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Original Article

Rubia cordifolia, Fagonia cretica linn and *Tinospora cordifolia* exert anti-inflammatory properties by modulating platelet aggregation and VEGF, COX-2 and VCAM gene expressions in rat hippocampal slices subjected to ischemic reperfusion injury.

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Summary: The formation of cerebral edema and central nervous system (CNS) inflammation are a result of cerebral ischemia. Pharmacological strategies to reverse or minimize acute ischemic brain injury include "antiplatelet" agents, anticoagulants, and thrombolytics. However, these therapies have either exhibited undesirable side effects or are not cost-effective for the common people. We report here the neuroprotective effects of three herbs Rubia cordifolia (RC), Fagonia cretica linn (FC) and Tinospora cordifolia (TC) as potent anti-inflammatory agents in view of their ability to downregulate the expressions of COX2 and VCAM genes and upregulate VEGF expression and inhibit platelet aggregation induced by multiple agonists in hypoxic-ischemic hippocampal slices. All the three herbs exhibited appreciable anti-inflammatory properties.

Industrial relevance: The above work will lead to development of new anti-inflammatory drugs with less toxic preparations and has the potential to generate employment among people who will go farming of such medicinal plants.

Keywords: Rubia cordifolia, Fagonia cretica linn, Tinospora coridfolia, inflammation, COX2, VCAM, VEGF, platelet aggregation, ischemia, hippocampus.

Introduction

Stroke is one of the most leading causes of death and disability worldwide. Inflammation is one of the important factors for progression and severity of stroke (Chamorro et.al., 2006, Samson et.al., 2005, Quing et.al., 2007). Reperfusion after ischemia leads to formation of reactive oxygen species (ROS), which stimulates ischemic cells and neurons to secrete inflammatory cytokines and chemokines causing leucocyte recruitment and up-regulation of adhesion molecules in the cerebral vasculature (Quing et.al., 2007, Li et.al., 2000). Since inflammation appears to contribute to ischemic pathology, anti-inflammatory strategies have therefore become therapies of choice.

Cell adhesion molecules (CAM), expressed and generated during inflammation also play a role in the pathogenesis of cerebral ischemia (Frijns & Kapepelle, 2002, Fassbender et.al., 1995, Blann et.al., 1999). As potent mediators of inflammation, arachidonic acid metabolites contribute to post-ischemic brain inflammation via activation of cycloxygenase-2 (COX-2) and production of thromboxanes (Sachez-Moreno et.al., 2004, Nogowa et.al., 1997). Pharmacological interventions to reverse or minimize acute ischemic brain injury include antiplatelet aggregatory agents, anticoagulants, and thrombolytics, of which antiplatelet therapy has been the most preferred mode of treatment (Lipton, 1999, Lancet, 1997a, Lancet, 1997b, Lancet, 1995, Verstraete, 1991). However, these therapies have exhibited toxic side effects and are quite expensive for the common man.

Keeping with the aforementioned difficulties in therapeutic stroke management and considering the effects of the herbs Rubia cordifolia (RC), Fagonia cretica linn (FC) and Tinospora cordifolia (TC) as described by us in our earlier studies (Rawal et.al., 2004, Rawal et.al., 2008), in the present study we tested their effect on platelet aggregation using various platelet-agregatory agents and their anti-inflammatory properties by

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evaluating the gene expression of COX2 a potent inflammatory mediator, VCAM the vascular cell adhesion molecule and an angiogenic factor VEGF.

Materials and Methods

Preparation of Hippocampal Slices & induction of hypoxic ischemia: Method for preparation of hippocampal slices and induction of hypoxic ischemic by oxygen-glucose deprivation (OGD) was as described by Taylor & Weber (Taylor & Weber, 1993).

Study Groups: Hippocampal slices were divided into 3 groups for present study.

a) The overall control group consisted of the slices stabilized for 2 hours at 22-26 ^{o}C in normoxic medium.

b) The experimental control consisted of stabilized slices subjected to ischemic insult by OGD for 30 min and then reperfused with normoxic medium for 24-48 hours.

c) The experimental group consisted of stabilized slices, given ischemic insult by OGD and then treated with the herbal preparation for 24-48 hours.

