T.C.
DOKUZ EYLÜL UNIVERSITY
IZMIR INTERNATIONAL BIOMEDICINE AND GENOME INSTITUTE

MICRORNA MEDIATED REGULATION OF NLRP3 INFLAMMASOME IN N9 MICROGLIA THROUGH AMPK-SIRT1 PATHWAY

BEDIR IREM ELTUTAN

DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

MASTER OF SCIENCE THESIS

IZMIR – 2020

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SUPERVISOR: PROF. DR. ŞERMİN GENÇ

Dokuz Eylül Üniversitesi İzmir Uluslararası Biyotıp ve Genom Enstitüsü Genom Bilimleri ve Moleküler Biyoteknoloji Anabilim Dalı,
Moleküler Biyoloji ve Genetik Yüksek Lisans programı öğrencisi Bedir Irem Eltutan
'MICRORNA MEDIATED REGULATION OF NLRP3 INFLAMMASOME IN N9 MICROGLIA THROUGH AMPK-SIRT1 PATHWAY'

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'MICRORNA MEDIATED REGULATION OF NLRP3 INFLAMMASOME IN N9 MICROGLIA THROUGH AMPK-SIRT1 PATHWAY'on the date of 27 / 01 / 2020

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LIST OF ABBREVIATIONS

LPS: Lipopolysaccharide

ATP: Adenosine triphosphate

AD: Alzheimer disease

PD: Parkinson disease

MS: Multiple Sclerosis

NLRP3: NOD-like receptor family, pyrin domain containing 3

RSV: Resveratrol

Sirt1: Siruin 1

AMPK: Adenosine Monophosphate Activated Protein Kinase

miRNAs: microRNAs

PRRs: Pattern recognizing receptors

RLRs: RIG-like receptors

TLRs: Toll-like receptors

NLRs: NOD-like receptors

CNS: Central nervous system

ALS: Amyotophic Lateral Sclerosis

SOD: Superoxide dismutase

CSF: Cerebrospinal fluid

Pre-miRNAs: Precursor miRNAs

RISC: RNA-induced silencing complex

JNK: c-Jun N-Terminal Kinase

SOCS1: Suppressor of cytokine signalling-1

MCP-1: Monocyte chemoattractant protein 1

CC: Compound C

NF-kB: Nuclear Factor kappa-B

ROS: Reactive oxygen species

FBS: Fetal bovine serum

GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase

DCFDA: 2 ', 7' dichlorofluorescindiacetate

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This thesis is dedicated to my unique mother, Sakin DENİZ ELTUTAN,

who always inspires me.

Bu tez bana her zaman ilham veren eşsiz annem,

Sakin DENİZ ELTUTAN'a adanmıştır.

ABSTRACT

One of the most pivotal parts of the natural immune system is NLRP3 inflammasome, which modulates pro-inflammatory cytokine responses. Its activation is underlying mechanism in inflammasome-mediated diseases. Therefore, its suppression is important in the treatment of diseases and to investigate the underlying pathogenesis.

Resveratrol is known to ability to suppressed NLRP3 inflammasome however it has not been clarified signalling mechanisms that used in its anti-inflammatory features. Studies have illustrated that resveratrol has the ability to control different regulatory pathways by altering the expression and consequently regulatory effects of non-coding RNA, that is microRNAs which are consisting of 22 recently identified nucleotides.

This thesis has suggested that resveratrol possess its anti-inflammatory features on NLRP3 inflammasome through AMPK-Sirt1 pathways by regulating microRNA. In our study, the effect of resveratrol on cell death and protection against reactive oxygen species has been demonstrated by LDH, WST, PI, DCFDA and MitoSOX assay. Resveratrol have been exhibited to activate Sirt1 and AMPK pathways with a increase in protein. Also, inhibition of these pathways has caused a notable increased in NLRP3, IL-1β mRNA and caspase-1 activity. Additionally, it has been shown that inhibition of AMPK-Sirt1 pathways significantly upregulated miR-155 expression while downregulated by resveratrol. Our results demonstrated that resveratrol suppresses the NLRP3 inflammasome through AMPK and Sirt1 pathways by regulating the miR-155.

As far as our knowledge, this thesis is representing the first one to establish the pathways and its regulatory mechanism that resveratrol possesses anti-inflammatory features on NLRP3 inflammasome in N9 microglia cell line.

ÖZET

NLRP3 inflamazomu, doğal bağışıklık sisteminin pro-inflamatuar sitokin yanıtlarını düzenleyen anahtar bileşenlerinden biridir. Aktivasyonu birçok inflamazom bağlantılı hastalığın altında yatan mekanizmalardandır. Bu nedenle baskılanması birçok hastalığın tedavisinde ve altında yatan patogenizin araştırılmasında önemlidir.

Resveratrolün NLRP3 inflamazomunu baskılayıcı etkisi bilinmesine rağmen anti-inflamatuar özelliklerini hangi sinyal yolakları aracılığı ile gösterdiği henüz açıklanmamıştır. Çalışmalar, resveratrolün birçok farklı yolu kontrol etme yeteneğine, kodlayıcı olmayan RNAlar olan 22 nukleotidden oluşan mikroRNAların ifadelerini düzenleyerek sahip olduğunu göstermiştir.

Bu tezde, resveratrolün anti-inflamatuar özelliklerini NLRP3 inflamazomu üzerinde AMPK ve Sirt1 yolaklarını mikroRNAlar aracılığı ile düzenleyerek gösterdiği önerilmiştir. Bu çalışmada resveratrolün hücre ölümüne etkisi LDH, WST ve PI teknikleri ile gösterilirken, reaktif oksijen bileşenlerine karşı koruyucu etkisi DCFDA ve MitoSOX yöntemleriyle belirtilmiştir. Farklı dozlarda resveratrolün AMPK ve Sirt1 yolaklarını aktive ettiği protein seviyesindeki anlamlı derecede artış ile gösterilmiştir. Dahası, bu yolakların inhibisyonunun resveratrolün anti-inflamatuar etkisinin NLRP3 bant yoğunluğu üzerinde geri döndürdüğü belirlenmiştir. Bunlara ek olarak yolakların inhibisyonunun, resveratrol ile önceden muamele edilen gruplara göre NLRP3 ve IL-1β seviyesini ve kaspaz-1 aktivitesini arttırdığı gösterilmiştir. Bu sonuçlar resveratrolün NLRP3 inflamazomunu baskılarken AMPK ve Sirt1 yolaklarını kullandığını göstermektedir. Ayrıca resveratrol ile ekspresyon seviyesi azalan miR-155'in AMPK ve Sirt1 yolaklarının inhibisyonu ile regulasyonunun arttığı da belirlenmiştir. Sonuçlarımızın resveratrolün NLRP3 inflamazomunu miR-155'i regüle ederek AMPK ve Sirt1 yolaklarına bağlı olarak baskıladığını göstermektedir.

Bildiğimiz kadarıyla, resveratrolün NLRP3 inflamazomunu baskılayan sinyal yolakları ve bunları regüle eden mekanizmaları N9 mikroglia hücrelerinde ilk olarak bu çalışmada gösterilmiştir.

1. INTRODUCTION AND AIM

1.1. Statement and importance of the problem

Inflammasome activation is a process which initiates inflammatory responses. Lipopolysaccharide (LPS) + Adenosine triphosphate (ATP) induction is used as a inflammasome model in microglial N9 cell line. Inflammasome activation not only leads to IL-1β and IL-18 cytokine release from cells and also conducts to activation of caspase-1. In the cell, activation of inflammasome furthermore conductive to pyroptotic cell death.

Microglial activation, which occurs in response to immunopathogens, brain injury, or neurotoxins, is the primary response in this defense mechanism of brain (Tang and Le 2016, Lee, Park et al. 2019). Anextend and unresolved inflammatory response leads to imbalances in microglial activation, which eventually originates chronic neuroinflammation. This process causes neuronal cell death and and the progression of neurodegenerative disorders for instance in Alzheimer disease (AD) nor Parkinson disease (PD) (Lee, Park et al. 2019).

Imbalance in microglial activation results in excess inflammation and contributes to neurodegenerative diseases including AD, PD, dementia and Multiple Sclerosis (MS) (Colonna and Butovsky 2017). Resveratrol (RSV) as one of the phytochemical that controls the activation of microglia by suppressing inflammasome and has protective properties in the progression of diseases.

1.2. Aim of the study

The aim of this study is to clarifies the signaling pathways which RSV has anti-inflammatory properties on suppressing NOD-like receptor family, NLRP3 inflammasome and the mechanisms of controlling these signaling pathways. Anti-inflammatory effects of RSV on NLRP3 inflammasome have been already demonstrated. The goal of this study is to explore the effects of Siruin 1 (Sirt1) and Adenosine Monophosphate Activated Protein Kinase (AMPK) pathways on RSV suppression of NLRP3 inflammasome and the effects of microRNAs (miRNAs) on regulation of these pathways.

1.3. Hypothesis of the study

RSV suppresses the NLRP3 inflammasome in murine microglia by regulating the Sirt1 and AMPK pathways via miRNAs.

2. GENERAL INFORMATION

2.1. Inflammasome

Inflammasome is one of the mechanisms that initiate inflammatory responses. Under normal circumstances, the immune system has the ability of recognize foreigners, pathogens, dead or dying cells, and and discriminate them from living and healthy cells. This feature of the immune system enable that the body does not produce an immune response by itself and possible damage to nearby tissues is prevented. Functions of the immune system that make up the primary response is to recognize and detect microbes, coordinate immune responses, and generate protection systems against pathogens and dying cells (Poon, Hulett et al. 2010). Cells or molecules that are foreign to the body are identified by the pattern recognizing receptors (PRRs) which are membrane-bound or cytoplasmic receptors of immune system cells. These receptors are RIG-like receptors (RLRs), C-type lectin receptors, Toll-like receptors (TLRs), purinergic receptors and NOD-like receptors (NLRs) (He, Hara et al. 2016, Próchnicki, Mangan et al. 2016). NLRs are found in the cytoplasm of cells, there are 23 different NLRs in human and 34 one in mouse (Kumar, Kawai et al. 2011). NLRs consist of three main regions. They are grouped into five main groups, namely NLRP, NLRC, NLRB, NLRX, and NLRA on the authority of the types of the N-terminus region (Kersse, Bertrand et al. 2011). The subtypes in each group are specialized to recognize different signals. In this way they able to recognize DAMP and PAMP molecular patterns and form inflammasome complexes (Bauernfeind, Rieger et al. 2012).

2.2. Inflammasome Activation in Microglia

Microglial activation, which occurs in response to immunopathogens, brain injury, or neurotoxins, is the primary response in this defense mechanism of brain (Tang and Le 2016, Lee, Park et al. 2019). Microglia are located in the central nervous system (CNS) as the innate immune cells that is responsible for homeostatic processes, cellular debris, and pathogens phagocytosis, also the release of chemokines and cytokines. (Walter, Kemmerling et al. 2017). Activated microglia can mediate neuroinflammatory responses by produce and secrete pro-inflammatory cytokines and mediators.

Microglial cells are cells of myeloid origin that has a critical part in the formation of a primary immune reply in the CNS and express TLR and NLR (Hanamsagar, Hanke et al. 2012). It is the first cell type shown to have activation of inflammasome in the CNS. It has been exhibited in

the in vivo and in vitro transgenic mouse Amyotophic Lateral Sclerosis (ALS) model that activation of inflammasome is take a part in the pathogenesis of another neurogenerative disease (Meissner, Molawi et al. 2010). In this microglial cell culture study, it was shown that the mutant superoxide dismutase (SOD) caused the activation of inflammasome which terminated with IL-1β release. The caspase-1 or IL-1β mutation slowed down the course of the disease in SOD defective mice (Meissner, Molawi et al. 2010). These findings advocated that suppression of inflammasome activation in ALS may be a new treatment option. Another important finding is that it has demonstrated in an animal study where chronic stress or glucocorticoid treatments cause an increase in hippocampal inflammatory markers (Frank, Hershman et al. 2014). In a similar finding, one of the depression models that chronic unpredictable stress model, was demostrated the activation of NLRP3 inflammasome in microglial cells without an increase in IL-1β in serum or cerebrospinal fluid (CSF) (Pan, Chen et al. 2014).

