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Effect of Liver Ischemia-Reperfusion Injury on Genotoxicity in Rabbits: Experimental Study

Tavşanlarda Karaciğer İskemi-Reperfüzyon Hasarının Genotoksisite Üzerine Etkisi: Deneysel Çalışma

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ABSTRACT Objective: Ischemia-reperfusion (I-R) injury is defined as the paradoxical exacerbation of cellular dysfunction and death following restoration of blood flow to ischemic tissues. In our study, it was aimed to examine the potential DNA injury effects of liver IR injury with an experimental animal model. **Material and Methods:** In the study, modeling was done with seven male New Zealand rabbits. Blood samples were taken before the experimental IR model, 30 minutes after ischemia, and 60 minutes after reperfusion. The DNA damage in the blood of the rabbits was measured using Tail Length, Intensity, and Moment techniques. Statistical significance was determined using one-way analysis of variance (ANOVA) and Tukey's post hoc test. **Results:** There are significant differences between control-ischemia, control-reperfusion and I-R groups in all 3 measurements. Tail length; increased by 51.84%, 54.16% after ischemia and reperfusion, respectively. Tail length increased by 134.09% between control and reperfusion. Similarly, tail density and tail moment were increased by 78.95% (after ischemia), 77.96% (after reperfusion), 85.54% (after ischemia), 165.52% (after reperfusion) respectively. **Conclusion:** Tissue blood flow disruption is known to occur tissue hypoxia that triggers anaerobic respiration. Restoring blood flow to a hypoxic-tissue results in an increase in reactive oxygen species production. Literature stated I/R-related DNA damage may result from the formation of oxygen radicals during the reperfusion period. Moreover, it induces oxidative damage and exceeds the antioxidative capacity of circulating leukocytes, leading to DNA damage. In our study, DNA lesions characteristic of DNA damage mediated by free radicals were detected at a significantly increased level during reperfusion.

Keywords: Comet assay; DNA damage; ischemia; reperfusion injury; rabbits

ÖZET Amaç: İskemi-reperfüzyon (I-R) hasarı, iskemik dokulara kan akışının yeniden sağlanmasını takiben, hücresel işlev bozukluğunun ve ölümün paradoksal alevlenmesi olarak tanımlanır. Çalışmamızda, deneysel hayvan modeli ile karaciğer I-R hasarının potansiyel DNA hasarı etkilerinin incelenmesi amaçlanmıştır. **Gereç ve Yöntemler:** Çalışmada, 7 adet erkek Yeni Zelanda tavşanı ile modelleme yapılmıştır. Deneysel I-R modeli öncesi, iskemi sonrası 30. dk ve reperfüzyon sağlandıktan 60 dk sonra kan örnekleri alınmıştır. Tavşanların kanlarındaki DNA hasarı Kuyruk Uzunluğu, Yoğunluk ve Moment teknikleri kullanılarak ölçüldü. İstatistiksel anlamlılık, tek yönlü varyans analizi (ANOVA) ve Tukey'in post hoc testi kullanılarak belirlendi. **Bulgular:** Her 3 ölçümde hem kontrol-iskemi hem kontrol-reperfüzyon hem de I/R grupları arasında anlamlı farklılıklar vardır. Kuyruk uzunluğu; iskemi ve reperfüzyon sonrasında sırasıyla %51,84 ve %54,16 artmıştır. Kuyruk uzunluğu, kontrol ve reperfüzyon arasında %134,09 artmıştır. Benzer şekilde, kuyruk yoğunluğu ve kuyruk momenti iskemi ve reperfüzyon sonrası sırasıyla %78,95 (iskemi sonrası), %77,96 (reperfüzyon sonrası) ve %85,54 (iskemi sonrası), %165,52 (reperfüzyon sonrası) artış göstermiştir. **Sonuç:** Doku kan akımının bozulmasının anaerobik solunumu tetikleyen doku hipoksisi oluşturduğu bilinmektedir. Hipoksik bir dokuya kan akışının yeniden sağlanması reaktif oksijen türlerinin üretiminde artışa neden olur. Literatürde, I-R ile ilişkili DNA hasarı, reperfüzyon periyodu sırasında oksijen radikallerinin oluşumundan kaynaklanabilir. Ayrıca oksidatif hasara neden olur ve dolaşımdaki lökositlerin antioksidan kapasitesini aşarak DNA hasarına yol açar. Çalışmamızda, serbest radikallerin aracılık ettiği DNA hasarının özelliği olan DNA lezyonlarının, reperfüzyon sırasında önemli ölçüde arttığı tespit edilmiştir.

