Involvement of Rat Hippocampal Astrocytes in β-Amyloid-Induced Angiogenesis and Neuroinflammation

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Abstract: Although Alzheimer's disease (AD) is considered a neurodegenerative disorder, in the last few years a large amount of evidence has suggested that it is also a vascular pathology characterized by increased capillary density and expression of angiogenic factors. In AD the endothelium degenerates, promoting local neuroinflammation and activation of brain endothelium, perivascular microglia, pericytes, astrocytes. Excess tumor necrosis factor (TNF) in the cerebrospinal fluid (CSF), at a concentration of 25 times higher than in the control group, has been demonstrated in AD. Recent studies provide evidence that treatment with TNF-α antagonists may result in a rapid cognitive improvement in AD patients. In the present work we investigated the role of astrocytes in AD angiogenesis and neuroinflammation by means of conditioned media of untreated and Aβ-treated rat hippocampal astrocytes (RHAs) on rat microvascular endothelial cells (RCECs). The results demonstrate that RHA media increase RCEC proliferation and capillary-like structure formation. Moreover RHAs secrete IL-1β and, only after the $A\beta_{1-42}$ treatment, TNF-α promotes RCEC release of IL-1β, IL-6 and TNF-α. The removal of IL-1β, TNF-α and/or VEGF, a strong angiogenic inducer highly over-expressed in AD brains, by means of specific antibody-coated beads in RHA media affects RCEC release of IL-1β, IL-6 and TNF-α. We hypothesised that astrocytes contribute to AD angiogenesis and neuroinflammation by the direct release of pro-inflammatory cytokines. The effect of an anti-inflammatory agent, such as etanercept, decreased RCEC *in vitro* cytokine release. This could be compared to the effect found in our experiments with antibody anti TNF-α-coated beads.

Keywords: Amyloid-β peptide, angiogenesis, astrocytes, endothelial cells, iinflammation, interleukin-1β, interleukin-6, tumor necrosis factor-α.

INTRODUCTION

Recent neuropathological studies have demonstrated that patients with Alzheimer's disease (AD) have concomitant cerebrovascular pathology [1]. In AD amyloid is associated with irregularities in the arterial wall structure and increased microcapillary density in the region of amyloid-β (Aβ) deposits [2-4]. A study using electron microscopy showed that alterations of the capillaries are a common finding both in vascular disease and in AD, suggesting that vascular factors may also play a role in the pathogenesis of AD [5, 6]. Increased vessel diameter, aberrant vessel branching, enhanced endothelial cell proliferation and an irregular basement membrane have been observed in animal models of AD [7, 8] as well as in AD patients [9]. Indeed, a regionally increased capillary density was noticed in AD [10]. Brinda *et al.* [11] demonstrated an increase in vascular density in the hippocampus of AD subjects compared with control subjects, suggesting that angiogenesis earlier in the disease resulted in vessel formation. *In vivo* experiments demonstrate considerable angiogenesis 7 days post-injection of $A\beta_{1-42}$ in rat hippocampus and the association of vascular endothelial growth factor (VEGF) immunoreactivity with astrocytes, but not with ECs, involving astrocytes in proliferation and activation of ECs [12].

High levels of several inflammatory cytokines in serum, plasma, peripheral blood and autopsy specimens of AD patients were found [13]. AD has been linked to inflammation, and inflammatory mediators, tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), and interleukin-8 (IL-8), also possess pro-angiogenic properties [14, 15]. Further support for an Aβ-inflammation-angiogenesis link in AD comes from the analysis of expression levels of several thousand genes using DNA array and proteomic technologies [16-18].

It has been hypothesised that microglia activated by Aβ releases various inflammatory mediators, neurotoxic cytokines [19] and chemokines [20]. It may also recruit astrocytes that actively enhance the inflammatory response to extracellular Aβ deposits, explaining the pro-inflammatory state and damage found on AD brains [19]. Activated glial cells also seem to contribute to pro-angiogenic activity in a peptide-injected brain [20]. In particular high concentrations of proinflammatory cytokines increase oxidative stress, down-reg-ulate endothelial NOS (eNOS) bioactivity and induce EC apoptosis [21]. Apoptotic signals have been reported to be implicated in a variety of cellular functions, including cell proliferation, survival, differentiation and inflammatory response [22].

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The aim of the present study is to estimate the *in vitro* effects of conditioned media of untreated and Aβ-treated rat hippocampal astrocytes (RHAs) on rat microvascular endothelial cells (RCECs). We hypothesised that astrocytes contribute to AD angiogenesis and neuroinflammation by the direct release of pro-inflammatory cytokines. However, *in vivo* assays are essential to confirm our *in vitro* results because of the complex nature of vascular response during angiogenesis [23].

MATERIALS AND METHODS

Rationale

Unconditioned medium (UM): the medium collected from plates without RHAs.

Conditioned control medium (CCM): the medium collected from untreated RHAs.

Conditioned treated medium (TCM): the medium collected from Aβ-treated RHAs.

The subscript number indicates the incubation time (e.g. CCM_{24h} stands for conditioned control medium collected after 24 hour incubation) while the subscript letter "s" refers to all the experimental time points (e.g. CCM_S stands for conditioned control media collected after 24 and 48 hour incubation).

Cells and Culture Conditions

The experimental protocol was approved by the Ethics Committee of the University of Padua for Animal Testing (CEASA).

In this study rat hippocampal astrocytes (RHAs) derived from 1 to 3-day-old Sprague Dawley rat pups (Charles-River, Como, Italy) and rat cerebral endothelial cells (RCECs) derived from adult Sprague Dawley rat (Charles-River, Como, Italy) were used. Primary cultures of RHAs were prepared according to McCarthy [24]. Pups were killed by cervical dislocation, hippocampus was removed, stripped of meninges and cut into small pieces. Cells were enzymatically treated with trypsin solution (0.8 mg/ml) and, after the subsequent treatment with trypsin inhibitor solution (0.5 mg/ml), plated on poly-lysine-coated Petri dishes at a density of $0.8x10^5$ cells/cm^2. The culture medium consisted of Eagle's basal medium (BME) (Sigma Aldrich Corp., St. Louis, MO) supplemented with 2 mM glutamine (Sigma Aldrich Corp., St. Louis, MO), 20 mM $NaHCO_3$, 25 mM KCl, 10% fetal calf serum (FCS) (PromoCell, Heidelberg, Germany) and streptomycin (0.1 mg/ml) (Sigma Aldrich Corp., St. Louis, MO). After the cultures reached confluence, an enriched population of type-1 astrocytes was prepared by means of a 20 hours continuous shaking.

RCECs were isolated and cultured as previously described [25]. Briefly, the grey matter of rat brains was dissected, chopped and centrifuged to separate microvessel fragments. In order to separate microvessels from other components, tissue was digested by means of 0.1% collagenase/dispase (Roche Applied Science, Penzberg, Germany) solution for 1 hour at 37° C, cells were resuspended in 25% BSA and the mixture was centrifuged. The cells were resuspended in Endothelial Cell Growth *Medium* MV2 (PromoCell, Heidelberg, Germany) and seeded on Petri dishes coated with fibronectin (1 μg/cm^2). At confluence, the cultures were *purified by immunoseparation* using Dynabeads M-450 Tosyl-activated (Oxoid, Hampshire, UK) coated with mouse anti-rat CD31 antibody (AbD Serotec Ltd, Oxford, UK).