2.3. miRNAs

miRNAs are molecules that are composed of approximately 22 nucleotides in their mature form and are mediators of the post-transcription and translation level regulation mechanism (Großhans and Filipowicz 2008, Erson-Bensan 2014, Tüfekci, Meuwissen et al. 2014).In the cell cytoplasm they able to selectively reduce protein expression with binding to specific recognition sites in target mRNAs, by suppressing the initiation and progression of mRNA degradation or translation. miRNAs are first generated as primary transcripts (Pri-miRNA), these transcripts have one header and one polyA tail. Pri-miRNAs are cut by the Drosha ribonuclease III enzyme and are converted into stalk-loop-shaped, precursor miRNAs (Pre-miRNAs) which are 70 nucleotides in extent. The pre-miRNAs sustained to the cytoplasm via the exportins have interacted with the endonuclease Dicer to formed mature miRNA.

MiRNAs are RNA molecules that do not have single-stranded coding functions, consisting of 22 recently identified nucleotides. miRNAs control translation by binding to the 3'-UTR region in the target RNA molecule. The miRNAs are encoded by the gene of interest as primary miRNA and then converted into the pre-miRNA by the 'Drosha' enzyme in the nucleus. The pre-miRNA is then transported to the cytoplasm with the "Exportin 5" molecule and processed by the "Dicer" enzyme to form a mature miRNA molecule of 19-24 nucleotides (Perez, Pham et al. 2009, Tüfekci, Meuwissen et al. 2014). At that time the mature miRNA is transported to the target RNA molecule by the RNA-induced silencing complex (RISC) (Tüfekci, Meuwissen et al. 2014).

It has been known that miRNAs are count in numerous cellular and organism level functions for instance proliferation, cell viability and death, tumor formation, vascular formation, organ development, metabolism, circadian rhythms.

2.4. miRNAs and Inflammasome

miRNAs participate in noteworthy functions in cell proliferation and differentiation, signal transduction, apoptosis and organ development as well as in the development and regulation of the immune system. Because of these properties, miRNAs can also be used as therapeutic drug targets (Perez, Pham et al. 2009, Catalanotto, Cogoni et al. 2016).

miRNAs affect many biological processes such as induction and suppression in response to inflammatory stimulus and cause pro-inflammatory or anti-inflammatory effect. miRNAs count in the inflammation process are called "inflammir". The leading miRNAs which are count in the inflammation process are mir-146a and miR-155. In macrophage and dendritic cells, miR-155 expression is induced by Nuclear factor kabba-B (NF-kB) and c-Jun N-Terminal Kinase (JNK) pathways with inflammatory stimuli (O'Connell, Taganov et al. 2007, Tili, Michaille et al. 2007, Cardoso, Guedes et al. 2016). In addition, an increase in miR-155 expression and a decrease in miR-125 expression is observed with LPS stimulation in an in vivo model. Besides, suppressor of cytokine signalling-1 (SOCS1), which acts as an inhibitor in the inflammatory process, is regulated by miR-155 (Wang, Hou et al. 2010).

Although the roles of miRNA molecules in the innate immune system have been identified primarily in the TLR4 pathway, their effects on activation of inflammasome have recently been demonstrated. In one study, miR-223 was indicated to inhibit its expression of NLRP3 mRNA by binding to the 3'-UTR region (Bauernfeind, Rieger et al. 2012). It was also reported that miR-BART15 encoded by Epstein-Barr virus showed similar effect by binding to the same territory of NLRP3 mRNA (Haneklaus, Gerlic et al. 2012). Furthermore, in a recent study examining the regulatory effects of miRNAs on activation of inflammasome, miR-20a has been shown to regulate activation of inflammasome. It was shown that miR-20a expression decreased significantly in the synoviocytes obtained from rheumatoid arthritis patients in which pathogenesis was influenced by inflammasome activation correspond to the control group and it was found that NLRP3, caspase-1 and ASC protein ranges were suppressed by miR-20a overexpression (Li, Shen et al. 2016).

Regulation of inflammatory pathways are related with miRNAs role in the modulation of adaptive and innate immune responses. While miR-155 and miR-132 have extensive pro-

inflammatory properties, contradictory regulators of inflammation in cell classification are mostly miR-125b and miR-146a. The induction of miR-155 in the cerebellum have indicated in an in vivo study that TLR4-dependent however they could not find any relation with miR-155 and induction of mature-IL-1β (Lippai, Bala et al. 2013). Current studies have demostrated that miR-155 is due to activation of NLRP3 inflammation (Artlett, Sassi-Gaha et al. 2017). It has been established that overexpression of miR-155 causes to enhancement caspase-1, IL - 1β and IL - 18 levels, contrarly degradation of miR-155 reduces the cell death of HK - 2 cells causes by inflammasome (Wu, Chang et al. 2018).

Identification of regulatory molecules that able to modulate NLRP3 inflammasome will be effective to shed light on the mechanism of onset of inflammation in answer to activation, cellular stress, or oxidative damage, and to clarified beneficial prospects for new therapeutic solutions.

2.5. Resveratrol

RSV (3,5,4′-trihydroxy-trans-stilbene), a polyphenolic natural compound, has been found in a diverse range of food such as peanuts, soy beans, pomegranates, mulberries and especially abundant in the skin red of grapes (Lu, Ma et al. 2010, Thiel, Rössler et al. 2016). RSV has been the focus of research area since the French Paradox that suggests RSV-containing red wine has cardioprotective effect (Thiel, Rössler et al. 2016). RSV is widely-known because of its pharmacological benefits in life threaten disease such as cardiovascular and cancer preventive features (Thiel, Rössler et al. 2016). Studies have exhibited that RSV may have protective effects in the CNS alongside cardiovascular protection, especially in neurodegenerative diseases (Sui, Xie et al. 2016) .

It has been known that RSV has a property which allows it to proceed the blood brain barrier and interact with neuronal and glialcells. Besidesall, RSV is also known with its anti-oxidant, anti-cancer, anti-obesity, anti-aging and anti-inflammatory effects (He, Li et al. 2017) . Recently, the anti-inflammatory effects of RSV have been the focus of research in many studies. RSV posses anti-inflammatory activity by reducing the biosynthesis of pro-inflammatory mediators and provoking the expression of anti-inflammatory proteins.

A multi-protein complex, NLRP3 inflammasome, is which generally associated with control of IL-1β and IL-18 release and caspase-1 activity in the innate immune system (Chang, Ka et al. 2015). In vivo and in vitro studies have pointed that RSV reduced expression levels of NLRP3, ASC and also procaspase-1 and pro-IL-1β which are dependent on NLRP3 inflammasome (Fu,

Wang et al. 2013, Huang, Lai et al. 2014, Chang, Ka et al. 2015) Acummulating evidences suggests that NLRP3 inflammasome is activated by cellular oxidative stress. Since RSV has anti-oxidant activity, experimental studies have indicated that RSV acts as an inhibitor in NLRP3 inflammasome (Zhang, Wu et al. 2017). The suppressive effect of RSV on NLRP3 inflammasome has been a pioneer in the investigation of its protective effects on inflammasome-mediated diseases.

2.6. Resveratrol and miRNAs

Despite a series of studies investigating the signaling and transcription pathways of the protective effect of RSV on various topics, the mechanisms of pleiotropic effect are still not fully understood (Delmas, Solary et al. 2011). However, some recently published studies have found that RSV has the ability to control so many different regulatory pathways by altering the expression and consequently regulatory effects of non-coding RNA, that is miRNAs. The protective effects of RSV, which provide pleiotropic beneficial effects in many different areas, are associated with the ability to regulate endogenous miRNA populations (Michaille, Piurowski et al. 2018).

Up to date studies have clarified that RSV, which has anti-inflammatory properties, has the ability of the regulation both of the pro- and anti-inflammatory miRNAs expression (Tili and Michaille 2011). In various diseases, miRNA biosynthesis and expression levels can be affected by dietary polyphenols including RSV. Apoptosis, suppression of growth, cell viability inhibition, migration and diverse types of cancer have caused invasion by modulation of specific miRNAs by resveratrol (Kumar, Rimando et al. 2017).

The effects of RSV on endogenous miRNA populations were first demonstrated in a human cell line (SW480) which is used for colon cancer modelling. In this study, it was indicated that resveratrol reduced the levels of oncogenic miRNAs namely as miR-17, miR-21, miR-25, miR-92a-2, miR-103-1, and miR-103-2 (Tili, Michaille et al. 2010).

RSV is thought to proceed by manipulating miR-663 levels in malignancies associated with high miR-155 levels to enhance its anti-inflammatory and anti-tumor effects. Study in human monocytic cells, THP-1, have demostrated that RSV increases an anti-inflammatory miRNA, miR-663 expression which is also has tumor-suppressor feature and transcriptional activity of AP-1 decreases so reduces its up-regulation by LPS via targeting JunB and JunD transcripts. But also they shown that RSV weaken the up-regulation of miR-155 which is pro-inflammatory and oncogenic miRNA by LPS in a miR-663-dependent manner (Tili, Michaille et al. 2010).

Recent study in BV2 microglial cell line have indicated that after cerebral ischemia miR-155 expression was increased in injured brain, however RSV pretreatment have downregulated miR-155 level and promoted M2 microglia polarization. This study have proved that RSV improved neuroinflammation caused from LPS activation by inhibiting miR-155 (Ma, Fan et al. 2019).

In an another study, daily administration of RSV for six months gained the upregulation of miR-663, miR-181b as well as miR-21 and miR-30c and also provided diminish levels of inflammatory cytokines which includes IL-1β, chemokine (C-C motif) ligand 3 (CCL3), IL-6, and also tumor necrosis factor α (TNF-α). Inflammatory cytokine gene expression and also downregulation of inflammation controlled by regulative effects of TLR and NF-kB pathways and was associated with an increase in these miRNAs (Smoliga and Blanchard 2013).

2.7. AMPK and Sirt1 Pathway

Sirtuin 1, a NAD + dependent class III histone deacetylase, is a mammalian homologue of Sir2. The critical role of Sirt1 has been reported in various pathophysiological processes in example cell growth and metabolism modulation as well as anti-carcinogen and anti-inflammation (Howitz, Bitterman et al. 2003, Ford, Jiang et al. 2005, Kojima, Ohhashi et al. 2008).The Sirt1 gene is known to play a mediating role in the regulation of pro-inflammatory cytokines. Decrease or knockout of Sirt1 gene expression increases cytokine release, while activation of Sirt1 not only inhibits TNF-α, but also inhibits monocyte chemoattractant protein 1 (MCP-1) and in addition IL-8 production (Yeung, Hoberg et al. 2004, Yang, Wright et al. 2007, Rajendrasozhan, Yang et al. 2008).

RSV can suppress NLRP3 inflammasome by different mechanisms and pathways. The most commonly accepted mechanism is that RSV has its antioxidant and anti-aging effects by imitating the Sirtuin 1-mediated caloric restriction (Corpas, 2018). An early study has reported that RSV triggers the activation of Sirt1, which modulates and inactivates the acetylation of inflammatory proteins (Sui, Xie et al. 2016). Increasing evidences, have viewed RSV as a potent sirtuin1 activator, which alters and inhibits the acetylation of inflammatory proteins. The important fact about Sirt1 activation is its protective effects in neurodegeneration (Mancuso et al., 2014; Sui et al., 2016b). In most of the studies RSV inhibits NLRP3 inflammasome via enhancing autophagy by activating Sirt1. For example (He, Li et al. 2017) has showed that Sirt1 knockdown blockages the impact of RSV on autophagy triggering and NLRP3 inhibition. Furthermore (Qi et al., 2018) has reported that while Sirt1 decreasing in Alzheimer Disease model, RSV treatment increased Sirt1 which contributes improvement on neurodegeneration.

Apart from that, (Yang and Lim 2014) also has demonstrated that RSV balances metabolic processes with its feature to ability act as a Sirt1 activator which relating to NLRP3 inflammasome. RSV dramatically upregulate the hepatic expressions of Sirt1 in high-fat-diet model which were decreased before RSV treatement.

