RESEARCH ARTICLE

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Evaluation of the Effects of Rapamycin Treatment on Antioxidant Enzyme Changes and AgNOR in Testicular Torsion

ABSTRACT

Objective: Testicle torsion/detorsion cause ischemia. Rapamycin has immune suppressive and antioxidant defense mechanisms. Nucleolar-organizing regions (NORs) are loops of ribosomal DNA.

Methods: To evaluate mean AgNOR number and total AgNOR area/total nuclear area (TAA/TNA) ratio and the relation between these proteins and rapamycin in Torsion/Detorsion process of testes. The six groups as control, sham, early and late torsion-detorsion (ETD&LTD) groups, and early and late rapamycin treatment groups (ETD+R&LTD+R) were included. The TAA/TNA and mean AgNOR number of testes cells and biochemical analysis of GPx, SOD and TBARS activities were detected.

Results: Significant differences were detected among the groups for mean AgNOR number and TAA/TNA($p<0.05$). For both mean AgNOR number and TAA/TNA, significant differences were found between control and ETD, between control and ETD+R, between control and LTD, between control and LTD+R. Also, statistically significant relation between both of mean AgNOR numbers and TAA/TNA of testes cells and all of antioxidant enzymes (SOD, TBARS and GPX) were detected ($p<0.05$).

Conclusions: We may obtaine information about the levels and duration of testes injury considering the levels of these proteins. Thus it can be said that, these proteins may be used the developing of the new and more effective therapeutic approaches to prevent the negative effects of the T/D injury.

Keywords: Testes Torsion, NOR, AgNOR, rDNA, Rapamycin

Testis Torsiyonunda Rapamisin Tedavisinin Antioksidan Enzim Değişiklikleri ve AgNOR Üzerindeki Etkilerinin Değerlendirilmesi

ÖZET

Amaç: Testis torsiyonu/detorsiyonu iskemiye neden olur. Rapamisin, immün baskılayıcı ve antioksidan savunma mekanizmalarına sahiptir. Nükleolar düzenleyen bölgeler (NOR'ler) ribozomal DNA'nın döngüleridir.

Gereç ve Yöntem: Testislerin Torsiyon/Detorsiyon işleminde ortalama AgNOR sayısı ve toplam AgNOR alanı/toplam nükleer alan (TAA/TNA) oranını ve bu proteinler ile rapamisin arasındaki ilişkiyi değerlendirmek. Kontrol, sahte, erken ve geç torsiyon-detorsiyon (ETD & LTD) grupları ve erken ve geç rapamisin tedavi grupları (ETD+R & LTD+R) olmak üzere altı grup dahil edildi. TAA/TNA ve testis hücrelerinin ortalama AgNOR sayısı ile GPx, SOD ve TBARS aktivitelerinin biyokimyasal analizi tespit edildi.

Bulgular: Gruplar arasında ortalama AgNOR sayısı ve TAA/TNA açısından önemli farklılıklar tespit edildi ($p<0.05$). Hem ortalama AgNOR sayısı hem de TAA/TNA için, kontrol ile ETD arasında, kontrol ile ETD+R arasında, kontrol ile LTD arasında, kontrol ile LTD+R arasında önemli farklılıklar bulundu. Ayrıca testis hücrelerinin ortalama AgNOR sayıları ile TAA/TNA ile tüm antioksidan enzimler (SOD, TBARS ve GPX) arasında istatistiksel olarak anlamlı ilişki saptandı ($p<0.05$).

Sonuç: Bu proteinlerin seviyelerine göre testis hasarının seviyeleri ve süresi hakkında bilgi edinebiliriz. Bu nedenle, bu proteinlerin, T/D hasarının olumsuz etkilerini önlemek için yeni ve daha etkili terapötik yaklaşımların geliştirilmesinde kullanılabileceği söylenebilir.

Anahtar Kelimeler: Testis Torsiyonu, NOR, AgNOR, rDNA, Rapamisin

INTRODUCTION

Rotation of the testicle with its own pedicle disrupts the blood flow, resulting in ischemia, and if not corrected immediately, it may result in organ loss (1). Despite the restoration of oxygen supply and nutrition, a new handicap emerges: ischemia reperfusion injury, which if not managed well can result in infertility (2,3). I/R injury causes anoxia in particular, resulting in large amounts of ROS, proinflammatory cytokines, lipid peroxidation and cell adhesion molecules, followed by necrosis caused by activation of the apoptosis pathway, leading to more severe ischemic tissue damage (4). Proinflammatory neutrophil infiltration and ROS formation happen in the early stage of I/R injury, are important in the pathogenesis of said injury, and in the early control of the reperfusion phase to reduce its damage (5,6).