Cells were characterized by morphology and immunophenotype; in particular, we tested out RHA cultures the expression of glial fibrillary acidic protein [26] and RCECs the expression of von Willebrand factor and capillary-like structure formation [25].

Preparation of the $A\beta_{1-42}$ Peptide

Rat $A\beta_{1-42}$ peptide (Calbiochem, La Jolla, CA) (Aβ) tested on cells was dissolved in sterile distilled water (10^{-3}M) and used at 10^{-7}M final concentration. Only fresh Aβ preparations were used for biological experiments.

For the experimental plan see Supportive material (1).

RHA Culture Media Collection

Our previous experiments on RCECs [27, 28] demonstrated that Aβ effects take place within 24 hours of treatment. Therefore in the present work all the experiments were performed within 48 hour incubation.

RHAs were seeded (6.25×10^4 cells/cm^2) on polylysine coated plates (2.5 μg/cm^2) and grown for 24 hours at 37°C, 5% CO_2 in BME 10% FBS. Then media were replaced with BME 2.5% FBS and different experimental conditions were assessed: plate without cells, plate with untreated RHAs and plate with Aβ-treated RHAs for 6, 12, 24 or 48 hours. At the end of each incubation time media were collected (UM, CCM and TCM respectively), centrifuged and stored at -20°C until use.

They were tested on RCECs with the same corresponding times: viability (6, 12, 24 and 48 hours), proliferation (24 and 48 hours), capillary-like structure formation (24 and 48 hours), determination of released cytokines (6, 12, 24 and 48 hours).

RCEC Proliferation

The proliferation was evaluated using Cell Proliferation ELISA BrdU assay (Roche Applied Science, Penzberg, Germany). RCECs were seeded on 96-well plates and cultured for 24 hours in MV2. 18 hours before the end of the incubation time BrdU was added to the samples. All the subsequent steps were performed according to manifacturer's instructions. The reaction product was quantified by measuring the absorbance at 405nm (reference wavelength 490nm) using Microplate Autoreader ELISA (Bio-Tek Instruments, Winooski, VT).

RCEC Morphogenesis on Matrigel

Growth factor Reduced Matrigel™ Matrix (Benecton-Dickinson Labware, Bedford, MA) was defrosted overnight at 4°C and added (50 μl/cm^2 growth area) on ice-cold plates. RCECs (2.5×10^4 cells/cm^2) were then cultured in 500 μl of RHA media previously collected at 24 and 48 hours at 37°C, 5% CO_2. At the end of each incubation time, samples were washed with PBS and fixed with 10% formaldehyde solution

in PBS for 24 hours. Five random fields were captured using a camera connected to Laborlux S microscope (Leitz, Wetzlar, Germany) (magnification 50x).

Image analysis was carried out using the software Leica Imaging System Ltd (Leica Imaging System) and the dimensional (area % covered by endothelial cells and the total length of the cellular network per field) and the topological parameters (the number of meshes and the number of branching points per field) were estimated and used to characterized the topological structure of endothelial cells according to Guidolin and co-workers [29].

RCEC Viability

RCECs were seeded on 96-well plates and grown for 24 hours in MV2. Media were replaced with RHA media previously collected at 6, 12, 24 or 48 hours. 1 hour and 30 minutes before the end of the incubation, MTT solution was added in an amount equal to 10% of culture medium volume. At the end of incubation cell culture media were removed and MTT crystals were dissolved adding acidified isopropanol (100 µl/well). Plates were gently stirred by gyratory shaker until the MTT formazan crystals have completely dissolved. The reaction product was quantified by measuring the absorbance at wavelength 570nm using Microplate Autoreader ELISA (Bio-Tek Instruments Inc., Winooski, VT).

Determination of Released Cytokines in Culture Media

RCECs were seeded on 96-well plates and cultured for 24 hours in MV2. Media were replaced with 100 µl RHA media for 6, 12, 24 or 48 hours. At the end of incubation, media were collected in polypropylene tubes, centrifuged and stored at -20°C until quantification of cytokines. UM_S were used as blank. The concentration of interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) was determined, using Rat Cytokine/Chemokine Milliplex Kit (Millipore, Billerica, MA) a Luminex X-MAP technology based multiplexed analysis kit. The amount of cytokines contained in cell culture media was determined according to manifacturer's instructions. Assay was carried out on Bio-Plex 100 (BioRad Laboratories) and data were obtained by Bioplex Manager 4.1 software, using a five parametric logistic curve fitting.

Beads Preparation

Magnetic polystyrene beads coated with a specific antibody anti-IL-1β, IL-6 or TNF-α were used to obtain IL-1β-, IL-6- and TNF-α-deprived CCM_S and TCM_S. Firstly beads were equilibrated in appropriate buffer (0.1 M phosphate buffer pH 7.4) allowing beads to be ready for coating. Then 10 µg pure antibody/10^7 beads (rabbit anti-rat TNF-α and rabbit anti-rat IL-1β, Millipore Milano, Italy; rabbit anti-rat VEGF, AbD Serotec Ltd, Oxford, UK) were added to beads solution (concentration 10^7 beads per ml final coating solution) and continued to vortex for 24 hours at 37°C. After incubation coated beads were washed in 0.1% BSA saline solution, re-suspended in buffer solution and kept at 4°C.

Our preliminary cytokine determinations showed that in CCM_S and TCM_S the IL-1β and TNF-α concentration range was approximately 15 pg/ml while VEGF was about 1500 pg/ml. Different coated-bead concentrations were assayed using an excess of cytokines (40 pg/ml for IL-1β and TNF-α and 2000 pg/ml for VEGF) and (10^6 beads)/(100µl culture medium) were needed to remove more than the concentration of the three cytokines in the CCM_S and TCM_S Fig. (**1**).

Beads were added to RHA collected media. After 60 minutes of incubation at 4°C beads were removed by a magnetic particle concentrator and the media were collected and stored at -20°C until required.

The experimental conditions tested on RCECs were the following: complete medium; VEGF deprived medium ((-) VEGF); IL-1β deprived medium ((-) IL-1β); TNF-α deprived medium ((-) TNF-α); VEGF and TNF-α deprived medium ((-) VEGF (-) TNF-α); VEGF and IL-1β deprived medium ((-) VEGF (-) IL-1β); TNF-α and IL-1β deprived medium ((-) TNF-α (-) IL-1β); VEGF, IL-1β and TNF-α deprived medium ((-) VEGF (-) IL-1β (-) TNF-α).

