THE EFFECTS OF ETHANOL EXTRACT PROPOLIS ON EXTRINSIC AND INTRINSIC PATHWAYS OF APOPTOSIS IN PRIMARY RAT ASTROCYTE CELL CULTURE.

Master Thesis

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Biology Department

Prof. Dr. Nedzvetskyi Viktor

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REPUBLIC OF TURKEY
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This master thesis on 30.01.2017 by the following jury memebers (The appropriate one will be stay, the other will be deleted) **Unanimously has been accepted.**

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Dedication

I would like to dedicate this thesis to my brother Raber and Dr. Mudhir Sabir who has been a constant source of support throughout the years of my postgraduate study. In addition, I would like to thank my family, especially my mother and father for constant support and encouragement.

To my Mother and Father

To my wife

To my Brothers and Sisters

Aso Nadhim HIDAYAT

January 2017

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LEST OF ABBREVIATION

4OHQ	:	4-hydroxyquinazolon
JNKs	:	c-Jun N-terminal kinases
FBS	:	Fetal bovine serum
EDTA	:	Ethylenediaminetetraacetic acid
MTT	:	Methylthiazolyl diphenyltetrazolium bromide
DMSO	:	Dimethyl sulfoxide
TEMED	:	N,N,N'N' tetramethylethylenediamine
GBM	:	Glioblastoma
TMZ	:	Temozolomide
TRAIL	:	Tumor necrosis factor-related apoptosis- inducing ligand
PVDF	:	PolyVinylidene Difluoride
ECL	:	Electrogenerated chemiluminescence
GAPDH	:	Glyceraldehyde-3-phosphate dehydrogenase
SiRNA	:	Small interfering RNA
NAD	:	Nicotinamide adenine dinucleotide
MKP-1	:	MAP kinase phosphosphatase-1
TNF	:	Tumor Necrosis Factor
TRAIL	:	Tumor Necrosis Factor (TNF) Related Apoptosis Inducing Ligand
Fas	:	Fatty Acid Synthase
CAPE	:	Caffeic Acid Phenyl Ester

LPS	:	Lipopolysacharide
DGaIN	:	D-galactosamine
GFAP	:	Glial Fibrillary Acidic Protein
NOS	:	Nitric Oxide Synthase
PTZ	:	Pentylenetetrazole
ROS	:	Reactive Oxygen Species
CC	:	Cerebral Cortex
CB	:	Cerebellum
TNFα	:	Tumor necrosis factor alpha
BS	:	Brain Stem
BBB	:	Blood Brain Barrier
IL-6	:	Interleukin 6 receptor
TGF-B1	:	Transforming Growth Factor-B1
NOS-2	:	Nitric Oxide Synthase-2
COX-2	:	Cyclooxygenase-2
TLR 4	:	Toll-like receptor 4
PAMPs	:	Pathogen-Associated Molecular Patterns
TBI	:	Traumatic Brain Injury
FGF	:	Fibroblast Growth Factor
FGFRs	:	Fibroblast growth factor receptors
CNTF	:	Ciliary neurotrophic factor
TGF- β	:	Transforming growth factor beta
STAT3	:	Signal transducer and activator of transcription 3
SOCS3	:	Suppressor of cytokine signaling 3
Camp	:	Cyclic adenosine monophosphate

OM	:	Outer Membrane
TM	:	Transmembrane
BH	:	BCL-2 homology
MOMP	:	Mitochondrial Outer Membrane Permeabilization
PTP	:	Permeability Transition Pore
MTP	:	Mitochondrial Transmembrane Potential
VDAC	:	Voltage-Dependent Anion Channel
CypD	:	Cyclophilin D
PCD	:	Programmed cell death
AIF	:	Apoptosis Induce Factor
DNA	:	Deoxyribonucleic acid
FADD	:	Fas-Associated Protein With A Death Domain
DISC	:	Death-Inducing Signaling Complex
DMEM	:	Dulbecco's modified Eagle's medium
AMID	:	Apoptosis-Inducing Factor-Homologous Mitochondrion-Induce Of Death
PARP	:	Poly-ADP-Ribose Polymerase
HI	:	Hypoxic–ischemic
EEP	:	Ethanol Extract Propolis
ADP	:	Adenosine diphosphate
AIF		Apoptosis Inducing Factor

PRIMER RAT ASTROSIT HÜCRELERINDE PROPOLIS ETANOL EKSTRESİNİN IÇ VE DIŞ APOPTOZ YOLAKLARI ÜZERİNDEKI ETKILERI

ÖZET

Çalışmamızda, propolisin etanol ekstresinin (EEP) veya EEP bileşenlerinin farklı konsantrasyonlarına bağlı olarak hücre canlılığı ve morfolojisine etkisi incelemektir. EEP'nin 100µg/ml'si ile ilgili sonuçlar immumoblot yöntemiyle elde edilerek değerlendirildi. Birincil astrosit hücrelerinde canlılığının güçlü düşüşü gözlemlendi. Birincil astrosit hücrelerinde immünoblot sonuçlarına göre GFAP ekspresyonunda EEP 10 µg/ml 'den yaklaşık %29, EEP'den 25µg/ml 'den sonra %22 ve EEP 100µg/ml uygulamasından sonra %79'luk bir artış gözlendi. Üstelik birincil astrosit hücrelerinde EEP tarafından indüklenen apoptozun iç yolağında markerlerinin modülasyonunda özellikle de kaspaz3, BAX ve Bcl-2 ifadelerinin aktivasyonu gözlemlendi. EEP, birçok biyokimyasal ve hücresel reaksiyonda düzgün işleyişi ve düzenlenmeyi sağlamada gerekli olduğu bilinen yüksek miktarda antioksidan madde içerir. Bununla birlikte, fazla dozda EEP'ye maruz kalma kaspaz-3 ve BAX ekspresyonunda daha dejeneratif baskılamaya yol açtığı görüldü. Apoptoz ile ilgili olarak EEP+LPS içeriğine göre, % 79'luk kaspaz-3 ve BAX %66 sonuçları gözlendi. Elde edilen sonuçlara göre primer astrosit hücre kültürü EEP'nin apoptotik yolakları durdurmaya yönelik etkilerini çalışmak için iyi bir modeldir. EEP'nin farklı dozları apoptozun iç ve dış yolaklarının markörlerinin ekspresyonları üzerinde doza bağlı lineer olmayan etki göstermiştir. EEP'nin hücrelere eklenmesi, astroglial reaktiviteyi 10-100 µg/ml konsantrasyon aralığında arttırmıştır. GFAP ekspresyonunun fazla olması ve glial hücrelerinin fazla büyümesi reaktivitenin artmasında etkili olmuştur.

EEP'nin apoptozun mitokondriyel iç yolağı üzerindeki modülayonu, Bcl-2 ekspresyonu üzerinden gösterilmiştir. Ek olarak, 25µg/ml EEP'nin diğer dozlara göre maksimum astroglial reaktivite sağladığı ilk olarak bu çalışmada gösterilmiştir. Bütün veriler dikkate alındığında bulunan sonuçlar, EEP'nin apoptotik yolaklar ve hücre reaktivasyonunu kontrol eden sinyal mekanizmaları üzerindeki etkilerini anlamaya yönelik önemli katkı sağlamıştır.

Anahtar kelimeler: Astrojiyosis, EEP, Apoptoz, Caspase-3, BAX, GFAP.

THE EFFECTS OF ETHANOL EXTRACT PROPOLIS ON EXTRINSIC AND INTRINSIC PATHWAYS OF APOPTOSIS IN PRIMARY RAT ASTROCYTE CELL CULTURE

ABSTRACT

Presented study was to examine the effect of ethanol extract of propolis (EEP), dependently on their concentrations on the viability and morphology of exposition to treated EEP or its ingredients. Results about 100µg/ml ingredients of EEP were evaluated by Immumoblot. We obtained the strong decline of viability of primary astrocytes cell. The results of Immunoblot shown an increasing of GFAP expression in primary astrocyte cell culture approximately to 29% after EEP 10µg/ml, 22% after EEP 25µg/ml and 79% after EEP 100µg/ml application. Moreover, the modulation of expression markers of apoptotic ways, especially, caspase3, BAX, Bcl-2 may indicate activation of intrinsic pathway of apoptosis induced by EEP in primary astrocyte cell culture line. EEP contain high amount antioxidant known to be essential for maintaining the proper function and regulation of many biochemical and cellular reactions. However, high exposure to levels of EEP in occupational can lead to more degenerative suppression caspase-3,BAX. Related to apoptosis, according to caspase-3 content on 79%, BAX 66% (EEP+LPS). Observed results showed that primary astrocyte cell culture is valid experimental model for the study of EEP effects on switching off apoptosis pathways. Different doses of EEP have a non linear dose-depend effect on expressions of both extrinsic and intrinsic apoptotic pathways markers. After treatment with EEP primary astrocyte cell culture induce astroglial reactivity in 10–100 µg/ml range concentration. This astrocyte's reactivation associated with GFAP overexpression and hypertrophy of glial cells.

The involvement of the intrinsic mitochondrial pathway of apoptosis was demonstrated with modulation by EEP a level of Bcl-2 expression. In addition, our data also shown for the first time that 25µg/ml EEP induce maximum astroglial reactivity compared with 10 – 100µg/ml EEP. Taking together, these findings contribute to a deeper elucidation of the molecular signaling mechanisms underlying EEP for regulation apoptotic pathways and cell reactivation.

Key words: Astrogiosis, EEP, Apoptosis, Caspase-3, BAX, GFAP.

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1. INTRODUCTION

Propolis is the resinous mixture that bees accumulate from various parts of plants such as bark gum and use for sealing the pore and protecting the microbe in bee hive (Khacha-anand aand Tragoolpua and Chantawannakul and Tragoolpua 2013). Propolis contains many compounds including phenolic, flavonoid, flavones, fatty acid, which have the therapeutic effects such as antimicrobial, antioxidant, immunostimulant and wound healing activities. The essential oil of propolis was found to inhibit the microbial infection and bee wax could supply moisture to human. However, propolis showed strong anti-free radical activity, which resulted from propolis including caffeic acid, ferulic acid, and caffeic acid phenyl ester (Ahn Kumazawa Hamasaka Bang and Nakayama 2004). Propolis can inhibit the superoxide anion and hydroxyl radical, which are the essential and dangerous reactive oxygen species. Free radicals are generated from an external source and biological process in the human body, and it can damage the biomolecule including protein, lipid, and genetic material. However, free radicals disturb the homeostasis such as DNA repair, inflammation and cell proliferation (Kryston Georgiev Pissis and Georgakilas 2011). Many different methods have been used for extraction of active component in propolis, for example, maceration, soxhlet extraction, ultrasonic extraction (sonication) and microwave extraction. For maceration technique, the organic solvent is used to dissolve the component in propolis directly without producing heat so this method is suitable for heat labile and heat stable substance (Khacha Ananda et al. 2013). Propolis, also named bee putty or bee glue, is a substance produced by bees from the resin collected from trees and shrubs, which combines with beeswax and secretions from the bee's salivary glands rich in enzymes. It can be yellow, brown or almost black, depending on the plants from which the resinous substance is collected. The use of propolis via humans has a long history. The Egyptians applied it for embalming the body because it was the perfect plastic material that further protected the mummy from bacteria, fungi, and viruses. Propolis has been the subject of several studies due to its antibacterial, antifungal, antiviral and hepatoprotective activity. Water- or alcohol-soluble

Propolis and its several compounds have been used in the treatment of inflammation, for immune stimulation, and as an anticancer agent. Above mentioned characterizes of propolis make it an unusual material of natural origin, described via a particular composition (Sawicka Car Borawska, and Niklinski 2012). propolis has been demonstrated its antibacterial, antifungal, antiviral, hepatoprotective and immunostimulatory activity. The anticancer effects of ethanol extract propolis (EEP) have been presented in various cell lines such as mammary carcinoma, epithelial carcinoma, variant carcinomas, human pancreatic cancer. The organic solvents were used to extract the chemical composition from propolis such as water, ethanol, methanol, hexane and acetone. The organic solvent can dissolve the different chemical compounds according to a polarity of substance. Ethanol was applied to extract propolis to generate the fatty acid and flavonoids, while acetone extraction made monosaccharide, glycerol, and caffeic acid. Alkane, alcohol and bee wax were found in hexane fraction of propolis (Prytzyk et al. 2003). Also, the extraction time, light and temperature affected propolis extraction. Therefore, the aims of this study to compare anti-free radical activity, the phenolic compound, flavonoid and antiproliferative activity of propolis extracts from two extraction methods (khacha Canada et al. 2013). The molecular mechanisms and the effects of honeybee bioactive compounds are actual questions of both cell viability and death regulation. The astrocyte culture is wide used as the model of cell response to several kind factors. Morphological changes of astroglial cell line after treatment with EEP appearing as a changing form of mitochondria with a dense matrix were observed in cells contained significant amounts of ADP (Dahout Gonzalez et al. 2006). Such the increased metabolism, enhanced demand for energy, but also may suggest induction of apoptosis in the astroglial cell line. These morphological changes may indicate over activity of cells. Morphological and physiological alterations including cell shrinkage, condensation of chromatin, nuclear fragmentation, cytoskeleton rearrangement, cytoplasmic membrane blisters or apoptotic bodies formation are an important component of cell pathology. There is no precise mechanism, which is responsible for a trigger apoptotic and necrotic death of cells. The releasing of proteins from mitochondrial intermembrane space to the cytosol is only small part of complete occurs switching the pathways into cells. The apoptotic factors such as apoptosis-inducing factor (AIF), second mitochondria derived activator of caspase direct IAP-binding protein with low PI (Smac/Diablo) and endonuclease G, are released from the mitochondrial intermembrane

space to cytosol through that channel. This event is necessary for triggering the mitochondria dependent apoptosis (Marzo Brenner and Kroemer 1998). Recent data of research demonstrate that EEP may induce apoptosis through release of cytochrome c from mitochondria to the cytosol and trigger caspases cascade in the result of pro-apoptotic proteins activity such as Bcl-2 associated x protein (Bax), Bcl-2 homologous antagonist/killer (Bak), protein (p53), and (p21). Besides, it has been presented after application of propolis that the extrinsic pathway of apoptosis in cancer cells can be stimulated by tumor necrosis factor (TNF) related apoptosis inducing ligand (TRAIL) or due to activation of fatty acid synthase (Fas) receptors and inhibition of NF-κB activity. Another study presented protective effect of CAPE against neuronal damage induced by the combination of lipopolysaccharide (LPS) and D-galactosamine (LPS/DGaIN) (Korish and Arafa 2011). These results suggest anti-inflammatory activities of propolis since it can inhibit the NF-κB activity. The unfavorable effect of EEP and its ingredients including chrysin and CAPE on the survival of human astroglial cell line (SVGp12) should be taken into account in considering therapeutic effects of propolis. Probably, apoptosis induced by EEP is linked to the mitochondria. The Precise mechanism of obtained results requires further investigations. Primary cell cultures of the rat are well suited to detect a neurotoxic potential and to differentiate its underlying mechanisms. Damage of the cytoskeleton, intercellular matrix proteins, modulation of proteases activity may be considered as an endpoint mechanistically related to both pathogenic neuroprotective effects in the brain.

