Dicle Tıp Dergisi/ Dicle Medical Journal (2016) 43 (4) :561-568 DOI10.5798/diclemedj.0921.2016.04.0729

ORIGINAL ARTICLE

The protective effects of acetyl L-carnitine which added into Histidine-Tryptophan-Ketoglutarate solution on donor uterus

Histidin-Triptofan-Ketoglutarat (HTK) solüsyonuna eklenen asetil L-karnitinin donör uterusu üzerindeki koruyucu etkileri

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Abstract
Objective: At the present times uterus transplantation is an alternative therapy for women with untreatable uterine-based infertility factors. Before transplantation, the donor organ must stored in some solutions, but they may not adequate for protection. In this study, we investigated the potential protective effects of acetyl L-carnitine, added into histidine-tryptophan-ketoglutarate (HTK) solution, on rat uterus.
Methods: We divided 24 female Wistar Albino rats into four groups (n=6). Their uterine tissues were stored into four different solutions at different periods at 4oC ; Group 1 HTK, 4 hours; Group 2. HTK + acetyl L- carnitine, 4 hours, Group 3. HTK, 24 hours; Group 4. HTK + acetyl L- carnitine, 24 hours. Then tissues from uterus were used for histological and biochemical examination.
Results: In the study the number of TUNEL positive cells in group 4 was lower than group 3. Biochemically evaluated TBARS and NOS levels were highest in group 3 however CAT level was highest in group 2.
Conclusion: In conclusion, addition of acetyl L-carnitine to HTK solution reversed the histological alterations after 24 hours cold storage on rat uterus.
Key word: acetyl L-carnitine, uterus, transplantation, infertility, antioxidant

Özet
Giriş: Günümüzde uterus transplantasyonu, tedavi edilemeyen uterus temelli infertilite faktörleri olan kadınlar için alternatif bir tedavidir. Transplantasyon öncesinde donör organların bazı solüsyonlarda saklanmalıdır ama bu solüsyonların sağladığı koruma yetersiz kalabilmektedir. Bu çalışmada Histidin Triptofan Ketoglutarat (HTK) solüsyonuna eklenen asetil L-karnitinin rat uterusu üzerindeki potansiyel koruyucu etkilerini araştırdık.
Yöntemler: 24 adet dişi Wistar Albino rat 4 gruba (n=6) ayrıldı. Uterus dokuları 4 farklı solüsyonda farklı periyotlarda 4oC de saklandı. 1.grup 4 saat HTK , 2. grup 4 saat HTK + asetil L-karnitin, 3. grup 24 saat HTK , 4. grup 24 saat HTK + asetil L-karnitin. Daha sonra uterustan alınan dokular histolojik ve biyokimyasal analizler için kullanıldı.
Bulgular: Çalışmada TUNEL pozitif hücre sayısının 4. grupta 3. gruptakinden daha az olduğu görüldü. Biyokimyasal olarak değerlendirilen TBARS ve NOS seviyelerinin 3. grupta en yüksek bununla birlikte CAT seviyelerinin ise 2. grupta en yüksek olduğu tespit edildi.
Sonuç: Sonuç olarak HTK solüsyonu içerisine eklenen asetil L-karnitin 24 saat soğuk saklama periyodunda histolojik değişiklikleri geri çevirdiği görülmüştür.
Anahtar kelimeler: asetil L-karnitin, uterus, transplantasyon, infertilite, antioksidan

Received:07 Nov 2016; Revised: 15 Nov 2016; Accepted:28 Nov 2016

INTRODUCTION

Nowadays most of the man and woman infertility reasons are treatable. Some uterine infertility factors such as absence of the uterus and implantation abnormalities are not curable [1,2]. For these patients in some countries surrogate motherhood is accepted, if their ovarian capacity is adequate. But in most countries this procedure is illegal because of religious, ethical and legal reasons [3,4]. Uterus transplantation is an alternative for uterine factor infertility treatment [5,6].