RESULTS

RCEC Proliferation

Proliferation effect of CCM_S and TCM_S on RCECs was assessed by BrdU assay. Treatment with CCM_{24h} did not affect RCEC proliferation in the following 24 hours while at 48 hours of CCM_{48h} proliferation rate increased ($p<0.05$) in comparison to UM_{48h} on RCECs. TCM_{48h} increased proliferation rate of RCECs in comparison to UM_{48h} and to CCM_{48h} ($p<0.01$) Fig. (**2**) see Supplementary material (**1**).

RCEC Morphogenesis on Matrigel

To assess whether CCM_S and TCM_S affected capillary-like structure formation at 24 and 48 hours, cells were seeded on growth factor-reduced Matrigel. Quantitative analysis demonstrated that CCM_S increased dimensional parameters, such as percent area ($p<0.01$) covered by cells and the total length ($p<0.05$ at 24 hours; $p<0.01$ at 48 hours) of the cell network per field, and topological parameters, such as mesh number ($p<0.05$ at 24 hours; $p<0.01$ at 48 hours) and branching points ($p<0.01$ only at 48 hours of treatment) per field compared to UM_S.

TCM_S increased the dimensional parameters, such as percent area ($p<0.05$ only at 48 hours of treatment) covered by cells and the total length ($p<0.05$ only at 24 hours of incubation) of the cell network per field, and topological parameters, such as mesh number ($p<0.05$ at 24 hours; $p<0.01$ at 48 hours) and branching points ($p<0.05$ only at 48 hours of treatment) per field compared to UM_S. Moreover they decreased the percent area ($p<0.05$ only at 24 hours of treatment) covered by cells, the total length ($p<0.05$ only at 48 hours of incubation) of the cell network per field, and the mesh number ($p<0.01$ only at 48 hours of incubation) compared to CCM_S Fig. (**3**) see Supplementary material (**2**).

RCEC Viability

MTT assay was performed to test whether CCM_S and TCM_S affected RCEC viability at 6, 12, 24 and 48 hours. No effects were observed at 6 and 24 hours of exposure with 6 and 24 hours media respectively while CCM_{12h} and TCM_{12h} increased RCEC redox activity in comparison to UM_{12h}

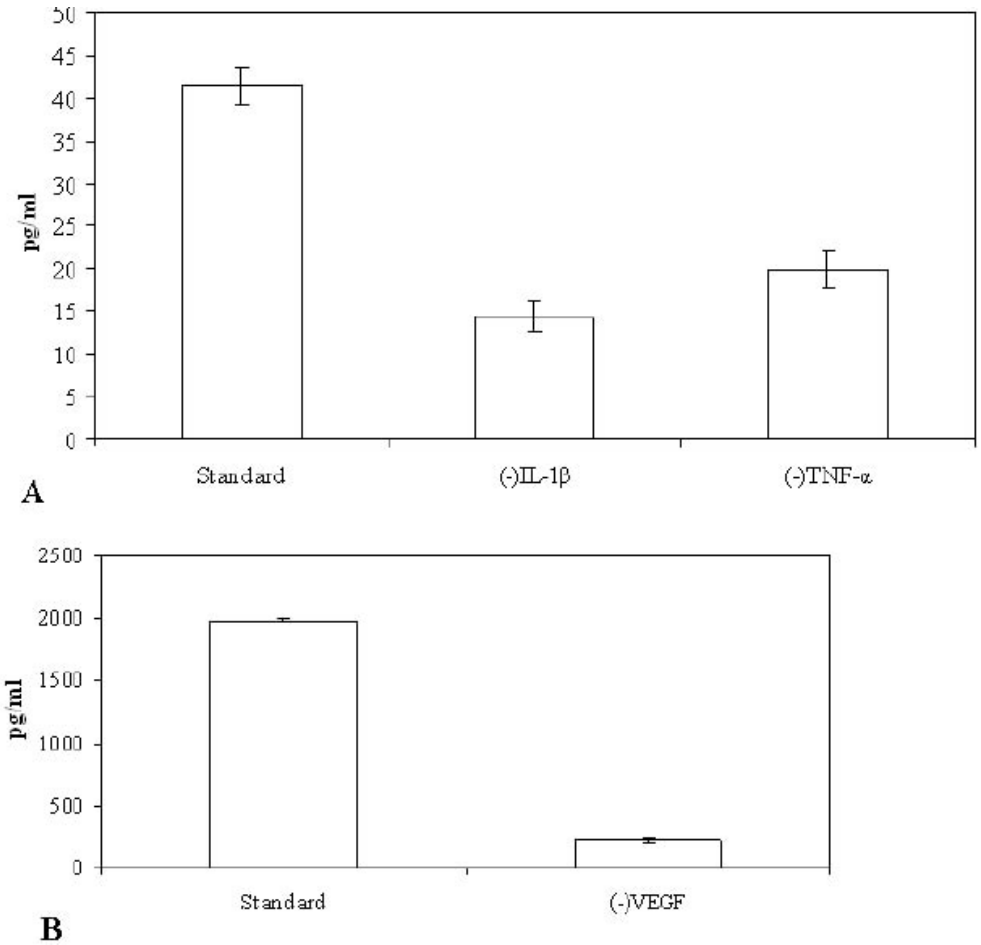

Fig. (1). effect of IL-1β and TNF-α-coated beads on IL-1β and TNF-α removal respectively (**A**). Effect of VEGF-coated beads on VEGF removal (**B**).

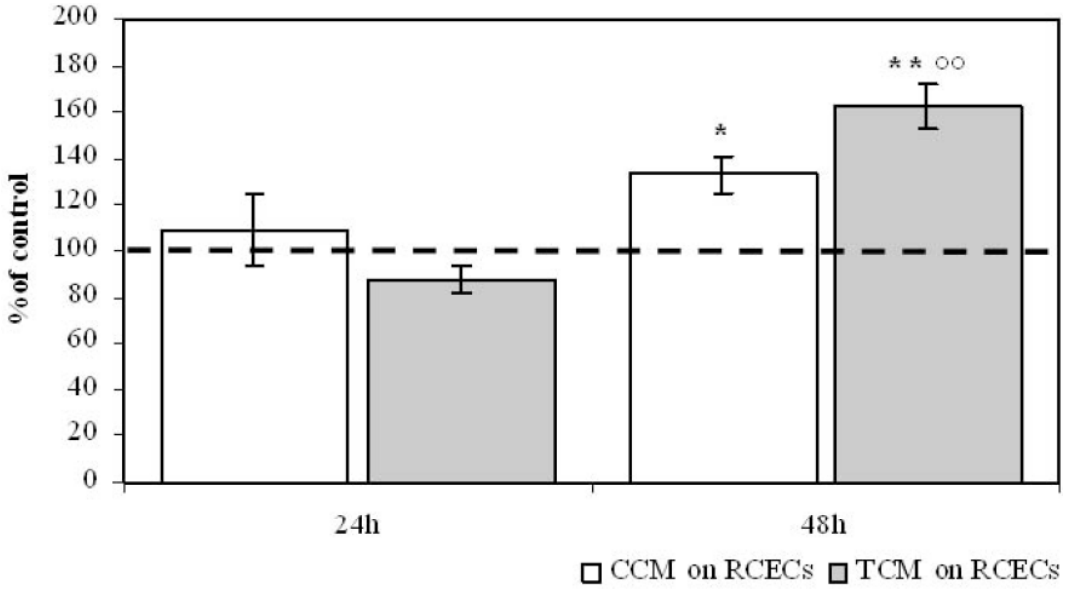

Fig. (2). BrdU assay performed on RCECs after 24 or 48 hour treatment with $CCM_{24h,\ 48h}$ and $TCM_{24h,\ 48h}$. 24 hour RHA medium did not affect the proliferation rate of RCECs at 24 hours while CCM_{48h} increased proliferation of RCECs at 48 hours in comparison to UM_{48h}. The broken lines represent the effect of UM_S on RCECs. * $p<0.05$ and ** $p<0.01$ to UM on RCECs; °° $p<0.01$ to TCM. Statistical analysis was performed by ANOVA followed by the t-test of Student-Newman-Keuls as post-hoc test. Bars are means ± standard deviation of six separate experiments.

