have been suggested to repair the damages induced.[53] Gemst could improve the microenvironmental conditions for transplantation of these cells in order to improve the functional recovery of the infarcted region.

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