Reverse transcription Polymerase chain reaction (RT-PCR): Total RNA was isolated from control and treated cells using TRIzol reagent (GIBCO-BRL), as per manufacturer's instructions. Moloney murine leukemia virus Reverse Transcriptase (Promega, UK) was used to reverse transcribe cDNA from 2µg of mRNA according to the manufacturer's instructions.

COX-2: for-5'-TCCCGGATCCCCAAGGCACAAATA-3',

rev-5'-TCAGACCCGGCACCAGACCAAAGA-3'.

PCR conditions were 35 cycles of denaturation at 95°C for 20 s; annealing at 63°C for 20 s, and extension at 72°C for 20 s with a final extension at 72°C (product size 582bp).

VCAM: for-5'AGTGGTGGCCTCGTGAATGG-3',

rev- 5'CTGTGTCTCCTGTC TCCGCT-3'.

PCR comprised of 35 cycles 95^{o}C 1 minute, 62 ^{o}C 45 s and 72^{o}C for 1 minute with a final extension of 10 minutes (product size 700bp).

VEGF: for-5'CACCGCCTTGGCTTGTCACA,

rev-5'-GCTCTCTTGGGTGCACTGGA.

PCR conditions were set to 34 cycles of 95 °C 30 s, 58 °C 45 s, 72 °C 60 s and final extension at 72 °C for 5 minutes (product size 431bp).

PCR products were separated by electrophoresis through 2% agarose containing 0.5 µg/mL ethidium bromide. The resolved bands were visualized and scanned by a white/UV transilluminator (Ultra Violet Products, Cambridge, UK) and quantified by densitometry.

Platelet aggregometry: Platelet aggregation studies were performed using human platelets via turbidometric analysis using two-channel platelet aggregometer (Chronolog Ca560, Labmedics, Stockport, UK). Signals were processed by a MacLab/4e analogue-digital converter and displayed through Chart software (AD Instruments, Sussex, UK). Aliquots (0.5ml) of platelet rich plasma (PRP) were equilibrated at 37^{o}C before induction of platelet aggregation by collagen, U46199 (a thromboxane B2 analogue), A23817 (calcium ionophore), ADP and PMA. The drugs were added in conjunction with the induction of platelet aggregation. Aggregation was monitored for 5 min and the maximum response recorded.

Statistical analysis

All data were expressed as mean ± SEM. Means were compared by ANOVA and a two way unpaired student's t-test (Skewness analysis showed the data to be normally distributed), followed by Tukey's post hoc test for multigroup comparisons and Bonferroni post-hoc analyses. $p<0.05$ was considered significant.

Results

RC, FC and TC exhibit preferential specificity towards mediators of platelet aggregation (PA)

Only RC and not RC and TC, exhibited partial inhibitory effects on A23817 (a calcium ionophore) mediated PA, suggesting that the herbs may not be acting via calcium dependent pathways at least in the initial phases of platelet aggregation (Fig.1a). However, FC and RC and not TC, effectively masked collagen induced platelet aggregation as depicted in Fig 1b. While, U46619 (a thromboxane A2 analogue) –induced platelet

aggregation was decreased by FC and RC, no such effect was observed for TC (Fig.1c). On the other hand all the three herbs RC, FC and TC failed to inhibit ADP induced platelet aggregation significantly (Fig. 1d), suggesting that the three herbs may not act via ADP receptor. Furthermore, as evident from figure 1-e, FC, RC and TC only partially inhibited PMA induced platelet aggregation.