The heterotrimer AMPK consists of a catalytic and two regulatory fractional monetary units. It is a cellular stress sensor that is energized by upstream kinases by phosphorylation in reaction to diminish ATP nor intracellular calcium levels (Carling, Sanders et al. 2008, Steinberg and Kemp 2009). Several metabolic targets including cholesterol and protein synthesis as well as fatty acid and energy homeostasis upregulate by AMPK activation via phosphorylated α-subunit of Thr172 (Hardie and Hawley 2001, Xiao, Heath et al. 2007). AMPK is a common kinase that has been endogenously activated by AMP and ADP. In addition, AMPK is exogenously regulated by natural components, which are also used as antidiabetic drugs such as RSV (Zhang, Zhou et al. 2009, Hardie 2011, Price and Dussor 2013). It has been known as an energy sensor for many years (Hardie and Hawley 2001). AMPK acts as a regulatory mechanism in plasticity, neurodegeneration and also neuronal function (Melemedjian, Asiedu et al. 2011, Price and Dussor 2013).

AMPK activation which has been regulated by RSV administration, has been shown to alleviate trigeminal neuralgia by inhibiting glia activation and alleviating glia-mediated neuroinflammationin BV2 microglial cells (Yang, Hu et al. 2016). In an up to date research, it was concluded that RSV exerted a therapeutic effect by AMPK pathway activation in RAW 264.7 cells which were treated with LPS (Yi, Jeon et al. 2011).

Although recent studies have shown that RSV suppresses NLRP3 inflammasome using AMPK and Sirt1 pathways, it has not been previously reported that RSV uses these pathways to demonstrates the anti-inflammatory effects in N9 murine microglia cells. It is also unknown how RSV regulates these pathways. In this study, it was shown that RSV regulates the AMPK and Sirt1 pathways with miRNAs, namely miR-155, and suppresses the NLRP3 inflammasome in this way.

3. MATERIALS AND METHODOLOGY

3.1. Research type

This study is an *in vitro* study.

3.2. Time and place of the study

This study was proceed at İzmir Biomedicine and Genome Institute, between Nowember 2018 and December 2019.

3.3. Research material

N9 mouse microglial cell line, generously supplied from Dr. Paola Ricciardi-Castagnoli (Cellular Pharmacology Center, Milan, Italy) was used.

3.4. Variables of the research

LPS/ATP and RSV, Compound C (CC), Ex527 were the independent variables of the study. On the other hand, cell viability and death rates, reactive oxygen species (ROS) measurements, mRNA and protein levels and cell death type are the dependent variables in this study.

3.5. *In vitro* experiment model

In purpose to determined the effect of RSV on the inflammasome suppressor role in cell death, oxidative stress and NF-kB pathway; the cells were incubated with RSV (10 µM, 1 hour) then ultra-pure LPS (1000ng / ml, 4 hours), pursued by ATP usage (5 mM, 1 hour). Unless otherwise specified, our classical test model was carried out in this way.

To evaluate whether the effect of RSV on the activation of NLRP3 inflammasome in LPS+ATP inducted microglial cells is intercede by the AMPK and Sirt1 pathways; prior to classical experiment model N9 cells were incuvated with Compound C (20µM,1 hour) which is AMPK inhibitor and Sirt1 inhibitor Ex527 (20 µM, 1 hour).

3.6. Cell culture

N9 mouse microglial cell line, were cultured in a moistened incubator with conditions at 37°C and 5% CO^2–95% air environment. Cellsmaintained in RPMI 1640 completed with 2mM L-Glutamine, 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin.

3.7. Determination of cell death via LDH Assay

Microglial cells with 1 x 10^4 cells/well cell density were seeded in to 96-well plate and incubated 24 hours under 37°C and 5% CO2. In experiment day, cells were treated by LPS (1

μg/ml) for 4 h and followed by 1 h ATP (5 mM) before with and without 1 h RSV incubation. After treatment, cell-free supernatant collected and release of LDH obtained spectrophotometrically by Cytotoxicity Detection Kit LDH (Roche, Basel, Switzerland) as manufacturer's guideless. Absorbance of each well was calculated at 492 nm with at 630 nm, which is regarded wavelength, on a microplate reader. Cell cytotoxicity was expressed as the percentage of the maximum LDH activity.

3.8. Determination of cell viability with WST-8

Tetrazolium based colorimetric assay was used for the determination of cell viability, namely WST-8, Cell Proliferation Assay Kit, (Sigma Aldrich, St Louis, USA). N9 cells (1×10^4/well) were incubated in 96-well plates during overnight. After incubation, cells were treated for 4 h by LPS (1 μg/ml) and then 1 h with ATP (5 mM) before with and without 1 h RSV incubation. After that period, WST-8 solution in 10 μl by volume was supplied each well and cell were incubated for 2 h in a humidified incubator. Absorbance was achieved at 450 nm with refer to 630 nm wavelength by utilizing a microplate reader (Varioskan, Thermo Scientific, USA). Cell viability was presented as a percentage of the untreated cells.

3.9. Determination of cell death via PI staining

Cells with 3×10^5/well cell density were incubated in 48-well plates for overnight. After incubation classical experiment model was performed with and without RSV treatment. 15 min before the termination of experiment, 20 μl propidium iodide (PI) was adjected each well in the dark and incubated till end. Phase-contrast microscope was used to detect dead cells, which were marked by PI dye. Propidium iodide is not permeant to live cells, it allows to determine percentage of viable cells.

3.10. Determination of ROS by MitoSOX staining

This assay was performed both immunocytochemically andfluorometrically. For immunocytochemically procedure; cells were cultured in 48-well plates with 30,000 cell density and incubated overnight. The next day, a classical experimental model was applied. MitoSOX vials were first vortexed in 13 μl DMSO and dissolved. The concentration at this stage was 5 mM. The final concentration was diluted with PBS to 5 μM and added to the wells. Hoechst dye (1/1000 dilution was performed) was also dilution to a final concentration of 20 μM was utilized with PBS and applied to the wells. Cells were incubated with conditions at 25°C for 10 min and then images of cells were taken via Olympus IX-71, an inverted fluorescent microscope (Olympus, Japan). For fluorometrically procedure; murine microglial cells seeded

with 1 × 10^4/well cell density into 96-black-well plates and incubated 24 h. After incubation a classical experimental model was applied. Mitochondrial ROS production in N9 cells were measured using MitoSOX (Molecular Probes, Invitrogen, USA) staining (with an administration dose of 5 µM for 15 min at 37 °C). The MitoSOX fluorescence (Ex 530/Em 590) was measured using a Varioskan Flash (Thermo Scientific, USA) fluorescence plate reader.

3.11. Determination of ROS by DCFDA

Murine microglial cells with 1 × 10^4/well cell density were seeded into 96-black-well plates for overnight. In experiment day, RSV pre-treatment was performed. Prior to the LPS treatment, 2 ', 7' dichlorofluorescindiacetate (DCFDA), a fluorogenic dye, was incubated for 1 hours. After incubation, cells were treated with LPS for 23 hours and ATP for 1 hour. At the end of the experiment, the fluorescence intensity of each well in the plate was measured by 495 nm excitation and 532 nm emission fluorometrically by Varioskan Flash (Thermo Scientific, USA) fluorescence plate reader. Fluorescence values of the samples were normalized to control groups and analyzed quantitatively.

3.12. Signal pathway assays for anti-inflammorty effects of RSV on NLRP3 inflammasome

For Western Blotting cells were seeded into T75 cell culture flasks with 3 x 10^6 cells density and incubated overnight under 37 °C and 5% CO_2 incubation conditions. After incubation experimental treatment were performed. Next, cells were harvested via scrapper and total protein were isolated from cells. Cells were precipitated to obtain total protein, then pellet was dissolved with RIPA lysis solution containing protease andphosphatase inhibitor (Thermo Scientific, USA). Cells were vortexed for 15 minutes on ice. Then, protein was obtained by centrifugation at 12000 rpm along with 15 minutes and concentrations were evaluated by BCA protein analysis method.

An identical amount of proteins whih was calculated from the concentration, were separated by 8, 10, 12 or 15% SDS-PAGE gel and transferred to PVDF membrane. Membrane incubation in blocking buffer followed by transfer step. Membranes were incubated in blocking buffer which was BSA or milk with required percentages within TBST washing solution for 1 h. Further blocking, the membranes were treated with primary antibodies which were specified for the desired protein according to antibodies protocol at 4 ° C for overnight. Next, membranes were treated with the horseradish peroxidase (HRP)-conjugated secondary antibodies (mouse or rabbit) related to primer antibody. Followed by membranes were washed by using TBST

solution (4 x 15 min) and then prepared for imaging. Supersignal West Dura ECL reagent (Thermo Scientific, USA) based on chemiluminescence method was used for imaging and band densities were evaluated densitometrically with ImageJ.

3.12.1. Determination of NF-kB at protein level by Western Blotting

In order to clarification of NF-kB protein levels by Western Blotting our classical experimental model was applied for the examination of the effect of RSV on expression level of NF-kB protein. When the experiment was teminated, cells were collected from flasksvia cell scrapers by scrapping. Proteins were separated by nucleic and cytosolic using NEPER kit for protein isolation. For this experiment nucleic and cytosolic proteins loaded to gel seperately. NF-kB primaryantibodies was used. For normalization lamin was used for nucleic proteins and actin was used for cytosolic proteins as housekeeping protein.

3.12.2. Determination of AMPK and Sirt1 activation at protein level by Western Blotting

In order to demostrate AMPK and Sirt1 activation in protein level cells were applied with RSV (10μm, 20 μm, 50 μm) for 6h alone. After the termination of the experiment total protein was isolated and Western Blotting method was performed. For AMPK activation membranes first incubated with phospho (p)-AMPK and then AMPK primary antibody.

3.12.3. Determination of AMPK and Sirt1 inhibition effect on NLRP3 inflammasome at protein level by Western Blotting

In order to clarified anti-inflammatory properties of RSV on NLRP3 inflammasome mediated with AMPK and Sirt1 pathways, prior to classical experiment model cells were treated with AMPK and Sirt1 inhibitors. After the experiment, total protein was isolated and Western Blotting method was performed. For that purpose, NLRP3 primary antibody was used.

3.13. Determination of AMPK and Sirt1 inhibition effect on NLRP3 inflammasome at gene level by quantitative RT-PCR

For quantitative RT-PCR, N9 microglial cells were seeded into T25 cell culture flasks (1 x 10^6 cells/flask) and maintained overnight under 37 °C and 5% CO_2 incubation conditions. Followed by the incubation for overnight, cells were treated with Compound C and Ex527 then our classical experiment model was applied.

After the experiment, cells were collected by scrapping. For Total RNA extraction from cells zymogen RNA and microRNA purification kit (Thermo-Fisher Scientific) were used following manufacturer's protocol. The purity and also concentration of the collected RNA samples were evaluated by spectrophotometric measurement with Nanodrop. High Capacity RNA-to-cDNA Reverse Transcription Kit (Applied Biosystems, Thermo, USA) was utilized to conducted reverse transcription. Quantitative real-time PCR was executed in LightCycler 480 Instrument II (Roche Life Science, USA) with the usage of GoTaq qPCR Mastermix (Promega, USA) in directed manufacturer's protocol. The primers used in the qPCR reactions are listed in Table 1. Melting curve analysis were used for the examinatiın of the specificity of PCR products. Quantifications of the mRNA's relative expression level with endogenous normalization to the median amounts of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) which is housekeeping gene were performed by using the $2^{-(\Delta\Delta Ct)}$ method.

