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Effects of glucosamine on LPS/IFN-γ induced RAW 264.7 macrophage apoptosis *

Glukozaminin LPS/IFN-γ ile İndüklenen RAW 264.7 Makrofaj Apoptozu Üzerine Etkileri

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**** Bu çalışma, TÜBİTAK tarafından 110S240 proje numarası ile desteklenmiştir.***

Aim: Apoptosis is a genetically programmed cell death mechanism which plays important roles in normal physiology such as tissue homeostasis regulation and pathophsiology of various diseases. iNOS enzyme activation and nitric oxide synthesis in various cell types as a defense mechanism against microbial and viral pathogens plays an important role in inflammatory and immune pathologies such as atherosclerosis, rheumatoid arthritis, diabetes, septic shock, multiple sclerosis and highly uncontrolled production of nitric oxide leads to cell death. Recently, glucosamine which is acclaimed to be beneficial for inflammatory disorders such as osteoarthritis is widely used clinically. There is limited information regarding the effect mechanisms of glucosamine on apoptosis of mainly immune cells, macrophages. In this study, effects of different concentrations of glucosamine were tested on LPS/IFNγ activated RAW 264.7 macrophages.

Materials and methods: RAW 264.7 macrophage cell line was treated with or without glucosamine before LPS/IFNγ stimulation. Nitrite levels, cell viability, caspase-3 activity, mitochondria membrane potential and flow cytometric analysis with Annexin V-PI cell staining was performed.

Results and discussion: Glucosamine inhibited nitrite levels, increased mitochondrial membrane potential, decreased caspase-3 enzyme activity significantly ($p<0.05$) and exhibited antiapoptotic effects. As the NO inhibitor effect of glucosamine on activated cells is not potent, the antiapoptotic effects of glucosamine is partly nitric oxide dependent and nitric oxide independent pathways are also thought to be responsible. In this regard, further elaborate studies are needed to clarify the effects of glucosamine on specific signaling pathways.

Key Words: ***Apoptosis, glucosamine, nitric oxide, macrophage.***

Amaç: Apoptoz, doku homeostazisinin düzenlenmesi gibi normal fizyolojide ve çeşitli hastalıkların patofizyolojisinde önemli rol oynayan genetik olarak programlanmış bir hücre ölüm mekanizmasıdır. Çeşitli hücre tiplerinde mikrobiyal ve viral patojenlere karşı bir savunma mekanizması olarak ortaya çıkan iNOS enzim aktivasyonu ve nitrik oksit sentezi, ateroskleroz, romatoid artrit, diyabet, septik şok, multipl sklerozis gibi inflamatuvar ve immün patolojilerde önemli rol oynamakta, kontrolsüz yüksek düzeydeki nitrik oksit üretimi hücre ölümüne yol açmaktadır. Yakın zamanda, osteoartrit gibi inflamatuvar bozukluklar için yararlı olduğu iddia edilen glukozamin günümüzde klinik olarak kullanılmaktadır. Glukozaminin, başlıca immün sistem hücreleri olan makrofajların apoptozu üzerine olan etki mekanizması hakkında sınırlı bilgi bulunmaktadır. Bu çalışmada, farklı konsantrasyonlardaki glukozaminin, LPS/IFNγ ile aktive edilmiş RAW 264.7 makrofajlar üzerindeki etkileri belirlenmiştir.

Materyal ve metod: RAW 264.7 makrofaj hücre serisi, LPS/IFNγ ile uyarım öncesinde glukozamin varlığında ve yokluğunda kültür edildi. Nitrit düzeyleri, hücre canlılığı, kaspaz-3 aktivitesi, mitokondri membran potansiyeli tayini ve Annexin V-PI hücre boyaması ile akım sitometrik analizler gerçekleştirilmiştir.

Sonuçlar ve tartışma: Glukozaminin, nitrit düzeylerini inhibe ettiği, mitokondri membran potansiyelini arttırdığı, kaspaz-3 enzim aktivitesini azalttığı ve anlamlı olarak antiapoptotik etkiler sergilediği ($p<0.05$) belirlenmiştir. Glukozaminin güçlü bir nitrik oksit inhibitör etkisi sergilemediğinden antiapoptotik etkilerinin kısmen nitrik oksit bağımlı olduğu, ancak bu etkilerinde nitrik oksit bağımsız yolakların da sorumlu olabileceği düşünülmektedir. Bu bağlamda, glukozaminin spesifik sinyal yolakları üzerindeki etkilerini aydınlatmak üzere daha ileri çalışmaların yapılmasına ihtiyaç duyulmaktadır.