Astroglial cells produce a host of trophic factors, which are crucial for the survival of neurons and the regulation its pathways. Though, activated astroglia become hypertrophic, exhibit increased production of glial fibrillary acidic protein (GFAP). This phenomenon named astrogliosis and associated with different kind neural tissue disturbance.

Thus, the study of the effects of ethanol extract propolis on extrinsic and intrinsic pathways of apoptosis in primary rat astrocyte cell culture can clarify the mechanisms that underlay in therapy with natural propolis. The elucidation of apoptotic activation ways with ethanol extract propolis is the aim of study in primary rat astrocyte cell culture.

2. LITERATURE REVIEW

2.1. Neurodegenerative Diseases

The specific mechanisms involved in onset and progression of neurodegenerative diseases are still unsuccessfully defined, but excitotoxicity is founded as one of the mechanisms included. Excessive neuronal excitation involving the excitatory glutamate receptors are known as an important underlying mechanism in neurodegenerative disorders. Moreover, the exact mechanism of how excitotoxicity is implicated in neurodegeneration still needs further examination. Kainic acid (KA) induced status epilepticus was linked with both apoptotic and necrotic cell death and induction of heat sensitive proteins in the hippocampus and cortical regions of the rodent brain. The exact mechanisms contributing to the enhanced concentration of nitric oxide (NO) in excitotoxicity are not well established. Prior studies reported that nitric oxide synthase (NOS) knockout mice were high seriously affected by epileptic activity than the controls and that the response to NO during epilepsy depends on its concentration. It was also particular that NO may be viewed as an anticonvulsant and pro-convulsant substance about convulsions induced by pentylenetetrazole (PTZ). Excitotoxicity is commonly begun in experimental animals by KA, a 30-fold more full glutamate agonist. Effects of KA are mediated via activation of the kainite receptors that respond to the neurotransmitter glutamate and such the induction of inflammatory responses, production of cytokines and neuronal death. The molecular mechanisms by which KA induces excitotoxicity and cell death to remain unclear; moreover, oxidative stress and the activation of proinflammatory cytokines are main contributors. The cytokines and other inflammatory molecules released by activated glial cells can modify the outcome of disease progression. Thus, antioxidant and anti-inflammatory treatment prevent KA-induced neurodegeneration. Additionally, KA promotes neuronal excitability, production of reactive oxygen species (ROS), and lipid peroxidation. Microglial activation and astrocytes reproduction are the other characteristics of KA-induced neurodegeneration.

Both in-vitro and in vivo studies exhibit that KA induces cell death by the growth of intracellular calcium, which stimulates ROS production and mitochondrial dysfunction, thereby leading to neuronal cell damage. There is occurring for stimulation of calpain- and caspases-induced neural apoptosis following KA exposure. Also oxidative stress and intracellular calcium overload, KA can activate molecular mechanisms leading to decrease of neuronal energy stores and thereby enabling the alternative cell death pathways. Honey bee propolis has been widely applied as a folk medicine and proposed to be preserved on neurodegenerative disorders. It has been demonstrating to have broad biological activities, which are principally attributed to the presence of flavonoids, and caffeic acid phenyl ester (CAPE). The prevailing opinion is that the full biological activities of flavonoids and CAPE are related in part, to their anti-inflammatory and antioxidant actions. Recent studies demonstrated that propolis supplementation reduced the oxidative stress and nitric oxide levels in KA-mediated excitotoxicity (Swamy et al. 2014). Hence the present study was conducted to assess the neuroprotective effects of the honeybee product propolis, by estimating the concentration of TNF-α, and NO, along with activities of NOS and caspase-3 in cerebral cortex (CC), cerebellum (CB), and brain stem (BS) of rats supplemented with honey bee product and subjected to KA-mediated excitotoxicity (Swamy Suhaili Sirajudeen Mustapha and Govindasamy 2014).

2.2. Glial Cells

In the late 1800's, neuroglia was recognized as distinct cellular components that included all supporting cells in the central nervous system (CNS). Neuroglial cells are subdivided into various classes: astrocytes, oligodendrocytes, and, more recently, NG2 cells (i.e. oligodendrocyte precursor cells). Today, the term glia is used to refer to neuroglia, Schwann cells, and microglia. Occasionally, ependymal cells (also called ependymoma) are included in the term glia since they are derived from radial glia and share astrocytic properties. One of the accepted roles for astrocytes is their house keeping functions maintaining a viable nervous system environment for neurons. The incorporates buffering excess potassium and neurotransmitters, providing nutrients and structural support around synapses, and contributing to the integrity of the blood-brain barrier (BBB). Astrocytes are also known to release molecules necessary for neuronal survival and neurite arrangement. Some of the emerging functions of astrocytes have been clearly

demonstrated, others remain speculative and controversial. Changes in intracellular calcium (Ca^{2+}) dynamics upon neuronal activity provide a mode of excitability to astrocytes. One late study reported that Ca^{2+} transients in individual astrocytes are functionally coupled to neuronal activity with remarkable spatial specificity in the ferret visual cortex in vivo. Besides, they demonstrated an unambiguous coupling between the astrocyte response to visual stimuli and local blood flow. However, intercellular Ca^{2+} waves allowing astrocyte-to-astrocyte communication have not been seen in acute slices or in vivo. The occurrence of intercellular Ca^{2+} waves may be more expected in pathological situations as proposed. Novel time-lapse imaging studies clearly revealed that astrocytes in acute slices shape the structural plasticity of synapses. However, their instructive role at synapses, in particular, their quick release of gliotransmitters controlling synaptic activity, remains controversial. In particular, issues will be raised regarding the methodologies used to empower astrocytes. Cells communicating the astrocytic marker glial fibrillary acidic protein (GFAP) (D D Wang and Bordey 2008).

2.3. Astrocytes

Astrocytes, the primary glial cell in the central nervous system (CNS), participate in all essential CNS functions, such as neuronal survival and differentiation blood flow regulation, energy metabolism, ion and water homeostasis, immune defense, neurotransmission, and adult neurogenesis. As one of the primary responders to injury, perturbation, cellular stress, and infections of the CNS, activated astrocytes can secrete some important cytokines affecting the cell state of surrounding cells including microglia and neurons, and astrocytes themselves, leading to excitotoxicity, inflammation, neurodegeneration, and apoptosis. For example, cytokines such as IL-1b, TNF-a, IL-6, and transforming growth factor-β1 (TGF-β1) can act to up or down-regulate another pro- and anti-inflammatory genes including nitric oxide synthase-2 (NOS-2) and cyclooxygenase-2 (COX-2). In comparison to moderate astrocytes activation, which is crucial in the recovery of injured CNS via secreting reparatory neurotrophic factors, a rapid, severe and prolonged process may initiate a massive inflammatory response ultimately leading to neuronal destruct, neuronal circuit impairments, and even neuronal death, resulting in neurodegenerative diseases. Thus, inhibition of astrocytes activation and the subsequent inflammatory process may help identify strategies to eliminate the

deleterious effects of astrocytes and ultimately preserve cognitive function. TLR4, a pattern recognition receptor that recognizes distinct pathogen-associated molecular patterns (PAMPs), can recruit signaling adaptors including lipopolysaccharide (LPS), and cytokines, resulting in the activation of NF-κB and the release of inflammatory cytokines. More and more studies have revealed the important role of TLR4 in the activation of astrocytes and the following inflammatory response. As a pathogen-associated molecular pattern recognizing receptor on the surface of astrocytes, TLR4 can identify pathogenic microorganisms and endogenous molecules released after stress or tissue destruct thereby causing the expression of downstream signaling proteins and leading to the massive release of inflammatory mediators. For example, the activation of TLR4 in spinal cord astrocytes triggers a signaling cascade leading to NF-kB activation. The latter, in turn, increases the expression of pro inflammatory and stress response mediators, exemplified by TNF-α, COX-2, and iNOS. Thus, inhibition of TLR4 can inactivate proinflammatory downstream signaling pathways by suppressing differential target gene expression and cellular responses. Lithium, an effective mood stabilizer for the treatment of bipolar disorder, is a neuroprotective and neurotrophic agent efficacious in the treatment of several neurodegenerative conditions. Besides, previous animal experiments indicated that lithium induced up-regulation of the production and survival of new cells in the hippocampus could progresses behavioral disorders in rats after transient global cerebral ischemia. Besides, it can significantly ameliorate the progression of AD-like pathology and rescue memory impairments. On the other hand, various studies have shown that lithium exerts strong anti-inflammatory effects by attenuating expression of proinflammatory cytokines and chemokines in vivo (Li et al. 2016). Traumatic brain injury (TBI) is a serious public health problem affecting millions of people in the world. Each year, TBI contributes to a substantial number of deaths and cases of permanent disability. TBI initiates a series of bio-physiological and pathological reactions, such as, activation of excitatory amino acids receptor, Ca^{2+} overload, mitochondrial injury and energy metabolic blockage, production of oxyradical, caspases activation, and activation of inflammatory reaction that contribute to subsequent tissue damage, and associated neuronal cell death, including apoptosis, necrosis, necroptosis, and autophagy (M Zhang et al. 2014).

2.4. Reactive Astrogliosis

Astrocytes play central roles in normal CNS function, and that reactive astrocyte is primary responders to injury and disease, the concept of reactive astrogliosis seems elusive, with no commonly agreed upon definition or model. It is, therefore, useful to begin a review of recent studies into the functions and mechanisms of reactive astrogliosis by presenting a working model that defines various aspects of astrogliosis in a manner parsimonious with currently available information while recognizing that any such model will require updating as new information accrues. Reactive astrogliosis that integrates four interdependent key features. (1) reactive astrogliosis is a spectrum of changes in astrocytes that occur in response to all forms and severities of CNS injury and disease including subtle perturbations. (2) The modifications underwent by reactive astrocytes different with the nature and gravity of the insult along a graduated continuum of following alterations in molecular expression, progressive cellular hypertrophy and, in severe cases, proliferation and scar formation (Figures 2.1]. (3) The altars of astrogliosis are regulated in a context-specific manner by specific signaling events that have the potential to modify both the nature and degree of those changes. (4) the modifications undergone during reactive astrogliosis have the potential to alter astrocyte activities both through gain and loss of functions that can impact both beneficially and detrimentally on surrounding neural and non-neural cells. According to this definition and model, reactive astrogliosis is not an all or none response, nor is it a single uniform process, nor is it ubiquitously synonymous with scar formation. Instead, reactive astrogliosis is a finely graduated continuum of progressive changes in gene expression and cellular changes [Figures 2.2]. That are subtly regulated via complex intercellular and intracellular signaling. In its mild and moderate forms, reactive astrogliosis exhibits the potential for resolution if the triggering mechanism has resolved, in which the cells return to an appearance similar to that of healthy tissue [Figure 2.1]. The extent to which tumors molecular changes resolve or persist is not well known. In this context, it is interesting to note that, in healthy tissue, the extensive network of finely branched processes of individual astrocytes occupy contiguous non-overlapping domains. In mild or moderate reactive astrogliosis, there appears to be the protection of the different nonoverlapping areas of reactive astrocytes in spite of the hypertrophy of the cell body and processes [Figures 2.2]. At its extreme level of activation in response to overt tissue damage and

inflammation, reactive astrogliosis involves scar formation that incorporates newly proliferated cells and in which astrocyte processes overlap in ways not shown in the healthy fabric [Figure 2.2]. It deserves mention that astrocytes interact with other cell types, in particular, fiber is meningeal and other glial cells (such as NG2-positive glia) to generate multiple glial scars in the CNS [Figure 2.1]. The structural changes associated with scar formation are long-lasting and persist long after the triggering insult might have resolved [Figure 2.2]. The striking potential various along the continuum of reactive astrocyte responses to insults of different types are of importance when considering the functions and loss of reactive astrogliosis on CNS functions (Sofroniew 2009). Reactive astrocytes divide and become hypertrophic with long and thick processes. The expression of the glial fibrillary acidic protein (GFAP) in reactive astrocytes promotes in response to a variety of insults, such as lipopolysaccharide (LPS), and causes glial scar tissues to form around the injury site. Consequently, the injury site is sealed, and the spread of inflammation or damage to the adjacent intact tissues is minimized. Moreover, reactive astrocytes are detrimental because they physically block neuronal regeneration and therefore inhibit functional recovery. The mechanisms underlying the morphological changes in astrocytes under injury conditions, and their biological implications about neuronal regeneration, remain unclear. The fibroblast growth factor (FGF) family is a group of ligands that play multiple roles in central nervous system (CNS) development. Ten of the 18 members of the FGF family are expressed in the CNS and conduct diverse biological functions. Also, three of the four FGF receptors (FGFRs), FGFR1, FGFR2, and FGFR3, are expressed in the brain. Elimination of the multiple FGFRs lower progenitors and results in reduced formation of neurons and oligodendrocytes, while downregulation of a single FGFR displays relatively minor defects. FGFR3, a primary receptor for the FGF8 ligand, is selectively expressed in astrocytes and their progenitors, indicating that FGF8-FGFR3 signaling may play more specific roles. The primary roles of FGFs during neural development have been extensively investigated over the last several decades. However, their roles in the postnatal CNS remain unclear.revealing more sophisticated roles in synaptic functions, behaviors, and neurological disorders.