Table 1. List of mRNA primers

IL-1β	F	5'-TTCTTTTCCTTCATCTTTGAAGAAG-3'
	R	5'-TCCATCTTCTTCTTTGGGTATTGTT-3'
NLRP3	F	5'-TGCCTGTTCTTCCAGACTGGTGA-3'
	R	5'-CACAGCACCCTCATGCCCGG-3'
GAPDH	F	5'-ACCACAGTCCATGCCATCAC-3'
	R	5'-TCCACCACCCTGTTGCTGTA-3'

3.14. Determination of AMPK and Sirt1 inhibition effect on NLRP3 inflammasome at caspase-1 by caspase-1 activity assay

In order to examinate Caspase-1 activity, murine microglial cells (1×10^4/well) were incubated for 24 h in 96-well plate. After overnight incubation, in vitro experimental model was applied prior to that cells were incubated with specific AMPK and Sirt1 inhibitors. In the end of the treatment, supernatant of the samples was taken into 96-white-well plate and Caspase-1 activitydetermined via luminometric Caspase-Glo-1 Inflammasome Assay (Promega, USA) as described by the manufacturer. The measure experimental samples Centro XS3 lb 960 named microplate luminometer (Berthold Technologies, Germany) was used.

3.15. Determination of miR-155 expression levels by quantitative RT-PCR

To perform RT-PCR, N9 microglial cells were seeded into T25 cell culture flasks (1 x 10^6 cells/flask). Cells were maintained overnight under 37 °C and 5% CO_2 incubation conditions. After incubation, our classical experiment model was realized with and without AMPK and Sirt1 inhibitors. Then, cells were collected via scrappers. In accordance with the protocol of miRNeasy kit (Qiagen) using TRIzol reagents isolation of total RNA was conducted. cDNAs were synthesized with usage of the miScript II RT Kit (Qiagen). For real-time PCR, PCR was followed through with primers and control RNA primers specific for miR-155.

3.16. Statistical analysis

Mann-Whitney U test was used for intergroup analysis. P values which were less than 0.05 ($p < 0.05$) noted as numerically significant.

3.17. Limitations of the Study

The first limitation of our study is that the study was only performed on the N9 microglial cell line. The results of the study should also be repeated in an in vivo model. Another limitation of our study is that we only investigated the miR-155. The study should have included other inflammatory miRNAs.

4. RESULTS

4.1. Determination of cell death via LDH Assay

The effect of RSV pre-treatment on cell death by administration of LPS + ATP was determined via LDH assay. According to the results, the cell death ratio of LPS + ATP group was 29.36 ± 4.1% and RSV pre-treatment group was 11.65 ± 3.30%. As seen in the Figure 1. RSV significantly suppressed LPS + ATP-induced pyroptotic cell death. (** p = 0.0079; ## p= 0.0079)

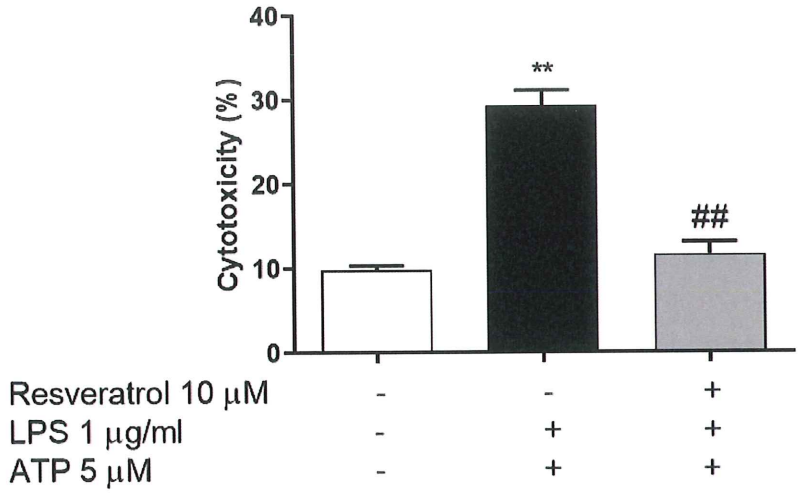

Figure 1: Determination of RSV protective effect on cell death via LDH. *LPS and ATP activated inflammasome increased pyroptotic cell death and RSV pre-incubation decreased pyroptotic cell death. Results were given as mean ± SD.*

4.2. Determination of cell viability with WST-8

The effect of RSV on cell viability was conducted by WST-8 cell viability test. Cell viability was 37.55 ± 5.7% in the LPS + ATP group and 74.05 ± 2.16% in the RSV pre-treatment group. As shown in Figure 2, RSV significantly increased the cell viability in microglia which was decreased with LPS + ATP.(** p = 0.0079; ## p = 0.0079)

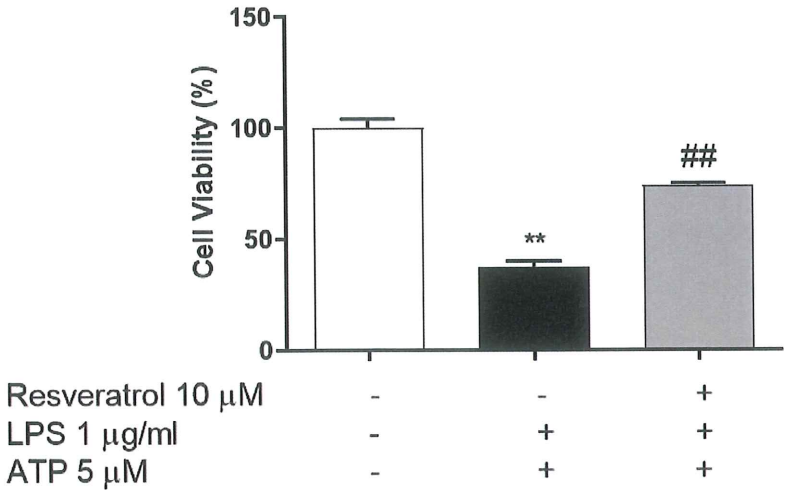

Figure 2: Determination of RSV protective effect on cell viability via WST-8. *LPS and ATP activated inflammasome decreased microglial cell viability and RSV pre-incubation increased cell viability. Results were given as mean ± SD.*

4.3. Determination of cell death via PI Staining

The effect of RSV on pyroptotic cell death was demonstrated by Propidium Iodide (PI) staining. Microglial cells were stained with PI dye after applied classical experiment model. Then images were taken via fluorescence microscope. The cells which were stained red show the cells that died in the pyroptotic way. According to the results, the percentage of PI positive cells in the group incubated with LPS and ATP was 74.29 ± 5.58. On the other hand, there was a dramatically reduce in the RSV pre-treatment group with the values of 40.84 ± 4.28 which was shown in Figure 3. (** $p = 0.0079$; ## $p = 0.0079$)

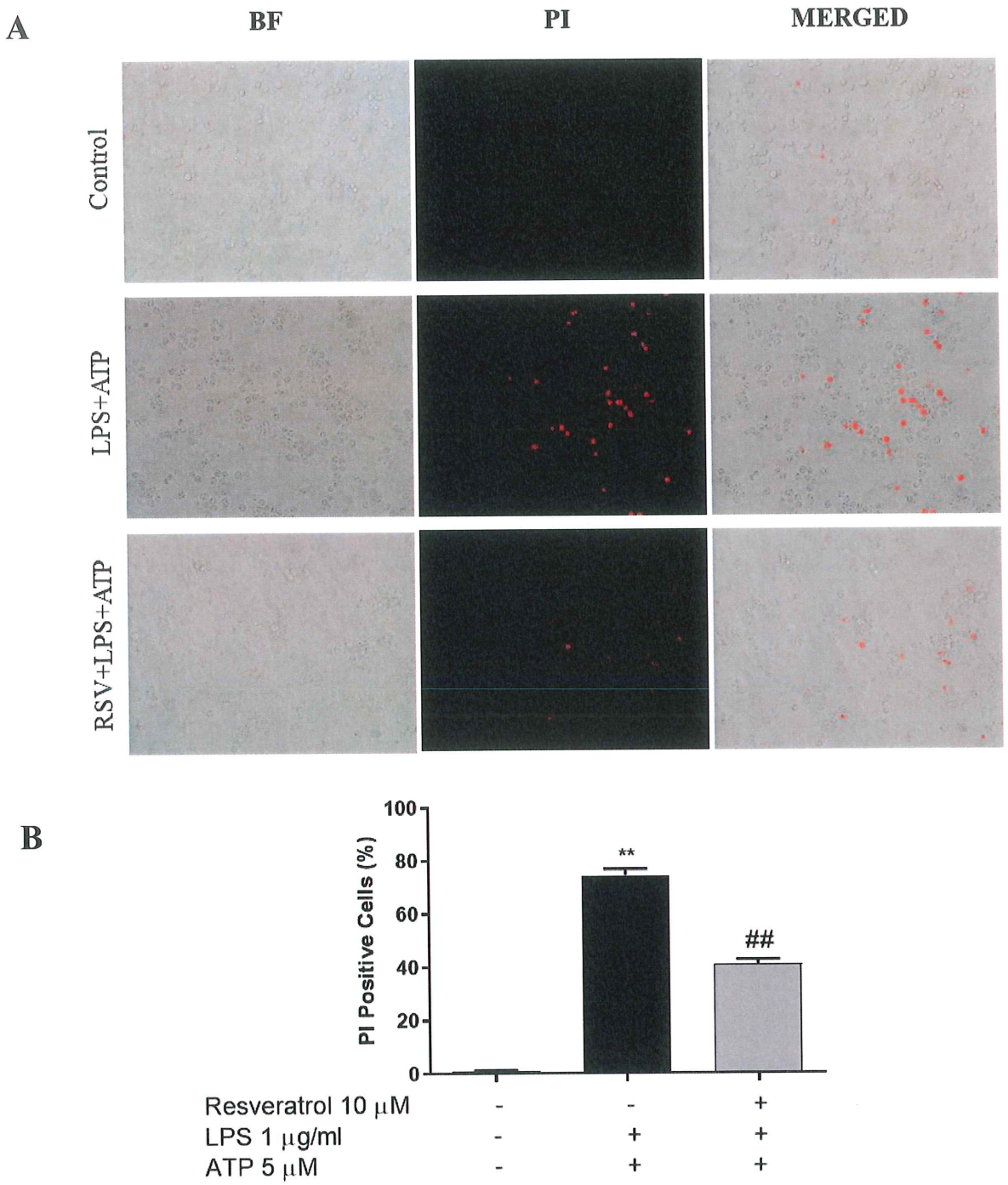

Figure 3: Determination of pyroptotic death in NLRP3 Inflammasome model. *According to PI staining results, LPS and ATP activated inflammasome dependent cell death, RSV pre-incubation decreased pyroptotic cell death. Results were given as mean ± SD.*

4.4. Determination of ROS by MitoSOX staining

The mitochondrial superoxide level, which is thought to increase in the cell by LPS + ATP applied to microglial cells, was shown by MitoSOX immunofluorescence staining in Figure 4. MitoSOX staining images under fluorescence microscopy of the microglia cells in LPS and ATP treated cells showed an increase in red color which was indicate ROS and a statistically significant decrease in RSV+LPS+ATP group. In LPS+ATP treated group ROS level was significantly increased as mean with standard deviation 22.08 ± 4.76 meanwhile it was significantly decreased in RSV pre-treated group 6.62 ± 2.77.(** $p = 0.0079$; ## $p = 0.0079$)

