The Effect of L-NAME Treatment on Leptin Associated Retinal Nitrosylation in Hypercarbic Oxygen Induced Retinopathy in Newborn Rats

Yenidoğan Sıçanlarda Hiperbarik Oksijenle İndüklenen Retinopatide Leptin İlişkili Retinal Nitrozilasyon Üzerine L-NAME Tedavisinin Etkisi

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ABSTRACT Objective: To investigate the biochemical effects of leptin and leptin plus L-NAME on retinal nitrotyrosine, malondialdehyde, and superoxide dismutase levels in a model of hypercarbic oxygen induced retinopathy in rats. **Material and Methods:** Forty newborn rat pups were divided into four groups consisting of leptin, leptin plus L-NAME, hyperoxia and sham groups. All groups, except the sham group, were exposed to ten minutes of hypercarbia followed by ten minutes of hyperoxia twice a day with an interval of 30 minutes. This protocol was performed for three days and then pups were sacrificed. The retinas were dissected and retinal nitrotyrosine, superoxide dismutase and malondialdehyde levels were measured. **Results:** The highest retinal nitrotyrosine levels were measured in the leptin group followed by the hyperoxia group ($p< 0.05$). Leptin plus L-NAME and the sham group showed similar results, indicating an antioxidative role for L-NAME ($p< 0.05$). Superoxide dismutase levels were also lower in the Leptin plus L-NAME group when compared to the hyperoxia group ($p< 0.05$). **Conclusion:** This study showed that leptin-induced retinal oxidation may be prevented by nitric oxide synthase inhibitors. We showed the protective role of L-NAME against nitric oxide mediated peroxynitrite toxicity. Further research should be carried out to investigate the role of nitrotyrosine on oxygen induced retinopathy.

Key Words: Leptin; NG-nitroarginine methyl ester; nitric oxide; retinopathy of prematurity

ÖZET Amaç: Sıçanlarda hiperbarik oksijenle indüklenen bir retinopati modelinde leptin ve leptin artı L-NAME'nin retinal nitrotirozin, malondialdehid ve süperoksit dismutaz seviyeleri üzerine biyokimyasal etkilerini araştırmak. **Gereç ve Yöntemler:** Kırk yenidoğan sıçan yavrusu leptin, leptin artı L-NAME, hiperoksi ve plasebo olarak dört gruba ayrıldı. Plasebo grubu hariç tüm gruplar günde ikişer kez 30 dakika arayla on dakikalık hiperkarbi sonrası on dakika hiperoksik duruma tabi tutuldular. Bu protokol üç gün uygulandıktan sonra sıçan yavruları sakrifiye edildiler. Retinaları diseke edilerek retinal nitrotirozin, süperoksit dismutaz ve malondialdehid düzeyleri ölçüldü. **Bulgular:** En yüksek retinal nitrotirozin düzeyleri leptin grubunda ve ikinci olarak da hiperoksi grubunda belirlendi ($p< 0.05$). Leptin artı L-NAME ve plasebo grubunda benzer sonuçlar elde edildi, bu da L-NAME'nin antioksidatif bir rolünün olduğuna işaret etmektedir ($p< 0.05$). Aynı zamanda süperoksit dismutaz düzeyleri de leptin artı L-NAME grubunda hiperoksi grubunda daha düşüktü. **Sonuç:** Bu çalışma sonucunda leptinle indüklenen retinal oksidasyonun nitrik oksit sentaz inhibitörlerince önlenebileceği gösterildi. Biz L-NAME'nin nitrik oksit aracılı peroksinitrit toksisitesine karşı koruyucu rolü olduğunu gösterdik. Oksijene bağlı retinopatide nitrotirozinin rolünün araştırılması amaçlı ileri çalışmalara gerek bulunmaktadır.