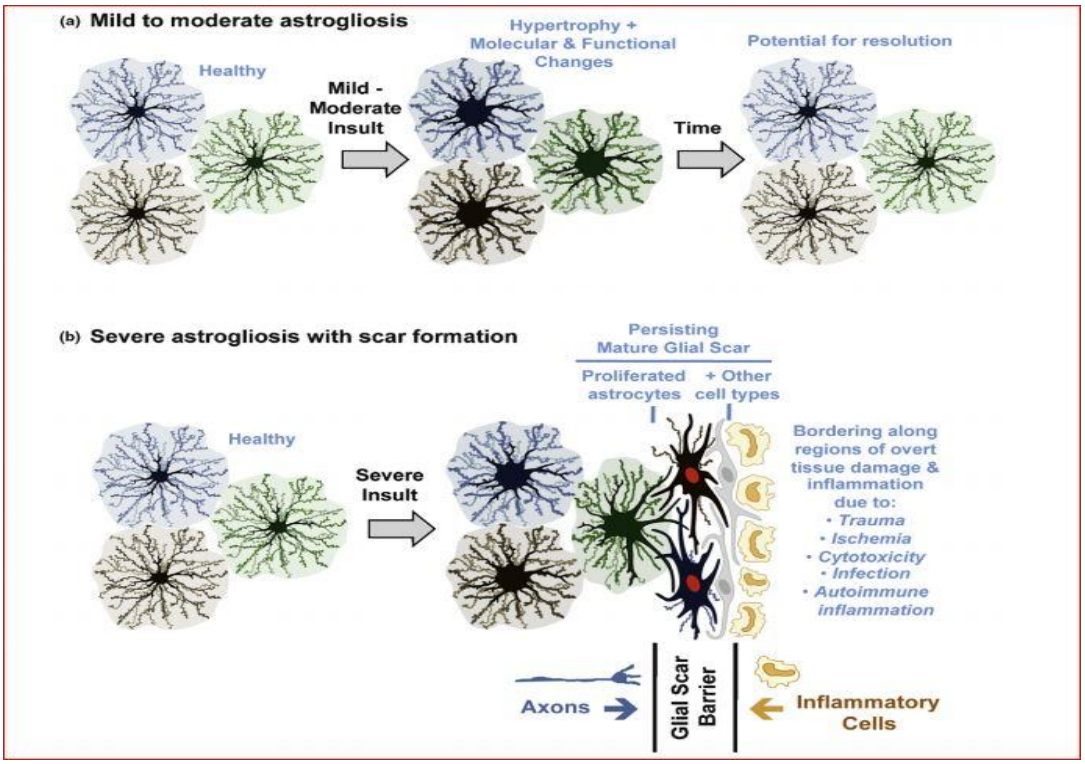

Figure 2.1. Schematic representations of different gradations of reactive astrogliosis that vary with insult severity (Sofroniew 2009)

Moreover, the postnatal functions of the classical FGF ligands remain unclear despite a wealth of reports confirming their presence in adult brains. Several lines of evidence suggest a plausible link between FGF and reactive gliosis. First, there is a high similarity between the expansion of astrocytes during gliogenesis within the first few postnatal weeks and hypertrophic and mitotic reactive astrocytes in an injured condition. Both actively propagate in response to mitotic signals, and reactive astrocytes predominant display glial progenitor markers as a sign of dedifferentiation. Second, primary astrocytes are capable of receiving FGF signals, and the expression of these signals is induced when reactive gliosis events. Finally, FGF ligands guide morphological changes in neurons that increase axon branching or migration, which requires the dynamic rearrangement of cytoskeletons. Thus, pathological responses of reactive astrocytes may adopt neurodevelopmental mechanisms including FGF signaling (Kang Lee Han Choi and Song 2014). Mild to moderate reactive astrogliosis comprises variable changes in molecular expression and functional activity together with variable degrees of cellular hypertrophy. Such changes occur after mild trauma or at sites distant from a more severe injury, or

after moderate metabolic or molecular insults or milder infections or inflammatory activation. These changes vary with abuse severity, involve little anatomical overlap of the processes of neighboring astrocytes and exhibit the potential for structural resolution if the triggering insult is removed or resolved. (b) Severe reactive astrogliosis with persisting scar formation occurs along borders to areas of clear cell and tissue damage and inflammation. Glial scar formation includes newly proliferated astrocytes (with red nuclei in the figure) and other cell types (with gray nuclei in the figure) such as fibro meningeal cells and other glia. In the mature glial scar, astrocytes no longer occupy discrete domains and instead have overlapping processes. Mature glial scars tend to persist for long periods and act as barriers not only to axon regeneration but also to inflammatory cells in a manner that protects healthy tissue from nearby areas of intense inflammation (a) The appearance of ‘normal' astrocytes in the healthy cerebral cortex of an untreated mouse. Note that the territories of astrocyte processes do not overlap. (b) Moderately reactive astrogliosis in mouse cerebral cortex in response to intracerebral injection of the bacterial antigen, lipopolysaccharide (LPS). Note that the territories of moderately reactive astrocyte processes also do not overlap. (c) Severe traumatic injury and inflammation.

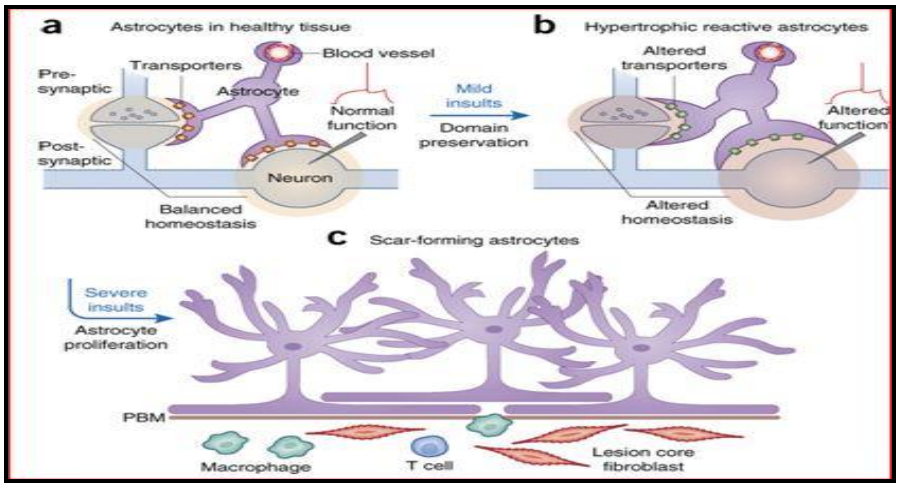

Figure 2.2. Scheme of interaction astrocytes in healthy tissue and of different gradations of reactive astrogliosis and glial scar formation after tissue insults of the various types and different severity (Khakh and Sofroniew 2015)

2.5. Molecular Triggers And Regulators Of Reactive Astrogliosis And Glial Scar Formation

Many different types of intercellular signaling molecules can trigger reactive astrogliosis or to regulate specific aspects of reactive astrogliosis, such as (1) large polypeptide growth factors and cytokines such as IL6, LIF, CNTF, TNFa, INFc, Il1, Il10, TGFb, FGF2, (2) mediators of innate immunity including lipopolysaccharide (LPS) and other Toll-like receptor ligands. (3) neurotransmitters such as glutamate and noradrenaline, (4) purines such as ATP. (5) reactive oxygen species (ROS) including nitric oxide (NO). (6) hypoxia and glucose deprivation. (7) products associated with neurodegeneration such as b-amyloid. (8) Molecules associated with systemic metabolic toxicity such as NH4, and (9) regulators of cell proliferation such endothelin-1. Such molecular mediators of reactive astrogliosis can be released by all cell types in CNS tissue, including neurons, microglia, oligodendrocyte lineage cells, pericytes, endothelial, and other astrocytes, in response to all aspect of CNS insults, ranging from subtle cellular perturbations that release some of the specific factors just listed, to cell stretching as might be encountered during acceleration/deceleration CNS injury and which releases ATP, to intense tissue injury and cell death that release various intracellular molecules that signal severe tissue damage [Figure 2.2]. It is also becoming clear that real molecular, morphological, and functional changes in reactive astrocytes are precisely controlled by inter and intra cellular signaling mechanisms that reflect the specific contexts of the stimuli and produce specific and graduated responses of reactive astrogliosis. For example, the different intracellular signaling pathways related with STAT3, NFjB, SOCS3, Nrf2, cAMP, Olig2 are implicated in mediating various aspects or varying degrees of reactive astrogliosis such as GFAP up regulation, cell hypertrophy, proliferation, and pro- or anti-inflammatory effects. Molecular triggers that lead to the spread of reactive astrocytes in vivo are incompletely distinguished but include EGF, FGF, endothelin1, and ATP (Sofroniew and Vinters 2010).

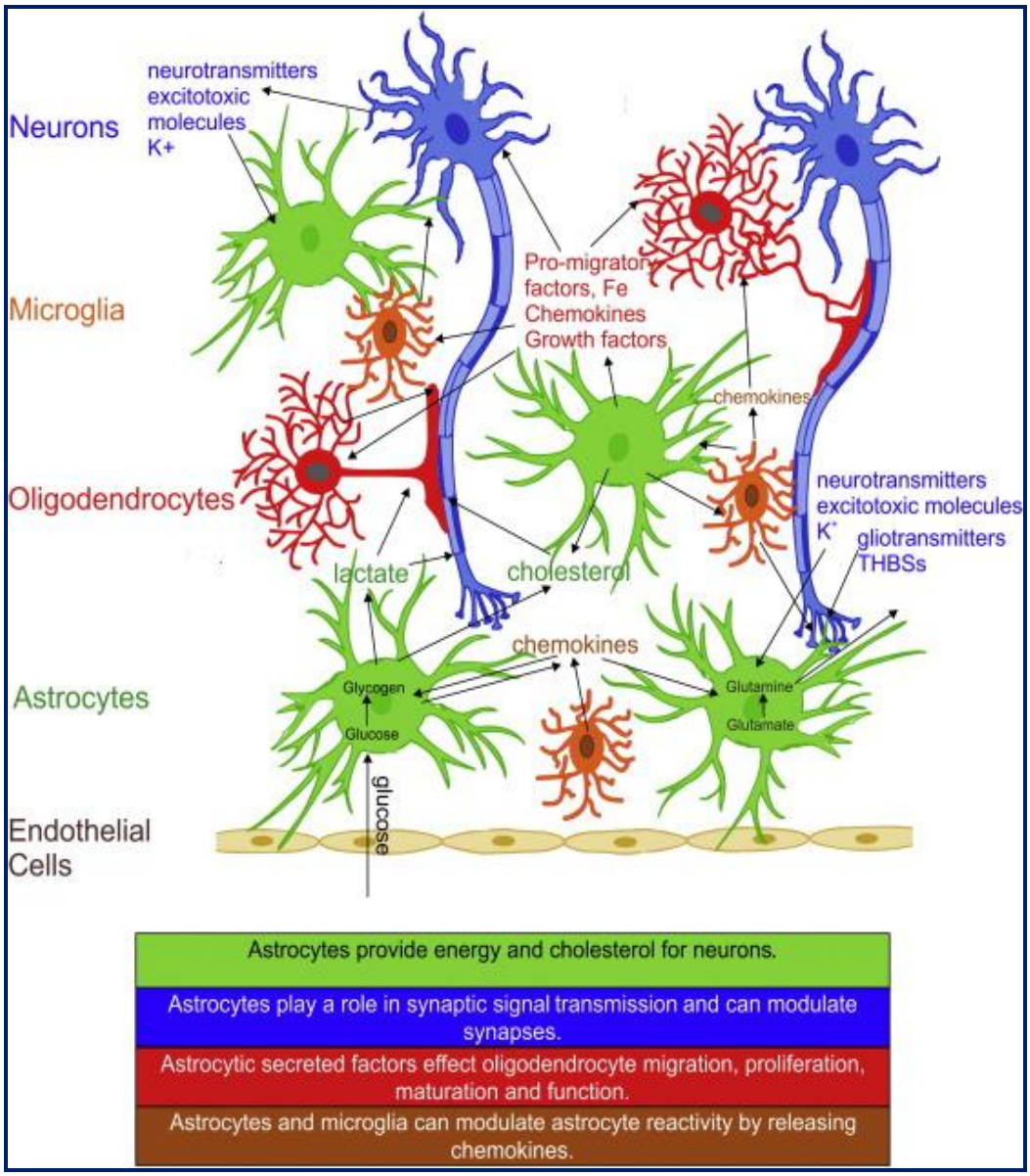

Figure 2.3. Astrocytic effects on re/myelination can be classified into four main groups. They contribute to re/myelination by Providing an energy source (lactate) and cholesterol for neurons. Glucose taken up by endothelial cells lining the blood brain barrier is later-transferred to astrocytes that transform it into glycogen, which can then be used to produce lactate. Playing a role in synaptic signal transmission by regulating the fluid, pH/ion (e.g. potassium, K^+), glial/neurotransmitter homeostasis and contributing to synapse modulation through secreted molecules, such as thrombospondins (THBSs). Affecting the survival, proliferation, and maturation of oligodendrocytes by secreting growth factors, some of which are regulated by iron homeostasis provided by astrocytes. Chemokines may also influence oligodendrocyte membrane ensheathing of axons. Altering reactivity status through their release of chemokines/cytokines, which in turn affects the cross-talk between all neural cells including microglia (Kiray Lindsay Hosseinzadeh and Barnett 2016)

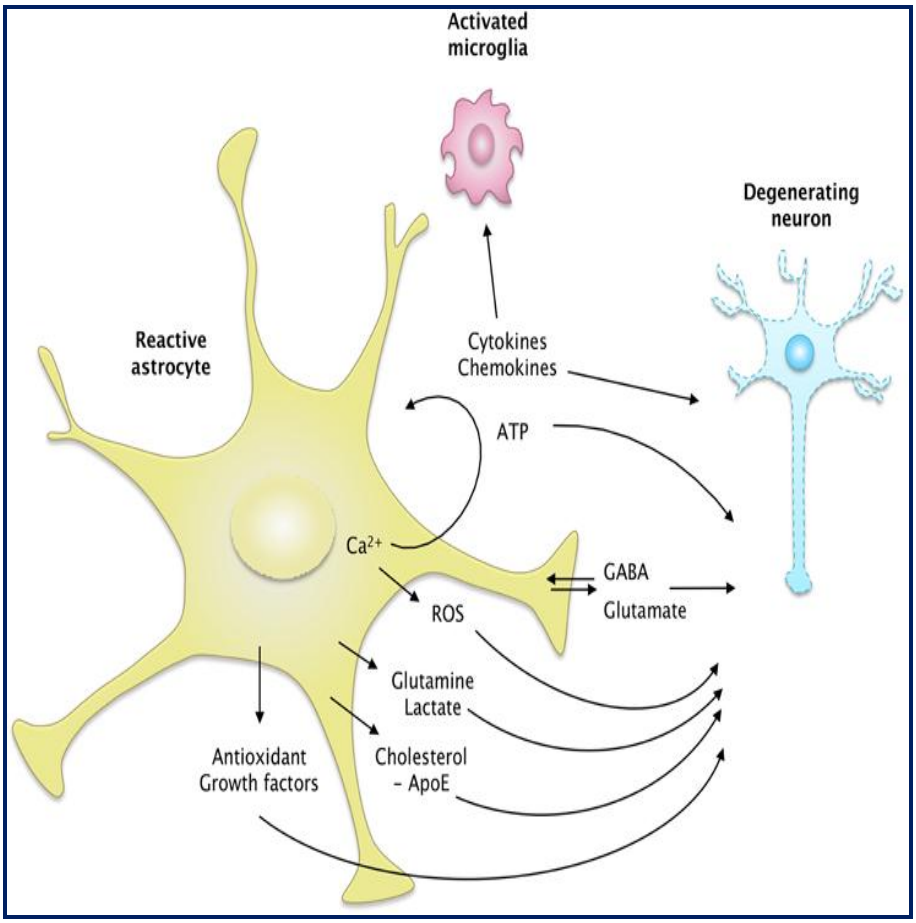

Figure 2.4. The secretome of reactive astrocytes. Astrocytes secrete many active molecules that influence neuronal survival and synaptic activity. Reactivity affects the pattern of secreted molecules, and thus alters neuron astrocyte communications. In ND, reactive astrocytes may secrete higher levels of antioxidants, such as glutathione and its precursors or metabolic substrates. These changes would promote neuron survival. However, reactive astrocytes may also release fewer trophic molecules such as cholesterol, growth factors or glutamine and produce more ROS than resting astrocytes. The regulation of glutamate and GABA homeostasis may also be altered by reactivity, due to a change in their release but also their uptake. Intracellular Ca^{2+} levels are deregulated in ND, which may stimulate the release of gliotransmitters such as glutamate and ATP. Reactive astrocytes also produce more cytokines, which activate microglial cells or act as paracrine factors, maintaining glial cells in a chronically reactive state (Ben Haim Carrillo de Sauvage Ceyzeriat, and Escartin 2015)

2.6. Functions Of Reactive Astrogliosis And Glial Scar Formation

Notions about reactive astrogliosis have long been dominated by the recognition over 100 years ago that scars formed by reactive astrocytes inhibit axon regeneration and via the interpretation that this scar formation was the main impediment to functional recovery

after CNS injury or disease. The ensuing over 100-year-long emphasis on glial scar formation as an inhibitor of axon regeneration has often attended to a generalized negative show of reactive astrogliosis per se, and there has been a tendency among certain authors to typecast the entire process of reactive astrogliosis as a uniformly negative and maladaptive phenomenon that unavoidably causes neurotoxicity, inflammation, or chronic pain. This stereotyped viewpoint has sometimes led to the simplistic notion that complete inhibition of reactive astrogliosis can be regarded as a therapeutic strategy. This negative view of reactive astrogliosis is no longer tenable, and it is now clear from many different lines of experimental evidence that there is a normal process of reactive astrogliosis that exerts essential beneficial functions and does not do harm. Many studies using transgenic and experimental animal models provide compelling occur that reactive astrocytes protect CNS cells and tissue by (1) uptake of potentially excitotoxic glutamate, (2) preservation from oxidative stress via glutathione production, (3) Neuroprotection by adenosine release, (4) preserve from NH4, toxicity, (5) neuroprotection via degradation of amyloid-beta peptides, (6) facilitating blood-brain barrier repair, (7) decreasing vasogenic edema after trauma, stroke or obstructive hydrocephalus, (8) stabilizing extracellular fluid and ion balance and decreasing seizure threshold, and (9) limiting the spread of inflammatory cells or infectious agents from areas of disease into healthy CNS parenchyma (Sofroniew and Vinters 2010).