For a long time, detailed research efforts have sought to find effective tactics and agents to protect the testicle from I-R damage or to minimize that damage. However, to date, no method has been able to successfully apply in clinical practice (7,8). Rapamycin, on the other hand, continues its adventure as an antifungal drug and continues as an immunosuppressive agent and the objections about this identity are increasing. Are rapamycin and its analogues immunosuppressive or immunomodulatory drugs? Theoretical and practical applications suggest that rapamycin may be effective against hypertension, atherosclerosis and hyper-coagulation diseases (such as myocardial infarction and stroke), cancer, osteoporosis, autoimmune disorders, diabetes, macular degeneration, Alzheimer's and Parkinson's diseases and even obesity (9). Rapamycin, a TOR antagonist, has also been proven to prevent aging (10). Could it show these effects in preventing I/R damage through Nucleolar regulating regions (NOR) proteins? Is there a relationship between the TOR pathway and NOR, ribosomal DNA (rDNA) rings that make up the functional subunits of the nucleolus and are copied into ribosomal RNA?

Nucleolar-organizing regions (NORs) are loops of ribosomal DNA (rDNA) that functional subunits of the nucleolus and are transcribed into ribosomal RNA which becoming a part of mature ribosomes. Some of these are argyrophilic features and stained with silver. Various studies about the significance of the interphase AgNOR quantity in different cells were done (11-30). To our knowledge, any study about the evaluation of mean AgNOR number and total AgNOR area/total nuclear area (TAA/TNA) ratio and the relation between AgNOR protein amounts and rapamycin was conducted on Torsion/Detorsion of testes. So, we performed the current study.

MATERIAL AND METHODS

Experimental Design and Animal Groups: This study was carried out with the approval of Duzce University Local Animal Experiments Ethics Committee (2020/11/1). 90-day-old adult male albino Wistar rats (n=30) weighing approximately 275-325 g were used in this study. The rats were kept in plastic cages under a 12-hour light and 12-hour dark cycle and under temperature-controlled place (21-22 °C). They were fed with standard rodent diet and filtered drinking water at the Experimental Animals Application and Research Center of Duzce University. The experimental animals were divided into 6 groups of 5 rats each: control, sham, early and late torsion-detorsion (ETD&LTD) groups, and early and late rapamycin treatment groups (ELTD+R&LTD+R). In the control group, no surgical procedure or medication was applied but in the sham surgery group, the right testicle was exposed for a short time with a surgical incision without any rotation and was replaced again. While just surgical procedure was performed in the TD groups, 0.2 mg/kg rapamycin was administered by oral gavage once a day for 3 days in addition to the surgical procedure in the TD+R groups.

In the torsion and treatment groups, the right testis, which was taken out of the body with a midline scrotal incision, was torsioned by turning it 720° clockwise after opening the tunica vaginalis. It was fixed to the scrotum with a 5/0 silk suture to maintain the torsion position (7). After 4 hours of ischemia, the testis was detorsioned by rotating it counterclockwise and brought to its natural position. Rapamycin TD+R groups (RAPAMUNE® Oral Solution 1 mg/ml Pfizer PFE) were administered to rats three times by oral gavage; 30 minutes before and 24 and 48 hours after corrective surgery (detorsion). Sampling was performed at the end of the 3rd day (72nd-hour postop) in the ETD and ETD+R, and on the 10th day (postop 240th-hour) in the LTD and LTD+R groups.

AgNOR Staining: The testes samples of each group fixed individually in a 4% formaldehyde solution, were embedded in paraffin blocks (about dimensions of approximately 1x1x1 cm3) and 4 μm sections were obtained from the paraffin blocks. The tissue sections of testes were deparaffinized in xylene and rehydrated in graded alcohol solutions. After rehydratation, the slides were air-dried at room temperature for 15 min and fixed in absolute methanol for 5 min. Then each slides were silver stained with slight modification of Benn and Perle protocol (31) and Lindner (32). For this purpose, the solution made by mixing one volume of 2% gelatine in 1% aqueous formic acid and two volumes of 50% silver nitrate were dropped on the slides and incubated at 37 0C for fifteen min in the dark. Then the slides were rinsed with bi-distilled water.