Anahtar Kelimeler: Leptin; NG-nitroarjinin metil ester; nitrik oksid; prematürite retinopatisi

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Retinopathy of prematurity (ROP) has been a topic of research among ophthalmologists.[1] The disease is characterized by the obliteration of vessels followed by pathological intravitreal neovascularization.[2] It

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is a significant problem leading to irreversible blindness and responsible for a large proportion of visual loss, especially in developed countries.[1]

Oxygen may be responsible for this disease, as is in many pathological processes.[3,4] When it was noticed that premature babies exposed to hyperbaric oxygen therapy were more likely to develop the disease, a special role has been attributed to oxygen toxicity. This finding has led to the use of oxygen to create ROP animal models.[5] Neonatal rats are extensively used as an experimental oxygen-induced retinopathy (OIR) model to investigate oxygen dependent proliferative retinopathy.[6,7] Sometimes carbondioxide is also added to this hyperoxic environment to strenghten the model, which is called as hypercarbic OIR.[8,9] Exposure to high concentrations of oxygen to animal models has successfully generated an environment of vitreoretinal proliferations similar to that seen in ROP.[7]

Recently many researches have focused on nitric oxide (NO) metabolism as well as oxidative stress in the pathogenesis of OIR.[10-12] NO is a unique molecule acting in many physiological and pathological processes. Moreover, leptin is a hormone reported to have a possible cytoprotective effect in ischemia reperfusion injury.[13,14]

Although many studies exist about OIR, none of them has described the role of NO, leptin and L-NAME in oxygen-dependent retinopathy. The principal topic of this research was to reflect some perspective into the biochemistry of oxygen-related retinopathy. We have investigated whether nitrotyrosine, an oxidative stress marker, has some impact on OIR and whether leptin hormone affects this pathological phenomenon. To test those effects, we designed a rat model of hypercarbic OIR in this study.

MATERIAL AND METHODS

All experiments were approved by the institutional Committee for Animal Use in Research and Education and conformed to the Statement for the Use of Animals in Ophthalmic and Vision Research (ARVO). Oxygen-induced retinopathy was induced in newborn mice starting from the first day according to the method described below. All rat pups were weighed and recorded. Forty pups were randomly grouped into four groups. Sham group and hyperoxygenation group did not take any medication. Leptin group was given leptin hormone in a dose of 20 μg/kg and leptin+L-NAME group was given 20 μg/kg leptin and 25 mg/kg L-NAME, both intraperitoneally.

To create oxygen induced retinopathy, all groups were put into custom built chambers. The chambers were made up of transparent glass with a cover having two ports for the pipes coming from the oxygen and CO_2 tubes. The chambers were evacuated off air first and then filled with 100% CO_2 for 10 minutes. During this stay, bluish color change of pup skin was confirmed visually, representing a true hypoxic environment for the subjects. After this, CO_2 was evacuated and 100% oxygen was given to the chamber and the pups breathed high oxygen concentration for the next 10 minutes. This allowed the pup skins to gain a pink hue. The pups were taken out and allowed to stay in room air for 30 minutes. After this, the cycle of hypercarbia followed by hyperoxia was repeated for the next 20 minutes once again. This protocol was applied for three days in all groups except the sham group. The same protocol was applied to the sham group but the chambers were given only normal room air. All rat pups were returned to their mother's cage after the experiment and kept in a normothermic environment (21°C- 23°C). They were allowed to receive breast milk and water ad libitum and kept under 12 hours of light and dark cycle. After the third day, the pups were decapitated, their retinas were dissected and prepared for investigation.

SAMPLE PREPARATION

All test specimens were washed with saline and were left to dry. Then the samples were homogenized in a phosphate buffered distilled water (0.01 mol/L, pH: 7.0) in 1:10 ratio (weight/volume, g/mL). After the samples were centrifugated at 8.000 rpm for 30 minutes. Supernatants were used for the biochemical analyses.