2.7. Reactive Astrogliosis, Glial Scar Formation And CNS Inflammation

The functions of reactive astrocytes are recent evidence that reactive astrogliosis and glial scar formation play essential roles in regulating CNS inflammation. In response to numerous kinds of stimulation, reactive astrocytes can make many different kinds of molecules with either pro or anti-inflammatory potential and reactive astrocytes can exert both pro- and anti-inflammatory effects on microglia. A large body of experimental studies investigate that reactive astrocytes can use both pro- and anti-inflammatory regulatory functions in vivo in a context dependent manner that is regulated by specific molecular signaling pathways. A functional model, which may reconcile the obvious paradox that reactive astrocytes have the potential to exert both pro- and anti-inflammatory activities, is that reactive astrocytes use different activities at different times after insults, or in various geographical locations about lesions, as determined via

context specific signaling mechanisms. For example, reactive astrocytes may exert pro-inflammatory roles at early times after insults and in the center or immediate vicinity of lesions, but exert anti-inflammatory functions at later times and the borders between lesions and healthy tissue. In cases of severe lesions, reactive astrogliosis may form scars that act as cell migration barriers around the borders of areas where intense inflammation is needed and thereby restrict the spread of inflammatory cells and infectious agents into adjacent healthy tissue [Figure 2.5]. Together, these findings indicate that there is a normal process of reactive astrogliosis and glial scar formation that exerts various beneficial functions including protecting neural cells and function, restricting the spread of inflammation and infection, and promoting tissue repair. It is important to differentiate this conventional process from the potential for dysfunction of reactive astrocytes to contribute to CNS disorders (Sofroniew and Vinters 2010). Astrocytesin healthy CNS tissue. Mild to moderate reactive astrogliosis comprises variable changes in molecular expression and functional activity together with variable degrees of cellular hypertrophy. Such changes occur after mild trauma or at sites distant from a more severe injury, or after moderate metabolic or molecular insults or milder infections or inflammatory activation. These changes vary with insult severity, involve little anatomical overlap of the processes of neighboring astrocytes and exhibit the potential for structural resolution if the triggering insult is removed or resolved. Severe diffuse reactive astrogliosis includes changes in molecular expression, functional activity, and cellular hypertrophy, as well newly proliferated astrocytes (with red nuclei in the figure), disrupting astrocyte domains and causing long-lasting reorganization of tissue architecture. Such changes are found in areas surrounding severe focal lesions, infections or regions responding to chronic neurodegenerative triggers. Severe reactive astrogliosis with compact glial scar formation occurs along borders to areas of apparent tissue damage and inflammation and includes newly proliferated astrocytes (with red nuclei in the figure) and other cell types (gray in the figure) such as fibro meningeal cells and other glia, as well as deposition of dense collagenous extracellular matrix. In the compact glial scar, astrocytes have densely overlapping processes. Mature glial scars tend to persist for long periods and act as barriers not only to axon regeneration but also to inflammatory cells, infectious agents, and non CNS cells in a manner that protect healthy tissue from nearby areas of high inflammation that are long-lasting and persist long after the triggering insult may have resolved. The findings summarized here show that there are pronounced differences

along the continuum of potential responses of reactive astrocyte to insults of different kinds and severities. These differences are likely to be of consequence when considering the functions and impact of reactive astrogliosis on CNS disorders and pathologies.

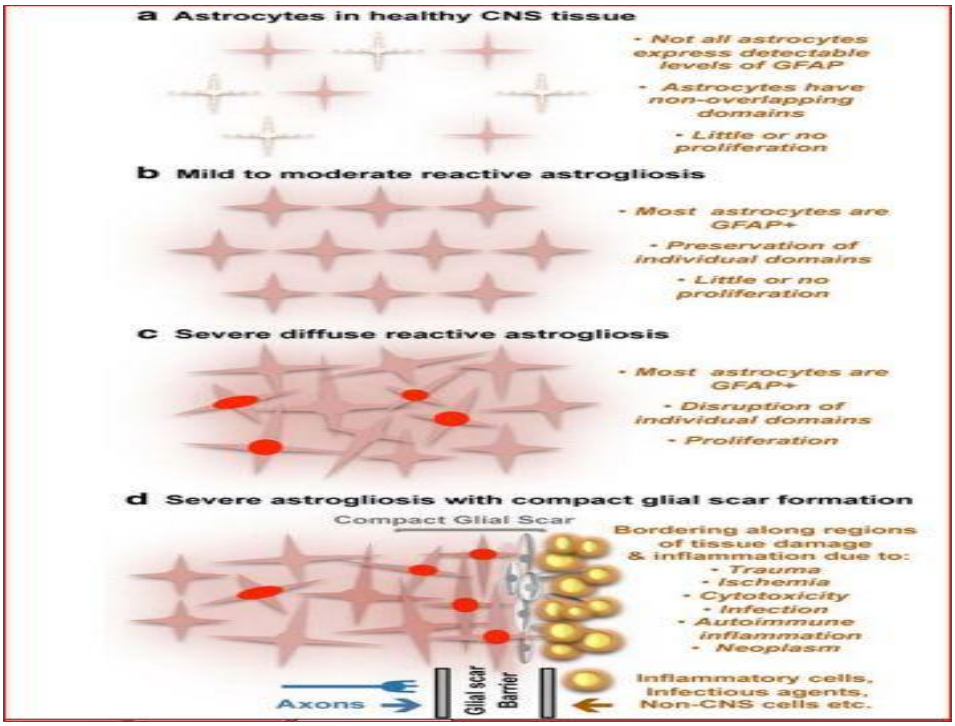

Figure 2.5 Schematic representations that summarize different gradations of reactive astrogliosis (Sofroniew and Vinters 2010)

2.8. The Intrinsic Pathway Of Apoptosis

The intrinsic or mitochondrial pathway of apoptosis is regulated via the Bcl-2 protein family, with two members, Bak and Bax, required to permeabilize the mitochondrial outer membrane (OM). During OM permeabilization, Bak and Bax undergo significant confirmation alter involving exposure of N-terminal epitopes and homo- oligomerization to form an as yet undefined pore. Bak conformation convert also includes transient exposure of the BH3 domain that then binds to the hydrophobic groove of another activated Bak molecule to form symmetric dimers, with the same process also occur for Bax. Symmetric dimers of Bak and Bax can then be connected interface into higher order oligomers that likely constitute the apoptotic pore. Bak and Bax are regulated via other Bcl-2 family members. They are activated via direct binding of BH3 only proteins (e.g.

Bim and tBid) and sequestered via binding to prosurvival proteins (e.g. Mcl-1 and Bcl-2). Specific binding results in Bak being guarded mainly by Bcl-xL, Mcl-1, and Al, while Bax is responding primarily via Bcl-2, Bcl-xL, Bcl-w, and A1. This specific binding can result in either Bak or Bax preferentially leading apoptosis. For example, Bak-driven apoptosis can be initiated by luck of the relatively labile Mcl-1 and Bcl-xL following UV, actinomycin D or cycloheximide. Bak and Bax composed a C-terminal hydrophobic region that inserts as a transmembrane (TM) domain into the mitochondrial OM. The C-termini of Bak and Bax can target GFP to mitochondria and their truncation in the local proteins can block membrane insertion and proapoptotic function. Two or more basic residues in the extreme C- terminus (i.e. within the C-segment) may assist insertion of the TM domain across the OM, as viewed for other mitochondrial tail anchored proteins. Whether the TM domain inserts spontaneously penetrate, the mitochondrial membrane remains con- controversial. Moreover, peptides equivalent to the C-termini of Bak (24 residues) and Bax (24 residues) can integrate into model membranes in the absence of chaperones or receptors. Bak is linked in the OM in healthy cells, whereas Bax is mostly cytosolic until its translocation to mitochondria after apoptotic signaling. A portion of Bax that is peripherally contacted to mitochondria in healthy cells can retrotranslocation upon binding Bcl-xL. Cytosolic Bax is proposed to sequester its TM domain in a hydrophobic surface groove through an interaction containing hydrogen bonding between S184 in the TM domain and D98 in the groove. While others have studied how the C-termini of Bax, Bcl-xL, and Bcl-2 control mitochondrial targeting, this has not been examined for Bak. To understand how Bak is targeted to mitochondria, and to address whether variances in Bak and Bax localization might contribute to their differential regulation, we mutated the C- terminus of Bak. Removing the C-segment (C-terminal six residues), or the basic residues within, decreased mitochondrial targeting and protein stability, thereby decreasing proapoptotic function. Notably, replacing the C-segment of Bak with that from Bax converted Bak to a relatively stable, semi-cytosolic protein (named Bak/BaxCS) that could translocate to mitochondria. Translocation of both Bak/BaxCS and Bax following apoptotic signaling suggests similar activation mechanisms for Bak and Bax. Furthermore, the semi-cytosolic localization of Bak/BaxCS did not alter its regulation by Mcl-1 (Anderova et al. 2014).

2.9. Extrinsic Pathway Of Apoptosis

Since it was first described more than 40 years ago, tremendous proceed has been made in our understanding of programmed cell death (Hans Jürgen Rode 2008).It's a conserved cell death mechanism required for normal development and tissue homeostasis in multicellular organisms (Zamorano et al. 2012). Apoptosis is a type of programmed cell death and events actively in multicellular organisms under physiological and pathological conditions (Chen et al. 2014). Although apoptosis presumably participates in the development of most cell lineages, alterations in the expression of apoptosis regulatory proteins is implicated in the initiation of different human diseases, including autoimmunity, immunogenic- science, cancer, and neurodegenerative diseases, among others. The BCL-2 family of proteins is a group of the upstream arrangement of the caspase cascade, comprised of both pro and anti apoptotic components. BCL-2 family members are defined by the presence of up to four a-helical conserved BCL-2 homologies (BH) domains. Proapoptotic BCL-2 family members can be further subdivided into more highly conserved, "multidomain" members displaying homology in the BH1, BH2 and BH3 domains (i.e. BAX and BAK), and the ‘‘BH3-only" members which contain a single BH domain critical for activation of apoptosis.

Genetic and biochemical studies indicate that BAX and BAK function in concert as a central core of the intrinsic apoptosis pathway at the mitochondria. Upstream BH3-only proteins respond to specific apoptotic signals and subsequently trigger the conformational activation of BAX and BAK, inducing their intramembranous homo-oligomerization and resultant mitochondrial outer membrane permeabilization (MOMP). MOMP is an essential step for the release of cytochrome c and the accumulating of the apoptosome. In addition, the BH3-only proteins can be functionally separated into two subtypes (1) activators (i.e. tBID, BIM particular, and PUMA) that directly engage BAX and BAK to trigger cytochrome c release, but are sequestered by anti-apoptotic BCL-2 molecules; and (2) sensitizers or inactivators (i.e. BAD and NOXA) that only bind to and antagonize anti-apoptotic BCL-2 members to release activator BH3-only proteins Alternatively, differential binding to anti-apoptotic proteins may explain the action of activator and sensitizer/inactivator BH3 only proteins or assembling of both models. Under certain conditions, cytochrome c release events independent of BAX and BAK through the

opening of the mitochondrial permeability transition pore (PTP), a non-specific pore in the inner mitochondrial membrane.

The opening of the PTP is observed under conditions of mitochondrial calcium overload, especially when accompanied by oxidative stress, increased phosphate concentrations, and adenine nucleotide depletion, enabling free passage into the mitochondria of molecules 1.5 kDa. The opening of the PTP leads to dissipation of the mitochondrial transmembrane potential (DYm) and an influx of solutes. This causes expansion of the matrix, resulting in sufficient swelling to rupture the outer mitochondrial membrane and cytochrome c release. Moreover, dissipation of DYm can also lead to a sudden decrease in ATP levels, triggering necrotic cell death. Although the molecular identity of PTP remains uncertain, different constitutes are proposed including voltage-dependent anion channel, the adenine nucleotide translocator, the mitochondrial phosphate carrier, and Cyclophilin D (CypD), a cyclosporin A target. Studies using knockout cells for putative constitutes of the PTP confirmed only a functional role for CypD in PTP-mediated cell death in vitro and in vivo. Remarkably, physical interactions between BCL-2 family members and constitutes of the PTP are also reported, suggesting that BCL-2 related proteins may facilitate PTP under certain conditions, possibly forming mixed protein complexes with membrane permeabilizing activity (Chen et al. 2014).

Study of apoptosis was focused on the caspase, a family of cysteine protease. While using the caspase inhibitor to block the apoptosis pathway, the researchers discover that the apoptosis still happen. So another pathway that is caspase-independent was found. Now, apoptosis is classified to type I, Type II, Type III PCD: type I PCD is the classic apoptosis, the well know caspase-dependent apoptosis; type II PCD's morphology characters are the appearance of the autophagic and double membrane of vacuole; type III PCD occurs without the condensate chromatin and has not been well-known. Type II and type III PCD are the caspase-independent apoptosis. For example, the apoptosis induces factor (AIF) (Hongmei 2012). AIF is required for mammalian development, knocking out AIF in the mouse is lethal before birth (Fabienne T Schulthess I et al 2009).