Image Analysis of Mean AgNOR Number and Total AgNOR area/total Nuclear Area (TAA/TNA) Ratio: Fifty nuclei for per slides have

been evaluated. Silver stained testes cells of each rats were photographed using a light microscope (Eclipse 80i; Nikon. Tokyo. Japan) via digital camera attachment (Digital Sight DS-Fi1c; Nikon) and evaluated using ImageJ version 1.47t image processing software (33). The mean AgNOR number was detected by counting and the TAA/TNA ratio was detected using ‘‘freehand selection’’ tool for each nucleus.

Biochemical Assays: After decapitation, blood samples were taken from the aortic abdominalis and placed in heparin plastered tubes. Plasma obtained by low speed centrifugation (2000×g, 15 minutes) was stored at –80 °C for biochemical analysis of GPx, SOD and TBARS activities. GPx, SOD and TBARS activities were evaluated with Cayman® Elisa kits without ignoring the manufacturer's instructions.

Statistical Analysis: Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS. Inc. Chicago. Illinois. USA) for Windows 23.0. The comparison of all groups (more than two) was done using the Kruskall-Wallis test. For pairwise comparison of all groups, Mann-Whitney U test was carried out. The polynomial regression were performed for the relationship between the variable. The results were given as n, median, range and mean ± SD. The $p<0.05$ was accepted as statistically significant.

RESULTS

The groups, mean AgNOR Number and TAA/TNA ratio of each group were given in the Table 1. Demonstrastive examples of silver stained NOR (a:Control, b:Sham, c:ETD, d: ETD+R, e: LTD f: LTD+R) for testicular cells were given in the Figure 1.

Table 1. The groups, mean AgNOR Number and TAA/TNA ratio of each group

Groups	Mean AgNOR Number ± SD (n=250)	Mean TAA/TNA ± SD (n=50)	Mean AgNOR number of Group/Median (Range) (n=250)	Mean TAA/TNA of Group/Median (Range) (n=250)	$\chi2$	p
C1	1.276±0.532	0.030±0.012	1.269±0.019/1.276	0.030±0.001/0.298		
C2	1.289±0.493	0.029±0.01	(0.048)	(0.001)		
C3	1.278±0.451	0.029±0.011				
C4	1.241±0.423	0.030±0.01				
C5	1.259±0.431	0.030±0.011				
S1	1.17±0.351	0.030±0.071	1.228±0.065/1.220	0.030±0.001/0.030		
S2	1.22±0.423	0.030±0.016	(0.140)	(0.002)		
S3	1.16±0.331	0.029±0.011				
S4	1.29±0.557	0.030±0.012				
S5	1.3±0.471	0.031±0.01				
ETD1	2.56±1.150	0.110±0.047	2.659±0.214/2.560	0.112±0.002/0.112		
ETD2	2.98±0.949	0.114±0.028	(0.540)	(0.006)	25.258*	0.000*
ETD3	2.765±1.116	0.108±0.040				
ETD4	2.55±0.994	0.112±0.133				
ETD5	2.44±1.191	0.113±0.086				
ETD+R1	1.937±0.982	0.094±0.025	1.789±0.464/1.655	0.080±0.001/0.078		
ETD+R2	2.52±1.147	0.072±0.032	(1.163)	(0.023)		
ETD+R3	1.357±0.638	0.071±0.031				
ETD+R4	1.655±0.637	0.078±0.034				
ETD+R5	1.474±0.684	0.086±0.038				
LTD1	2.08±0.703	0.104±0.038	2.242±0.225/2.100	0.104±0.003/0.105		0.000&
LTD2	2.59±0.891	0.105±0.047	(0.510)	(0.007)		
LTD3	2.35±0.667	0.100±0.031			27.194&	
LTD4	2.09±0.785	0.106±0.033				
LTD5	2.1±0.881	0.107±0.031				
LTD+R1	2±1.125	0.096±0.044	1.868±0.218/1.980	0.089±0.008/0.089		
LTD+R2	1.84±0.866	0.089±0.866	(0.520)	(0.017)		
LTD+R3	1.5±0.707	0.081±0.023				
LTD+R4	2.02±0.589	0.08±0.026				
LTD+R5	1.98±0.589	0.098±0.024				