SUPEROXIDE DISMUTASE (SOD)

SOD activity was determined as described by Fridovich.[15] This method employs xanthine and xanthine oxidase to generate superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitro phenol-s-phenyl tetrazolium chloride) to form a red formazan dye. SOD activity is then measured with spectrophotometer in 230 nm (Shimatzu 1201-UV Japan) by the degree of inhibition of this reaction. Results were expressed in unit/mg-protein for SOD.

MALONDIALDEHYDE (MDA) MEASUREMENT

The degree of lipid peroxidation in tissue homogenates was assessed by the method of Ohkawa et al.[16] The principle of this method is based on measuring the concentration of pink chromogen compound that forms when malondialdehyde couples to thiobarbituric acid. Color solution was measured with spectrophotometer in 532 nm. Results were calculated in nanomoles of MDA per milligram of protein.

PROTEIN MEASUREMENT

The protein content of homogenates was determined with spectrophotometry according to the procedure of Lowry et al.[17]

NITROTYROSINE MEASUREMENT

Nitrotyrosine levels were measured with enzyme linked immunosorbent assay (ELISA) (BIOXYTECH-OXIS International Portland, USA). This method is principally a sandwich ELISA in which antigen captured by a solid phase monoclonal antibody is detected with a biotin-labeled goat polyclonal anti-nitrotyrosine. A streptavidin peroxidase conjugate then binds to the biotinylated antibody. The tetramethylbenzidine substrate is added and the yellow product is measured at 450 nm. Absorbance values were calculated against to standard curve as nanomoles of nitrotyrosine and the results were given as nanomoles per mg of protein.

STATISTICS

Data were expressed as median (minimum-maximum). The statistical analyses were done using Kruskal Wallis analysis of variance and Mann-Whitney U test, as appropriate. A threshold value of $p< 0.05$ was taken as significant.

RESULTS

The weight of the pups was between 7.1-8.0 gr and was similar among the groups ($p> 0.05$). All pups in the study completed the experiment. The median nitrotyrosine level was the highest in leptin group, which was 2.09 nmol/mg-prot ($p< 0.001$). Superoxide dismutase levels were the highest in sham group and the lowest in leptin group ($p< 0.001$). Leptin + L-NAME group was similar to sham group in nitrotyrosine, MDA and SOD levels ($p> 0.05$). The results are presented in the Table 1.

DISCUSSION

The SOD level was lower and MDA level was higher in hyperoxia and leptin groups in our study. MDA level is an indicator of lipid peroxidation in the tissue, derived from oxygen radicals. We produced oxidative damage in the retina of rat pups in our experimental design. The radicals generated in the environment, attack molecules and deplete antioxidant enzymes such as superoxide dismutase, and lead to the failure of scavenging capacity.

TABLE 1: The median levels (minimum-maximum) of SOD, nitrotyrosine and MDA according to the groups.

Groups	SOD U/mg prot	Nitrotyrosine nmol/mg-prot	MDA nmol/mg-prot
Sham	2858.02 (2498.12-3157.28)	0.17 (0.11-0.25)	9.62 (7.93-17.79)
Hyperoxia	801.70 (605.74-850.52) *p< 0.001	0.86 (0.34-1.37) *p< 0.001	34.40 (19.24-58.93) *p< 0.001
Leptin	443.18 (384.37-477.94) *p< 0.001 ¥p< 0.001	2.09 (1.94-2.90) *p< 0.001 ¥p< 0.001	53.60 (27.80-74.36) *p< 0.001 ¥p= 0.042
Leptin+L-NAME	2275.32 (1409.55-2706.04) §p> 0.05	0.23 § (0.20-0.33) §p> 0.05	12.62 § (8.89-27.85) §p> 0.05

* significant from the sham group,
¥ significant from the hyperoxia group,
§ nonsignificant from the sham group,
SOD: Superoxide dismutase,
MDA: Malondialdelnyde.