Anahtar Sözcükler: ***Apoptoz, glukozamin, nitrik oksit, makrofaj***

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Apoptosis is a programmed cell death mechanism which is important for tissue remodelling, organ size control, normal embryonic development and homeostasis of multicellular organisms (1, 2). One of the pathways leading to apoptosis is in tinsic pathway which is activated with direct or indirect action of death signals on the mitochondria and subsequent release of proapoptotic proteins from mitochondrial intermembrane space (3). Among various stimulants of apoptosis via intrinsic pathway is nitric oxide which is an important cellular messenger molecule involved in many physiological and pathological processes (4). Nitric oxide is synthesised by nitric oxide synthase enzyme family which contains izozymes eNOS, nNOS and iNOS. iNOS is responsible for high levels of nitric oxide production as a defense to various pathogen agents and therefore an important participant of immune system (5). On the other hand, macrophages are the primary cells of the immune system which synthesise NO as a result of iNOS activation after exposure to foreign agents (6, 7). Macrophages are activated with bacterial endotoxin lipopolysaccharide and cytokine IFN-γ which constitutes the classical activation model (8, 9, 10). NO dependent death of murine peritoneal macrophages have shown to be mediated by apoptosis (11). Nitric oxide dependent apoptosis of activated macrophages occurs via loss of mitochondrial membrane potential, cytochrome c release from mitochondria (12), stimulation of p53 expression (13) and accumulation of pro-apoptotic Bcl-2 family members (14). Subsequently, PARP cleavage occurs as a sign of caspase activation (15). High levels of nitric oxide synthesized by iNOS can effect surrounding tissues, playing cytotoxic roles at septic shock and various autoimmune diseases (16, 17). Abnormal or chronical macrophage activation contributes to pathogenesis of various diseases including arthritis, atherosclerosis and insulin resistance which are closely related to disordered inflammatory regulation (18) and excessive and long time production of pro-inflammator mediators may be detrimental to host cells (19). Therefore, developing treatment options by manipulating life spans of immune system cells for controlling inflammation has gained importance. In recent years, natural or chemically synthesized diet supplements have been widely used for prevention or treatment of diseases. Among these supplements is glucosamine which is acclaimed to be beneficial for osteoarthritis treatment (20). Glucosamine, which serves as an important precursor for biosynthesis of connective tissue macromolecules is a naturally produced hexosamine sugar in humans (21). Among various studies examining the biochemical action mechanism of glucosamine, researchers postulated that different forms of glucosamine inhibited COX-2 and iNOS expression in LPS-stimulated RAW 264.7 macrophages (22, 23) and therefore can be used as an antiinflammatory agent. In another study it was found that NO production in mouse peritoneal macrophages was significantly augmented by glucosamine treatment and glucosamine exerted immunostimulating properties (24). Glucosamine has been tested for its effects on apoptosis and shown to induce apoptosis in different cancer cell lines (25, 26, 27) whereas it exhibited protection against ischemia-perfusion injury in cardiomyocytes (28) and impact induced cell death in chondrocytes (29). These literatures indicate that glucosamine may promote or inhibit cell viability depending on cell type and the active specific biochemical pathway. There is limited research regarding the effects of glucosamine on apoptosis of macrophages which are the principle immune cells. Since glucosamine has been widely used for treatment of arthritic conditions characterised with chronic inflammation, it is important to identify its effects on apoptotic behaviour of macrophages. Therefore, in this study, effects of glucosamine on LPS/IFN-γ induced RAW 264.7 macrophage apoptosis was investigated.

MATERIALS AND METHODS

Chemicals

RPMI-1640 medium was from Sigma, L-glutamine, penicilin-streptomycin was obtained from PAA, N-(1 naphtyl) ethylenediamidedihydrochloride, bovine serum albumin (BSA), Annexin V-FITC, propidium iodide, phosphate-buffered saline (PBS), glucosamine hydrochloride, 4, 5-dimet hylthiazo l-2yl)-2, 5-diphe nyltetrazolium bromide (MTT) dimethyl sulfoxide (DMSO), Giemsa-Accustain, glucosamine hydrochloride, LPS (E. Coli) were obtained from Sigma; IFN-γ was obtained from R&D, Rhodamine 123 and Caspase-3 substrate Ac-DEVD-AMC were obtained from Alexis Biochemicals, bradford reagent was obtained from Bio-Rad.

Cell culture

RAW 264.7 macrophage cell line (a gift from Beatriz de Las Heras-University Complutense-Madrid) was cultured in RPMI-1640 medium containing 10% heat inactivated fetal bovine serum and antibiotics (100 U/ml penicilin and 100 μg/ml streptomycin) at 37 ^{0}C in an atmosphere of 5% CO2. Cells were seeded to 96 well plates and 6 well plates for 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) assay, nitrite assay, Rh123 assay, caspase enzyme activity assay and Annexin V-PI staining with flow cytometry.

Nitrite assay

Nitrite production in the medium was measured with colorimetric Griess assay. Principle of Griess assay is conversion of sulfanilic acid to a diazonium salt with nitrite under acidic conditions and coupling of this diazonium salt to N-(1 naphthyl) ethylenediamide dihydrochloride to form an azo dye which can be measured spectrophotometrically. RAW 264.7 macrophages were seeded to 96 and/or 6-well plates for one night incubation, the next day fresh medium was added and cells

were treated with glucosamine for one hour and then stimulated with LPS (10 ng/ml)/IFNγ (1 ng/ml). Medium was mixed with an equal volume of Griess reagent [0.1% N-(1 naphthyl) ethylenediamide dihydrochloride, 1% sulfanilamide in 5% phosphoric acid] and absorbance was measured spectrophotometrically (Molecular Devices Spectra-max Gemini). Nitrite concentrations were determined by absorbance of standard sodium nitrite solutions at 550 nm (30).