Mitochondrial intermembrane flavoprotein that can be released from mitochondria to translocate into the nuclear and lead to many high molecular weight DNA fragmentation and chromatin condensation in cells, this type of apoptosis is in a caspase-independent

manner. No matter the type I, Type II or type III PCD, the apoptosis will assist the host to defense the outer or inner aliens and toxic constitutes and help the organism survive (Hongmei 2012). Apoptotic cells may not increase inflammation because phagocytes usually ingest them before releasing their intracellular contents. An essential biochemical event leading to oncotic /necrosis, as opposed to apoptosis, is a rapid decrease of intracellular ATP. The assessment of oncotic is frequently neglected, although it is an essential pre-lethal phase that follows a serious cell injury and, unlike in necrosis, some mechanisms possibly exist for reversing the process (Balvan et al. 2015). Various apoptotic genes produce pro-apoptotic or anti-apoptotic isoforms depending on the regulation of their alternative splicing (Vicente Crespo et al. 2008). Gene expression plays a critical role in the response of cells to death-inducing stimuli. A growing body of evidence indicates that the levels of several death-related genes can be induced during apoptosis (Wilhelm Pellay Benecke and Bell 2008). ER stress induced apoptosis is mediated by the activation of BAX and BAK at the mitochondria through upregulation of the upstream BH3 only proteins leading to mitochondrial outer membrane permeabilization, cytochrome c release, and subsequent apoptosome assembling. Under certain conditions such as mitochondrial calcium overload and oxidative stress, cytochrome c release can occur independently of BAX and BAK through the opening of the mitochondrial permeability transition pore (PTP), which is form by several components including CypD and VDAC. The opening of the PTP leads to the expansion of the mitochondrial matrix, resulting in sufficient swelling to rupture the outer mitochondrial membrane and cytochrome c release. Stimulation of ER stress in conjunction with mild serum withdrawal triggers apoptosis in a BAX/BAK and CypD-independent manner. The activation of caspase-9 mediates this alternative death pathway. Under normal conditions growth factor signaling may inhibit this alternative cell death pathway at the level of (1) mitochondria, or by (2) blocking the activation of a particular ER-dependent event.

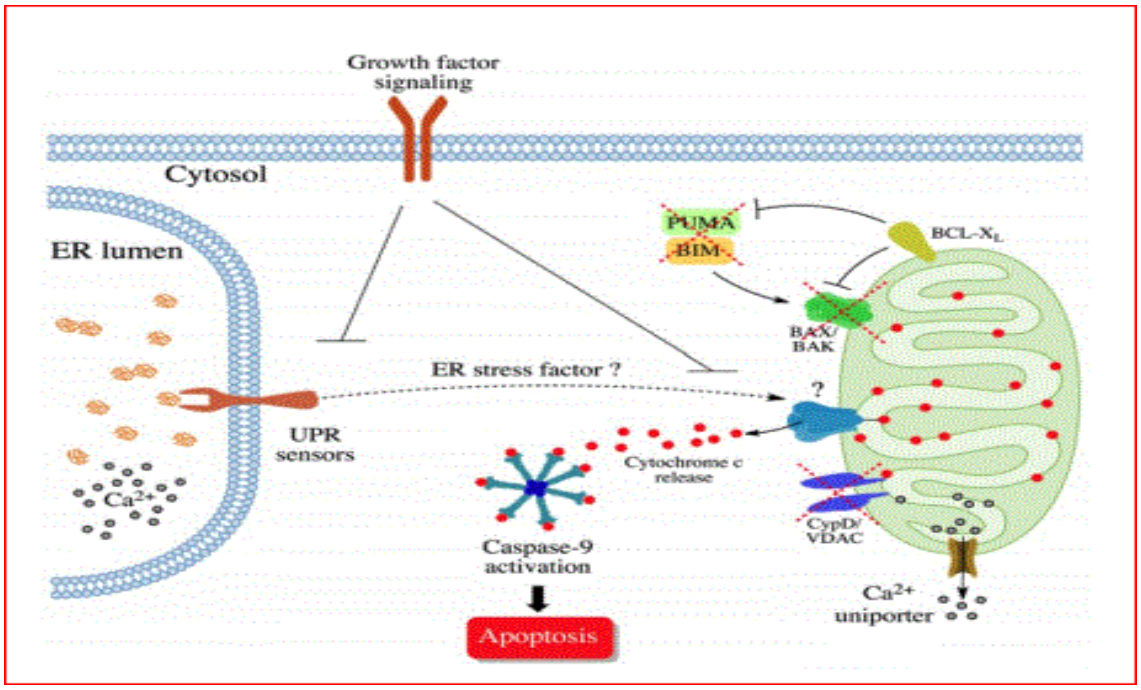

Figure 2.6. Working model ABAX/BAK and CypD-independent intrinsic apoptosis pathway (Zamorano et al. 2012)

2.10. Mechanism Of Apoptotic Signaling Pathways

Apoptotic signaling pathways are induced via activation of caspases which then cleave key protein substrates resulting in cell death. Based on their structure, caspases can be divided into two classes. Caspases-2, -8, -9, and -10 contain long amino- terminal domains and normally function as initiators of apoptotic pathways, whereas caspases-3, -6, and -7 have only small areas and function as the influence of cell death. The activation of the initiator caspase-9 in the intrinsic mitochondrial apoptosis pathway involves BH3 proteins of the Bcl-2 family that works as monitors of cellular damage. In response to cellular damage, these proteins increase activation of the pro-apoptotic activities of Bax and Bak, inducing the release of cytochrome c, and subsequent formation of the apoptosome, which is a multi- subunit caspase scaffold that activates the caspase-9-dependent apoptotic pathway. In the death receptor-mediated apoptosis pathway, a protein complex recruiting the Fas-associated protein with a death domain (FADD), and procaspase-8 and -10 invites the death-inducing signaling complex (DISC). The procaspases-8 and-10 in the DISC are activated by oligomerization followed by

proteolytic self-processing enabling them to activate downstream effector caspases including caspase-3 (Zhang et al. 2011).

Mitochondria are also known to contain other FAD-containing oxidoreductases, namely the apoptosis-inducing factor (AIF) and the apoptosis-inducing factor-homologous mitochondrion-associated inducer of death (AMID), which have been assigned key roles in caspase-independent apoptosis (Carneiro Duarte and Videira 2012). Recent studies of the mitochondrial apoptosis pathway observed that caspase-8 and-10 can also be activated downstream of the mitochondria by caspase-3, indicating the existence of so-called amplification loops where caspase-8 or -10 activate caspase-9 and -3. In this context, it should be observed that activated caspase-8 and -10 can also proteolytically activate pro-apoptotic Bcl-2 family member Bid generating tBid. tBid causes the release of mitochondrial cytochrome c resulting in the activation of caspase- 9 which can further promote caspase-3 activity to complete the apoptotic process. To evaluate whether and to what extent CAAP is involved in the regulation of apoptosis, we examined caspase activation and apoptosis signaling in the presence and absence of CAAP in several tumor models. Our study revealed that CAAP exerts a prominent anti-apoptotic function that critically depends on the presence of caspases-3 and -10. Also, we demonstrated that treatment of MCF-7/casp3-10b cells with staurosporine and etoposide triggered knockdown of the CAAP expression concur- rent with the induction of apoptosis. These data suggest that CAAP may be a target site for chemotherapy since it does not require siRNA to knockdown the expression of this anti-apoptotic protein (Zhang Y et al. 2011).

Nemours apoptotic signaling pathways have been identified, including the Fas/FasL pathway, the caspase family pathway, the cytochrome C signaling pathway, and the mitochondrial pathway. Of these apoptotic signaling pathways, the caspase family pathway is considered to be of great essential because many signaling pathways ultimately activate caspase cascades. Caspases are cysteine protease family members and play a significant role in apoptosis. Activated caspases can cause primary protein degradation and cell apoptosis irreversibly by cleaving substrate proteins such as poly-ADP-ribose polymerase (PARP). Rho family proteins participate in the regulation of polarity, proliferation, adhesion, spreading, migration, cytoskeleton organization, and apoptosis of cells. Rho GDP dissociation inhibitor alpha (RhoGDIa) is frequently

overexpressed in human tumors and chemoresistant cancer cell lines, raising the possibility that RhoGDIa is an anti-apoptotic molecule in cancer cells. In healthy cells, a previous study demonstrated that RhoGDIa plays a critical role in low shear stress-induced apoptosis of vascular smooth muscle cells. Hence, it was theoretical that RhoGDIa may participate in apoptosis of other healthy cells such as human PDL cells (L Wang Pan Wang Song and Chen 2013).

2.11. Hypoxic Postconditioning Reduces Microglial Activation, Astrocyte And Caspase Activity, And Inflammatory Markers

Neonatal hypoxic ischemic (HI) brain injury is a primary cause of neonatal mortality due to absent of oxygen and blood flow to the brain, causing approximately one million neonatal deaths every year. Currently, the only clinical treatment available for these infants is hypothermia, which has limited success in reducing mortality and neurodevelopmental disabilities. Thus, there is a need to progress additional therapeutic strategies to minimize brain damage and potentially promote brain repair after HI. After HI injury, microglial activation occurs, followed by the release of inflammatory mediators, including the cytokines interleukin (IL) 1β and tumor necrosis factor α (TNF-α). An IL-1β receptor antagonist can eliminate levels of IL-1β increase after HI and excitotoxic brain injury in newborn rats.

Similarly, TNF-α and IL-1β gene expression in the brain were promoted after a systemic inflammatory and central excitotoxic insult in neonatal mice, and this was reduced in TNF-α knockout mice and by TNF-α blockade using an- except. Preventing microglial activation with minocycline reduced levels of IL-1β, TNF-α, and restricted brain tissue absent after HI in the neonatal rat. These studies suggest that microglial activation and proinflammatory mediators play a significant role in mediating brain injury responses and moderating inflammatory responses can eliminate the extent of the injury.

Astrocytes have important roles in blood–brain barrier function, metabolism, uptake of glutamate, and the formation of a glial scar during injury. Studies using glial fibrillary acidic protein (GFAP) and vimentin knockouts have shown that reactive gliosis surrounding a lesion site can protect against damage and delay functional recovery

following stroke in mice. There is substantial occur suggesting that astrocyte activation can inhibit normal restorative processes at later stages of injury to the adult central nervous system. In the neonatal brain, preventing astrogliosis via knocking out these intermediate filaments did not affect the overall brain injury but increased cortical neurogenesis after HI, highlighting the complex role of astrocytes in normal and pathological processes in the brain. Therefore, in the developing brain, regulating astrocyte activity might offer beneficial influences after an HI brain injury. Delayed cell death is involved in neuronal pruning, which is an important process that occurs in the brain during development. The development of HI injury, apoptosis closely for lows necrosis which happens due to energy failure. Apoptosis is triggered by a cascade of proapoptotic initiator and effector caspase activity, caspase-3 in particular. Pharmacological inhibitors of apoptosis can decrease damage and progresses sensorimotor function in neonatal models of brain injury. Hence, reducing apoptosis by limiting caspase 3 activity might prove to be a viable target in reducing the damage sustained as a result of neonatal HI injury. Mild hypoxic precondition reduced tissue damage following HI in the neonatal rat brain, and this protection may be mediated via reduced apoptosis and inflammatory mediators. While animal studies have shown robust preserve effects of hypoxic pre- conditioning, the bench to bedside application remains limited due to the unpredictable nature of HI brain injury in human newborns. Neuroprotective effects of exposure to hypoxic postconditioning (PostC) following a brain injury in rodents. The current study investigates the potential mechanism of neuroprotection by hypoxic PostC after a neonatal HI brain injury in rats. Here, we have examined the response of different brain cell populations such as neurons, microglia, astrocytes, and inflammatory mediators in the cortex of the brain following PostC and HI (Teo Morris and Jones 2015).

2.12. Glioblastoma As Cell Model For Apoptosis Pathways Study

Glioblastoma (GBM) are the most common initial brain tumors, with a worldwide annual incidence of around 7 cases per 100,000 individuals. More than 20,000 cases are examined every year only in the USA and gliomas have a disproportionately high mortality rate of more than 70% of cases in two years after diagnosis. Among the primary brain tumors, GBM, classified as grade IV via the World Health Organization, is

the most frequent and biologically aggressive type, corresponding to around 65% of cases. The high malignancy of GBM is due to their intense cell proliferation, spread infiltration, high resistance to apoptosis and robust angiogenesis, in which cells from the tumor form part of the endothelium possibly cause of reprogramming. GBM Cancer Stem Cells (gCSC) have received much attention in glioma biology, and this type of cell is highly related with high aggressiveness, being fundamental for the maintenance and recurrence of GBM. It was recently demonstrated that gCSCs participate in the formation of the tumor endothelium, promoting the invasiveness of the tumor and leading to the impedance to radiotherapy through many mechanisms. The primary therapy for GBM consists of surgery followed via radio and chemotherapy with temozolomide (TMZ), which is in clinical use since 2005.

Despite this multimodal approach, the prognosis has only slightly repaired. Among the potential alternatives that have emerged for treating GBM are some natural products which present high antitumoral efficiency without some of the harmful side effects of conventional chemotherapies. Resveratrol (Rsv) (3,4,5-trihydroxy-trans-stilbene) is a polyphenolic phytoalexin extremely present in plants and enriched in red wine, peanuts, and other sources. This component exerts useful functions in healthy cells both in vitro and in vivo, by inducing mitochondrial biogenesis and neuroprotection in reverse conditions, such as oxygen-glucose deprivation and traumatic brain.

On the other hand, it is cytotoxic for the majority of malignant cells, blocking the three top stages of carcinogenesis (i.e. initiation, promotion and progression in many types of cancer cells and models, like breast, colon, melanoma, uterine, lung and leukemia. Rsv exerts its toxicity through modulation of many pathways and induction of different mechanisms of cell death and growth inhibition. It induces program cell death in colon cancer cells, necrosis in prostate carcinoma cells, growth arrest in myeloma cells and autophagocytosis in ovarian cancer cells. In gliomas, Rsv stimulates signs of necrosis, apoptosis and senescence in C6 (rat) cells, apoptosis in U251 and U87 (human) cells in high dosages and autophagy in U251 cells. In C6, U138 (human) and GL261 (mouse) glioma cells lines, we have previously shown that Rsv inhibits cells growth, through mechanisms that involve but are not restricted to apoptosis and senescence.

Growth inhibition and induction of cell death are among the primary objectives of anti-cancer therapies. Some types of cancers frequently develop resistance to apoptotic cell death, among which we highlight primary gliomas. Two essential pathways mediate part of this resistance in these tumors: PTEN/Akt/PI3K pathway, which is over activated in GBM cells through damage of PTEN, overexpression of EGFR (a typical alteration of primary gliomas) and/or promote of PI3k/Akt activity due to mutations in its regulators; and NF-kB pathway, which is constitutively activated in a large proportion of GBMs and is increased by cells in response to cytotoxic drugs, favoring cell survival by inducing the expression of anti-apoptotic genes. Thus, inhibition of these pathways may be a way to decrease GBM intrinsic- and drug-induced resistance, sensitizing GBM cells to apoptotic cell death. On the other hand, induction of other non-apoptotic mechanisms of cell death is central for the elimination of apoptosis resistant GBM cells. Thus, the progress of drugs that induce multiple mechanisms of cell death like senescence, mitotic catastrophe, para ptosis, auto schisis and specially autophagy and autophagic cell death are fundamental to overcome this resistance. Autophagy is a genetically programmed, evolutionarily conserved process coordinated via a family of genes, called At, that lead to the degradation of organelles and proteins. It involves the formation of double-membrane vesicles, containing cellular components, that merge to lysosomes, forming the autophagolysosome, where the components are degraded and the products generated are reused by the cell. Autophagy acts as a prosurvival mechanism, mainly in adverse conditions including nutrient and oxygen deprivation.