Anahtar Kelimeler: Comet assay; DNA hasarı; iskemi; reperfüzyon hasarı; tavşanlar

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The goal of treating ischemia-reperfusion (I-R) injury is to limit cell death and keep organs functioning normally. Tissue hypoperfusion is what we mean when we talk about ischemia. Tissue hypoperfusion can be brought on by a number of different medical issues, including sepsis.[1] Ischemia, and hypoperfusion can be facilitated by a number of physiological factors, acute myocardial infarction.[2] Reduced oxygen levels and mitochondrial dysfunction result from obstructed arterial blood flow. Anaerobic metabolism, malfunction of sodium-potassium pumps, and ribosome segregation are all brought on by a drop in mitochondrial adenosine triphosphate (ATP) synthesis. A decline in ATP and antioxidant material synthesis results from anaerobic metabolism. Metabolic acidosis is another possible outcome of lactic acid storage. There might also be issues with the cell surface pumps that transport sodium and potassium (Na+-K+-ATPase pumping) and calcium (Ca2+-ATPase pumping). When the Na+-K+-ATPase valves stop working, the cell retains sodium and releases potassium. When the amount of sodium inside a cell rises, Na+-H+ pumps become less efficient. Water enters the cytoplasm as a result of hyperosmolarity caused by the accumulation of sodium, calcium, and hydrogen ions, leading to cell enlargement. Reduced cellular pH caused by hydrogen retention inhibits enzyme activity and causes nuclear chromatin to aggregate. Red blood cells deliver oxygen after the reperfusion phase has been completed in ischemic tissue. At the same time, a decline in antioxidant levels in ischemia cells leads to an increase in reactive oxygen species (ROS) generation. Inducing endothelial dysfunction, DNA damage, and local inflammatory responses, ROS are the root cause of oxidative stress. Damage to cellular structures, brought on by inflammatory cascades and oxidative stress, can trigger a cytokine storm, leading to cell death.[2]

The reperfusion period is quite variable and may last many days. The potential for new treatment avenues and injury prevention is enhanced if we have a firm grasp on the intricate mechanisms that underlie I-R damage.

Eukaryotic cell DNA damage may be assessed rapidly and accurately using the comet test (single-cell gel electrophoresis SCG or SCGE test). It is predicated on the observation that electrophoresis may be used to quantify the amount of damaged DNA that leaves a cell's nucleus.[3] The operator can choose from a number of available algorithms that will calculate the comets' fluorescence properties. Tail length, intensity, moment and head intensity (usually measured as a percentage of DNA in tail), are the most often used parameters.[4]

Our primary objective in doing this work was to employ the Comet test to identify DNA breaks brought on by ischemia and reperfusion.

MATERIAL AND METHODS

SAMPLE COLLECTION AND LYMPHOCYTE ISOLATION

The Animal Experiments Ethics Committee of Ankara SUAM gave their consent to this investigation (date: July 1, 2022, no: 698). Ischemia reperfusion was performed on 7 male, white New Zealand Rabbits (Oryctolagus cuniculus L.). First, EDTA tubes were used to preserve blood from a healthy control group. After that, the Treitz ligament were dissected and the abdomen was opened. After the liver was dissected, ischemia was induced by clamping the portal triangle. After 15 minutes of ischemia, the clamp was released and post-ischemia blood was taken immediately. After 30 minutes have passed after the reperfusion blood was obtained, the rabbits were euthanized. The blood was sent to a laboratory as soon as possible and stored at $+4^0$C. Then, employing density gradient centrifugation, the lymphocytes were separated.[5]

COMET ASSAY

Singh et al. detailed the use of a slightly modified alkaline Comet test for DNA damage detection.[6] We also used this method in our study. At 37 degrees Celsius, 100 of lymphocytes combined with 1% low melting agarose, and then dispersed over a slide that had been coated with 1% normal melting agarose. For each sample, 2 slides were analyzed. Slides were stored in lysis solution at 4°C for a night after gel solidification. The DNA on the slides was dissolved by incubating them for 15 minutes in a cold

electrophoresis solution. The electrophoresis was run for 20 minutes at 4 degrees Celsius, 25 volts, and 300 milliamperes. After being washed with neutralization buffer, the slides were coated with ethidium bromide. A total of 100 cells per slide were chosen at random and viewed using a fluorescent microscope with a Comet assay III image analysis system for image processing.

STATISTICAL ANALYSES

The DNA damage in the blood of the rabbits was measured using tail length, intensity, and moment techniques. These measurements were made for each group (control, ischemia, reperfusion), median values were taken, and their percentage increases were noted. Statistical significance was determined using one-way analysis of variance (ANOVA) and Tukey's post hoc test. The statistical software utilized was IBM SPSS U.S.A. Statistics v22.0.