Cell viability assay

MTT assay was used for determination of cell viability. Briefly cells were seeded to 96 and/or 6-well plates for overnight incubation. The next day medium was aspirated, fresh medium and different concentrations of gluocosamine was added 1 hour before stimulation with LPS (10 ng/ml)/IFNγ (1 ng/ml). Cells without glucosamine treatment was used as control group. After incubation period, MTT (0.5 mg/ml) was added to medium and incubated at 37 ^{0}C for 1 hour, then culture medium was removed and DMSO was added to solubilize formazan crystals. Absorbance at 690 nm was measured with spectrophotometry and the color intensity was used as an implication of cell viability (31).

Flow cytometric analysis for apoptosis

Annexin V-PI staining was used as a biochemical marker of apoptosis. After treatment of cells with chemicals as explained above, cells were washed twice with PBS, pH 7.4, washed with binding buffer and resuspended in binding buffer containing PI and Annexin V-FITC. Cells were incubated in the dark at room temperature for 15 min and then analyzed with flow cytometry (Accuri. C6) according to the suppliers instructions (BD Biosciences).

Caspase-3 activity assay

After treatments, cells were lysed with lysis buffer cocktail and spesific caspase-3 activity was spectrofluorometrically (Perkin-Elmer LC 55) determined in cytosolic protein extracts using a specific substrate (Ac-DEVD-AMC) according to the supplier's instructions (Alexis Biochemicals).

Mitochondria membrane potential

Rh 123 was used to determine mitochondrial potential. After treatment of cells with chemicals as indicated above, cells were washed with PBS two times and incubated Rh123 for 15 minutes before measuring fluoresence intensity at Ex:495 Em:525 with a spectrofluorometer(32).

Morphological investigations

Cells were grown in 6-well plates. After adhesion overnight, cells were treated and stimulated according to experimental protocols, scraped off the culture plates, followed by washing with PBS two times and fixation with 1:5 volume of acetic acid/methanol. Cells were then soaked into 1:20 diluted Giemsa solution for 15 minutes and then washed with distilled water and visualised under a light microscope (Leica DM IL LED).

RESULTS

Effects of glucosamine on nitrite production and cell viability in unstimulated macrophages:

Effects of glucosamine on cell viability in unstimulated RAW 264.7 macrophages was examined and it was found that glucosamine concentrations higher than 4 mM was cytotoxic for RAW 264.7 macrophages ($p<0.05$) (Figure 1).

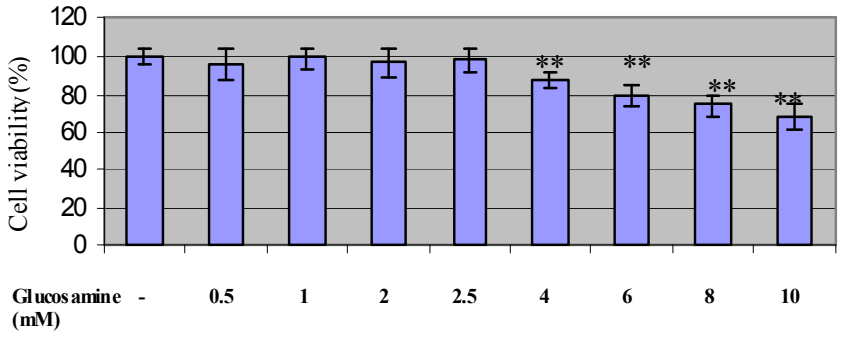

Figure 1: Effect of glucosamine on cell viability in RAW 264.7 macrophages. (**): $p<0.05$ versus cells not treated with glucosamine.

Nitrite concentrations were increased with glucosamine concentrations higher than 5 mM (Figure 2) and therefore a concentration range less than 4 mM which is non-toxic and does not induce an NO production was chosen for treatment of LPS/IFNγ stimulated RAW 264.7 macrophages.

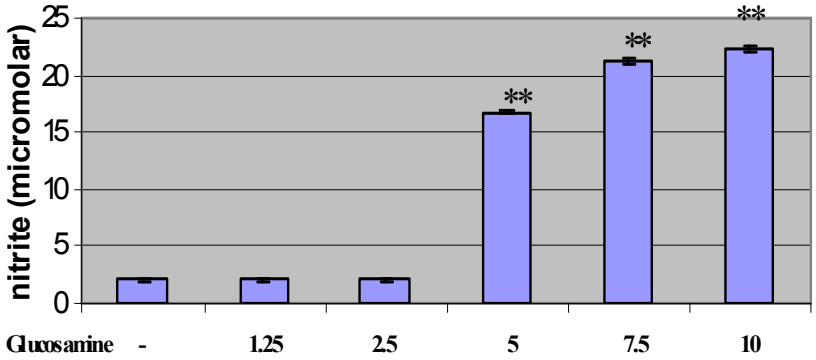

Figure 2: Effect of glucosamine on nitrite levels in RAW 264.7 macrophages. (**): p<0.05 versus cells not treated with glucosamine.