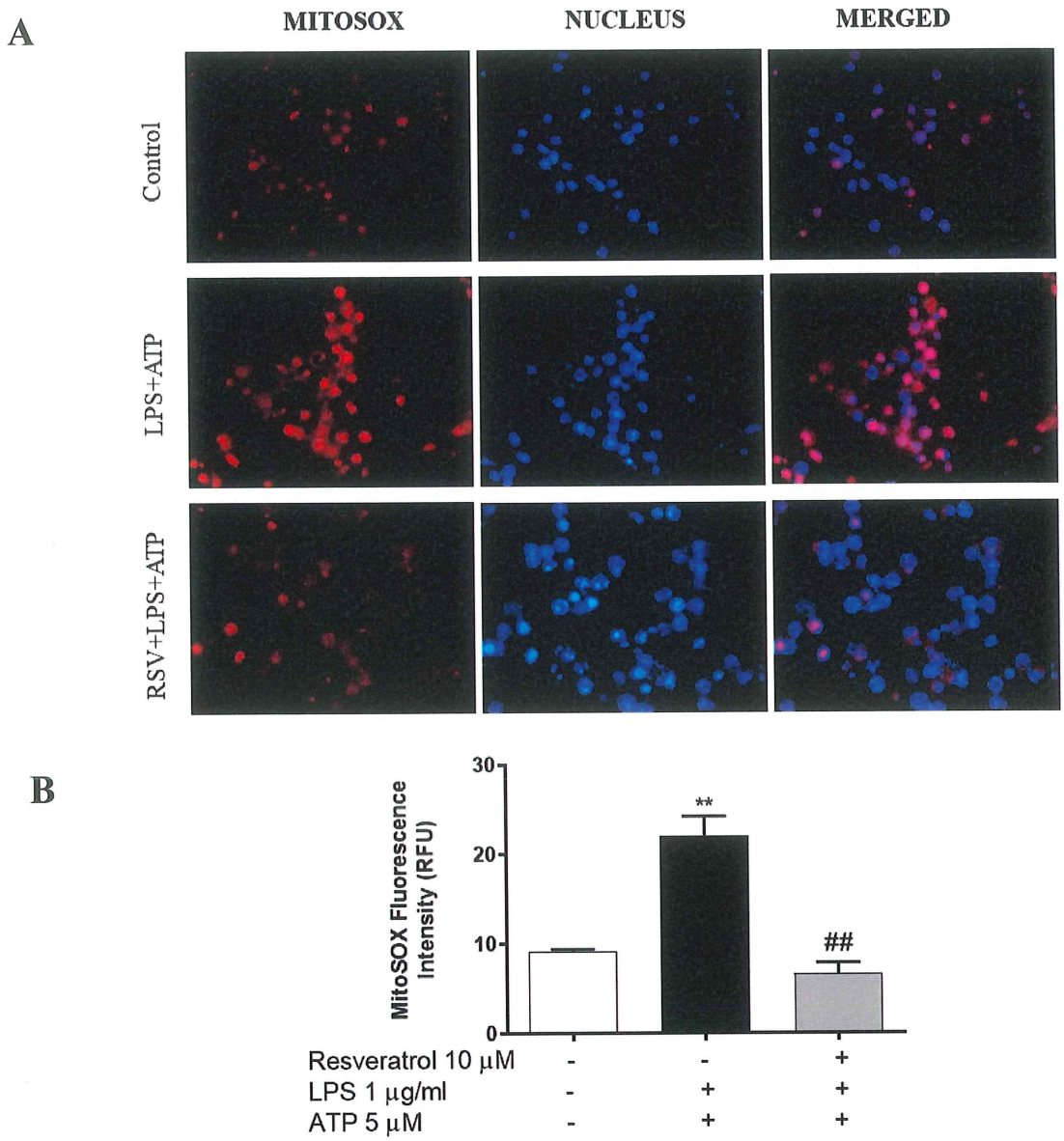

Figure 4: Determination of mitochondrial ROS in NLRP3 inflammasome model. *RSV significantly decreased mitochondrial ROS production compared to the LPS + ATP-activated inflammasome group. Results were given as mean ± SD.*

4.5. Determination of ROS by DCFDA

Cellular ROS level in microglial cells after LPS+ATP treatment was determined with use of permeant reagent DCFDA. As seen in the Figure 5. cellular ROS level in RSV pre-treated group was observed to decrease with 147.02±14.54 standard deviation from 199.38±26.39 which was increased in LPS+ATP treated group.(* $p = 0.0159$; # $p = 0.0286$)

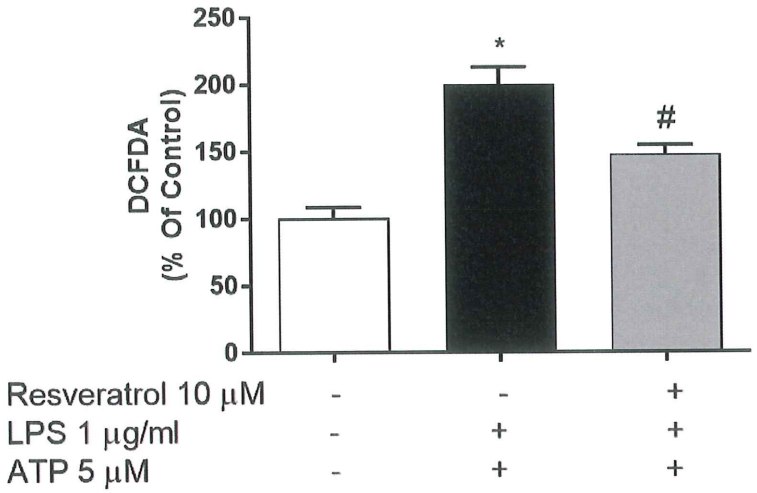

Figure 5: Determination of cellular ROS in NLRP3 inflammasome model. *RSV significantly decreased cellular ROS production compared to the LPS + ATP-activated inflammasome group. Results were given as mean ± SD.*

4.6. Determination of NF-kB at protein level

The effect of RSV on nuclear translocation of NF-kB protein was investigated by immunoblot method. LPS+ATP induction was increased band intensity of NF-kB protein as mean with standart deviation 0.78 ± 0.098 compared to the control group. However, as seen in Figure 6A. RSV pre-treatment decreased this increased band intensity as mean with standart deviation 0.56 ± 0.027. Figure 6B. showed that LPS+ATP treatment significantly increased the NF-kB protein level in nucleus and RSV pre-treatment significantly reduced this increase. (** p= 0.079, ## p=0.079)

Meanwhile, as seen in Figure 6C. NF-kB protein level decreased in cytosolic samples after LPS+ATP treatment with mean value 0.83 ± 0.05. In addition RSV treatment then reversed the reduction and increased NF-kB protein level in cytosolic samples with 1.15 ± 0.18 mean standart deviation. (** p= 0.079, ## p=0.079)

According to this results which was NF-kB translocation and showed in Figure 6D., LPS+ATP treatment significantly increased the ratio of nuclear and cytoplosmic samples at protein level and RSV treatment decreased this ratio. (** p= 0.079, ## p=0.079)

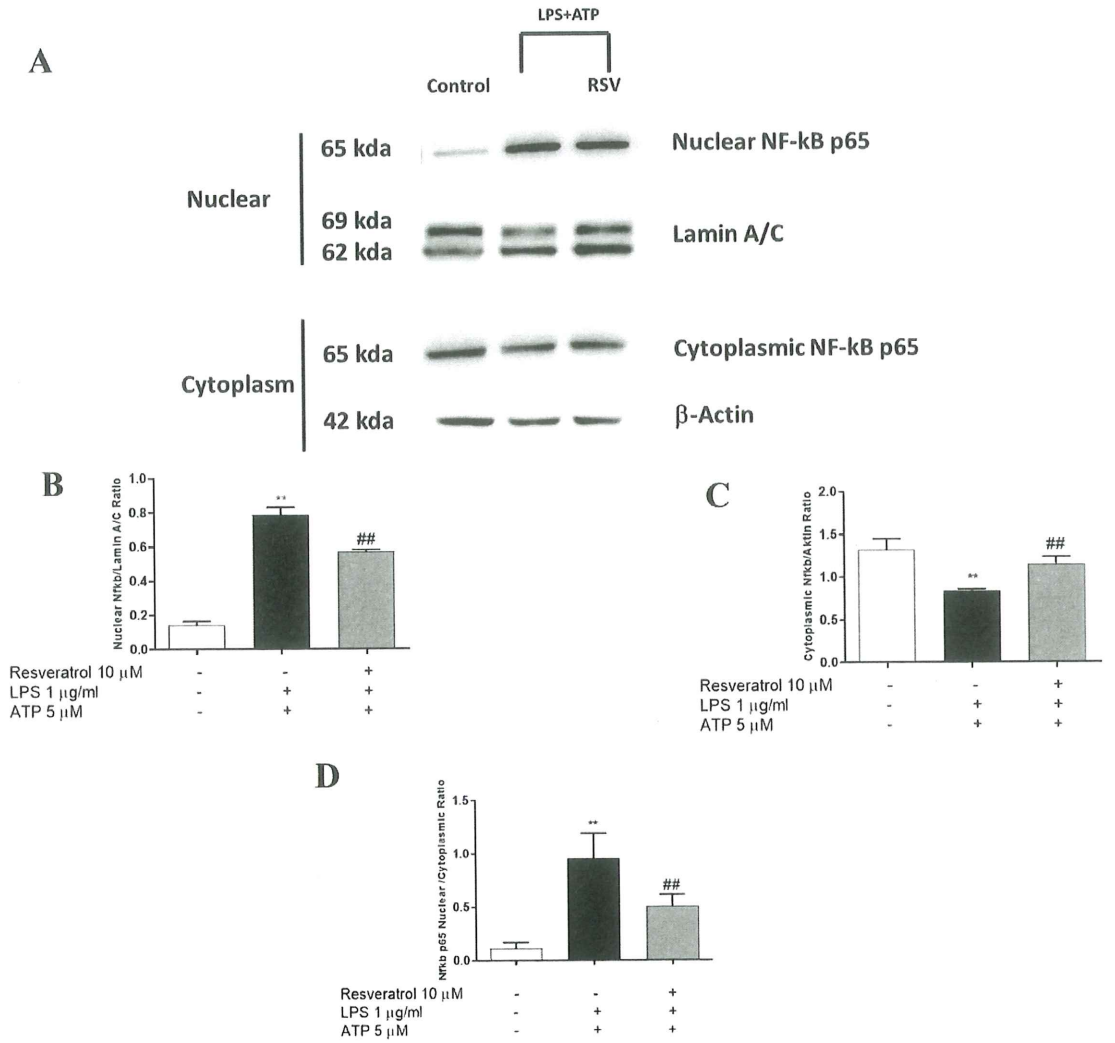

Figure 6: Determination of NF-kB via immunoblotting. *Nuclear and cytoplasmic NF-kB protein band intesities (A) Nuclear NF-kB (B) Cytoplasmic NF-kB (C) Nuclear/Cytoplasmic NF-Kb protein ratio (D)*

4.7. Determination of AMPK and Sirt1 activation at protein level

Immunoblotting was used to determine whether different doses of RSV (10 μM,20 μM,50 μM) activated AMPK pathway. As it was shown in Figure 7A. all of the RSV doses increased the band intensity of phospho AMPK. As seen in Figure 7B. phospho AMPK band intensity increased significantly with RSV pre-treatment with dose dependent manner respectively; 1.92±0.30, 2.02±0.57, 2.11±0.96 mean and standard deviation. (* p=0.037, ** p=0.0079, * p=0.0159)