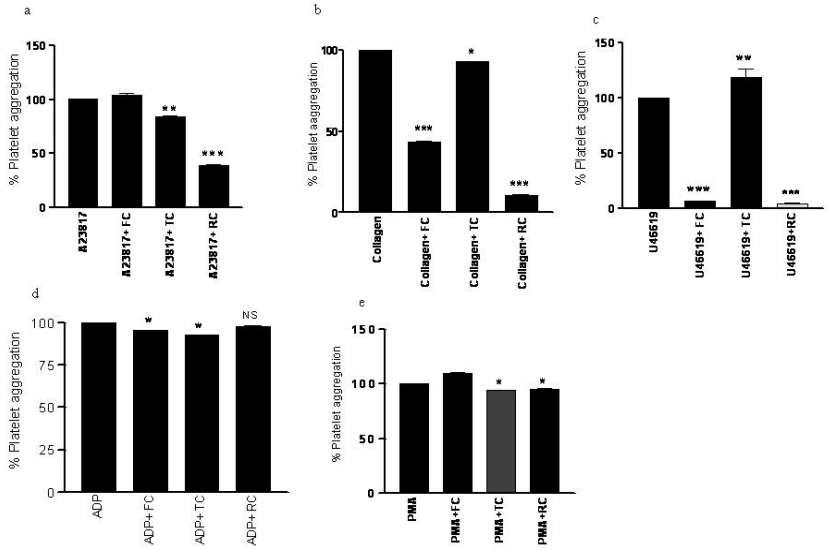

Figure 1- a) Effect of Fagonia cretica, Tinospora cordifolia and Rubia cordifolia on calcium ionophore - A23817 induced human platelet aggregation. b) Effect of Fagonia cretica, Tinospora cordifolia and Rubia cordifolia on collagen induced human platelet aggregation. c) Effect of Fagonia cretica, Tinospora cordifolia and Rubia cordifolia on human platelet aggregation induced by U46619. d) Effect of Fagonia cretica, Tinospora cordifolia and Rubia cordifolia on ADP mediated human platelet aggregation. e) Effect of Fagonia cretica, Tinospora cordifolia and Rubia cordifolia on Phorbol 12-myristate 13-acetate (PMA) induced human platelet aggregation. *p<0.001, **p<0.01 and ***p<0.05 versus respective agents.

RC, FC and TC exhibit anti-inflammatory properties by significantly lowering COX-2 gene expression

Figure 2 shows the effect of RC, FC and TC on COX-2 gene expression as assessed by RT-PCR, 24 and 48 hours post OGD (<0.05 and p<0.001 respectively, Fig 2a, and 2b) as compared to the control. Although the COX-2 gene expression was significantly suppressed by all the three herbs (p<0.001 for FC, RC and <0.05 for TC), the effect was more pronounced for FC and RC.

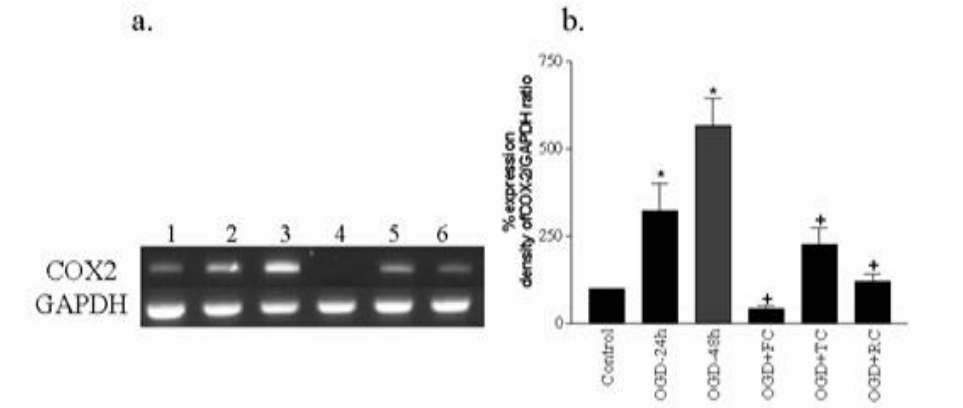

Figure 2- a) COX-2 mRNA expression in relation to GAPDH in control, OGD and OGD + drug treated rat hippocampal slices.(Lane 1-Control, Lane 2-OGD 24 hr, Lane 3 –OGD 48 hr, Lane 4 – OGD + FC, Lane 5-OGD+TC, Lane 6-OGD+RC. b) Percentage densitometric expression of COX-2 mRNA expression in relation to GAPDH in control, OGD and OGD + drug treated rat hippocampal slices. * p<0.001 compared to control and + p<0.001 compared to OGD 48 hrs.