Effects of glucosamine on nitrite production and cell viability in LPS/IFNγ stimulated macrophages:

We found a massive NO production in macrophages after 24 hours of incubation with LPS/IFNγ. 1 h pre-incubation with glucosamine upon activation decreased NO levels of LPS/IFNγ activated macrophages dose dependently (Figure 3) (p<0.05).

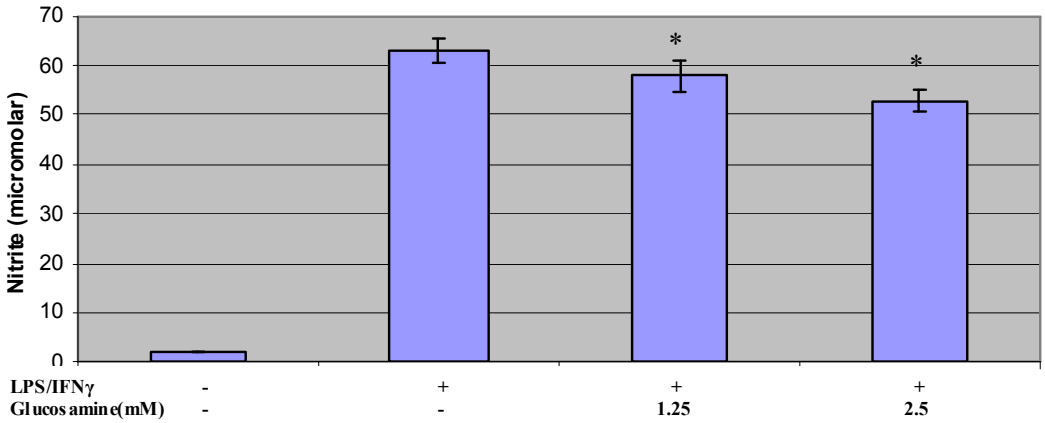

Figure 3: Effect of glucosamine on nitrite levels in RAW 264.7 macrophages. (*): p<0.05 versus LPS/IFN-γ treated macrophages.

Besides its inhibitor effects on NO production glucosamine inhibited the decrease in cell viability caused by LPS/IFNγ treatment (Figure 4) (p<0.05).

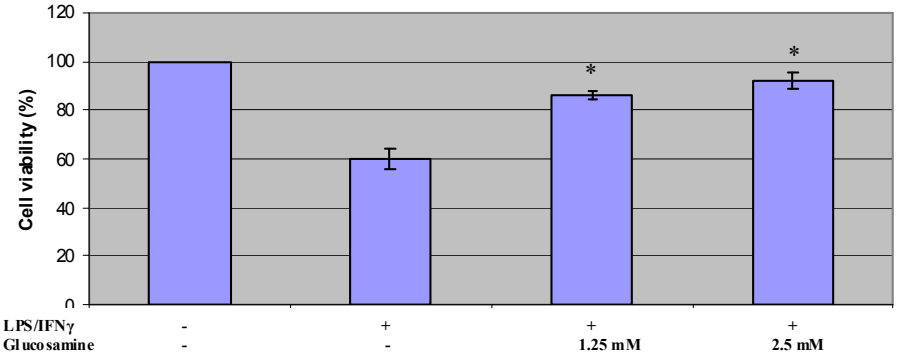

Figure 4: Effect of glucosamine on cell viability in macrophages. (*): p<0.05 versus LPS/IFN-γ treated macrophages.

Morphological assessment of stimulated macrophages:

Apoptotic morphology was observed in LPS/IFNγ activated macrophages and glucosamine treatment partially decreased the apoptotic morphology of activated cells (Figure 5).

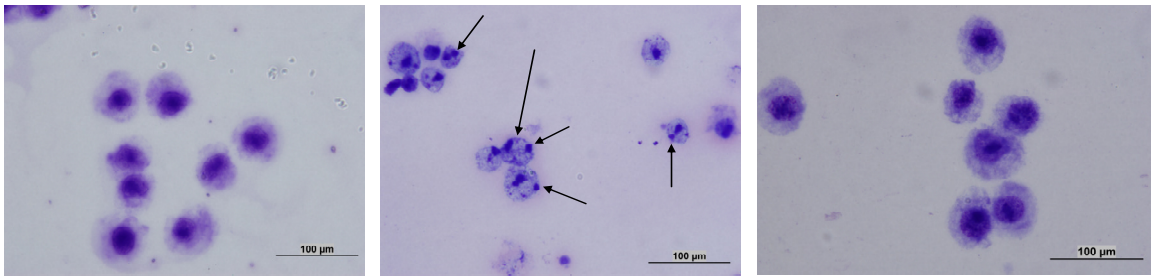

Figure 5: (a) RAW 264.7 macrophages without stimulation. **(b)** LPS/IFNγ stimulated RAW 264.7 macrophages. **(c)** RAW 264.7 macrophages treated with 2.5 mM glucosamine before LPS/IFNγ stimulation.

Flow cytometry for analysis of apoptosis:

We found that LPS/IFNγ treatment significantly increased number of Annexin V-FITC stained cells and number of apoptotic cells significantly ($p<0.05$) decreased with glucosamine treatment (Figure 6).