RESULTS

There are significant differences between control-ischemia, control-reperfusion and I-R groups in all 3 measurements. Tail length; increased by 51.84% (p=0.0001<0.01) and 54.16% (p=0.0001, p<0.01), respectively, after ischemia and reperfusion. Tail length increased by 134.09% (p=0.0001, p<0.001) between control and reperfusion. Similarly, tail density and tail moment were 78.95% after ischemia and reperfusion, respectively; 77.96% (p=0.0001, p<0.01) and 85.54%; increased by 165.52% (p=0.0001, p<0.01). An increase of 232.03% (p=0.0001, p<0.01) was detected between control and reperfusion. There was an increase of 372.54% (2.95 -13.94) between control and reperfusion (p=0.0001, p<0.01) (Table 1).

TABLE 1: The mean tail length, intensity, and moment values.

	Control (no injury) group (n=7)			First injury (hypoxia) (n=7)			Reperfusion (n=7)			
	Median	Minimum-maximum	Mean±SS	Median	Minimum-maximum	Mean±SS	Median	Minimum-maximum	Mean±SS	p value
Tail lenght mean	72.25	48.27-110.81	78.66±22.7	122.47	84.72-147.09	119.44±22	175.77	136.4-229.39	184.14±34.9	0.0001
Tail intensity mean	6.52	4.7-13.18	7.46±2.8	13.86	8.55-17.85	13.35±3.4	24.64	18.35-31.18	24.77±4.5	0.0001
Tail moment mean	2.06	1.99-7.92	2.95±2.2	4.62	3.89-7.75	5.25±1.4	13.38	10.86-19.57	13.94±3.5	0.0001

DISCUSSION

Tissue hypoxia, which causes anaerobic respiration, is known to affect blood flow to tissues. Inflammation brought on by trauma, I-R injury, or chemically produced injury is called "sterile inflammation" when no germs are present. Overproduction of reactive nitrogen species (RNS) and ROS during I-R damage can be detrimental, leading to symptoms including severe cytotoxicity and even multiorgan system failure.[7] It is possible for multisystem organ failure to develop if the inflammatory mediators like ROS and RNS, interleukin (IL)-1, and tumor necrosis factor, as well as IL-6 are not neutralized. It is well established that ROS and RNS may cause cellular damage and alter pathways of signaling in organs.[8] Restoring blood flow to a hypoxic tissue causes pathways to activate, leading to an increase in ROS generation due to the increased availability of molecular oxygen. Endothelial cell (EC), H/R-exposed monolayers had also demonstrated for being exceptionally accurate at replicating the diverse microvascular changes induced by reperfusion, including 1) an increased synthesis of ROS; 2) endothelial cells' elevated adherence to leukocytes as a result of elevated adhesion molecule expression, 3) decreased EC barrier function and 4) procoagulant/prothrombotic phenotype development.[9-12] It has become widely recognized that patients who experience a transient blood circulation interruption to a single tissue or several organs as a result of a medical procedure (such as

donor organs and thrombolytic treatment) or disease response may be at risk for reperfusion injury, which may slow their functional recovery.[13]

Oxidative damage to tissues after I-R has been connected to many ROS sources. Non-enzymatic ROS sources including hemoglobin and myoglobin have attracted considerable attention as potential inflammation molecules after reperfusion.[14] However, most investigations have linked the enhanced ROS generation in post-ischemic tissues to one or more enzymes able to convert molecular oxygen to create superoxide (and hydrogen peroxide) and subsequently release ROS into extracellular or intracellular compartments. A review of the scientific literature reveals that the enhanced ROS generation in postischemic tissues is most commonly attributed to the actions of the enzyme complexes xanthine oxidase, the electron transport chain in mitochondria, and unattached nitric-oxide synthase.[15]

Thiol/disulfide transfers, simple transfer of electrons incidents, and radical processes are all examples of redox activities that occur all over the cell and are essential for energy metabolism, inflammatory defenses of the host, and other cellular activities. In order to maintain optimal health, a living cell must constantly monitor, regulate, and protect its internal redox balance. However, I-R damage causes oxidative stress due to a lack of antioxidant defense systems and an increase in ROS.[16] Overproduction of ROS threatens the integrity of proteins, lipids, and DNA, and can cause tissue damage through peroxidation of membrane lipids, oxidation of proteins, and DNA molecule breaks. ROS play an important role in innate immunity as a defense mechanism.[17,18]