The first uterine transplantation from human to human was performed in Saudi Arabia in 2000 but the woman's body rejected the uterus. In 2013 the operation, performed in Turkey, was the world's first uterus transplant surgery providing pregnancy. But they terminated the pregnancy because of the failed gestational sac. In October 2014, for the first time, a healthy baby had been born after uterine transplantation, in Sweden [7-9]

Before transplantation it is important to maintain the cellular and functional ability of the organ. During transplantation there may be some changes occurring. The earliest reason that causes the organ damage is ischemia [10,11]. There are some protective solutions that provide the metabolism and vitality of the organ during the organ storage and decrease the ischemia-reperfusion damage after transplantation. The most common used solutions are; Ringer Lactate solution, Euro-Collins, Ross-Marshall, University of Wisconsin, Celsior and Kyoto, Histidine-tryptophan-ketoglutarate (HTK) solutions. Additionally, it was reported that acetyl L-carnitine is a strong antioxidant agent and free radical catcher. Because of this reason acetyl L-carnitine usage during transplant storage will have protective effect on ischemic process [12].

In this study we examined the potential protective effects of acetyl l-carnitine on rat uterus in cold stored HTK solution after 4 hours and 24 hours.

METHODS

Animals and experimental design

This study was performed in Experimental Research Centre of Ankara Education and Research Hospital. A total of 24 female Wistar Albino rats, weighing 200-250 gr, provided from Saki Yenilli Experimental Animal Product Laboratory, were used.

After rats were anesthetized with ketamine (50 mg/kg i.m.) uteruses were removed and rats were sacrificed by decapitation.

Animals were randomly divided into four groups (n=6) and uteruses stored into four different solutions at different periods; 1. HTK-4 hours, 2. HTK + acetyl L- carnitine (10-8 M) - 4 hours, 3. HTK - 24 hours, 4. HTK + acetyl L-carnitine (10-8 M) - 24 hours.

The samples from left uterine corns were fixed for 72 hours in 10 % neutral formaldehyde for histomorphological and Terminal Deoxynucleotidyl Transferase dUTP Nick End Labelling (TUNEL) method examinations. After routine histological methods paraffin blocks were prepared.

The samples from right uterine corns were washed into 0.9% NACI cold solution and stored at -80 °C until we performed biochemical analyses of Thiobarbituric Acid Reactive Substances (TBARS), Nitric Oxide Synthase (NOS) and Catalase (CAT).

TUNEL Assay

TUNEL method was used to assess DNA fragmentation in the cells. After deparaffinization and washing, 4 µm thick cross-sections were incubated with 20 µg/ml proteinase K (Roche Diagnostics, GmbH) at 37 °C for 25 minutes. Then samples were incubated with 3% hydrogen peroxide (Lab Vision, Fremont, USA) and Equilibration Buffer for 5 minutes at RT, respectively. TUNEL kit (77 µL Reaction Buffer, 33 µL TDT enzyme solution) (Millipore Apoptag Plus Peroxidase in Situ Apoptosis Detection kit) was applied for 60 minutes at 37 ˚C in the dark. Then Stop/Wash solution and Anti-Digoxin Peroxidase were

applied at RT, respectively. At last Diaminobenzidine (DAB) (147µL DAB Dilution Buffer, 3µL DAB Substrate) and Methyl Green were used for staining. Sections were evaluated under light microscope using a computer-supported imaging system to take photos using the Leica Q Vin 3 program.

Biochemical procedure

After uterine tissues removed and washed with %0.9 NaCl solution they stored at -80ºC. Tissues homogenised and get raw extracts were obtained by Heidolph DIAX900 homogenisator. Extracts were centrifuged and supernatants were reserved for enzyme and the other parameters till the experiment date under -80ºC.