RC, FC and TC inhibit post- ischemic injury by repressing VCAM gene expression

Figure 3-a shows the effect of RC, FC and TC on VCAM gene expression as assessed by RT-PCR, 24 and 48 hours post OGD ($p<0.001$ respectively, Fig 3-a, and 3-b) as compared to the control. All the three herbs significantly suppressed VCAM gene expression ($p<0.001$ for FC, RC and TC).

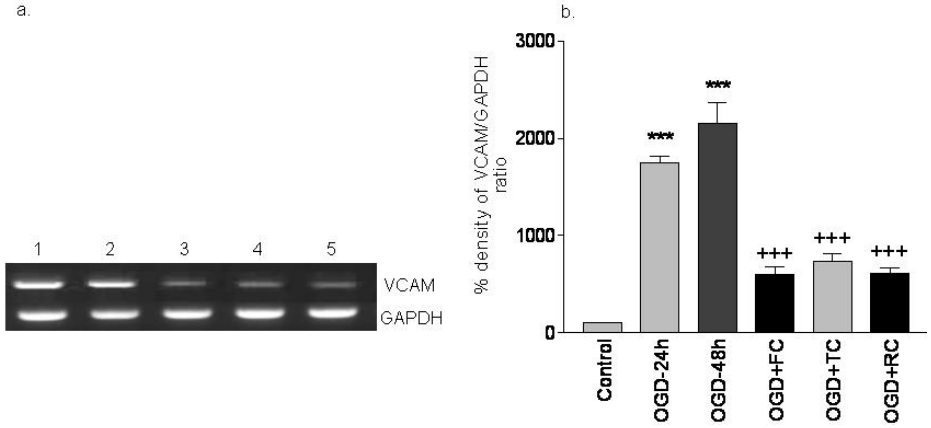

Figure 3- a) VCAM mRNA expression in relation to GAPDH in control, OGD and OGD+ drug treated rat hippocampal slices (Lane 1-Control, Lane 2-OGD 24 hr, Lane 3 –OGD 48 hr, Lane 4 – OGD + FC, Lane 5-OGD+TC, Lane 6-OGD+RC). b) Percentage densitometric expression of VCAM mRNA expression in relation to GAPDH in control, OGD and OGD+ drug treated rat hippocampal slices ($p<0.001$). ***$p<0.001$ compared to control and +++ $p<0.001$ compared to OGD 48 hrs.

RC, FC and TC significantly induce VEGF gene expression

Figure 4 shows the effect of RC, FC and TC on VEGF gene expression, assessed 24 and 48 hours post OGD ($p<0.001$ respectively, Fig 4-a, and 4-b) as compared to the control. The VEGF gene expression was significantly enhanced by all the three herbs ($p<0.001$ for all the drugs).