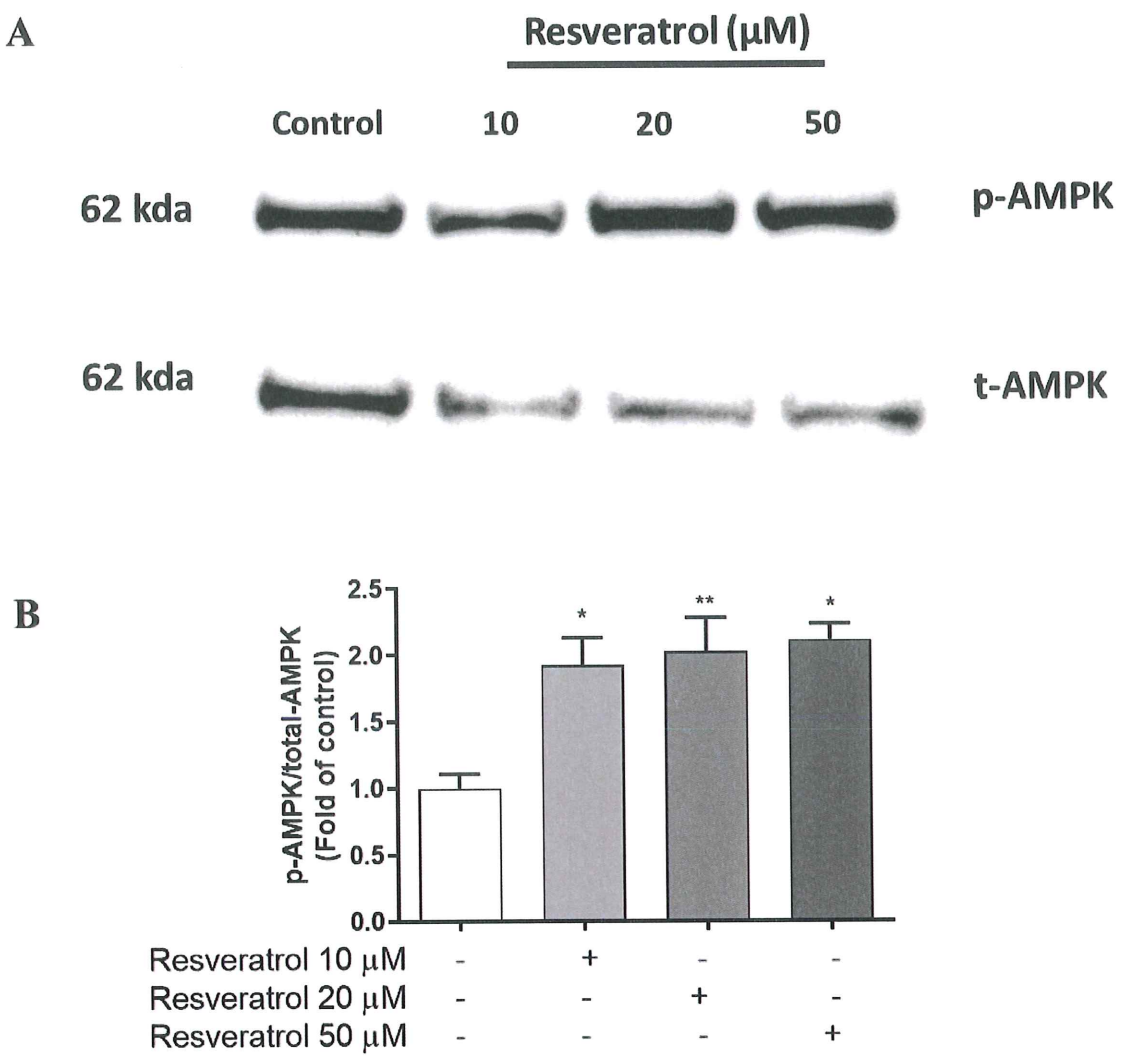

Figure 7: Determination of AMPK activation via Western Blotting. *Different doses of RSV increased phospho AMPK at protein level. Results were given as mean ±SD.*

Immunoblotting was used to determine whether different doses of RSV (10µM,20µM,50µM) activated Sirt1 pathway. As it was shown in Figure 8A. all of the RSV doses increased the band intensity of Sirt1 protein compared to the control group. As seen in Figure 8B.Sirt1 protein band intensity increased significantly with RSV pre-treatment with dose dependent manner respectively; 1.16±0.31, 1.21±0.37, 1.23±0.46 standard deviation. (* p=0.0480, ** p = 0.0047, * p=0.0480)

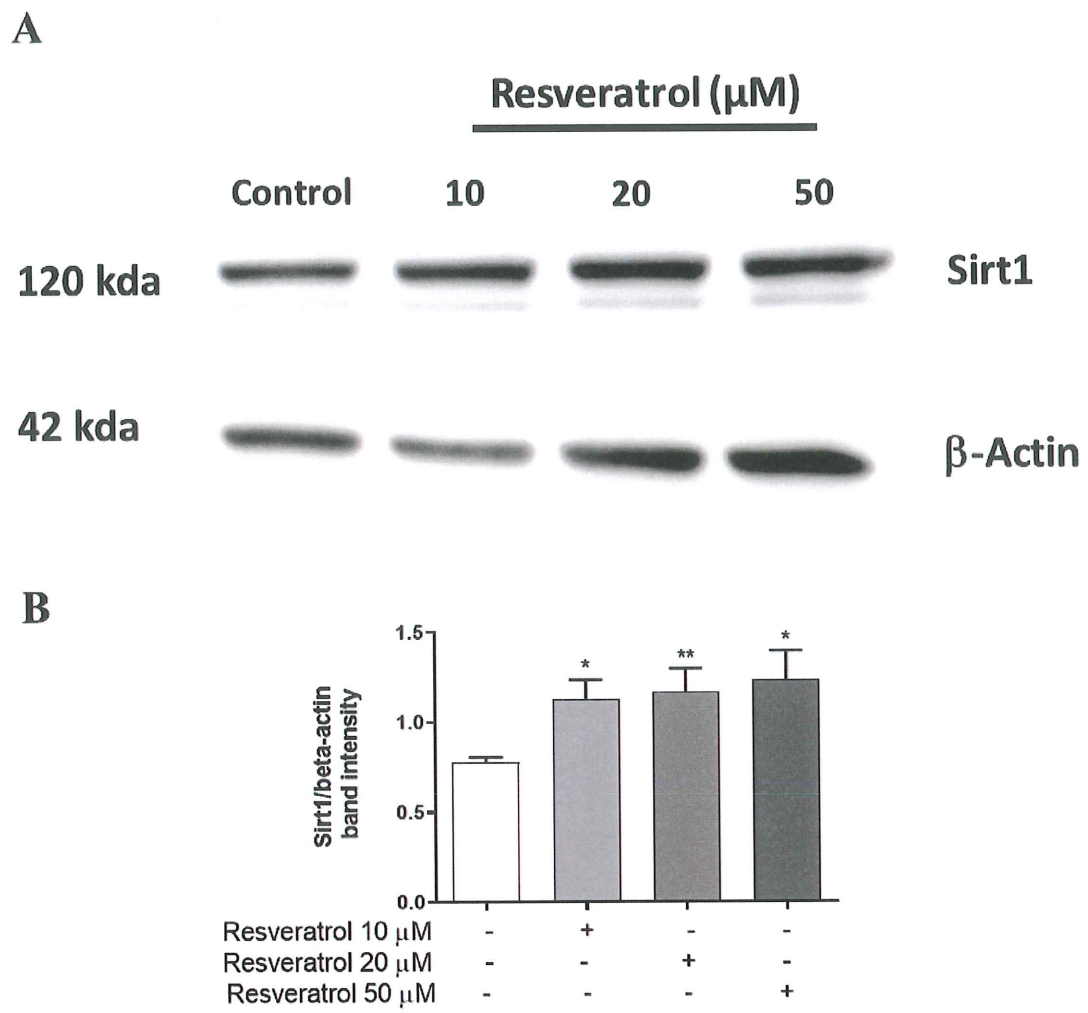

Figure 8: Determination of Sirt1 activation via Western Blotting. *Different doses of RSV significantly increased Sirt1 protein level. Results were given as mean ±SD.*

4.8. Determination of AMPK and Sirt1 inhibition on NLRP3 inflammasome at protein level

In order to determine whether RSV uses AMPK and Sirt1 pathways in it's anti-inflammatory effects, the level of NLRP3 protein, which is a critical marker of inflammasome, was evaluated using inhibitors of these pathways. Figure 9A. visualized and Figure 9B. demostrated that LPS+ATP treatment increased the NLRP3 protein level as mean with standard deviation 2.14 ± 1.08 while RSV pre-treatment significantly decreased this level to 0.74 ± 0.27 mean value. In

addition inhibition of the pathways reverses the decrease, which demonstrated in RSV pre-treatment group, in significant manner. Pre-treatment with CC increased the NLRP3 protein level into 1.49±0.76 as well as Ex-527 pretreatment 1.85±0.71. (** p = 0.0079, # p = 0.0159, $ p = 0.0317, $ p = 0.0317)

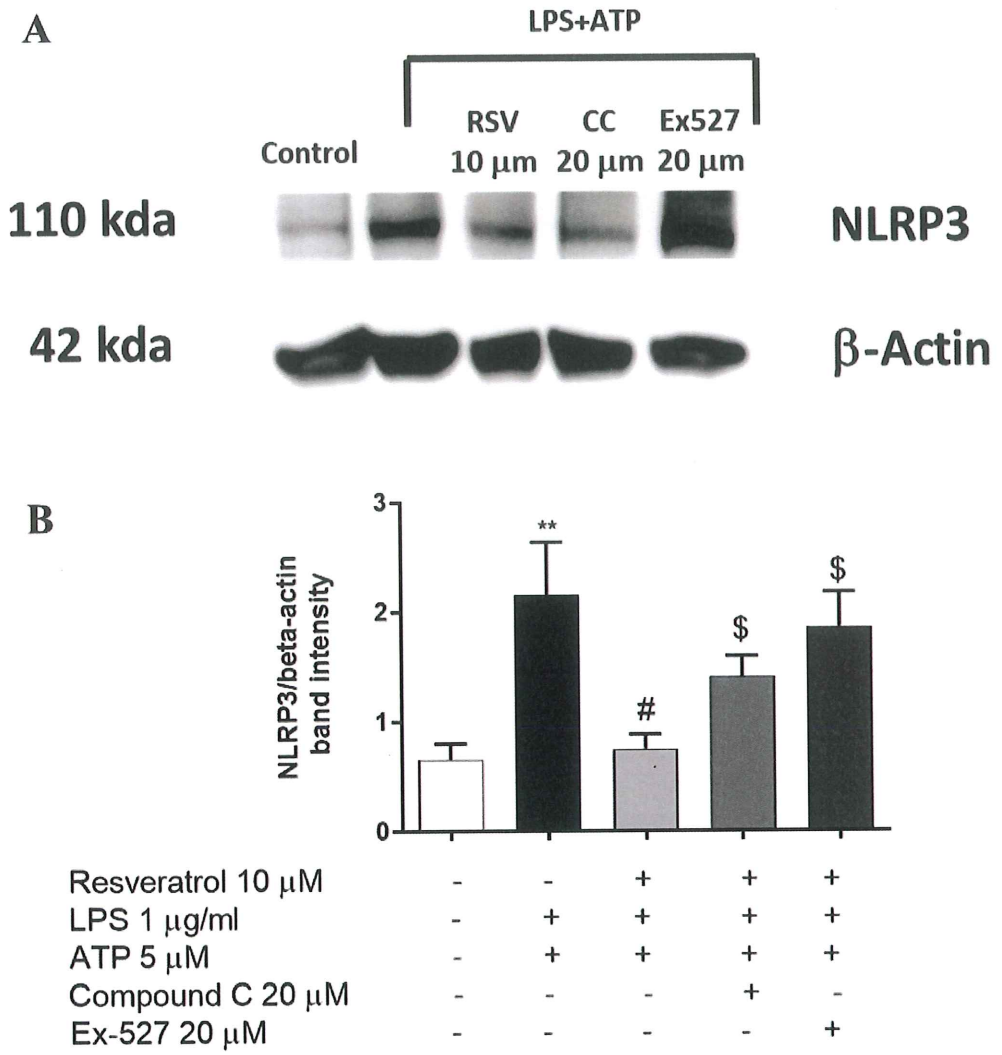

Figure 9: Determination of NLRP3 via immunoblotting. *NLRP3 protein level was determined to decrease with RSV pre-incubation, after LPS+ATP treatment. CC and Ex-527 pre-treatment significantly increased the NLRP3 protein. Results were given as mean ±SD.*

4.9. Determination of AMPK and Sirt1 inhibition on NLRP3 and IL-1β expression level

The effect of RSV on genes IL-1β and NLRP3 that identifier of the inflammasome, mRNA expression levels in LPS + ATP-induced microglial cells was examined with and without using AMPK and Sirt1 inhibitors.