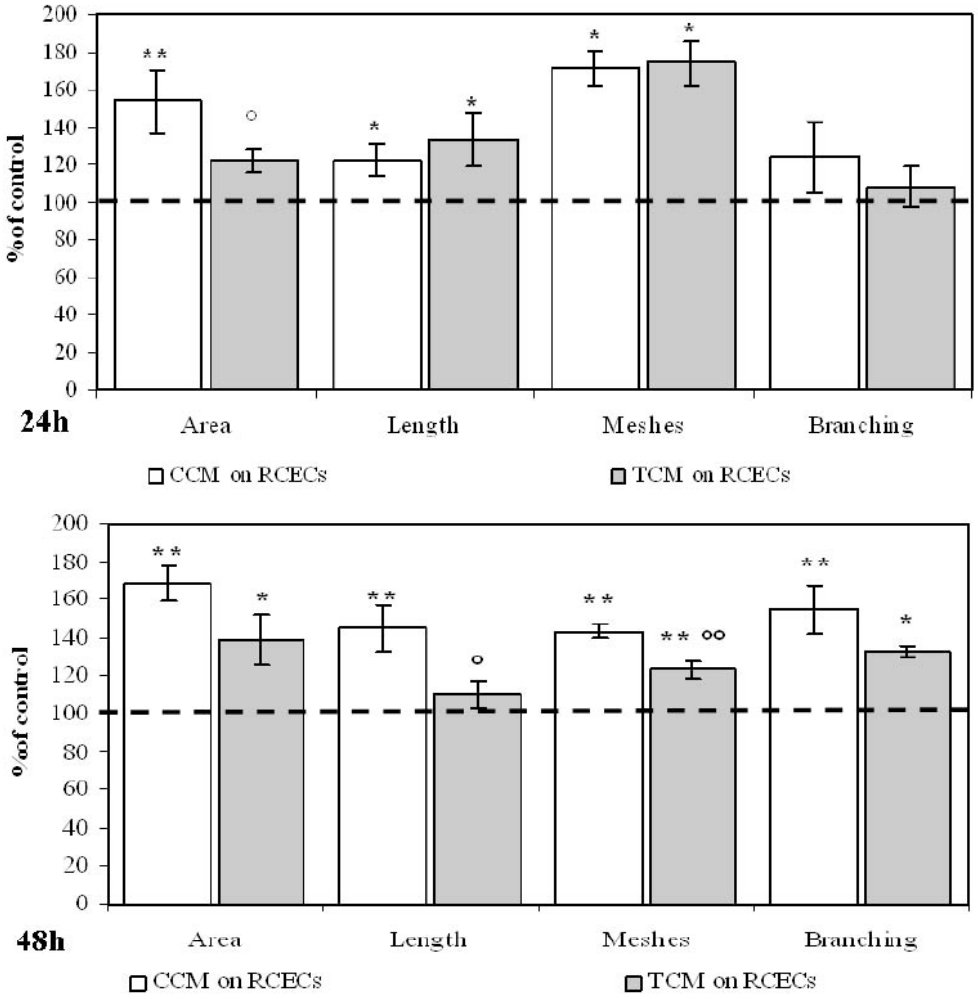

Fig. (3). Quantitative analysis of tube formation estimated as dimensional (percent of area covered by cells and total length of the cell network per field) and topological (mesh number and branching point per field) parameters indicates that both $CCM_{24h,\ 48h}$ and $TCM_{24h,\ 48h}$ increased the capillary-like structure formation in RCECs at 24 and 48 hours in comparison to $UM_{24h,\ 48h}$. The treatment with $CCM_{24h,\ 48h}$ increased all the considered parameters also in comparison to $TCM_{24h,\ 48h}$. The broken lines represent the effect of UM_S on RCECs. * $p<0.05$ and ** $p<0.01$ to UM_S on RCECs; ° $p<0.05$ and °° $p<0.01$ to CCM_S. Statistical analysis was determined by Student's t test unpaired data between mean values. Bars are means ± standard deviation of five separate experiments.

($p<0.05$ and $p<0.01$ respectively). The incubation of TCM_{48h} for 48 hours decreased ($p<0.01$) RCEC redox activity in comparison to UM_{48h} and CCM_{48h} ($p<0.01$) Fig. (**4**) see Supplementary material (**3**).

Determination of Released Cytokines in Culture Media

To determine the levels of the three cytokines (IL-1β, IL-6 and TNF-α) in CCM_S and TCM_S before and after the exposure to RCECs, Rat Cytokine/Chemokine Milliplex Kit based on Luminex method was used. Both RHAs and RCECs release low levels of the cytokines.

After 6 and 12 hours the level of IL-1β significantly increased in all the media tested in comparison to $CCM_{6h,\ 12h}$ ($p<0.05$ in RCEC medium treated with $CCM_{6h,\ 12h}$; $p<0.01$ in the other). Moreover after the exposure on RCECs, the $TCM_{6h,\ 12h}$ had higher levels ($p<0.01$) of IL-1β than $CCM_{6h,\ 12h}$. No differences were observed between $TCM_{24h,\ 48h}$ and $CCM_{24h,\ 48h}$ treatment Fig. (**5A**).

The level of IL-6 increased in CCM_S ($p<0.01$) and TCM_S ($p<0.01$ after 6 and 12 hours; $p<0.05$ after 24 and 48 hours) after the exposure to RCECs in comparison to CCM_S. No differences were observed in TCM_S in comparison to CCM_S at all the time points. After 6 hours of exposure to RCECs, the level of IL-6 in TCM_{6h} was higher than all the other media ($p<0.05$ to CCM_{6h} after RCEC exposure; $p<0.01$ to TCM_{6h}) Fig. (**5B**).

After 6, 12 and 48 hours the level of TNF-α significantly increased in $TCM_{6h,\ 12h,\ 48h}$ ($p<0.01$ after 6 and 12 hours; $p<0.05$ after 48 hours) and decreased in the other media in comparison to $CCM_{6h,\ 12h,\ 48h}$ ($p<0.01$). No effects were observed after 24 hours of treatment Fig. (**5C**) see Supplementary material (**4**).