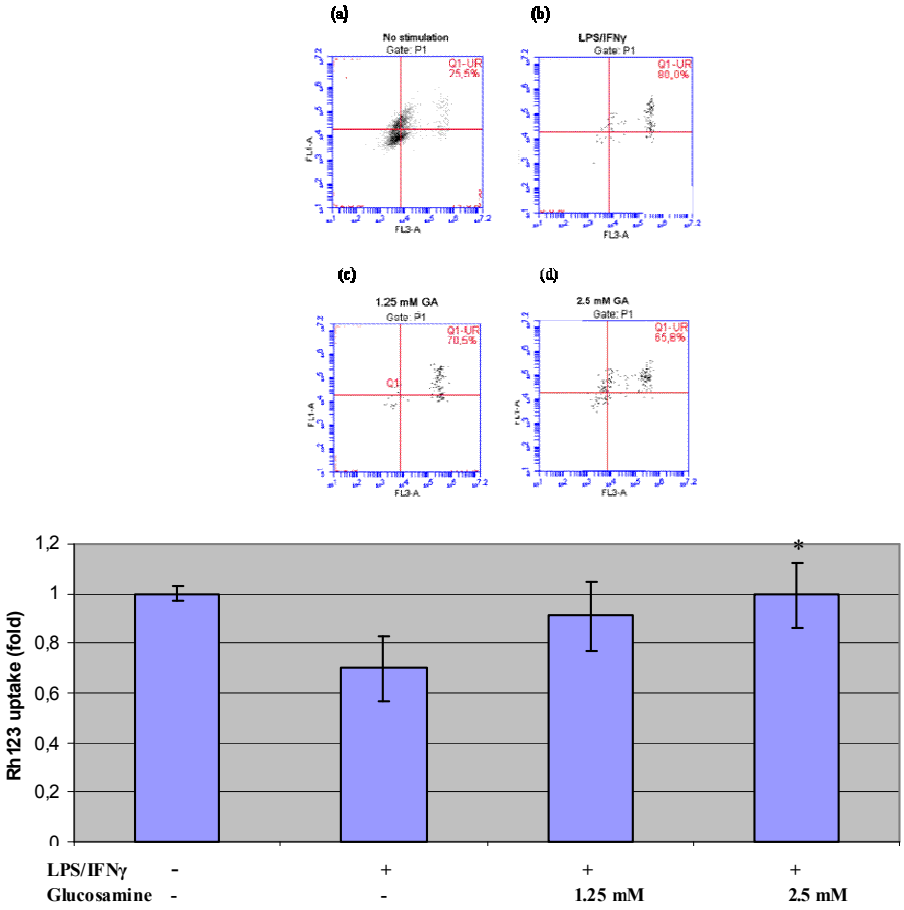

Figure 6 **(a)** Cells without stimulation, **(b)** Cells stimulated with LPS/IFNγ for 24 hours, Cells were pretreated with 1.25 mM **(c)** or 2.5 **(d)** mM for 1 hour and then stimulated with LPS/IFNγ for 24 hours. Each figure was chosen as a representative of at least three different experiments. Bar graph shows % mean of late apoptotic cells for each group. (*): $p<0.05$ versus LPS/IFNγ treated group.

Rh123 staining for mitochondria membrane potential:

It is found that LPS/IFNγ treatment deteriorated mitochondrial permeability of macrophages and glucosamine (2.5 mM) treatment restored mitochondrial membrane potential (Figure 7).

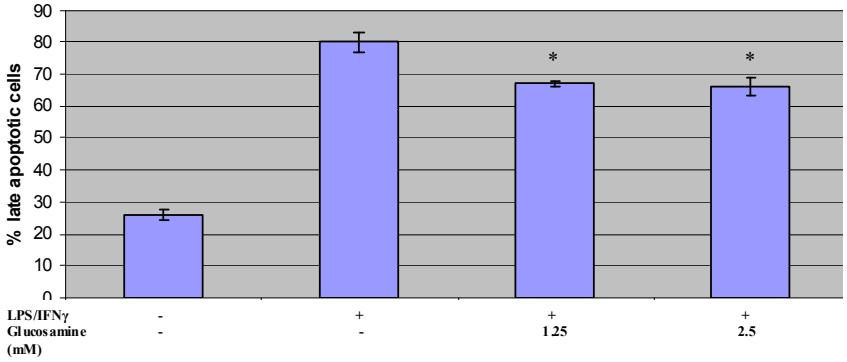

Figure 7: Effect of glucosamine on mitochondrial membrane potential. Rh123 uptake of unstimulated cells was estimated as 1 fold and other groups were determined according to unstimulated cells (*): p<0.05 versus LPS/IFNγ treated group.

Effect of glucosamine on caspase-3 activity:

After 1 h pre-treatment with or without glucosamine and 24 hours of stimulation with LPS/IFNγ we found that caspase-3 activity was significantly higher in LPS/IFNγ compared to unstimulated cells. Glucosamine treatment significantly decreased caspase-3 activity of stimulated cells (Figure 8).