Moreover, this process has a definite self-limiting character and may lead to cell death with autophagic features (or programmed cell death type II) when at high levels or duration. In cancer, it has been shown that autophagy may be an essential anti-cancer mechanism in vivo since the expression level of Beclin-1, a fundamental gene for autophagy is inversely correlated with the malignancy of brain tumors and is directly correlated with survival. However, autophagy is induced by efficient physical and chemical anti-cancer treatments in gliomas, like TMZ, rapamycin, c radiation, oncolytic adenoviruses, and others. However, it was shown that GBM cells are more sensitive to agents that induce autophagy than apoptosis, like TMZ, and autophagic structures were investigated in gliomas in vivo after treatments.

Also, the understanding of the complexity of the relationship between apoptotic cell death and autophagy (and other mechanisms of cell death and growth inhibition) in cancer is required for the understanding on how to tip the balance from tumor survival to death. The evaluated the actions of Rsv in glioma cells, focusing on the role of autophagy and their interaction with cell cycle regulation, apoptosis and the biology of gCSCs. Inhibition of basal autophagy decrease the stemness of GBM cells, while inhibition of Rsv-induced autophagy in U87 cells caused apoptotic cell death and, more interestingly, inhibited cell cycle arrest induced by Rsv, suggesting that, despite not being directly involved in the inhibition of cell growth by Rsv, autophagy plays an indirect, but fundamental, role in mediating the effects of Rsv in GBMs (Filippi Chiela Villodre Zamin Lenz 2011). Numerous cancers display a highly treatment-resistant phenotype. A prototype of these tumors represents Glioblastoma (GBM), which despite vast treatment efforts carries a grim prognosis as reflected by a median overall survival of fewer than 15 months. One mechanism via which GBM can evade therapy is resistance to apoptotic cell death. Restoring apoptotic sensitivity is therefore of paramount essential to render GBMs sensitive to drug treatment. One way to make treatment resistant cancers amenable to drug treatment is the administration of combinatorial drug regimens. Such treatments may overcome primary and acquired resistance to therapy. Virtually all GBMs develop secondary treatment resistance after administration of either Temozolomide (TMZ), radiation or the combination of TMZ with radiation.

Since the DNA repair enzyme poly(ADP-ribose) polymerase (PARP) is expressed at higher levels in tumor cells when compared to benign tissues and cells, PARP may, therefore, represent a tumor-specific treatment target. However, while assisting rapid dividing cancer cells with DNA-repair, PARP counteracts apoptotic cell death. Consistent with this idea, interference with PARP by RNA silencing or PARP inhibitors render cancer cells more prone to the cytotoxic effects of DNA-damage inducing treatment modalities, such as radiation, topoisomerase inhibitors or alkylating reagents (i.e. Temozolomide). The PARP inhibitor, which penetrates the blood-brain barrier and has already reached clinical trials in GBM patients. Our data demonstrate that Olaparib overcomes apoptotic resistance and sensitizes GBM cells for death receptor- mediated apoptosis induced by TRAIL (Tumor necrosis factor-related apoptosis-inducing ligand) through up-regulation of TRAIL receptor 2 (DR5) independent of their TP53 status.

Therefore, PARP-1 specific siRNA, as well as PJ34, another pharmacological PARP inhibitor, also increase extrinsic apoptosis in GBM cells in vitro and in vivo. Since TRAIL is known for its tumor specificity, the combination treatment of PARP inhibitors with TRAIL may be an ideal drug combination therapy with potential little side effects (Karpel Massler et al. 2014).

2.13. Clinically (ADP-ribose) Polymerase (PARP)

The enzyme poly(ADP-ribose) polymerase (PARP) Is induced at higher levels in tumor cells when compared to benign tissues and cells. PARP may, represent a tumor-specific treatment target. However, while assisting rapid dividing cancer cells with DNA-repair, PARP counteracts apoptotic cell death. Consistent with this idea, interference with PARP by RNA silencing or PARP inhibitors render cancer cells more prone to the cytotoxic effects of DNA-damage inducing treatment modalities, such as radiation, topoisomerase inhibitors or alkylating reagents (Temozolomide). The PARP inhibitor, which penetrates the blood brain barrier and has already reached clinical trials in GBM patients. Recent data shown that Olaparib overcomes apoptotic resistance and sensitizes GBM cells for death receptor mediated apoptosis induced by TRAIL (Tumor necrosis factor-related apoptosis inducing ligand) through up regulation of TRAIL receptor 2 (DR5) independent of their TP53 status. Also, PARP-1 specific siRNA, as well as PJ34, another pharmacological PARP inhibitor, also increased extrinsic apoptosis in GBM cells in vitro and in vivo. Since TRAIL is known for its tumor specificity, the combination treatment of PARP inhibitors with TRAIL may be an ideal drug combination therapy with potential little side effects (Karpel Massler et al. 2014).

2.14. Function of Poly- (ADP-ribose) polymerase (PARP)-1

PARP-1 is a high copy number nuclear enzyme which is activated by DNA-breaks and catalyzes the poly-ADP-ribosylation of nuclear proteins using NAD^+. Oxidative stress via the induction of DNA breaks can activate PARP leading to NAD^+ and ATP depletion followed by necrotic cell death. Besides, PARP activation through the destabilization of mitochondrial outer membranes promotes the release and nuclear translocation of Apoptosis Inducing Factor (AIF) and Endonuclease G leading to apoptosis. Hence, PARP

inhibitors can be used to prevent oxidative stress-induced cell death. Oxidative stress-induced activation of PARP promotes JNK and p38 APK activation while PARP inhibitors suppress their activation. The inhibition of PARP in oxidative stress activates the expression of MAP kinase phosphosphatase-1 (MKP-1/Dusp1) which is the major phosphatase, which dephosphorylates and inactivates the MAP kinases.

PARP inhibitors have the potential to protect different tissues from oxidative stress, and able to regulate a pleasant way MAP kinases and inflammatory processes. Hence, PARP inhibitors have protective effects in various oxidative stress related disease models by preventing compromised energy status and by preventing other cell death promoting effects of PARP activation. Excessive activation of PARP by stress stimuli, such as reactive oxygen species (ROS) formation has been associated with the pathogenesis of deferent diseases, including cerebral ischemia, Parkinson's disease, ischemic reperfusion (IR) induced cardiac dysfunction, the progress of diabetic complications and angiogenesis. Studying the renal graft dysfunction in acute rat rejection model we found PARP inhibitor 4-hydroxyquinazolone (4OHQ) has preserve effects. It prevents the disintegration of the interstitial tubule structures, decreases oxidative stress markers, increases antiapoptotic Bcl-2 levels, suppresses cell death by inducing JNK activation and activates the cytoprotective PI-3K-Akt pathway. Our data suggest PARP inhibitors to immunosuppressant regimens during kidney transplantation may be advantageous in the acute rejection period to protect the graft against ischemic reperfusion and other types of oxidative stress induced damages (Kalmar Nagy et al. 2013).

3. MATHERIALS AND METHODS

3.1. Chemicals

Fetal bovine serum (FBS), trypsin-EDTA, Puck's-D1 solution , penicillin, streptomycin were purchased calcium-free phosphate buffered saline (PBS) was from Biomed, methylthiazolyl diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich). Acrylamide bis-acrylamide solution, ammonium persulfate, bovine serum albumin, Lysis buffer , bromophenol blue, calcium chloride, glycine, glycerol, TMZ, sodium dodecyl sulfate (SDS), N,N,N'N' tetramethylethylenediamine (TEMED), trichloroacetic acid, Tris-Triton buffer, triton X-100, trizma base were obtained from Sigma-Aldrich. Hank's solution, Novus Biological (Littleton, CO), antiglial fibrillary acidic protein (GFAP rabbit polyclonal).

3.2. Characterization Of Raw Propolis Samples

Propolis is a complex mixture, containing resins, balsamic products, wax, essential oils, pollen, and microelements, aside from other components. The samples presented a characteristic aroma, balsamic and/or resinous, malleable (red) to rigid (brown and green) consistencies at room temperature, with a very heterogeneous granulometry. It is famous that the analysis of the physical-chemical composition is of great importance to determine the quality of the studied material, considering the incorporation of this matrix in food products. The results of the physical-chemical characterization of the different samples of propolis are various. The value of humidity and total solids varied among the samples. In relation to the contents of ash, protein, lipids and fibres, a significant variation was observed among the samples. The determination of the total ash content is specially important in samples of propolis commercialized in powder form, as this analysis can identify a possible adulteration of the material through the presence of impurities, or even residues from already extracted propolis. Among the microelements construe and

identified in the samples, we can highlight the high contents of potassium on the three samples of green propolis. Some studies show aluminium, vanadium, iron, calcium, silicon, manganese, strontium and potassium, as the major microelements present in propolis samples (Machado et al. 2016).

3.3. The Preparation Of Propolis For The Cell Line

There are three methods commonly applied for extraction with ethanol, methanol and water. The chemical composition or propolis is extremely complex: content of more than 300 components have been identified, and its composition is directly dependent with the composition of the vegetation of the region. Moreover, propolis composition is completely changeable creating a problem for the medical use and standardization. In the present work Bee products where collected green propolis from the bee hive in February to march from north-west of Iraq during winter 2016. The samples of propolis have been cleaned and saved with protection from the light. Ethanolic extraction was prepared by extraction of 30g of bee glue with 450 ml of ethanol 98% for 12 h of a darkened place in the shaker machine, then it was filtered by qualitative filter paper (125 mm and 110 mm), dried, and evaporated in rotary evaporation the yield of prepared extracted (% w/w) as that original product. The EEP was then placed in a glass container and left for approximately three days for the residual solvent to evaporate; as a result, a solid mass (162 g) with viscous appearance was obtained and stored. However, the extracts were filtered twice, dried and stored in sealed bottles at 4°C until use (Machado et al. 2016).

3.4. Surgical Specimens

Fresh rat pups normal brain tissue samples from a 24 h old were obtained from the Molecular Biology of Bingol University in Turkey. The tissue were collected immediately after a surgical resection. Every tissue was separated, homogenized, filtered with 150 μm sieve and seeded into tissue culture plates with a medium containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin The medium was changed 2–3 times a week. Under these conditions, the cells got attached and grew for a few weeks and were used after VI passages. Sub

confluent cells were detached with a trypsin-EDTA solution in PBS and counted in a Neubauer hemocytometer.

3.5. Cell Culture

Mixed cortical cell isolation for astrocyte cultures were performed from brain of rat pups. In order to achieve proper astrocyte density it is necessary to use 4 rat pup cortices per T75 tissue culture flask (Schildge Bohrer Beck and Schachtrup 2013). All experimental procedures conformed to the "Guidelines for Proper Control of Animal Experiments" approved via the local ethics regulations. Astrocytes are seed in 75-cm^2 flasks at a starting density $6{\cdot}10^5$ cells/ml (20 ml/flask) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin and incubated at 37°C in atmosphere containing 10% CO_2. After 9–10 days, these cell cultures should wash with Hank's solution (Ca_2, Mg_2-free; in mM: 50 NaCl, 5 KCl, 0.2 KH_2PO_4, 0.17 Na_2HPO_4, 5.0 glucose, 58.4 sucrose, pH 7.4), detached from the plastic with trypsin-EGTA (0.05%/0.02% wt/vol) solution. Then DMEM containing 10% (vol/vol) (FBS) is added to stop the action of trypsin. After the cells were centrifuged at 500 g for 5 min. The cells were plated for the experiments in DMEM with 10% (vol/vol) (FBS). For experiments cells were used between day 11 and 13 in culture. These cultures are characterized to contain more than 90% astrocytes. After discarding the supernatant, the pellet of cells was again suspended in a complete DMEM medium and then the inoculum of cells corresponding to the final concentration of $1x10^5$ cells/ml was calculated. After distributing the culture medium in plaques of 24 wells, the compounds were added to two concentrations of 50μg/ml and 100μg/ml, and the DMSO was applied as a diluting control and statistic parameter (Machado et al. 2016).

3.6. Preparation Of Proteine Samples

Cells should be rapidly frozen to eschew protease degradation of proteins or collected and lysed as quickly as possible. Solid tissue is mechanically broken down, usually using a homogenizer or via sonication in a lysis buffer. Tissue preparation can be performed on an ice at cold temperatures to avert denaturation and degradation of protein. Lysis buffer

was used to enable lysis of cells and to solubilize proteins. Lysis buffer contains 50 mM triz pH 6,8, 5% glycerol, 3% SDS, 2 mM dithiotreitol, 0,01% bromphenol blue. Lysis buffers used in sample preparation for western blotting should enable efficient protein extraction and maintain antisera recognition of the protein (MacPhee 2010). Importantly, quantification and comparison with other samples in western blot analysis is dependent on the protein lysates prepared for polyacrylamide gel electrophoresis. For cytoskeleton proteins the buffer should contains Tris-Triton buffer: 10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% deoxycholate. As soon as lysis occurs, proteolysis, dephosphorylation and denaturation begin. These events can be slowed down tremendously if samples are kept on ice or at 4°C at all times and appropriate inhibitors are added fresh to the lysis buffer.

3.7. Immunobot Method

PAAG electrophoresis. For optimal separation, it is important to determine the ideal bisacrylamide:acrylamide ratio prior to electrophoresis. The samples are loaded onto the gel. One lane should include a molecular weight marker that is applied to determine the molecular weight of the target protein. Another lane should include an internal control, ideally with a known concentration and molecular weight to determine. Polyacrylamide gels are formed from the polymerization of two compounds, acrylamide and N,N-methylenebisacrylamide (Bis, for short). Bis is a cross-linking agent for the gels. The polymerization is initiated via the addition of ammonium persulfate along with either DMAP or TEMED. The gels are neutral, hydrophilic, three-dimensional networks of long hydrocarbons cross linked via methylene groups. The separation of molecules within a gel is determined via the relative size of the pores formed within the gel. The pore size of a gel is determined via two factors: the total amount of acrylamide present (designated as %T) and the amount of cross-linker (%C). As the total amount of acrylamide increases, the pore size decreases. With cross linking, 5%C gives the smallest pore size. Any increase or decrease in %C increases the pore size. Gels are designated as percent solutions and will have two requisite parameters. The total acrylamide is given as a percentage (w/v) of the acrylamide plus the bis-acrylamide. A standard migration buffer (also called running buffer) for PAGE is 1X Tris-glycine: 25 mM Tris base, 190 mM glycine, 0.1% SDS, pH 8.3.