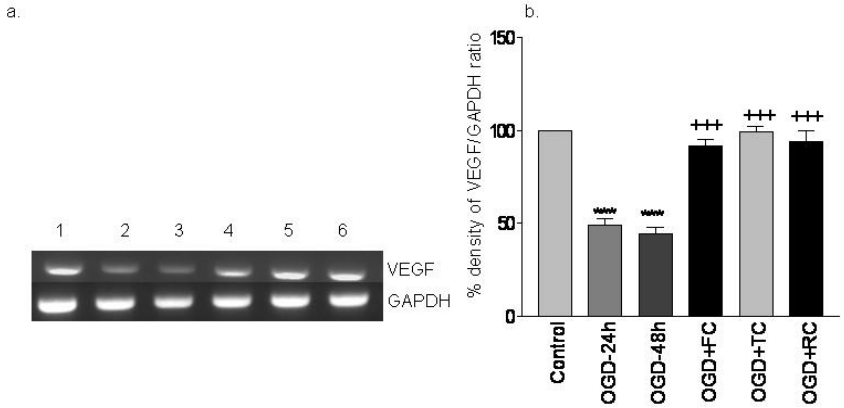

Figure 4- a) VEGF mRNA expression in relation to GAPDH in control, OGD and OGD+ drug treated rat hippocampal slices.(Lane 1-Control, Lane 2-OGD 24 hr, Lane 3 –OGD 48 hr, Lane 4 – OGD + FC, Lane 5-OGD+TC, Lane 6-OGD+RC. b) Percentage densitometric expression of VEGF mRNA expression in relation to GAPDH in control, OGD and OGD + drug treated rat hippocampal slices. +$p<0.001$ compared to OGD 48h.

Discussion

The progress of a stroke is related to the activation of endothelial cells, platelets, leucocytes and array of free radicals generated at the site of ischemic injury. In the present study we have demonstrated that RC, FC and TC could significantly enhance VEGF and markedly decrease the expression of pro-inflammatory mediators such as COX-2 and cell adhesion molecule such as VCAM. While RC and FC exhibited significant inhibition in platelet aggregation induced by U46619, A23871 and ADP, TC was least effective in preventing platelet aggregation.

Adhesion molecules play an important role in inflammatory disorders as well as in a variety of other pathophysiological processes including post-ischemic neuronal damage (Quing et.al., 2007). P-selectin is expressed on the plasma membrane after activation of endothelial cells and platelets, while E-selectin is observed on activated endothelium. Therefore, P-selectin serves as a marker of endothelial and/or platelet activation and E-selectin as an activated endothelial marker in various diseases (Frijns et.al., 1997, Gearing et.al., 1992, Abrams & Shattil, 1991). A recent in vivo study has presented evidence that platelet-endothelial cell adhesion molecule-1 (PECAM-1; CD31) expressed on endothelial cells may contribute to platelet adhesion and aggregation at a site of injured but not denuded endothelium (Rosenblum et.al., 1996, Bombeli et.al., 1998). Our

finding of RC, FC and TC significantly repressing the expression of VCAM is therefore an important observation and implies one of the mechanisms by which these herbs may impart their neuroprotective effect. Expression of adhesion molecules post ischemia also attracts a host of leucocytes to the site of focal ischemic injury (Clark et.al., 1991, Zhang et.al., 1995). There are evidences of probable involvement of neutrophils in ischemic injury in other tissues (Gumina et.al., 1997, Rabb & Martin, 1997), and that there is a cross talk between the platelets and the leukocytes, (Li et.al., 2000). This is supported by the reports that neutrophils damage the neuronal tissue by releasing inflammatory mediators and proteolytic hydrolases (Duilio et.al., 2001, Kochanek & Hallenbeck, 1992). Since all the three herbs have been reported to have appreciable antioxidant activity (Rawal et.al., 2004, Rawal et.al., 2008), it may therefore be surmised that inhibition of VCAM by RC, FC and TC is not only an anti-inflammatory effect but the herbs also protect the post-ischemic tissue from oxidant injury elicited by the phagocytes.

Platelets are capable of inducing chain reaction leading to hemostasis, coagulation, and thrombosis (Marcus, 1999). Thromboxane A_2 (TXA_2), a potent vasoconstrictor (Hamberg et.al., 1975), is generated by activation of platelet membrane phospholipase A_2 (PLA2) following adhesion of platelets to collagen and the basement membrane of exposed sub endothelium in the presence of von Willebrand factor (vWF) and platelet glycoprotein Ib (Ruggeri, 1997, Zuker, 1980). U-46619, a TXA_2 analogue, induces significant expression of adhesion molecules on the surface of human vascular endothelial cells such as ICAM-1 and VCAM-1 (Raychowdhury et.al., 1994, Ishizuka et.al., 1998). In light of the above, our observation of the ability of RC and FC but not TC to effectively block U46619 mediated platelet aggregation and to significantly repress VCAM gene expression provides a mechanism that the three herbs block the expression of cell adhesion molecules by interfering with the TXA_2 pathway.