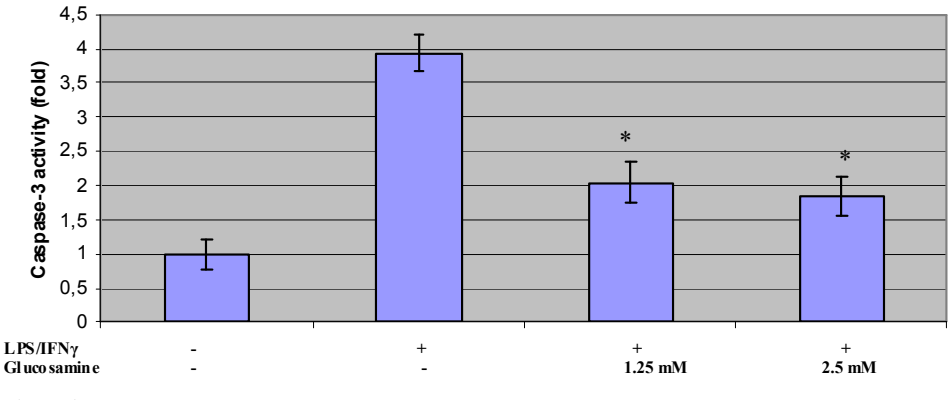

Figure 8: Effect of glucosamine on caspase-3 activity in RAW 264.7 macrophages. Caspase-3 activity of unstimulated cells was estimated as 1 fold and other groups were determined according to unstimulated cells. (*): p<0.05 versus LPS/IFNγ treated group.

Statistical analysis

Results were expressed as means ± standard deviation (SD) from at least three independent experiments. Statistical analysis was performed using one-way analysis of variance . A value of $p < 0.05$ was considered statistically significant.

DISCUSSION

Apoptosis is an essential process which plays important roles in development and tissue homeostasis of multicellular organisms (1, 2). Apoptosis regulation disorders are closely related to different pathologies including cancer, diabetes, cardiovascular and inflammatory diseases. Apoptotic pathways vary according to stimulus and cell type and manipulating apoptotic pathways might be a treatment option for different pathologies especially those which are linked with chronic inflammation (33, 34). Among stimulants of apoptosis is nitric oxide which plays important roles in various inflammatory pathologies (35, 36). Macrophages, one of the main immun system cells produce various biological mediators including nitric oxide as a defense mechanism against pathogens. Upon activation with LPS/IFN-γ, RAW 264.7 macrophages undergo nitric oxide dependent apoptosis. It has been shown that inhibitors of iNOS enzyme and/or nitric oxide scavengers might be benefical for disorders related with excess NO production, block cell damage and cell death (37, 38) and improve the outcome in sepsis (39). Therefore, in this study we explored the effects of glucosamine on NO-dependent apoptosis of RAW 264.7 macrophages which has presented an apoptotic model for nitrosative stress.

In order to identify the effects of glucosamine on LPS/IFN-γ stimulated macrophages, first we treated macrophages with only glucosamine to show its effects on basal NO levels and cell viability. We interestingly found that glucosamine increased NO levels and diminished cell viability in unstimulated macrophages at 4 mM and higher concentrations. This finding implied that glucosamine could induce immune function of macrophages. As inhibition of iNOS and NO production is a treatment strategy for various inflammatory pathologies, excess NO production may have detrimental and cytotoxic effects to macrophages themselves and surrounding tissues. Therefore concentration of glucosamine used is critically important and NO inducing effect of glucosamine should be considered carefully.

It was shown that NO dependent apoptosis in LPS/IFN-γ activated macrophages is characterised with up-regulation of p53, bax activation, mitochondria depolarisation, cytochrome c release and caspase activation (40, 34, 41, 42). In our study, we found consistent findings to those literatures and showed that RAW 264.7 macrophages exhibited apoptotic morphology, increased caspase-3 activity, diminished mitochodria membrane potential and increased staining with Annexin V/propidium iodide after stimulation with LPS/IFNγ for 24 hours. Next, we examined the effects of glucosamine on apoptosis in this model. We found that glucosamine inhibited NO production in activated macrophages in a dose dependent manner. Cell viability significantly decreased with a massive NO production in LPS/IFNγ treated cells when compared to unstimulated cells and glucosamine treatment increased cell viability in stimulated macrophages significantly. After identification of these findings, we examined the mechanism of viability boosting effects of glucosamine on macrophages. We found that glucosamine treatment restored the impairment in mitochondria membrane potential and decreased caspase-3 activity which are hallmarks of apoptosis. Glucosamine treatment also decreased Annexin V-FITC/Propidium iodide stained cell population as a marker of late apoptosis. Glucosamine was shown to supress proliferation and induce cell death in DU145 prostate cancer line (26), decrease cell viability in HepG2 hepatocyte cell line (43) or contrarily inhibit cardiomyocyte death by decreasing mitochondrial depolarisation (28) and decrease impact induced chondrocyte apoptosis (29). These literatures indicate that glucosamine may promote or inhibit cell viability depending on cell type and specific active biochemical pathway. In our study, glucosamine reversed the apoptosis of RAW 264.7 macrophages which are under nitrosative stress and therefore could be used for disorders related with excess NO-production and related cell death. We think that the antiapoptotic effects of glucosamine are partially mediated by its inhibitory action on NO production. It should also be noted that since glucosamine only partially inhibited NO production, there might be other pathways responsible for the diminishment in apoptosis by glucosamine.