Transferring of Proteins. Once electrophoresis is complete, the separated proteins should be transferred from within the gel onto a membrane (a western blot) made of nitrocellulose, polyvinylidene difluoride (Kurien and Scofield 2006). Transferring proteins from a gel to a membrane should realize 90 – 120 min with constant current 150 – 200 mA. Its major advantages are speed and completeness of transfer. This process uses an electric current to pull proteins from the gel onto the membrane. The effectiveness of protein transfer is dependent on the type of gel applied, the molecular mass of the protein, and the type of membrane. Visualization of protein sorption onto membranes: To check for success of transfer, wash the membrane in TBST. Staining with Ponceau Red: The stock is made of 2% Ponceau S in 30% trichloroacetic acid and 30% sulfosalicylic acid. Incubate on an agitator for 5 min. Wash extensively in water until the water is clear and the protein bands are well-defined. The membrane may be distained completely via repeated washing in TBST or water. When using a PolyVinylidene Difluoride (PVDF) membrane, reactivate the membrane with methanol then wash again in TBST. Blocking and Antibodies. To block nonspecific binding, the membrane is placed in a dilute solution of protein such as bovine serum albumin and nonfat dry milk. Blocking helps mask any potential nonspecific binding sites on the membrane. After blocking, the most popular method is to incubate the membrane with primary antibody, wash, reblock, and then incubate with secondary antibody and wash again. It is important to determine the optimal concentration of antibodies before running all the samples as optimization is a prime determinant of the sensitivity of the assay (Burnette 1981). The antibody concentration should be optimized to provide the best signal to noise ratio. Both monoclonal and polyclonal antibodies can be applies for western analyses, with advantages and disadvantages in using either type (MacPhee 2010). Detection the probes that are labeled and bound to the protein of interest need to be detected on the western blot. Chemiluminescent detection is applied most often and therefore, will be briefly described. Increased chemiluminescence (ECL) is a sensitive method and can be applied for relative quantitation of the protein of interest (Kurien and Scofield 2006 and MacPhee 2010). The primary antibody binds to the protein of interest and the secondary antibody linked to horseradish peroxidase, is applied to cleave a chemiluminescent agent. The reaction product produces luminescence, which is related to the amount of protein. Once exposures have been captured, blots can be washed in a buffer and then ‘‘stripped,’’ which involves removing bound antisera to enable reuse of the blot. Blots can then be

stored for future reprobing several more times. Moreover, subsequent reprobing can interfere with protein antigens, resulting in a decreased signal (Alegria Schaffer Lodge and Vattem 2009).

Relative quantification and analysis. The blot results can be analyzed via densitometry to measure the relative amount of a specific protein on the blot via comparing it with a control or specific time point. This quantification is necessary to compare samples. There are commercial software programs for image analysis of bands on film or membrane. Relative levels of protein expression can then be obtained via comparing ratios of intensities of a reference band (e.g. b-actin or glyceraldehyde-3-phosphate dehydrogenase [GAPDH]) or a band of known protein concentration. Relative optical density units can be plotted in a graph, and the appropriate statistical analysis can be performed on the samples that have been converted to optical density units (Jensen Wood and Keller Wood 2007).

3.8. Determination Of Protein Concentration

The protein concentrations of the samples to be loaded on a gel need to be determined. Quantification for total protein can be achieved via measuring samples at 280 nm on a spectrophotometer, but the buffer must not contain absorbing materials. When the buffer contains absorbing materials, the Bradford assay (Bradford 1976). Can be used where a standard curve is created to determine unknown sample concentrations.

3.9. Statistical Analysis

The statistical analysis performed with the Statistica software (GraphPad Prism, v.6.01). The obtained results were shown in the form of the mean and standard variations. A $P< 0.05$ was considered statistically significant, data sets that are significant at different levels: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

4. RESULTS

The results of western blot GFAP content shown the significant effect EEP for this cytoskeleton protein expression and astrocyte reactivity. Events of western blot the samples of primary astrocytes treated with EEP in a range of concentration 10 µg/ml - 100 µg/ml shown total increased of GFAP content in primary astrocyte cell culture. There was not direct depend on effect/dose for 24 hours later treatment with EEP. The small dose of EEP affected increased of expression GFAP contents about 22% to compare with control. On the opposite, the treatment EEP 25 µg/ml induced the increase of GFAP expression contents about 89% to compare with control [Figure 4.1]. At the same time, a treatment primary astrocyte cell culture with dose 100 µg/ml EEP during 24 hours lead to rising of the GFAP expression content on 67% to compare with control group. The concentration EEP 100µg/ml decrease level expression about 22% to compared with 25µg/ml EEP. The concentration 25µg/ml EEP+LPS 0.01µg/ml rising expression about 54% to compared with 25µg/ml EEP [Figure 4.1].

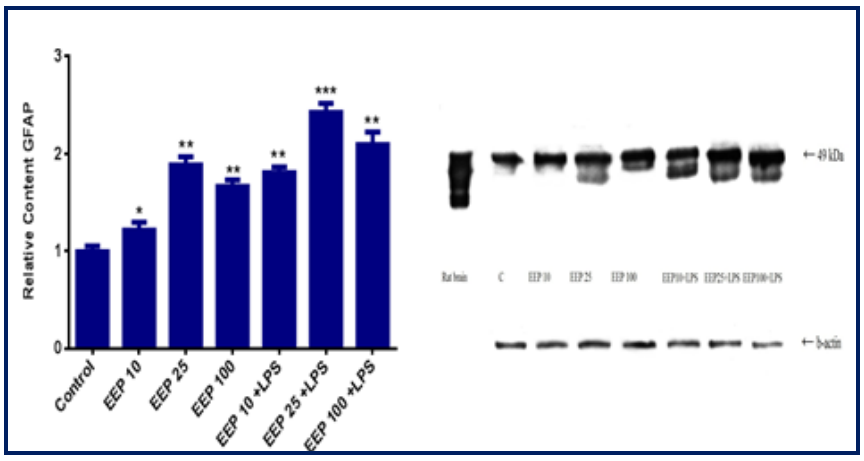

Figure 4.1. Relative content of GFAP in control and primary astrocyte cell Culture treated with EEP and EEP+LPS Significant differences * p<0.05, ** p<0.01, *** p<0.001

At the same time the treatment with concentration 100µg/ml EEP+LPS 0.01µg/ml lead to rising the level of GFAP more than two times compared with control.

The treatment with EEP+LPS induced similar results in all examined groups. The effects of EEP+LPS treatment on cleaved GFAP content for 10, 25 and 100 µg/ml treatments were 81%, 143% and 110% respectively [Figure 4.1]. These results shown that the treatment with 25mg concentration EEP produces more intense rising GFAP expression about 89% it may be cell atrophy and death. Obtained results may be related to indirect effects of bioactive EEP compounds on multifactorial pathways that modulate of generation cleaved GFAP It is understood that LPS activates astrocyte for generating proinflammatory factors and reactivation treatment primary astrocytes with EEP 10µg/ml stimulated not strongly pronounce rising of GFAP expression about 22% compared with EEP group it reverses the cytoskeleton to similar shapes. In addition, the results of western blot observed the samples of astrocytes treated with EEP in a range of concentration µg/ml 10-100 µg/ml shown total decreasing of cleaved caspase-3 (17 kDa), content in primary astrocyte culture.

However, there was not linear depend on effect/dose for 24 hours later treatment with EEP. The small dose of EEP treatment induced decreasing of cleaved caspase-3 contents about 29% to compare with control. On the contrary, the EEP treatment with 25µg/ml caused the decline of cleaved caspase-3 17 kDa contents about 22%. The same time a treatment primary astrocyte cell culture with dose 100 µg/ml during 24 hours lead to the dramatic decline of the cleaved caspase-3 17 kDa content on 79% to compare with the control group [Figure 4.2]. The treated of caspase-3 expression from primary astrocyte compared with GFAP expression. In all concentration caspase-3 decrease expression but the GFAP from all concentration EEP lead to increase expression. The treatment with EEP+LPS induced very similar results in the concentration EEP with and without LPS. The effects of EEP+LPS 0.01µg/ml treatment on cleaved caspase-3 (17kDa) content for 10, 25 and 100 µg/ml treatments were decreased 31%, 25% and 84% accordingly[Figure 7.2]. Observed results may be related to indirect effects of bioactive EEP compounds on multifactorial pathways that modulate of generation cleaved caspase-3 17 kDa. While the treated with concentration 100µg/ml EEP+ LPS0.01µg/ml caspase-3 compared with treated 100µg/ml EEP+LPS 0.01µg/ml for GFAP expression high decreased about 1.94

time than the GFAP expression in primary astrocyte ,it observed GFAP over expression at treated 100µg/ml EEP+LPS 0.01µg/ml [Figure 4.2].

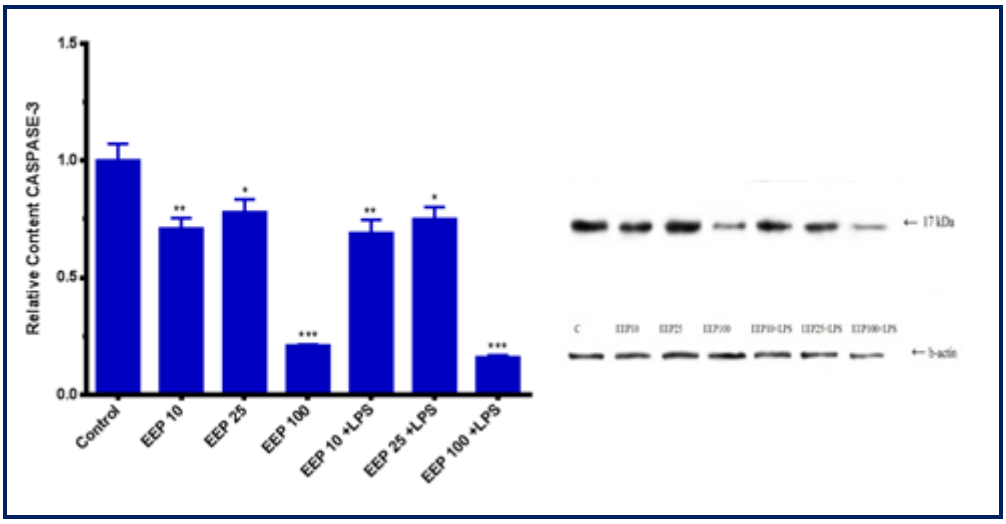

Figure 4.2. Relative content of caspase-3 in control and primary astrocyte cell Culture treated with EEP and EEP+LPS Significant differences * $p<0.05$, ** $p<0.01$, *** $p<0.001$

The effect of the treatment EEP concentration 10µg/ml caspase-3 compared with 10µg/ml EEP+LPS 0.01µg/ml in primary astrocyte was very similar related to decline of expression this apoptotic marker. The combined concentration of 100µg/ml EEP +LPS 0.01µg/ml more different for 100µg/ml EEP due to 100µg/ml EEP+LPS 0.01µg/ml dramatic decline to inhibition cell expression [Figure 4.2].

We observed non linear depend effect of EEP in our studies of astrocyte cell culture treated the concentration 100µg/ml EEP+LPS 0.01µg/ml the inhibition of cell growth. However, the concentration 100µg/ml EEP in GFAP more different compared with treated with 100µg/ml EEP in caspase-3 [Figure 4.2].

The results of western blot the samples of primary astrocytes treated with EEP in a range of concentration 10 µg/ml-100 µg/ml shown total increasing exactly 100 µg/ml EEP +LPS 0.01µg/ml decreased of cleaved BAX content in primary astrocyte cell culture.

Moreover, there was not linear depend effect/dose for 24 hours later treatment with EEP The small dose of EEP induced increasing of expression BAX contents about 76% to

compared with control [Figure 4.3]. On the contrary, the treatment EEP with 25µg/ml induced the rising of BAX contents about 27% compared with control [Figure 4.3]. The same time a treatment primary astrocyte cell culture with dose 100µg/ml EEP during 24 hours lead to expression of the BAX content on 52% to compare with control group. But at the same time effect of EEP treatment 25 µg/ml compared with EEP 10µg/ml the range of decrease 25µg/ml EEP 49% [Figure 4.3].

The combined treatment with EEP+LPS induced very similar results except in the studied 100 µg/ml groups. The effects of EEP+LPS treatment on BAX expression with 10, 25 and 100 µg/ml treatments were 62%, 32% and 66% compared with control [Figure 4.3]. Moreover, the treatment with 25µg/ml EEP low expression Bax to activated primary astrocyte cell culture.

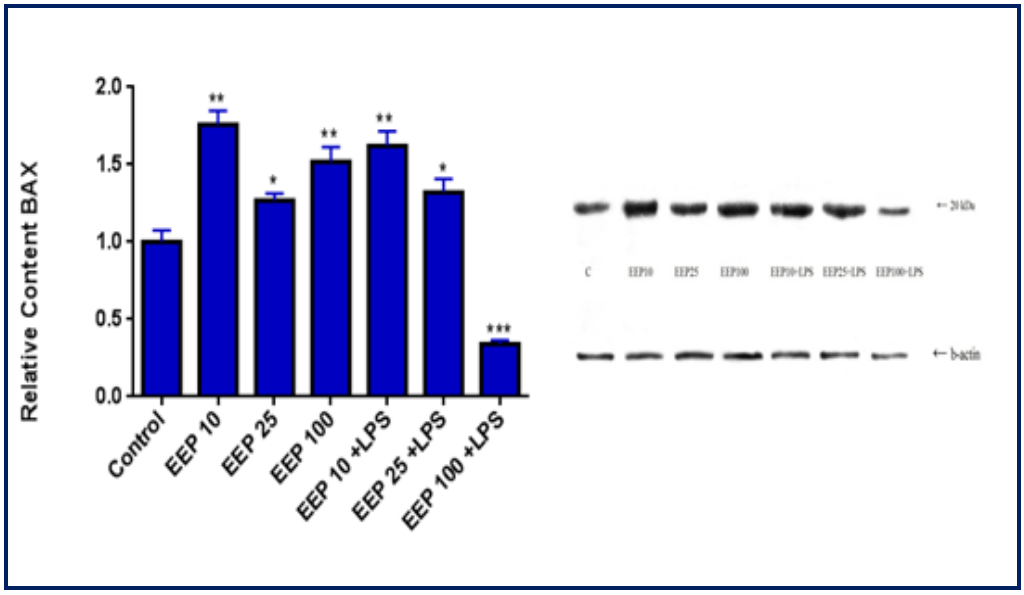

Figure 4.3. Relative content of Bax in control and primary astrocyte cell culture treated with EEP and EEP+LPS Significant differences * $p<0.05$, ** $p<0.01$, *** $p<0.001$

The treatment with EEP 100µg/ml+LPS 0.01µg/ml have a more effect of Bax expression, dramatically decrease 98%to compared with EEP 25 µg/ml+LPS. However, comparative analysis the modulation an expression of both GFAP and Bax treated by EEP+LPS shown some reciprocal dependence. The treated concentration EEP 100 µg/ml +LPS 0.01 increase expression GFAP 1.76 times than the Bax and the concentration EEP 10 µg/ml

of GFAP few rising 1.22 times to compared with control, EEP 10 µg/ml Bax 1.76 times compared with control [Figure 4.3].