To evaluate the effect of IL-1β, TNF-α and VEGF of conditioned $RHA_{6h,\ 12h}$ media on RCEC secretion of the interested cytokines, IL-1β, TNF-α and VEGF were removed from RHA culture media by means of specific antibody-

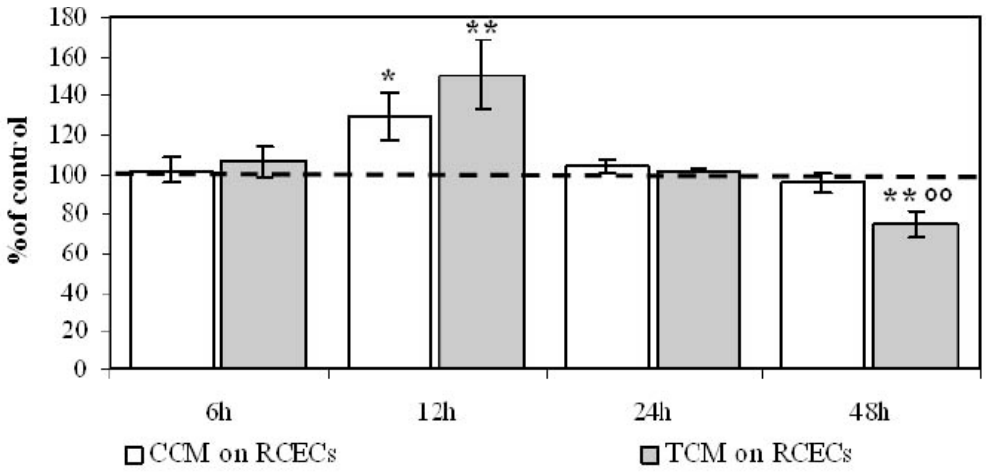

Fig. (4). Effect of 6, 12, 24 and 48 hours of CCM_S and TCM_S on the viability of cultured RCECs, as measured by MTT-reduction assay after 6, 12, 24 and 48 hours. The broken lines represent the effect of UM_S on RCECs. * $p<0.05$ to and ** $p<0.01$ to UM_S on RCECs; °° $p<0.01$ to CCM_S. Statistical analysis was performed by ANOVA followed by the t-test of Student-Newman-Keuls as post-hoc test. Bars are means ± standard deviation of four separate experiments.

coated beads and the obtained media were tested on RCECs. The levels of the three cytokines (IL-1β, IL-6 and TNF-α) were evaluated after 6 and 12 hours of incubation.

After 6 hours of exposure to RCECs, the level of IL-1β significantly increased in CCM_{6h} deprived of VEGF, or TNF-α, or IL-1β ($p<0.01$), or VEGF and TNF-α ($p<0.05$) or all the cytokines ($p<0.05$) in comparison to untreated coated-bead media. The levels of IL-1β in the RCEC media incubated with TCM_{6h} decreased in media in which IL-1β alone or in association with VEGF ($p<0.05$) were previously removed and they were absent in the medium in which TNF-α in association with VEGF and/or IL-1β were removed. After 12 hours of exposure to RCECs, in both CCM_{12h} and TCM_{12h} in which the cytokines alone or in association with the others were removed, IL-1β was absent Fig. (**6**) see Supplementary material (**5**).

After 6 hours of exposure to RCECs, in CCM_{6h} and TCM_{6h} the level of IL-6 decreased ($p<0.01$) in all the experimental conditions in comparison to media non-treated with coated-beads media. After 12 hours IL-6 significantly decreased in RCEC media treated with CCM_{12h} in which cytokines were removed ($p<0.05$ in media where VEGF, IL-1β, VEGF and TNF-α, TNF-α and IL-1β, all the cytokines were removed; $p<0.01$ where TNF-α, VEGF and IL-1β were removed) in comparison to CCM_{12h} after RCEC exposure. Similarly in cytokine deprived TCM_{12h} exposed to RCEC the levels of IL-6 decreased in all the experimental conditions ($p<0.01$) in comparison to TCM_{12h} in which no cytokines were removed. No effect was observed in CCM_{12h} and TCM_{12h} in which TNF-α in association with IL-1β were removed Fig (**7**) see Supplementary material (**6**).

After 6 and 12 hours of incubation of RCECs in $CCM_{6h,\ 12h}$ and $TCM_{6h,\ 12h}$ the levels of TNF-α decreased in all the experimental conditions ($p<0.05$ in the CCM_{6h} where VEGF was removed and $p<0.01$ in all the other experimental conditions; $p<0.01$ in TCM_{6h} where all the cytokines were removed and $p<0.05$ in all the other experimental conditions; $p<0.01$ in CCM_{12h} in all the experimental conditions; $p<0.05$ in TCM_{12h} where VEGF was removed and $p<0.01$ in all the other specimens) in comparison to both RHA media in which no cytokines were removed Fig (**8**) see Supplementary material (**7**).

DISCUSSION

AD is associated with vascular disorders that initiate pathology through cerebral microvascular abnormalities, including thinning and discontinuities within the vascular basement membrane, shrinkage of endothelial cells, pericyte degeneration, and luminal buckling [30]. Many studies on AD brain specimens demonstrate increased vascular density [5, 11, 31] and the over-expression of pro-angiogenic factors [32] in AD brains compared with controls. In the last few years the role of inflammatory processes in AD pathogenesis has generated great interest [33]. Brinda *et al.* [11] propose that an initial neuronal insult triggers an inflammatory response, resulting in angiogenesis allowing neurotoxic substances and immune cells to cross the blood-brain barrier (BBB), exacerbating tissue damage and perpetuating an inflammatory response.

Aβ activates microglia and astrocytes that release several pro-inflammatory and pro-angiogenic mediators [20, 21]. Moreover it possess pro-angiogenic activity [34, 35] as well as anti-angiogenic *in vitro* activity [36, 37]. In the present study 24 and 48 hour conditioned media of untreated and Aβ-treated RHAs were tested on RCECs with the same corresponding time trying to explain the possible involvement of astrocytes in promoting proliferation and capillary-like structure formations. Both 48 hour RHA media increase RCEC proliferation. The effect of TCM_S was higher than CCM_S. Moreover both 24 and 48 hour RHA media increase the formation of capillary-like structures on Matrigel and the effect of CCM_S is higher than TCM_S. RHAs promote both *in vitro* angiogenic assays probably because of pro-angiogenic molecule release in the media. However the effect is independent of Aβ treatment.