TBARS Activity Assay

TBARS level was determined by method of Van Ye et al. [13]. The reaction was performed at pH 2–3 at 90 °C for 15 min. The sample was mixed with two volumes of cold 10% (w/v) trichloroacetic acid to precipitate protein. The precipitate was pelleted by centrifugation, and an aliquot of the supernatant was reacted with an equal volume of 0.67% (w/v) TBA in a boiling water bath for 10 min. After cooling, the absorbance was read at 532 nm.

NOS Activity Assay

NOS activity was assayed by method of Durak et al. [14]. Sample was first incubated with arginine for 1 h at 37˚C and then, colour development reaction with HCl, N-(1-naphthyl)-ethylenediamine and sulfanilic acid was performed for 30 min. A blank assay was also carried out without arginine in the first incubation step. Then, sample absorbance were read at 540 nm.

CAT Activity assay

CAT activity was determined according to the method of Aebi [15]. The principle of the assay is based on the determination of the H_2O_2 decomposition rate at 240 nm.

Statistical analyses

TUNEL positive cells were counted among the 300 cells in 6 different areas of 6 different sections for each group. Then the averages were taken for statistically analyses. Mann Whitney U test was done for evaluation of pairwise comparison of TUNEL and comparing binary groups of TBARS, NOS and CAT results. p values <0.05 were accepted as significant.

RESULTS

Light Microscope

In group 1, subepithelial inflammation was observed distinctly. There were inflammatory cells and apoptotic cells between surface epithelial cells, and apoptotic cells in gland epithelium and underlying stroma. There were a few plasma cells in the stroma (Fig 1a-c).

In group 2, subepithelial inflammation wasn't observed. There were apoptotic cells in gland epithelium and underlying stroma and a few plasma cells in the stroma (Fig 1d-f).

In group 3, subepithelial inflammation and granulation tissue were distinct. There were inflammatory cells and apoptotic cells between surface epithelial cells. The number of apoptotic cells that located in gland epithelium and underlying stroma, were more than the other groups. Plasma cells were numerous in stroma that was the evidence of the inflammation. Muscular integrity was destroyed with connective tissue (Fig 1g-i).

In group 4, subepithelial inflammation was less than group 1 and 3. There wasn't any granulation tissue. Apoptotic cells and inflammatory cells in gland epithelium were less than the other groups. Also plasma cells in stroma were less than the other groups (Fig 1j-l).

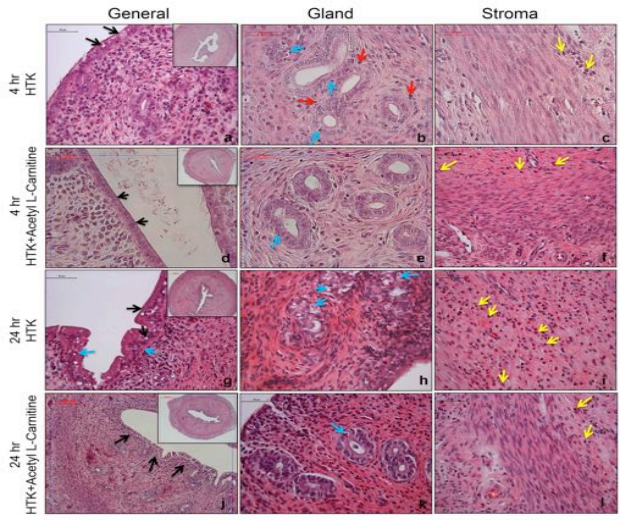


Figure 1. In group 1, subepithelial inflammation and apoptotic cells between surface epithelial cells (black arrow) (a), apoptotic cells in gland epithelium (blue arrow) and stroma (red arrow) (b), plasma cells in stroma (yelow arrows). In group 2, normal histologic uterine epithelium (black arrows) and subepithelium (d), a few apoptotic cells in endometrial gland epithelium (blue arrows) (e), less plasma cells (yellow arrow) than 4 hours HTK group (f). In group 3, subepithelial inflammation (black arrow) and apoptotic cells (blue arrows) (g), abundant apoptotic bodies (blue arrow) were observed distinctly in some endometrial glands (h), abundant plasma cells (yellow arrow) were observed distinctly in deep stroma (i). In group 4, subepithelial inflammation (black arrows) was less than 24 hours HTK group (j), apoptotic cells (blue arrows) in gland epithelium were less than 24 hours HTK group (k), the number of plasma cells (yellow arrows) was less than 24 hours HTK group (l). (H&E)