The results of our studies shown nonlinear changes of the markers astrogliosis and apoptosis in primary astrocyte culture treated with EEP range concentration 10 – 100 µg/ml. These modulations of expression specific markers may be related with indirect effects of bioactive EEP100 µg/ml with LPS µg/ml compounds on multifactorial pathways that modulate of generation expression BAX.

The treated concentration 10µg/ml in duced rising of Bax expression in 76% times compared with control.it could be result of total activation metabolism with all compounds EEP. It can be activate and control inhibition cell metabolism. But the concentration 100µg/ml EEP+0.01µg/ml LPS compared with control dramatic dicrease Bax. While the concentration 100µg/ml EEP+0.01µg/ml LPS Bax compared with 100µg/ml EEP+0.01µg/ml LPS for GFAP high decrease 1.67 time than the GFAP expression. When Bax effect for cytochrome C location it can modulate ATP production under treatment with EEP range concentration of 25µg/ml and 100 µg/ml EEP lead to metabolic changes effect more protein expression [Figure 4.3].

GFAP can be one of them dependent on maitochndreal modulate ATP production, ATP decreasing lead to expression of protein production. Thus, in concentration EEP 100µg/ml+LPS Bax dramatical decrease only in treated concentration 100µg/ml EEP+LPS this lead to decrease of GFAP expression. GFAP cytoskleton protein for 24h very stable prorein. The expression of Bax was dramatically decreasing while this regulate effect. Apoptosis control BAX, also known as bcl-2-like protein 4, is a protein that in humans the encoded via the BAX gene. The results of western blot the samples of primary astrocytes activate by LPS in a range of concentration 0.01µg/ml LPS. The GFAP little rising compares with the control in primary astrocyte culture. However, there was not linear depend on effect/dose for 24 hours later treatment with LPS. The same dose of LPS induced increased of GFAP contents about 44% to compare with control. On the contrary, the treatment with LPS 0.01 µg/ml induced the few rising of BAX contents about 12%. The same time a treatment primary astrocyte culture with dose LPS 0.01 µg/ml during 24 hours lead to little effect caspase-3 content on 5% to

compare with control group [Figure 4.4]. Observed result for modulation content of caspase-3 in primary astrocytes treated with LPS show no effect for 0.01µg/ml LPS. Gram-negative bacteria and their endotoxins may be causal about the complicating agent in common severe diseases. Bacterial lipopolysaccharides (LPS) could potentially act human pathogens. With the various disorders caused via LPS, neurodegenerative diseases such as Parkinson's are reported and are of high interest. Despite the occur on LPS-induced neurodegeneration, the exact mechanism is unknown.

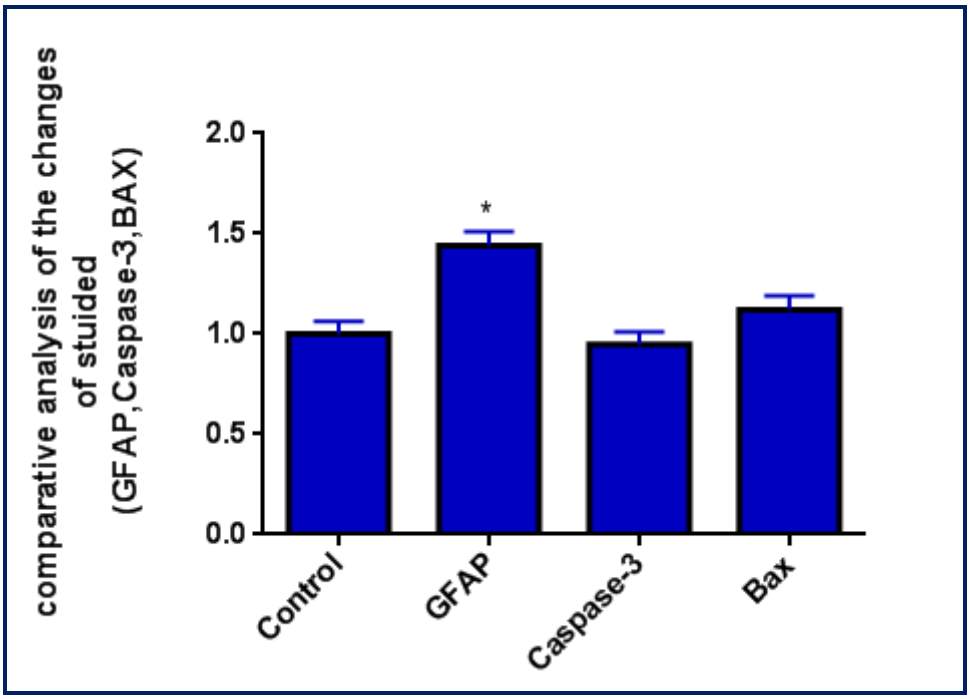

Figure 4.4. Comparative analysis of the changes of stuided GFAP, Caspase-3 and BAX treated with LPS Significant differences * $p<0.05$

LPS activated GFAP expression about 1.44 time more compared with unteatet cell [Figure 4.4]. This activation is only one way form multifactorial pathways affected with LPS for cell reactivity. The effects of LPS treatment on cleaved caspase-3 17 kDa content for µg/ml treatments were 5%, accordingly. Observed results may be related to indirect effects of LPS on multifactorial pathways that modulate of generation cleaved caspase-3 17 kDa.

5. DISCUSSION

GFAP is a key intermediate filament (IF) III protein responsible for the cytoskeleton formation of glial cells and for maintaining their mechanical strength while supporting neighboring neurons and the blood-brain barrier (BBB). GFAP is structurally similar to other non epithelial IF members (class III), containing vimentin, desmin, and peripherin, and has a head, rod and tail domains. Activated astrocytes take on the morphology of thickened and elongated processes and GFAP-through its involvement in the IF network-is critical to the preservation of such structure. GFAP gene activation and protein induction resemble to play a critical role in astroglial cell activation (astrogliosis) following CNS injuries and neurodegeneration. GFAP is including regulated both at the transcriptional and the post-translational levels, and how such regulations might impact on GFAP's healthy cytoskeletal functions and its involvement in maintaining the activated astroglial cell state (Yang and Wang 2015). Activated astrocytes expression various neurotrophic factors for neuronal survival, it is understood that rapid and harsh activation augments, initiates an inflammatory response, beginning with neuronal death and brain injury. GFAP raises at the periphery of ischemic injury after neurodegenerative insults. Though, the mechanism via the astroglial expression of GFAP is extended in neurodegenerative CNS (Brahmachari Fung and Pahan 2006).

Caspase-3 is a protein that interacts with caspase-8 and caspase-9. Caspase-3 have been identified in most kind mammals. The function of caspase-3 in the response of breast cancer cells to chemotherapeutic drugs remains a controversial issue. The loss of caspase-3 expression, as well as defaults in cytochrome C release from the mitochondria that is requested in most apoptotic pathways to activate caspase-3 within caspase-9 activation, are associated with multidrug resistance (Vegran Boidot Solary and Lizard Nacol 2011). Caspases are normally present in normal cells in inactive forms (zymogens) and know proteolytic processing during activation. The eleven caspases that have been recognized to date all exhibit the same basic structure – an N-terminal prodomain that is discarded

during activation followed via a large and a small subunit, both of which are required for proteolytic activity of the mature enzyme. Two large and two small subunits associate to form the actual tetrameric form of the enzyme which possesses two active sites (Rotonda et al 1996). Deposits within both the large and small subunits combine to form the substrate-binding pocket, describing the requirement for both subunits for enzymatic activity (Slee and Martin 1998) .The list of identified substrates of the caspase family is growing immediately (Martin and Green 1995 Nicholson and Thornberry 1997 Slee et al 1998). These substrates represent an eclectic group of proteins though most can be put into one of three classes; those that have a structural role, those that function as regulatory or signaling enzymes, and those that are included in DNA repair or gene expression. The consequences of these break events are still the subject of speculation at present, but it is likely that several of these directly result in some of the gross phenotypic as well as biochemical changes (such as plasma membrane blebbing, nuclear envelope collapse, nuclear fragmentation) that take place during apoptosis. For example, a recently characterized dimeric protein, DNA fragmentation factor (DFF Liu et al 1997). Which is a substrate of caspase-3 that when cleaved acts to initiate the internucleosomal degradation of DNA a classic hallmark of apoptosis. The murine homolog of this protein called caspase-activated deoxyribonuclease (CAD) has been cloned (Enari et al 1998).
Studies with CAD have conclusively demonstrated that this enzyme becomes activated during apoptosis as a consequence of caspase-mediated inactivation of its associated inhibitor (ICA). Thus evidence is growing to link specific caspase-mediated proteolytic events with the stereotypical destructive changes that have long been known to take place in the cell during apoptosis(Slee and Martin 1998).

The apoptotic factors are so as the apoptosis-inducing factor (AIF), second mitochondria-derived activator of caspase direct IAP binding protein with few PI (Smac/Diablo) and endonuclease G, that released from the mitochondrial intermembrane space to cytosol during that channel. This event is important for triggering the mitochondria-dependent apoptosis. Cytosolic cytochrome C makes a complex with the apoptotic protease activating factor 1 (Apaf)-1 and procaspase-9, resulting in activation of caspase-9, which then processes and stimulates caspase-3, initiating via that the biochemical execution of programmed cell death (PCD) (Benz 1994).

Bax is a pro-apoptotic fragment of the Bcl-2 protein family that remains in the outer mitochondrial membrane. It is controversial whether Bax increased cell death directly through its putative function as a channel protein versus indirectly via inhibiting cellular regulators of the cell death proteases (caspases). Here that interest of submicromolar amounts of recombinant Bax protein to isolated mitochondria can induce cytochrome C release, while a peptide representing the Bax BH3 domain was inactive. When placed into the purified cytosol, neither mitochondria nor Bax exclusively induced proteolytic processing and activation of caspases. In contrast, the combination of Bax and mitochondrial triggered the release of Cytocrome C from mitochondria and induced caspase activation in cytosols. Increase in Bax protein levels are produced in various clinically relevant distances where cell death occurs, including tumor cells as responses to chemotherapy and radiation, neurons developing cerebral ischemia (Jurgensmeier et al. 1998). The effects of Bax on cytochrome C release from mitochondria could be an incidental outgrowth of Bax induced activation of caspases that subsequently divide mitochondrial proteins and release cytochrome C from these organelles In the case of BAX insufficiency or Bcl-2 overexpression, an innovation of pro- or anti-apoptotic proteins has been reported to develop lymphocyte development.

BAX is a member of the Bcl-2. BCL2 family branches form hetero- or homodimers and act while anti- or pro-apoptotic regulators that are involved in a wide variation of cellular activities.This protein makes a heterodimer with BCL2 and functions as an apoptotic activator. This protein is reported to combine with and enhance the opening of, the mitochondrial voltage-dependent anion channel (VDAC), which leads to the loss of membrane potential and the release of cytochrome C. The expression of this gene is regulated by the tumor suppressor P53 and has been shown to be involved in P53-mediated apoptosis. Bax is an essential control of apoptosis that, under cell stress, aggregate at mitochondria, wherever it oligomerizes to mediate the permeabilization of the outer mitochondrial membrane leading to cytochrome c release including cell damage (Salvador Gallego et al. 2016).

The cytotoxic effect of LPS and also to examine the involvement of Bax, pro apoptotic, Bcl-2, anti-apoptotic, and caspase-3 (Sharifi Hoda and Noor 2010). In various cell types, apoptosis induced by LPS has been connected to the cytotoxic effect of iNOS-

derived NO. Because LPS effects the expression of iNOS in macrophages, we examined whether the production of NO also mediated the induction of apoptosis in bone marrow macrophages. The toxic effect of NO was observed via healing cell macrophages with the NO-donor SNAP55 that spontaneously produces NO after doing added to the culture (Xaus et al 2000). LPS including activated the three major MAP kinase cascades in macrophages, specifically ERK, p38, and JNK pathways. All three pathways must be linked to activation via LPS and following cytokine gene expression. As well as via LPS, the ERK pathway is activated via growth factors and differentiation signals. Activation of the p38 and JNK pathways is often linked to cell stress. Because LPS can activate different signal transduction pathways that result in the activation of MAP kinases, we decided to analyze the involvement of these MAP kinases in apoptosis induced by LPS in macrophages (Comalada et al. 2003).

That LPS activate proinflammation in defferent kind cells and especially in astrocytes. Obtained results may reflect the mechanisms of cell response that associated with rsing astrocyte reactivity and producing proinflammation factors into medium. Reactive astrogliosis is great established to be actively included in the entire pathological process of the PD condition. However, whether astrogliosis plays a beneficial or harmful role persists to be determined. While neurotrophic factors, so as glial-derived neurotrophic factor (GDNF), basic fibroblast growth factor, and brain-derived neurotrophic factor (BDNF), have been associated in certain activated astrocytes, a line of growing evidence has demonstrated that LPS or inflammation factors also trigger astrocytes to release the pro-inflammatory cytokines NO and ROS, that are detrimental to the adjacent neurons (Sun et al. 2016). Genomic analysis of an LPS mouse model revealed that the activated astrocytes present a detrimental phenotype. Consistently, we saw significant increases in the pro-inflammatory factors iNOS and ROS in the activated astrocytes the following stimulation with LPS in combination with other cytokines. Thus, the identification of new regulators that control inflammatory astrogliosis and the prevention of the progression of PD by the elimination of these activated astrocytes with detrimental properties may be essential (Sun et al 2016). These factors, especially ILs and TNF, can activate additionally with LPS cells into culture. Feedback between LPS and inflammative factors induce intensive growth of cell reactivation. In this condition many pathways can be modulated with intracellular signals that are results of LPS and its secondary messengers.

Treatments to response for astrocyte activate GFAP lead to caspase-3 few decline. The treatment with LPS induced similar results for modulation both GFAP and BAX expression studied groups. Following excluding the direct cytotoxicity of the LPS exposure of the primary astrocytes, we further studied the effects of mixtures of LPS and its affected products and TNF from microglial cells or lymphocytes in neuroinflammation events (Sun et al. 2016).

CONCLUSIONS

1. The ethanol extract propolis stimulate reactive astrogliosis in primary astrocyte cell culture with nonlinear dose/depend manner.

2. EEP with dose 100 µg/ml induce significant decreasing caspase-3 (17 kDa) generation in primary astrocyte cell culture compared with EEP 10µg/ml and EEP 25µg/ml dose.

3. EEP treatment leads to Bax overexpression with dose EEP 10µg/ml more than EEP 25µg/ml. This effect EEP may determinate the modulation apoptosis in primary astrocyte cell culture as well the producing proinflammation factors.

4. Treatment primary astrocytes with EEP dose 10µg/ml induce overxpression GFAP less in 1.5 times than the same dose EEP for astrocytes activated with (0.01) LPS.

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