According to the results, in the LPS + ATP group, IL-1β mRNA fold change was 6.95 ± 2.99, whereas RSV pre-incubation reduced this rate to 2.91 ± 1.19 value. As seen in Figure 10A. CC pre-treatment reversed this reduction and increased the IL-1β mRNA fold change 7.17 ± 2.2 mean deviation. As well as Ex-527 pre-treatment increased this mean into 8.25 ± 4.91. (* p = 0.0357, # p = 0.0286, $ p = 0.0159, $ p = 0.0317)

It was demostrated that in the LPS + ATP group, NLRP3 mRNA fold change was 5.59 ± 1.36, whereas RSV pre-incubation reduced this rate to 3.89 ± 0.17value.Likewise IL-1β, as seen in Figure 10B., CC and Ex527 pre-treatment was increased the reduction significantly as mean 9.69 ± 1.6 and 10.68 ± 2.46. (* p = 0.0357, # p = 0.0357, $ p = 0.0357)

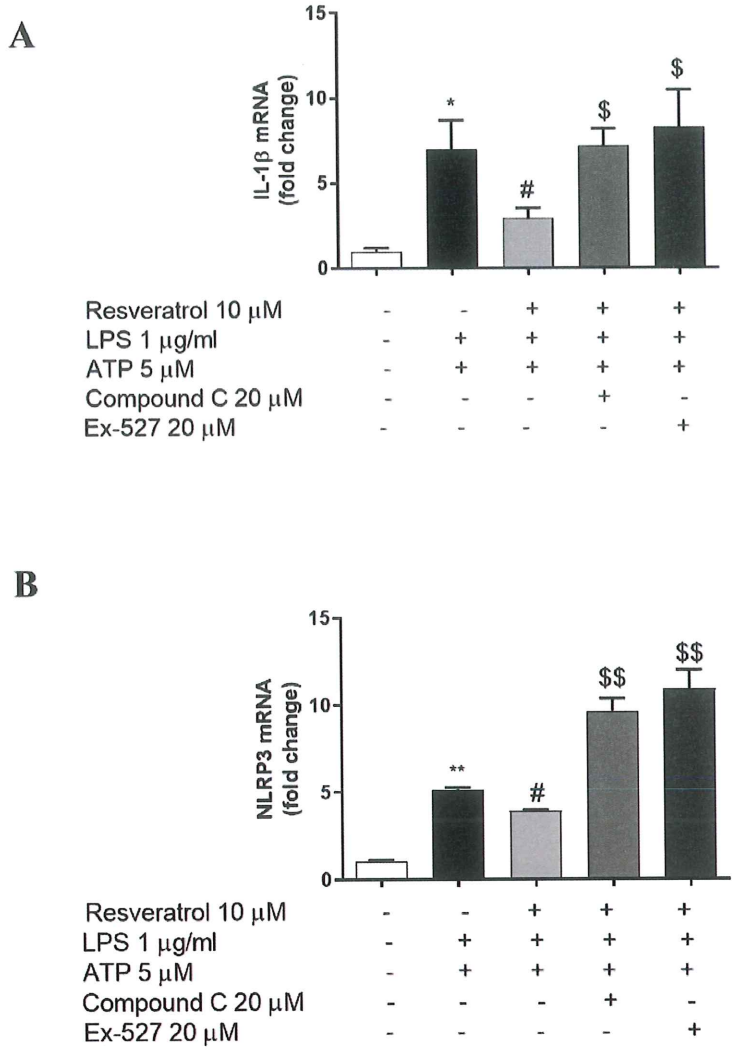

Figure 10: Determination of NLRP3 and IL-1β expression level. *NLRP3 (A) and IL-1β (B) expression was determined to downregulated with RSV pre-incubation. CC and Ex-527 pre-treatment significantly upregulated NLRP3 (A) and IL-1β (B) expression. Results were given as mean ±SD.*

4.10. Determination of AMPK and Sirt1 inhibition on caspase-1 activity

The activity of Caspase-1, which is one of the important indicators of the activated inflammasome in microglia cells, was conducted by the use of the Caspase-1 activity assay kit which was developed for this purpose. As shown in Figure 11, Caspase-1 activity in LPS + ATP-induced cells was 314.87 ± 39.88. Caspase-1 activity value decreased to 275.12 + 13.68 by pretreatment of RSV and increased to 346.68 + 29.52 after CC pretreatment as a result of inhibition of AMPK pathway. Likewise, with the inhibition of Sirt1 pathway, Ex527

pretreatment significantly increased the caspase-1 activity to 304.15 + 16.18 value.(** p = 0.0079; # p = 0.0317; $$ p=00.0079; $ p=0.0216)

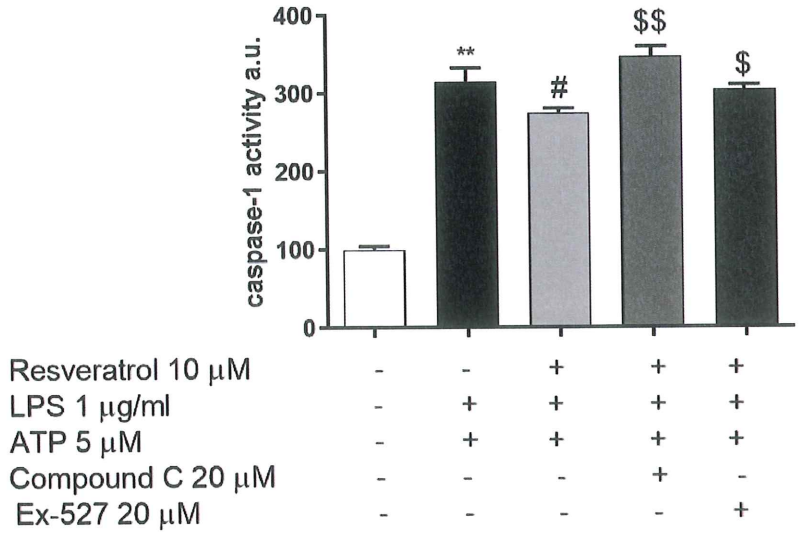

Figure 11: Determination of Caspase-1 via Caspase-1 activity assay. *Caspase-1 activity was determined to decrease with RSV pre-incubation, after inflammasome induction by LPS+ATP. CC and Ex-527 pre-treatment significantly increased the Caspase-1 activity. Results were given as mean ±SD.*

4.11. Determination of miR-155 expression levels

According to following result, after LPS+ATP treatment miR-155 expression level was upregulated compared to the control group as mean standart deviation 16.94 ± 1.82. Also, it was showed that RSV pre-treatment significantly downregulated miR-155 expression with 5.36 ± 1.21 value. (* p = 0.159, # p = 0.159)

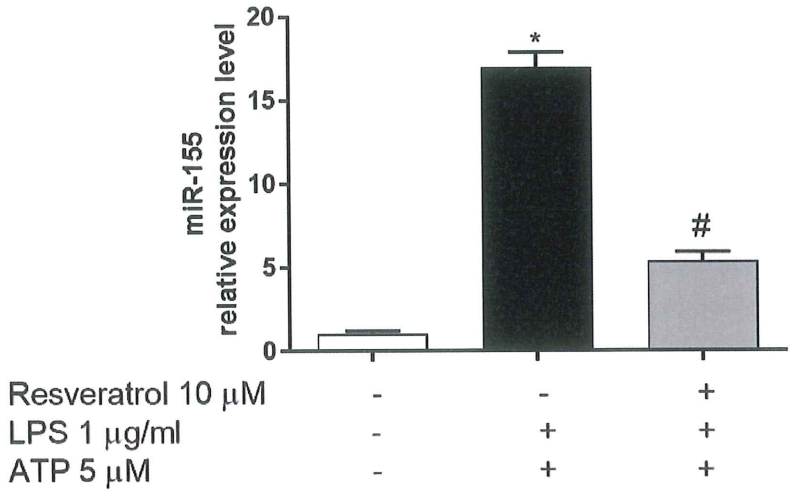

Figure 12: Determination regulative effect of RSV on miR-155 expression levels. *miR-155 epression level increased in LPS+ATP induced activated inflammasome model, RSV pre-treatment downregulated miR-155 expression. Results were given as mean ±SD.*

4.12. Determination of AMPK and Sirt1 inhibition on miR-155 expression levels

In order to determine whether RSV suppress NLRP3 inflammasomeby regulating the Sirt1 and AMPK pathways via miRNAs, Figure 14 was showed that miR-155 expression level. As seen in Figure 13, miR-155 upregulated after LPS+ATP treatment with 9.206 ± 7.2 mean value while RSV pre-treatment knockdown this regulation as mean standart deviation 0.57 ± 0.31. It was also demostrated that CC and Ex527 pre-treatment was increased the reduction significantly as mean 3.42 ± 2.36 and 3.78 ± 4.74. (* p= 0.0286, ## p= 0.0095, $ p =0.0238, $$ p =0.0043)

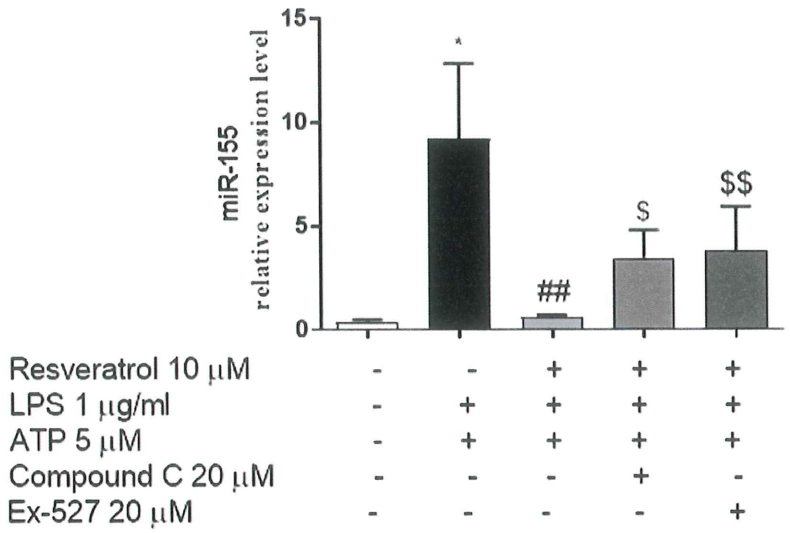

Figure 13: Determination AMPK Sirt1 inhibition on miR-155 expression levels. *miR-155 epression level increased in LPS+ATP induced activated inflammasome model, RSV pre-treatment downregulated miR-155 expression. CC and Ex-527 pre-treatment upregulated miR-155 expression. Results were given as mean ±SD.*

5. DISCUSSION

Large multimeric protein complexes are named as inflammasomes which are found in the immune cells's cytosol for detect and respond to threats from internal and external signals, such as pathogen infection and tissue damage. The first defense response to microbial infections is the activation of inflammasome. NLRP3 is one of the most researched inflammasome type in innate immune cells. Although many studies have been realized on NLRP3 inflammasome, the signaling pathways regulating its activation are still not fully understood.

An important link that is highly preserved in eukaryotic cells to detect and respond to cellular energy state is AMPK regulators of cellular energy metabolism. A heterotrimeric protein, AMPK, is consisting of 3 units namely as α, β and γ; the α subunit contains the catalytic domain in which it activates kinase with phosphorylation in threonine (Thr) 172. Activation of AMPK occurs if the cellular AMP / ATP ratio increases due to metabolic stresses or ATP consumption. Besides regulation of cellular metabolism, activation of AMPK regulates cell survival and proliferation. In a study with LPS-primed murine macrophages, revealed that ATP-induced inflammasome activation regulated by AMPK signaling. They have shown that AMPK inhibitor, Compound C suppressed the secretion of IL-1β in J774A.1 cells (Zha, Wei et al. 2016).Another study in LPS-primed macrophage cells, RAW 264.7, demonstrated that RSV increased phosphorylated AMPK in addition AMPK inhibitor CC attenuated TNF-α protein expression which is a potent pro-inflammatory cytokine (Yi, Jeon et al. 2011).Similar results have been revealed in a study with BV2 microglial cells. They have demonstrated that RSV enhanced AMPK phosphorylation in a dose dependent manner and attenuated of morphine-induced microglial activation. Furthermore they have indicated CC alter the suppressive effects of RSV (Han, Jiang et al. 2014).