REFERENCES

1. Kerr J F, Wyllie A H. and Currie A R. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* 1972; **26**, 239-257.
2. Golstein P, Ojcius DM and Young JD. Cell death mechanisms and the immune system *Immunol Rev* 1991; **121**, 29/65.
3. Orrenius S, Nicotera P Zhivotovsky B. Cell death mechanisms and their implications in toxicology. *Toxicol Sci* 2011; **119**:3-19.
4. Dimmeler S, Zeiher AM. Nitric oxide and apoptosis: another paradigm for the double-edged role of nitric oxide. *Nitric Oxide* 1997; 1:275-81.
5. Snyder SH, Bredt DS. Biological roles of nitric oxide. *Sci Am* 1992; **266**:68-71, 74-7.
6. Simmons ML, Murphy S. Induction of nitric oxide synthase in glial cells. *J Neurochem* 1992; **59**:897-905.
7. Magalhaes CR, Socodato RE, Paes-de-Carvalho R. Nitric oxide regulates the proliferation of chick embryo retina cells by a cyclic GMP-independent mechanism. *Int J Dev Neurosci* 2006; **24**:53-60.
8. Gordon, S. Alternative activation of macrophages. *Nat Rev Immunol* 2003; **3**:23-35.
9. Dalton DK, Pitts-Meek S, Keshav S, Figar IS, Bradley A, Stewart TA. Multiple defects of immune cell function in mice with disrupted interferon-gamma genes. *Science* 1993; **259**:1739-42.
10. Ehrt S, Schnappinger D, Bekiranov S, Drenkow J, Shi S, Gingeras T.R, Gaasterland T, Schoolnik G, Nathan C. Reprogramming of the macrophage transcriptome in response to interferon-gamma and Mycobacterium tuberculosis: signaling roles of nitric oxide synthase-2 and phagocyte oxidase. *J Exp Med* 2001; **194**:1123-40.
11. Albina JE, Cui S, Mateo RB, Reichner JS. Nitric oxide-mediated apoptosis in murine peritoneal macrophages. *J Immunol* 1993; **150**:5080-5.
12. Hortelano S, Alvarez AM, Bosca L. Nitric oxide induces tyrosine nitration and release of cytochrome c preceding an increase of mitochondrial transmembrane potential in macrophages. *FASEB J* 1999; **13**:2311-7.
13. Brockhaus F, Brüne B. p53 accumulation in apoptotic macrophages is an energy demanding process that precedes cytochrome c release in response to nitric oxide.*Oncogene* 1999; **11**; 18:6403-10.

14. Lei K, Nimnual A, Zong WX, Kennedy NJ, Flavell RA, Thompson CB, Bar-Sagi D, Davis RJ. The Bax subfamily of Bcl2-related proteins is essential for apoptotic signal transduction by c-Jun NH(2)-terminal kinase. *Mol Cell Biol* 2002; **22**:4929-42.

15. Messmer UK, Reimer DM, Reed JC, Brüne B. Nitric oxide induced poly(ADP-ribose) polymerase cleavage in RAW 264.7 macrophage apoptosis is blocked by Bcl-2. *FEBS Lett* 1996; **15**;384:162-6.

16. Moncada S. The L-arginine: nitric oxide pathway, cellular transduction and immunological roles. *Adv Second Messenger Phosphoprotein Res* 1993; **28**:97-9.

17. Nathan C. Nitric oxide as a secretory product of mammalian cells. *FASEB J* 1992; **6**:3051-64.

18. Odegaard JI, Ricardo-Gonzales RR, Red Eagle A, Vats D, Morel CR, Goforth MH., Subramanian V, Mukundan L, Ferrante AW, Chawla A. Alternative M2 activation of Kupffer cells by PPARdelta ameliorates obesity-induced insulin resistance. *Cell Metab* 2008; **7**:496-507.

19. Alexander WS, Hilton DJ. The role of suppressors of cytokine signaling (SOCS) proteins in regulation of the immune response. *Annu Rev Immunol* 2004; **22**:503-29.

20. Block JA, Oegema TR, Sandy JD, Plaas A. The effects of oral glucosamine on joint health: is a change in research approach needed? *Osteoarthritis Cartilage* 2010; **18**:5-11.

21. Miller KL, Clegg DO. Glucosamine and chondroitin sulfate. *Rheum Dis Clin North Am* 2011; **7**:103-18.

22. Rafi MM, Yadav PN, Rossi AO. Glucosamine inhibits LPS-induced COX-2 and iNOS expression in mouse macrophage cells (RAW 264.7) by inhibition of p38-MAP kinase and transcription factor NF-kappaB. *Mol Nutr Food Res* 2007; **2051**:587-93.