C: Control; **S:**Sham; **ETD:** early torsion detorsion; ETD**+R:** early torsion detorsion+rapamycin treatment; **LTD:** late torsion detorsion; **LTD+R:**late torsion detorsion+rapamycin treatment; *:For Mean AgNOR Number; &=For TAA/TNA; **TAA/TNA:** Total AgNOR area/total nuclear area

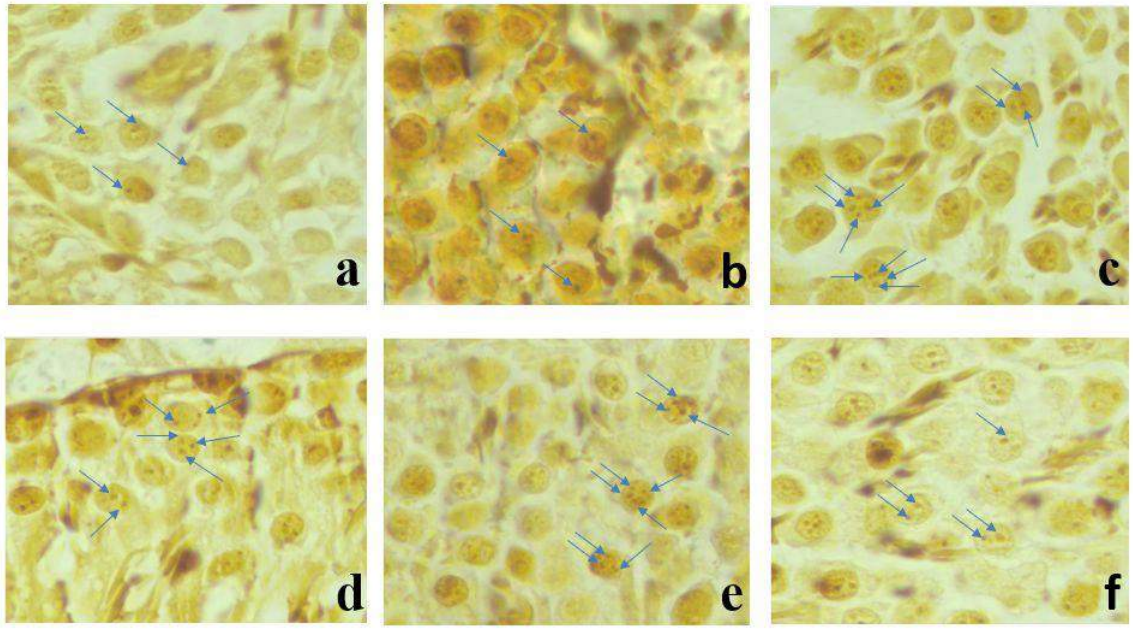


Figure 1. Demonstrative examples of silver stained NOR (**a:**Control, **b:**Sham, **c:**ETD, **d:** ETD+R, **e:** LTD **f:** LTD+R) for testicular cells

When the all groups to be considered, statistically significant differences were detected among the groups for both mean AgNOR number ($\chi 2$=25.258, p=0.000) and TAA/TNA ($\chi 2$=247.194, p=0.000), respectively (Table 1 and Figure 2). In order to understand the causes of these differences from which groups, binary comparison of the groups was performed.

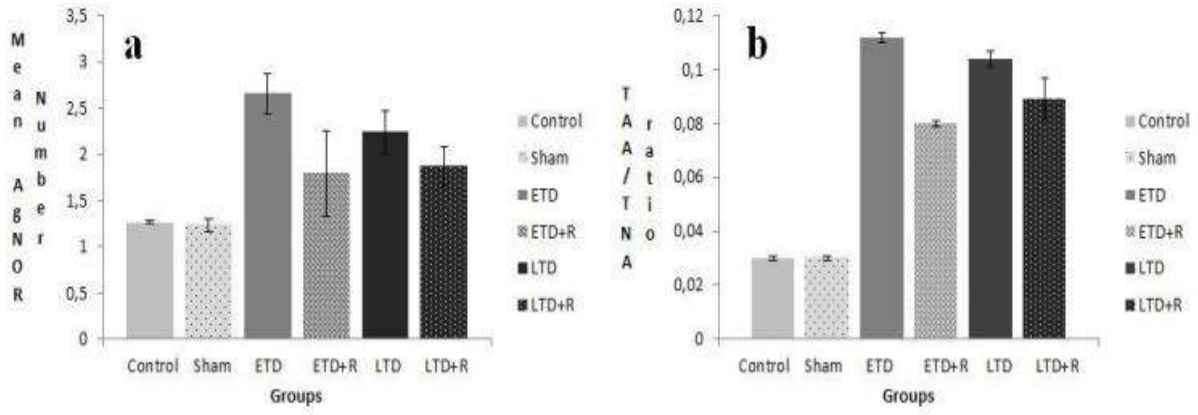


Figure 2. Comparison of mean AgNOR number and TAA/TNA ratio of all groups.