RSV has been recognized to carried protective properties through the AMPK pathway. Furthermore, AMPK activation of RSV has been proposed to indirectly activate the Sirt1 pathway by increasing intracellular NAD + levels (Mancuso, Del Valle et al. 2014). Sirt1 activation has been demonstrated to have therapeutic benefits against neurodegeneration through the modulation of several cellular pathways (Mancuso, Del Valle et al. 2014).It has been attributed that anti-inflammatory effects of RSV dependent to the Sirt1 activation (Li, Zhou et al. 2014). (Fu, Wang et al. 2013) have shown that RSV upregulates Sirt1 protein expression level in dose dependent way. Moreover they demostrated that pre-treatment with RSV weakened the expression of IL-1β and NLRP3, however knockout of Sirt1 expression with inhibitor NAM and siRNA significantly enhanced IL-1β and NLRP3 expression level in

mesenchimal stem cell(Fu, Wang et al. 2013).Another study with in vivo model have shown that RSV downregulated NLRP3 inflammasome but yet Sirt1 knockout reversed this downregulation (He, Li et al. 2017). Similar findigs have been indicated in traumatic brain injured-rat. They have indicated that RSV suppressed NLRP3 and reduced IL-1β and IL-18 secretion while elevated Sirt1 expression. Also they have demostrated that pre-treatment with Sirt1 inhibitor, Sirtinol, inhibited the anti-inflammatory effect of RSV and therefor enhanced NLRP3, IL-1β and IL-18 expression (Zou, Liu et al. 2018). In a study of microglial cells stimulated by LPS and ATP have been demonstrated that Sirt1 inhibitor NAM inhibited anti-inflammatory effects of RSV on NLRP3 inflammasome. They have shown that NLRP3 expression and IL-1β cleavage enhanced by Sirt1 inhibitor NAM treatment in BV2 cells (Sui, Xie et al. 2016).

In our study, effect of AMPK and Sirt1 pathways on NLRP3 inflammasome model has been investigated. First we have shown that RSV administration activated AMPK and Sirt1 in microglial cells. A statistically significant increase in their protein levels have indicated via immunoblotting as seen in Figure 8 and 9. In order to determined regulation of NLRP3 inflammasome through AMPK and Sirt1 pathway, expression grades of NLRP3 and IL-1β have been investigated with or without AMPK inhibitor and Sirt1 inhibitor. As seen in Figure 10, our results have determined that CC and Ex527 significantly upregulated NLRP3 and IL-1β level of expression which have been downregulated by RSV pre-incubation. Caspase-1 activity has been demonstrated to significantly increased with inhibitors while decreased in RSV treatment as seen in Figure 11. These results have indicated that RSV shows its anti-inflammatory effects on NLRP3 inflammasome through AMPK and Sirt pathways.

Involment of RSV in more than one signal pathway in immune system responses has been the subject of research for many years. RSV is thought to realize most of these effects by modifications it has made in the microRNA population within the cell (Michaille, Piurowski et al. 2018). MiRNAs, single-stranded noncoding small RNA molecules have the ability to regulate of translation or degradation of target mRNAs (Kutty, Nagineni et al. 2010). MiRNAs epigenetically regulate factors involved in diverse signal transduction pathways namely transcription factors, kinases, phosphatases or methylases (Michaille, Piurowski et al. 2018). In addition, miRNAs have been known that they are capable of modification the inflammatory responses (Kutty, Nagineni et al. 2010).For example, a study was conducted to investigate whether the antitumor and anti-inflammatory impact of RSV would depend on the expression cadence of pro- or anti-inflammatory miRNAs. In this study in THP1 cells, RSV has been

shown to disrupt the enhancement of oncogenic pro-inflammatory miR-155 by LPS treatment through the increase on the regulation of miR-663, a miRNA that partially targets JunB and JunD transcripts (Tili, Michaille et al. 2010). SOCS1 is pro-apoptotic and anti-inflammatory protein and also one of the targets of miR-155 which generates a negative feedback loop in LPS-primed signaling pathways through inhibiting JAK and STAT (Ma, Wang et al. 2017). In a study with murine macrophages which were stimulated by LPS, they have demonstrated that RSV suppress STAT activation and increase expression of SOCS1 by downregulate miR-155 (Ma, Wang et al. 2017). In an another study to look into the role and function of miRNAs in innate immune system responses, it was figured that miR-155 expression increased after stimulation with LPS in RAW264.7 and C57BL / 6 mice (Tili, Michaille et al. 2007). In a study with BV2 microglia cells, miR-155 has been shown to be upregulate after cerebral ischemia injury. In this study, RSV has been indicated to prevent neuroinflammation which caused by cerebral ischemia via down-regulating miR-155 (Ma, Fan et al. 2019).

Although it is known that RSV suppresses inflammasome and able to regulates miR-155, which is an inflammatory miRNA, there has not been demonstrated that how it regulates signalling pathways. In this thesis, we demonstrated in Figure 12 that RSV pretreatment downregulated miR-155 level which was upregulated by LPS+ATP induction in microglia cell line. Furthermore, to established which signalling pathways regulates this expression level, we determined miR-155 expression level with and without AMPK and Sirt1 inhibitors. As seen in Figure 13, pre-treatment with CC and Ex527 reversed the downregulation of miR-155 expression significantly compared to the only RSV treatment group. These results have suggested that RSV regulative effects on miR-155 expression are dependent on AMPK and Sirt pathways.

6. CONCLUSION AND FUTURE ASPECTS

In this thesis, the signaling pathways where RSV has anti-inflammatory properties through suppressing NLRP3 inflammasome and the mechanisms of controlling these signaling pathways has been investigated. In the first stage of the research, the effects of RSV on cell death, oxidative stress, NF-kB, Sirt1 and AMPK pathways has been determined. Following the RSV has been shown to activate Sirt1 and AMPK pathways, NLRP3 inflammasome parameters were investigated with pathway inhibitors in order to answer whether the suppression effects of RSV on the NLRP3 infammasome dependent to these pathways. Inhibition of Sirt1 and AMPK pathways has been shown to inhibit the anti-inflammatory effects of RSV on NLRP3 mRNA and protein, IL-1β mRNA state of expression and caspase-1 activity. In the second stage of this research, it has been determined that miR-155 upregulated by LPS+ATP induction while RSV pre-treatment reduced its upregulation. In addition, we have demonstrated that RSV effect on miR-155 regulation was significantly reversed by Sirt1 and AMPK pathway inhibition. As a consequences of our study, RSV has its anti-inflammatory effects on NLRP3 infammasome dependently on Sirt1 and AMPK pathways and its affect can be modulate with microRNA regulation.

To our knowledge this research is the first study that clarifies the signaling pathways which RSV has anti-inflammatory properties on suppressing NLRP3 inflammasome and the mechanisms of controlling these signaling pathways in murine microglia cell line. In further experiments, we are going to explore functional effects of miRNAs on NLRP3 inflammasome and its signalling pathways.

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DOKUZ EYLÜL ÜNİVERSİTESİ
GİRİŞİMSEL OLMAYAN ARAŞTIRMALAR ETİK KURUL KARARI

Sayın Prof.Dr.Şermin Genç

Araştırmanıza ilişkin Kurulumuz kararı aşağıda sunulmuştur.

Bilgilerinizi ve gereğini rica ederiz.

ETİK KOMİSYONUN ADI	DOKUZ EYLÜL ÜNİVERSİTESİ GİRİŞİMSEL OLMAYAN ARAŞTIRMALAR ETİK KURULU
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	ARAŞTIRMANIN AÇIK ADI	**N9 Mikroglial Hücrelerde AMPK-SIRT1 Yolaklarının MikroRNA aracılığı ile NLRP3 İnflamazomunun Düzenlenmesi**	
	ARAŞTIRMA PROTOKOL KODU		
	SORUMLU ARAŞTIRMACI ÜNVANI/ADI/SOYADI ve UZMANLIK ALANI	Prof.Dr.Şermin Genç İBG	
	ARAŞTIRMAYA KATILAN MERKEZLER	TEK MERKEZ ☐	ÇOK MERKEZLİ☒

	Belge Adı	Tarihi	Versiyon Numarası	Dili
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	ARAŞTIRMA İLE İLGİLİ LİTERATÜR	Mevcut		Türkçe ☐ İngilizce ☒ Diğer ☐
	BİLGİLENDİRİLMİŞ GÖNÜLLÜ OLUR FORMU	Mevcut		Türkçe ☒ İngilizce ☐ Diğer ☐
	OLGU RAPOR FORMU	Mevcut		Türkçe ☒ İngilizce ☐ Diğer ☐

KARAR BİLGİLERİ	Karar No:2020/06-37	Tarih:09.03.2020
	Prof.Dr.Şermin Genç'in sorumlusu olduğu "**N9 Mikroglial Hücrelerde AMPK-SIRT1 Yolaklarının MikroRNA aracılığı ile NLRP3 İnflamazomunun Düzenlenmesi**" isimli klinik araştırmaya ait başvuru dosyası ve ilgili belgeler araştırmanın gerekçe, amaç, yaklaşım ve yöntemleri dikkate alınarak incelenmiş, etik açıdan çalışmanın gerçekleştirilmesinin uygun olduğuna oy birliği ile karar verilmiştir.	

ETİK KURUL BİLGİLERİ	
ÇALIŞMA ESASI	Dokuz Eylül Üniversitesi Girişimsel Olmayan Araştırmalar Etik Kurulu İşleyiş Yönergesi İyi Klinik Uygulamaları Kılavuzu

ETİK KURUL ÜYELERİ

Unvanı/Adı/Soyadı	Uzmanlık Alanı	Kurumu	Cinsiyet	Araştırma ile İlişkili mi?		İmza
Prof.Dr.Sadık Kıvanç METİN (Başkan)	Kalp ve Damar Cerrahisi	DEU Tıp Fakültesi Kalp Damar Cerrahisi Anabilim Dalı	Erkek	E ☐	H ☒	
Prof.Dr. Sermin ÖZKAL (Başkan Yardımcısı)	Tıbbi Patoloji	DEÜ Tıp Fakültesi Tıbbi Patoloji A.D	Kadın	E ☐	H ☒	
Prof.Dr.Serkan YENER	Endokrinoloji	DEU Tıp Fakültesi İç Hastalıkları Anabilim Dalı	Erkek	E ☐	H ☒	
Prof.Dr.Pınar TUNCEL	Tıbbi Biyokimya	DEU Tıp Fakültesi Tıbbi Biyokimya Anabilim Dalı	Kadın	E ☐	H ☒	
Prof.Dr.Arzu GENÇ	Nörolojik Fizyoterapi - Fizik Tedavi ve Rehabilitasyon	DEU Fizik Tedavi ve Rehabilitasyon Yüksek Okulu	Kadın	E ☐	H ☒	
Doç.Dr.Nil Hocaoğlu AKSAY	Tıbbi Farmakoloji	DEU Tıp Fakültesi Tıbbi Farmakoloji Anabilim Dalı	Kadın	E ☐	H ☒	
Doç.Dr.Murat BEKTAŞ	Çocuk Sağlığı ve Hastalıkları Hemşireliği	DEU Hemşirelik Fakültesi Çocuk Sağlığı ve Hastalıkları Hemşireliği	Erkek	E ☐	H ☒	
Doç.Dr.Tufan ÇANKAYA	Tıbbi Genetik	Tıbbi Genetik Anabilim Dalı	Erkek	E ☐	H ☒	
Doç.Dr.Ayfer DAYI	Davranış Fizyolojisi	DEU Tıp Fakültesi Fizyoloji Anabilim Dalı	Kadın	E ☐	H ☒	
Doç.Dr.Korcan DEMİR	Pediatrik Endokrinoloji	DEU Tıp Fakültesi Çocuk Sağlığı ve Hastalıkları Anabilim Dalı	Erkek	E ☐	H ☒	
Doç.Dr.Mahmut Cem ERGON	Tıbbi Mikrobiyoloji	DEU Tıp Fakültesi Tıbbi Mikrobiyoloji Anabilim Dalı	Erkek	E ☐	H ☒	
Öğr.Gör.Dr.Kıvanç YÜKSEL	Biyoistatistik ve Tıbbi Bilişim	Ege Üniversitesi Tıp Fakültesi Biyoistatistik ve Bilişim A.D	Erkek	E ☐	H ☒	
Av.Esra FIRTINA	Avukat	DEU Rektörlüğü Hukuk Müşavirliği	Kadın	E ☐	H ☒	
Mehmet Erhan ÖZKUL	Sağlık mensubu olmayan üye	D.E.U Tıp Fakültesi İdari Mali İşler	Erkek	E ☐	H ☒	