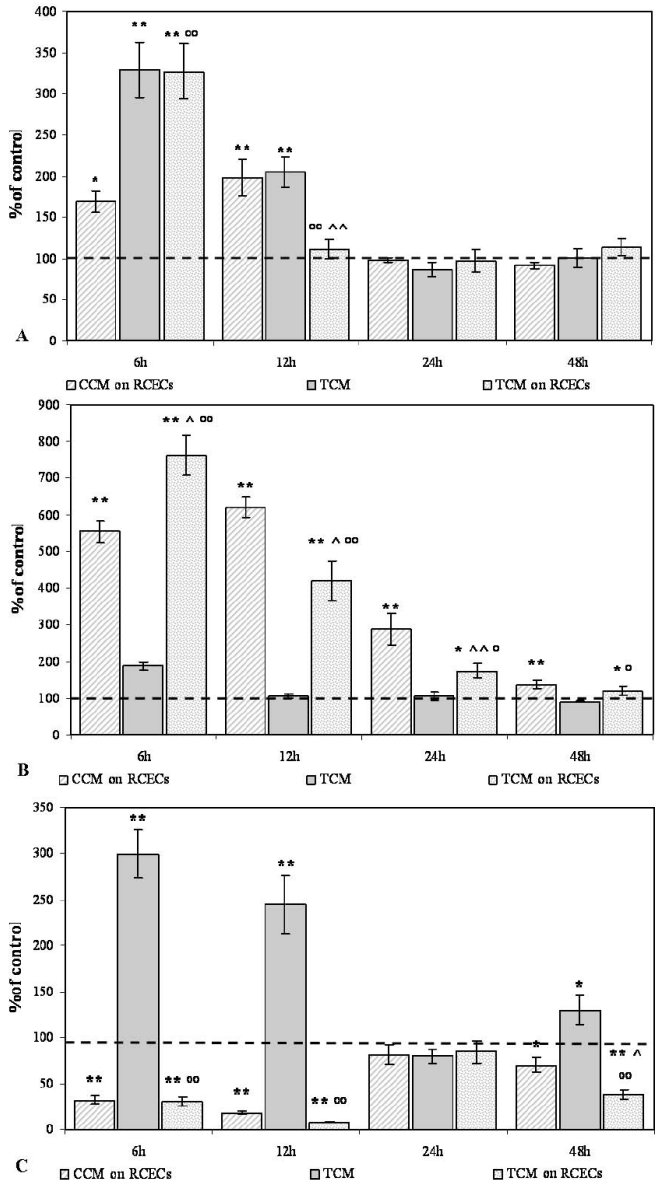

Fig. (5). Effect of 6, 12, 24 and 48 hours of CCM_S and TCM_S on the release of IL-1β (**A**), IL-6 (**B**) and TNF-α (**C**) before and after contact with RCECs, as measured by Luminex technique. The broken lines represent the cytokine levels in CCM_S. * $p<0.05$ and ** $p<0.01$ to CCM_S; ° $p<0.05$ and °° $p<0.01$ to TCM_S; ^ $p<0.05$ and ^^ $p<0.01$ to CCM_S after contact with RCECs. Statistical analysis was performed by ANOVA followed by the t-test of Student-Newman-Keuls as post-hoc test. Bars are means ± standard deviation of four separate experiments.

RCEC viability increased at 12 hour treatment with both CCM_{12h} and TCM_{12h} and decreased at 48 hour treatment with TCM_{48h}. Probably the secretion of RHA pro-angiogenic factors induces the release of RCEC pro-inflammatory cytokines that may decrease RCEC viability. Indeed high concentration of pro-inflammatory cytokines increases oxidative stress, down-regulates eNOS bioactivity and induces EC apoptosis [21]. Apoptotic signals have been reported to be implicated in a variety of cellular functions, including cell proliferation, survival, differentiation and inflammatory response [22].

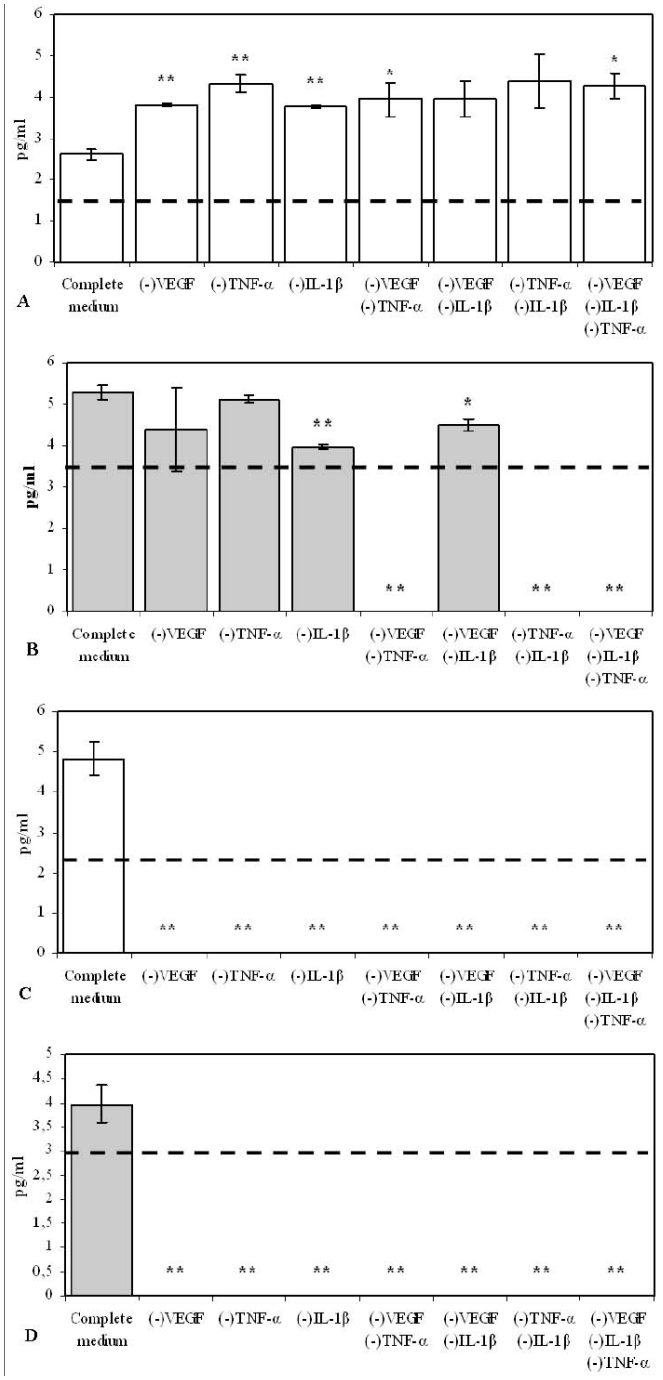

Fig. (6): Effect of 6 (**A** and **B**) and 12 (**C** and **D**) hours of CCM_S (**A** and **C**) and TCM_S (**B** and **D**) before and after the removal of VEGF, TNF-α and/or IL-1β on the release of IL-1β from RCECs, as measured by Luminex technique. The broken lines represent the concentration of IL-1β in CCM_S. * $p<0.05$ and ** $p<0.01$ to non-treated coated-bead RHA media on RCECs. Bars are means ± standard deviation of three separate experiments.