During intracellular signal transduction, ROS are used as secondary messengers by a variety of growth factors, cytokines, hormone modifications, and neurotransmitters.[19] The antioxidant element-targeting nuclear factor-E2-related factor 2 pathway, ROS detection, and the nuclear factor-kappa B (NF-KB) and activator protein-1 transcription factor families are all examples of redox-sensitive transcription factors. Redox regulation of transcription factor activation and/or modified DNA-binding as a result of oxidative modifications to the protein that acts as a transcription factor may be the mechanisms for altered transcription factor control, as oxidative damage to the DNA may reduce transcription factor binding to promoter regions.[20-24] Finally, the great reactivity of ROS means that, despite their physiological benefits, they may also cause damage, mutations, and even cancer. Therefore, proteins, lipids, and DNA-the 3 major types of biomolecules-are all vulnerable to destruction by ROS/RNS. DNA single strand breakage, endothelial cell damage, enhanced microvascular permeability, and neutrophil recruitment are only some of the proinflammatory consequences of superoxide anion.[25] Leukotriene B4 formation, neutrophil recruitment at the site of inflammation, lipid peroxidation, and DNA damage, as well as the release of cytokines, DNA harm and lipid peroxidation.[26-30] To contrast, High-mobility group (HMG) box molecules are able to zero in on specific DNA sites in chromatin by either recognition of preexisting DNA structures or protein-protein interactions. The HMG box molecule prevents cytokine activity by stimulating the release of proinflammatory cytokines from macrophages. In addition to its role as a chromatin-binding factor, the highly conserved nuclear protein HMG box 1 also causes DNA to curve and encourages the assembly of protein complexes involved in transcription at particular sites on the genome. Because it builds up near the locations of oxidative DNA harm in cells, HMGB1 is also an element of the first phase of the response to DNA damage.[31,32]

Although we did not examine the exact mechanisms of DNA damage, we found a dramatic increase in DNA lesions indicative of DNA damage mediated by free radicals regardless of the mechanisms, during reperfusion.

The creation of ROS is held responsible for the harm. Reperfusion damage can be avoided with careful detection of ROS origins.

Many biological origins of ROS generation have been identified, and their possible functions have been extensively studied in I-R (H/R)-exposed tissues and cells. In the beginning of this field's investigation, XO was thought to be the primary reservoir of ROS following I-R. The discovery of this enzyme's low to negligible expression or activity in

additional organs allowed researchers to investigate the role of other possible sources of ROS, such as Nox and mitochondria. Reperfusion injury has also been linked to free NOS and other enzymes that were not studied extensively in the original research. There is evidence in the literature to suggest that some sources of ROS formation are more important than others at certain times in some tissues, (such as gut, a metabolically engaged heart, and mitochondria in the brain), we cannot make definitive judgments at this time.[33]

The few studies that looked at how the body reacts to I-R with risk variables present found that reperfusion caused more ROS production and tissue damage. Whether or whether the proportional contributions of various ROS-producing proteins to I-R-induced oxidative harm are affected by the existence or lack of a risk indicator for cardiovascular disease remains to be determined. It's not just the field of reperfusion injury research that has this problem, though, it will be challenging for future researchers to enhance experimental models such that they better reflect the realities encountered by a large number of people with reperfusion damage. More research into this issue is required to find innovative ROS-targeted drugs for treating reperfusion injury effectively and paving the way for major breakthroughs in the translation of experimental results into clinical practice.

CONCLUSION

As a result, we can say that in vivo I-R damage can cause oxidative DNA damage in lymphoid cultured cells under laboratory conditions. More study is needed to determine whether antioxidants may prevent this damage and to learn the connection between I-R damage and DNA damage caused by oxidative stress.

Source of Finance

During this study, no financial or spiritual support was received neither from any pharmaceutical company that has a direct connection with the research subject, nor from a company that provides or produces medical instruments and materials which may negatively affect the evaluation process of this study.

Conflict of Interest

No conflicts of interest between the authors and / or family members of the scientific and medical committee members or members of the potential conflicts of interest, counseling, expertise, working conditions, share holding and similar situations in any firm.

Authorship Contributions

Idea/Concept: *Meltem Bektaş;* ***Design:*** *Çetin Kaymak;* ***Control/Supervision:*** *Ayşe Özcan;* ***Data Collection and/or Processing:*** *Ela Kadıoğlu;* ***Analysis and/or Interpretation:*** *Ela Kadıoğlu;* ***Literature Review:*** *Mert Nakip;* ***Writing the Article:*** *Meltem Bektaş;* ***Critical Review:*** *Ayşe Özcan;* ***References and Fundings:*** *Mehmet Çakırca;* ***Materials:*** *Mert Nakip.*

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