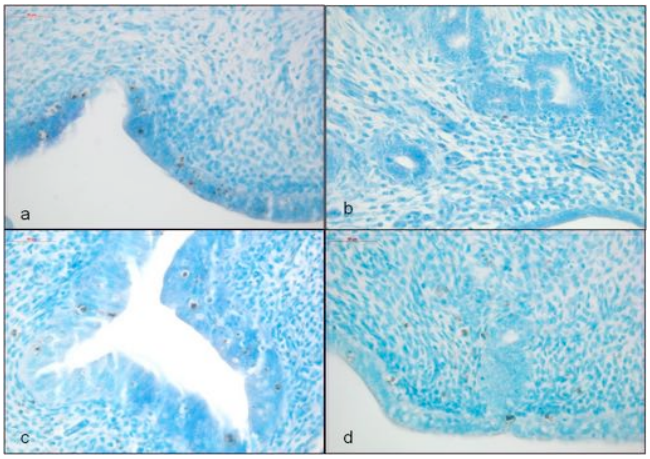
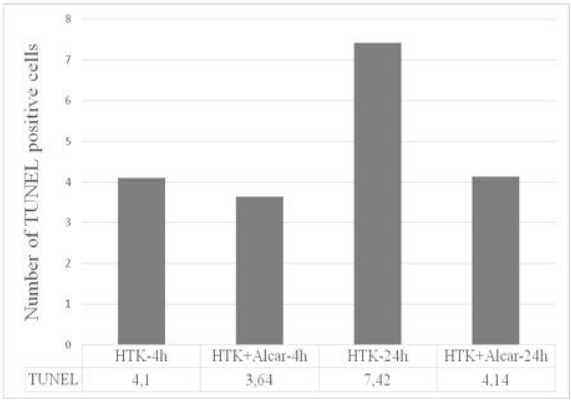


Figure 2. TUNEL marking (DAB, Hematoxylin). HTK 4 hr (a), HTK + Acetyl L-carnitine 4 hr (b), HTK 24 hr (c), HTK + Acetyl L-carnitine 24 hr (d)

TUNEL Results

TUNEL positive cells were counted and the averages were taken. (Figure 2, Graphic 1)

Graphic 1: The mean number of TUNEL positive cells

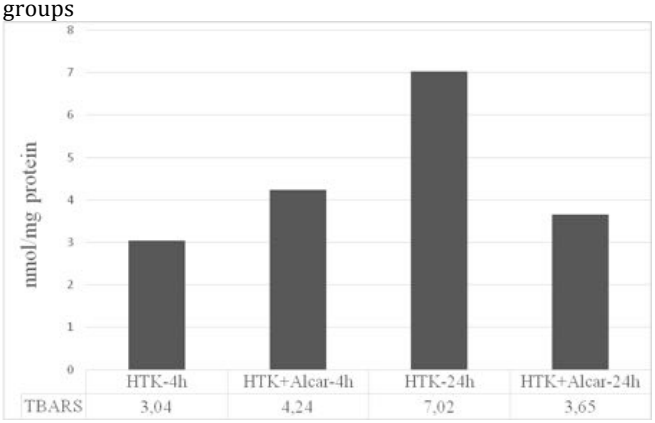


There was no significant difference in the number of TUNEL positive cells between group 1 and 2 (p=0.516), between group 2 and 4 (p=0.509). There was a significant difference between group 1 and 3 (p=0.001); between group 3 and 4 (p=0.002). The number of TUNEL positive cells was high in group 3.