Among many PA agonists, collagen is the most thrombogenic component of the subendothelial layer following vascular injury and its interaction with platelets is complex and mediated by several receptors (Kramer et.al., 1995, Saklatvala etal., 1996, Siess, 1989, Clemetson & Clemetson, 2001). Therefore, our findings of RC and FC to preferentially inhibit collagen induced PA suggests that a combination of RC, FC and TC may prove more effective rather than being used individually.

Arachidonic acid released from neuronal phospholipids during ischemia/reperfusion is converted to prostaglandin H_2 (PGH_2) by cyclooxygenase [(COX or PGHS (prostaglandin H synthetase)], which exists as PGHS-1 (or COX 1) and PGHS-2 (or COX 2), produced by two distinct genes (Kraemer et.al., 1992, Schwab et.al., 2002). PGHS-2 is an inducible enzyme involved in the overproduction of prostanoids during inflammatory disorders (Inoue et.al., 1994), and is regulated at the transcription level. It may be expressed by many nucleated cells upon challenge with PMA, LPS or inflammatory cytokines (Perkins & Kniss, 1997). Inhibition of COX2 has been a major therapeutic target for not only post-ischemic care but also in various other inflammatory diseases. It is therefore an important observation that a significant drop in the levels of COX2 gene expression post OGD in the rat hippocampal slices were achieved by RC, FC and TC. These findings become more important in light of the knowledge that most metabolic diseases in their course of development and progression enter inflammatory stage at one stage or the other, which further adds to the complexity of these diseases. Therefore the ability of the herbs to resolve the inflammatory status is an important finding from therapeutic point of view. This contention is furthered by the reports that COX-2 deficient mice exhibited reduced neuronal injury after N-methyl-D-aspartate (NMDA) exposure (Iadecola et.al., 2001), whereas COX-2 over-expression exacerbated brain injury (Dore et.al., 2003). Interestingly, it has recently been shown that COX-2 mediates its toxic effect through PGE_2 rather than ROS, even though COX-2 can generate both (Manabe et.al., 2004).

VEGF (vascular endothelial growth factor) is an angiogenic agent (Leung et.al., 1989, Keck et.al., 1989) and is induced during hypoxia (Shweiki et.al., 1992, Ogunshola et.al., 2000). Ischemia stimulates VEGF expression in the brain (Kovacs et.al., 1996) and VEGF promotes formation of new cerebral blood vessels Rosenstein et.al., 1998, Krum et.al., 2002, Marti et.al., 2002). Several studies have shown that cerebral ischemia increases VEGF expression (Kovacs et.al., 1996, Pichiule et.al., 2003). The release of VEGF from the activated platelets may be a protective mechanism of the brain during hypoxic/ischemic stress. Interestingly, VEGF has been used as therapeutic agent in post-ischemic subjects (Yang et.al., 2002, Wick et.al., 2002). Therefore our finding of a significant enhancement in the level of VEGF expression on treatment of hippocampal slices with RC, FC and TC, is again indicative of the therapeutic potential of the three herbs. However, the use of VEGF as a therapeutic agent to treat cerebral ischemia (therapeutic angiogenesis, neuroprotection) should be done with caution as VEGF is also reported to induce vascular permeability leading to formation of brain oedema (Schoch et.al., 2002).

Overall, RC, FC and TC appear to have good therapeutic potential for reperfusion injury as anti-inflammatory agents in view of their ability to downregulate the expressions of COX2 and VCAM genes and inhibit platelet aggregation induced in vitro by multiple agonists. RC, FC and TC also provide neuroprotective effects due to VEGF induction during hypoxia/ischemia. The later therapeutic role must be further investigated keeping in view that VEGF can also induce vascular permeability in the brain leading to cerebral oedema.

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