23. Rajapakse N, Kim MM, Mendis E, Kim SK. Inhibition of inducible nitric oxide synthase and cyclooxygenase-2 in lipopolysaccharide-stimulated RAW264.7 cells by carboxybutyrylated glucosamine takes place via down-regulation of mitogen-activated protein kinase-mediated nuclear factor-kappaB signaling. *Immunology* 2008;**123**:348-57.

24. Yan Y, Wanshun L, Baoqin H, Changhong W, Chenwei F, Bing L, Liehuan C. The antioxidative and immunostimulating properties of D-glucosamine. *Int Immunopharmacol.* 2007; **7**:29-35.

25. Wang Z, Liang R, Huang GS, Piao Y, Zhang YQ, Wang AQ, Dong BX, Feng JL, Yang GR., Guo Y. Glucosamine sulfate-induced apoptosis in chronic myelogenous leukemia K562 cells is associated with translocation of cathepsin D and downregulation of Bcl-xL. *Apoptosis* 2006; **11**:1851-60.

26. Chesnokov V, Sun C, Itakura K. Glucosamine suppresses proliferation of human prostate carcinoma DU145 cells through inhibition of STAT3 signaling. *Cancer Cell International.* 2009; **9**:25.

27. Zhang, L, Liu, WS., Han, BQ, Peng, YF., Wang, DF. Antitumor activities of D-glucosamine and its derivatives. *J Zhejiang Univ Sci B* 2006; **7**:608-14.

28. Huser CA, Davies ME. Effect of a glucosamine derivative on impact-induced chondrocyte apoptosis in vitro. A preliminary report. *Osteoarthritis Cartilage* 2008; **16**:125-8.

29. Champattanachai V, Marchase RB, Chatham JC. Glucosamine protects neonatal cardiomyocytes from ischemia-reperfusion injury via increased protein-associated O-GlcNAc. *Am J Physiol Cell Physiol* 2007; **292**:C178-87.

30. Choi HJ, Kang OH, Park PS, Chae HS, Oh YC, Lee YS, Choi JG, Lee GH, Kweon OH, Kwon DY. Mume Fructus water extract inhibits pro-inflammatory mediators in lipopolysaccharide-stimulated macrophages. *J Med Food* 2007;**10**:460–6.

31. Mossman B T. In vitro approaches for determining mechanisms of toxicity and carcinogenecity by asbestos in the gastrointestinal and respiratory tracts. *Environ Health Perspect* 1983; **53** : 155 – 161.

32. Yang YM, Liu GT. Damaging effect of cigarette smoke extract on primary cultured human umbilical vein endothelial cells and its mechanism. *Biomed Environ Sci* .2004; **17**:121-34.

33. Ward C, Dransfield I, Chilvers ER, Haslett C, Rossi AG. Pharmacological manipulation of granulocyte apoptosis: potential therapeutic targets. *Trends Pharmacol Sci* 1999;**20**:503-9.

34. Gilroy DW, Lawrence T, Perretti M, Rossi AG. Inflammatory resolution: new opportunities for drug discovery. *Nat Rev Drug Discov* 2004; **3**:401-16.

35. Messmer UK, Brüne B. Nitric oxide (NO) in apoptotic versus necrotic RAW 264.7 macrophage cell death: the role of NO-donor exposure, NAD+ content, and p53 accumulation. *Arch Biochem Biophys*. 1996; **327**:1-10.

36. Borutaite V, Brown GC. Nitric oxide induces apoptosis via hydrogen peroxide, but necrosis via energy and thiol depletion. *Free Radic Biol Med* 2003; **1**; 35:1457-68.

37. De Las Heras B, Hortelano S, Girón N, Bermejo P, Rodríguez B, Boscá L. Kaurane diterpenes protect against apoptosis and inhibition of phagocytosis in activated macrophages. *Br J Pharmacol* 2007; **152**:249-55.

38. Tsi CJ, Chao Y, Chen CW, Lin WW. Aurintricarboxylic acid protects against cell death caused by lipopolysaccharide in macrophages by decreasing inducible nitric-oxide synthase induction via IkappaB kinase, extracellular signal-regulated kinase, and p38 mitogen-activated protein kinase inhibition. *Mol Pharmacol* 2002; **62**:90-101.

39. Jean-Baptiste EJ. Cellular mechanisms in sepsis. *Intensive Care Med* 2007; **22**:63-72.

40. Calmels S, Hainaut P, Ohshima H. Nitric oxide induces conformational and functional modifications of wild-type p53 tumor suppressor protein.*Cancer Res.* 1997; **57**:3365-9.

41. Messmer UK, Reimer DM, Reed JC, Brüne B. Nitric oxide induced poly(ADP-ribose) polymerase cleavage in RAW 264.7 macrophage apoptosis is blocked by Bcl-2. *FEBS Lett* 1996; **15**;384:162-6.

42. Hortelano, S., Alvarez, A.M, Bosca, L. Nitric oxide induces tyrosine nitration and release of cytochrome c preceding an increase of mitochondrial transmembrane potential in macrophages. *FASEB J* 1999; **13**:2311-7.

43. Haas, M.J., Wong, N.C., Mooradian, A.D. Effect of glucosamine on apolipoprotein AI mRNA stabilization and expression in HepG2 cells. *Metabolism* 2004; **53**:766-7