Local inflammatory responses in AD involve microglia activation and inflammatory cytokine production [38, 39]. There is a cerebral spinal fluid (CSF) concentration increase of different pro-inflammatory cytokines [19], such as IL-1β, IL-6 and TNF-α [40], and pro-angiogenic factors, such as vascular endothelial growth factor (VEGF) and TGF-β [11],

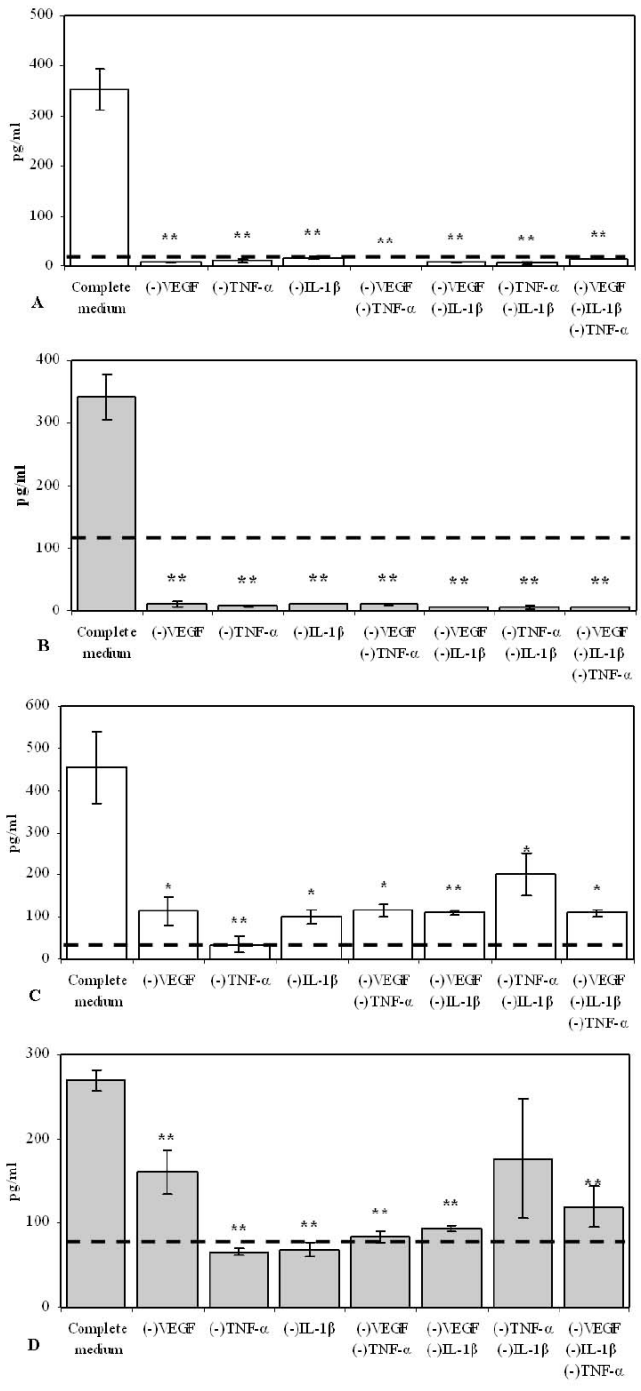

Fig. (7). Effect of 6 (**A** and **B**) and 12 (**C** and **D**) hours of CCM_S (**A** and **C**) and TCM_S (**B** and **D**) before and after the removal of VEGF, TNF-α and/or IL-1β on the release of IL-6 from RCECs, as measured by Luminex technique. The broken lines represent the concentration of IL-6 on CCM_S. * $p<0.05$ and ** $p<0.01$ to non-treated coated-bead RHA media on RCECs. Bars are means ± standard deviation of three separate experiments.

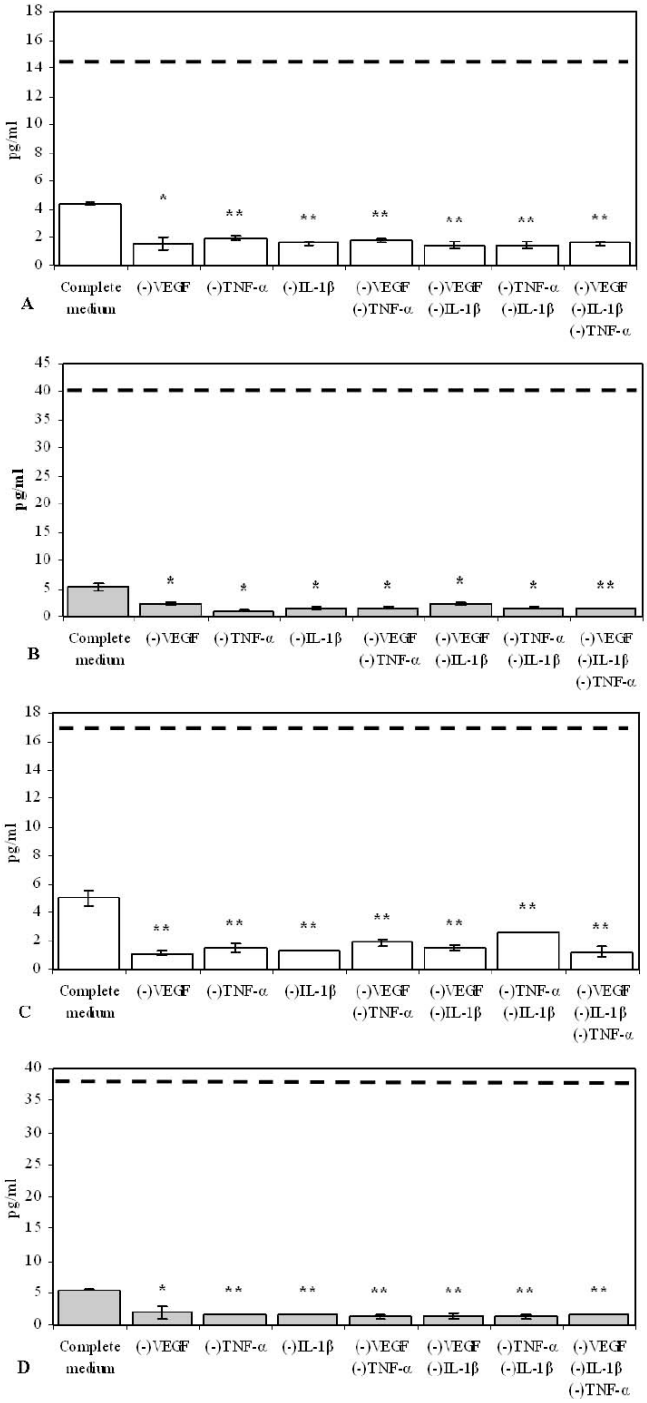

Fig. (8): effect of 6 (**A** and **B**) and 12 (**C** and **D**) hours of CCM_S (**A** and **C**) and TCM_S (**B** and **D**) before and after the removal of VEGF, TNF-α and/or IL-1β on the release of TNF-α from RCECs, as measured by Luminex technique. The broken lines represent the concentration of TNF-α on CCM_S. * $p<0.05$ and ** $p<0.01$ to non-treated coated-bead RHA media on RCECs. Bars are means ± standard deviation of three separate experiments.

in AD patients when compared to the controls [19]. Excess TNF in CSF, at concentrations 25 times higher than in controls, has been demonstrated in AD, and may predict disease progression [41]. Moreover, elevated levels of the soluble TNF receptors (sTNFR), a reliable indicator of the activation of the TNF system, has been found in both CSF and plasma of pre-clinical dementia in comparison to the age-matched control [42]. Thus IL-1β, IL-6 and TNF-α levels in CCM_S and TCM_S before and after the exposure to RCECs were evaluated. Both 24 and 48 hour RHA media increased the

release of IL-6 from RCECs after 24 and 48 hours respectively. The effect is again independent of Aβ treatment.