We found that nitrotyrosine levels were 10-fold higher in leptin group compared to the sham group, a result which is very striking. In this study, leptin-aggravated nitrosative stress in rat pups subjected to hyperoxia, indicating retinal toxicity caused by NO metabolism. Leptin stimulates NO production in endothelial cells by interacting with its receptor on the cell membrane.[18-21] Studies also indicated that leptin significantly increased intracellular reactive oxygen radical formation in endothelial cells and caused endothelial damage through oxidative stress.[22-24] Under the existence of increased oxyradicals, nitric oxide undergoes a rapid reaction with superoxide radicals to form peroxynitrite, which may be another mediator of oxidative stress. In ischemic conditions where both substrates are present in favorable concentrations, the formation of peroxynitrite is augmented. Peroxynitrite has numerous potential pathological consequences on ocular blood vessels and tissues such as vasospasm, platelet aggregation and lipid peroxidation, all of which result in impaired circulation and vasoobliteration.[25] Hence, in cooperation with other oxidants, it may take part in the pathogenesis of ischemic proliferative retinopathies such as ROP. The peroxynitrite anion also reacts rapidly with carbon radicals to increase tissue damage, which may be a cofactor for retinal damage such as the case in our study.

We have found that leptin is a stress inducer hormone under hyperoxic conditions in prematurely developed retina. It increased tissue toxicity in our study. However, controversial reports also exist about the beneficial role of leptin. Leptin is reported to have a protective effect against damage induced by ischemia reperfusion injury in the gastrointestinal system.[13] This may be attributed to the tissue specific effects of leptin.[26,27]

We showed that stimulated NO synthesis in a media rich of oxygen increased damage while the introduction of L-NAME decreased retinal nitrosylation significantly. L-NAME showed antioxidative effect, which was also reflected by the SOD levels. L-NAME is a structural analogue of L-arginine and it inhibits the production of NO through competition with L-arginine. The protective role of L-NAME has been reported by other authors.[12] NO mediated damage of leptin is suggested to be inhibited by L-NAME administration.[7,18,19,23]

The high production rate of oxgen radicals and peroxynitrite will lead to the failure of antioxidative capacity, and the protein attack of peroxynitrite produces protein tyrosine nitration.[28] Nitrotyrosine is a biomarker of reactive nitrogen species and an indicator of peroxynitrite formation.[12,29] It helps track the formation of reactive nitric oxide-derived species in diseases.[9,12,30] In this aspect, the high levels of nitrotyrosin in leptin and hyperoxia group found in our study reflects retinal damage.

Oxygen induced retinopathy is mediated by oxygen radicals and is similar to pathological events taking place in ROP. Adding hypercarbic therapy to this model was also proven to be successful for stimulating retinal neovascularization.[5,8] Although hyperoxia may still be the triggering insult, hypercarbia due to the reduced CO_2 removal can also be a confounding factor exacerbating the pathology.[8,9] We used a hypercarbic model of OIR in our experiments and succeeded to produce retinal damage.

Some questions may arise regarding to our study. We used 100% pure oxygen and carbondiondioxide for a short time to create an environment rich in oxygen and carbondioxide, simulating the medium seen during the OIR. Although we lack definite evidence that our design support OIR, we can assume that our model reflects some parts of this proliferative retinopathy accurately. Leske et al. tried to generate such an environment in their experiment by using relatively low levels of oxygen and carbondioxide but with a higher interval.[8]

Our primary focus was leptin-associated biochemical stress and retinal nitrosylation. To our best knowledge, this study is the first in this aspect. Suganami et al. suggested in their report that leptin antagonism might be a new approach for the prevention of ROP.[14] When leptin deficient mice were exposed to high concentrations of oxygen , extensive neovascularization developed; an experi-

ment indicating the provocative role of leptin in vascular proliferative retinopathies. In account with our findings, leptin antogonism may offer some help in of retinopathy prematurity.[14,31]

As an extension of our study, morphological alterations may be investigated concurrently with biochemical analyses. In this way, it would be better to show the biochemistry and histology at the same time and we would be able to correlate biochemical events to morphological changes. Further analyses are required to reach more decisive results about the pathophysiology of the disease.

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