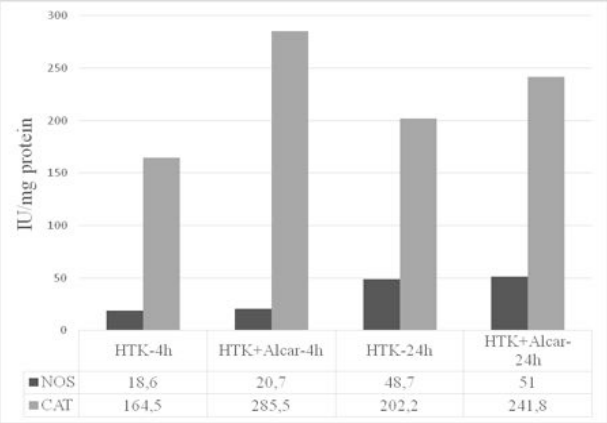
Biochemical Results

TBARS, NOS and CAT mean values were determined biochemically for each group. (Graphic 2, 3)

Graphic 2: The mean values of the TBARS level between the groups



Graphic 3: The mean values of the NOS and CAT levels between the groups



There was a significant difference in TBARS levels between group 1 and 2 (p=0.036). TBARS level was higher in group 2. There was a significant difference between group 1 and 3 (p=0.006) TBARS level was higher in group 3. There was a significant difference between group 3 and 4 (p=0.020). TBARS level was higher in group 3. There was no significant difference in TBARS levels between group 2 and 4 (p=0.490).

There was no significant difference in NOS levels between group 1 and 2 (p=0.621) and between group 3 and 4 (p=0.879). There was a significant difference in NOS levels between group 1 and 3 (p=0.020). NOS level was higher in group 3. There was a significant difference between group 2 and 4 (p=0.016). NOS level was higher in group 4.

There was a significant difference in CAT levels between group 1 and 2 (p=0.016). CAT level was higher in group 2. There was no significant difference in CAT levels between group 2 and 4 (p=0.265), between group 1 and 3 (p=0.302) and between group 3 and 4 (p=0.256).

DISCUSSION

The studies about uterus transplantation were done both with animal models and a few human bodies [16,17].

The main aim of the organ protecting is to maintain morphological, functional and biochemical properties of the organ with equilibrating solutions. With these solutions, we aim to provide hypothermia during transplantation, to prevent necrosis and tissue damage by free radicals and then to reduce ischemia-reperfusion damage [14,15,18-20].

HTK was first used as a cardiological transplant solution by Bretschneider in 1970s. Its electrolyte contents are similar as cytoplasm and it prevents liquid entrance into the cell and cell swelling. It contains mannitol, histidine, tryptophan and α-ketoglutarate. Mannitol acts as an antioxidant, histidine acts as a strong hydrogen ion buffer, tryptophan acts as a membrane stabilizer, α-ketoglutarate act as an energy substrate [13,18].

After organ transplantation, it was shown that ischemia-reperfusion damage has not been prevented yet. Recent studies indicated that reperfusion increases free radicals and decreases antioxidant enzymes. There isn't any method that already eliminates reperfusion damage but studies go on for removing free radical damages by antioxidants [21].

The protective effect of L -carnitine and derivations on the toxic effect of free radicals were showed by some studies. Some protective effects are related to the reduction of apoptosis and the others are related to the prooxidant effect and the reduction of free oxygen radical formation [22-24].

The antioxidant effects of carnitine and ascorbic acid were investigated on reperfusion damage after ischemia with rat skeletal muscle by Akar et al [25]. 4 hours after ischemia the damage reducing effect of carnitine was showed.

Barun et al. researched iloprost's effect on uterine transplantation, which is an antioxidant agent and added into HTK solution [2]. Uterus was found in normal when removed from 4 hours HTK solution and HTK+ iloprost solution but there were apoptotic cells in uterine epithelium when removed from 24 hours HTK solution. On the contrary uterus was normal when removed from 24 hours HTK+ iloprost solution.