IL-1β and TNF-α act as potent stimulators of cytokine and chemokine expression for both astrocytes and microglia. They can also stimulate IL-6 production by astrocytes which, in turn, acts in an autocrine manner to potentiate IL-6 release [43, 44]. Indeed 6 and 12 hour IL-1β, IL-6 and TNF-α levels in CCM_S and TCM_S before and after the exposure on RCECs were evaluated: IL-1β increased in all the media while IL-6 only in RCEC media treated with both CCM_S and TCM_S and TNF-α only in TCM_S. RHA media stimulated the secretion of IL-1β and TNF-α from RCECs within 24 hours while IL-6 at all the time points. The maximum cytokine release is at 6 hours.

Perispinal administration of etanercept, a genetically engineered fusion protein consisting of two recombinant human TNF p75 receptors linked to a Fc portion of human IgG1 fragment [45], resulted in clinical improvement in patients with AD. In particular a prospective clinical trial of six months duration involving AD patients ranging in severity from mild to severe, revealed improvements in verbal learning, memory and fluency. It has been hypothesised that perispinal administration of etanercept may ameliorate the effects of excess CSF TNF by passage across the blood-cerebrospinal fluid barrier in a therapeutically effective concentration [46-48]. TNF-α inhibits *in vitro* endothelial proliferation and tube formation [49] suggesting that TNF-α may indirectly induce angiogenesis activating other angiogenic regulators. TNF-α induces the sequential upregulation of angiopoientin (Ang)-2 and then Ang-1 and VEGF mRNA and protein expression in choroidal microvascular ECs and in HUVECs *in vitro*, exerting its profound angiogenic action *in vivo* by stimulating the appropriate sequence of endothelial-specific angiogenic factors [49]. Angiogenesis requires initial inactivation, or at least weakening, of constitutive tyrosine kinase with immunoglobulin and epidermal growth factor homology domains 2 (Tie2) signalling in ECs. It has been postulated that Ang-2 co-operates with VEGF at the leading edge of the vascular sprout by blocking the stabilizing or maturing function of Ang-1, thus allowing vessels to revert to, and remain in, a plastic state where they are more responsive to a sprouting signal by VEGF [50]. These data provide support for the importance of TNF-α and Ang-1/Ang-2 and their autocrine regulatory loops in neovascularisation. [49]. We hypothesized pro-angiogenic effect of RHA media is due to the stimulation of different factors acting together to promote angiogenesis: TNF-α activates RCECs and subsequently other cytokines, which are released by RHAs or by RCECs, promote angiogenesis. Thus we evaluated the IL-1β, IL-6 and TNF-α levels in 6 and 12 hour RCEC media treated with 6 and 12 hour CCM_S and TCM_S where IL-1β, TNF-α and VEGF (that is expressed by AD but not in control-derived microvessels [51]) were selectively removed. IL-1β, TNF-α and VEGF secreted by RHAs play a pivotal role in RCEC release of IL-6 and TNF-α at both 6 and 12 hours and of IL-1β only after 12 hours. The removal of TNF-α alone and together with the other cytokines prevented the 6 hour IL-1β release by RCECs. In contrast, the removal of just the other cytokines, leaving the TNF-α, did not however affect the production of the IL-1β.

IL-1β and TNF-α control survival, growth and effector functions of tissue cells. They require *de novo* synthesis and are often not stored in vesicles. Once released, cytokines mostly act locally in an autocrine or paracrine manner at low concentrations. IL-1β, produced by blood vessels in AD or by astrocytes in animal models and acting on its signalling receptor expressed by brain ECs and perivascular macrophages or hippocampal neurons, plays an important role in mediating neuronal damage [52]. TNF-α plays a pivotal role in the disruption of macrovascular and microvascular circulation both *in vivo* and *in vitro* experiments [53]. To identify heritable traits in middle age that contribute to AD, van Exel *et al.* demonstrate that the higher production capacity of the pro-inflammatory cytokines, including TNF-α, is likely to be part of this risk profile [54]. The up-regulated TNF-α contributes to age-induced endothelial alterations:oxidative stress by up-regulating/activating NAD(P)H oxidase, endothelial dysfunction, endothelial apoptosis, and up-regulation of pro-atherogenic inflammatory mediators, such as inducible nitric-oxide synthase (iNOS) and adhesion molecules [53, 55].

Aβ is able to localize on vascular endothelium and to inhibit endothelial nitric oxide synthase (NOS) activity causing endothelial dysfunction. More interesting, at the level of BBB, an increased flow of circulating free Aβ causes an up-regulation of the receptor for advanced end glycation products (RAGE) which is responsible for the increase of the soluble vascular cell adhesion molecule (sVCAM)-1 expression [56]. Our previous data suggest RAGE involvement, at least partly, in mediating Aβ effects on RCECs. In particular, the *in vitro* decrease of cell viability and functionality and nitrosative stress activation was inhibited by preventing Aβ-RAGE interaction [27]. The promoter region of the TNF-α gene contains a nuclear factor (NF)-κB binding element, and the ligation of RAGE is known to trigger a series of cellular signalling events, including the activation of NF-κB, leading to the production of proinflammatory cytokines [53, 57, 58].

CONCLUSION

Our *in vitro* study on astrocytes and microvascular endothelial cells could explain the link among AD-angiogenesis-inflammation.

We demonstrated the *in vitro* pro-angiogenic effect of conditioned media of untreated and Aβ-treated rat hippocampal astrocytes on microvascular endothelial cells. Astrocytes modify endothelial cell morphologic properties and inflammatory cytokine secretion confirming the *in vivo* observation of microglia activation, inflammatory cytokine production in AD [38, 39], and the disruption of macrovascular and microvascular circulation both *in vivo* and *in vitro* [53].

Astrocytic secretion of TNF-α plays a key role in endothelial cell pro-inflammatory release. In fact astrocytic media bereft of TNF-α decreased the endothelial cell release of IL-6, IL-1β and TNF-α cytokines. Our results could explain the rapid clinical improvement in etanercept administration, an anti-TNF-α agent, that penetrates into the CSF in a therapeutically effective concentration [47], in AD patients, suggesting the existence of rapidly reversible TNF-mediated pathophysiological mechanisms [48].

Our results need to be confirmed by *in vivo* experiments as the *in vitro* tests provide critical information to be validated. In fact endothelial cell chemokinesis, chemotaxis, proliferation, and tube formation are complementary assays that need to be verified *in vivo* [23].

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publishers Web site along with the published article.

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