In this study we evaluated the potential protective effects of acetyl L-carnitine on uterus during 4 and 24 hours storage periods with HTK solution. Compatible as literature uterine epithelium was normal and there were no subepithelial inflammation in group 2. There were apoptotic cells in epithelial cells and stroma in group 1 and group 2. Also there were plasma cells in superficial and deep stroma both in group 1 and group 2. The subepithelial inflammation in group 4 was less than in group 1 and group 3. In group 4, apoptotic cells, inflammatory cells in epithelium and also the plasma cells were less than all the other groups. Budzinski et al. researched prolactin's effect on liver transplantation, which is added into HTK [24]. And they evaluated the apoptotic effects of prolactin.

In our study it was determined that the number of TUNEL positive cells increased by the time in group 1 and 3 but decreased in group 4. It was asserted that hydrogen peroxide caused by activated neutrophils reached to cell nucleus by pass the membranes easily and caused DNA damage. It is evaluated that in second group DNA fragmented cell number was more than fourth group because of acetyl L-carnitine effected as a prooxidant and induced lipid peroxidation in short term. In this study it was assumed that acetyl L-carnitine reduced the number of TUNEL positive cells by decreasing oxidative stress in the long term.

Acetyl L-carnitine protects the activity of SOD, GPx and Catalase enzymes which involve in antioxidant defense mechanism. Acetyl L-carnitine has protective effect on lipids by increasing glutathione and decreasing last product of fatty acid peroxidation, MDA, ratio. It was showed that acetyl L-carnitine inhibits xanthine oxidase partially and because of that it prevents oxidative damage [22,26,27].

In a study the antioxidant effect of the agent, which added into HTK, solution was determined by biochemical analysis of Malondialdehyte an Nitric Oxide levels. With this results, it was determined that ischemia induced damage was prevented by 24-hour-iloprost added HTK solution. Mister et al. investigated the effect of propionyl-L-carnitine on ischemia-reperfusion damage after organ transplantation. They found that propionyl L-carnitine decreased lipid peroxidation, free radical production and protected cell structures. With these findings, they indicated that they brought a new perspective with this antioxidant effect on ischemia reperfusion damage. In our study as same as the literature it was determined that lipid peroxidation in uterine tissue was high in group 1, group 2 and group 3 but low in group 4. TBARS examinations, showing lipid peroxidation, supported TUNEL findings.

Trocha et al. evaluated simvastatin effects on antioxidant enzymes in rat livers which were treated into cold ischemia. Rats were divided into four groups [28]. In 2 groups intragastric simvastatin administrated 21 days and then liver tissues were removed. Then tissues were stored in 4°C HTK solution 24 hours. It was found that SOD and CAT activities and NOS protein concentration were higher in SV administrated groups.

In our study CAT levels were evaluated and it was found higher similar to literature in long period but there wasn't statistically significant difference. Catalase levels were found higher in short period when acetyl L-carnitine added short and long periods were compared but not statistically significant. This paradox was tried to be explained by NOS mechanism. Total NOS values, which induced by cytokine and endotoxin during inflammation and infection, were increased in parallel to time in short and long period HTK solutions. When acetyl L-carnitine added into HTK the NOS values increased in long period but this increase was not statistically significant. It was thought that this increment was because of reductant effect of superoxide. Superoxide anion has both of oxidising and reducing properties. When it effects as a reductant it loses one electron and oxidised into oxygen. It was assumed that in

this study superoxide which united with NO acted as a reductant in long period acetyl L-carnitine solution, protected tissues from ischemia reperfusion damage and inhibited the completion of apoptotic process.

CONCLUSION

As a result, during organ transplantation to protect and store organ for a long time and prevent tissue rejection because of reperfusion damage we planned this study and we found that 24 hours acetyl L-carnitine added HTK solution has protective effect on ischemia reperfusion damage in uterus.

Declaration of Conflicting Interests: The authors declare that they have no conflict of interest.

Financial Disclosure: This study was supported by Gazi University Scientific Project Unit Ankara, Turkey Grant number: 01/2011-90

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