When the two groups are compared in terms of mean AgNOR number, statistically significant differences were found between control and ETD (Z=-2.611, p=0.009), between control and ETD+R (Z=-2.611, p=0.009), between control and LTD (Z=-2.619, p=0.009), between control and LTD+R (Z=-2.619, p=0.009), between sham and ETD (Z=-2.611, p=0.009), between sham and ETD+R (Z=-2.611, p=0.009), between sham and LTD (Z=-2.619, p=0.009), between sham and LTD+R (Z=-2.611, p=0.009), between ETD and ETD+R (Z=-2.402, p=0.016), between ETD and LTD (Z=-1.984, p=0.047), between ETD and LTD+R (Z=-2.611, p=0.009), between LTD and LTD+R (Z=-2.611, p=0.009). But the difference between control and sham (Z=-0.522, p=0.602), between ETD+R and LTD (Z=-1.776, p=0.076), between ETD+R and LTD+R (Z=-0.940, p=0.347), were not significant (Table 2).

Table 2. Double comparison results of the groups for Mean AgNOR Number

Groups	C		S		ETD		ETD+R		LTD		LTD+R	
	Z	**P**	**Z**	**P**	**Z**	**P**	**Z**	**P**	**Z**	**P**	**Z**	**P**
C	-	-	0.522	0.602	2.611	**0.009**	2.611	**0.009**	2.611	**0.009**	2.611	**0.009**
S	0.522	0.602	-	-	2.611	**0.009**	2.611	**0.009**	2.611	**0.009**	2.611	**0.009**
ETD	2.611	**0.009**	2.611	**0.009**	-	-	2.402	**0.016**	1.984	**0.047**	2.611	**0.009**
ETD+R	2.611	**0.009**	2.611	**0.009**	2.402	**0.016**	-	-	1.776	0.076	0.940	0.347
LTD	2.611	**0.009**	2.611	**0.009**	1.984	**0.047**	1.776	0.076	-	-	2.611	**0.009**
LTD+R	2.611	**0.009**	2.611	**0.009**	2.611	**0.009**	0.940	0.347	2.611	**0.009**	-	-

C: Control; **S:**Sham; **ETD:**early torsion detorsion; ETD**+R:** early torsion detorsion+rapamycin treatment; **LTD:** late torsion detorsion; **LTD+R:**late torsion detorsion+rapamycin treatment; *:For Mean AgNOR Number

When the two groups are compared in terms of TAA/TNA, the statistically significant differences were found between control and ETD (Z=-2.611, p=0.009), between control and ETD+R (Z=-2.611, p=0.009), between control and LTD (Z=-2.611, p=0.009), between control and LTD+R (Z=-2.619, p=0.009), between sham and ETD (Z=-2.611, p=0.009), between sham and ETD+R (Z=-2.611, p=0.009), between sham and LTD (Z=-2.611, p=0.009), between sham and LTD+R (Z=-2.611, p=0.009), between ETD and ETD+R (Z=-2.611, p=0.009), between ETD and LTD (Z=-2.611, p=0.009), between ETD and LTD+R (Z=-2.611, p=0.009), between ETD+R and LTD (Z=-2.611, p=0.009) and between LTD and LTD+R (Z=-2.1611, p=0.009). Conversely the differences between control and sham (Z=-1.567, p=0.117), between ETD+R and LTD+R (Z=-1.567, p=0.117) were not meaningful for TAA/TNA (Table 3).

Table 3. Double comparison results of the groups for TAA/TNA ratio

Groups	C		S		ETD		ETD+R		LTD		LTD+R	
	Z	P	Z	P	Z	P	Z	P	Z	P	Z	P
C	-	-	1.567	0.117	2.611	**0.009**	2.611	**0.009**	2.611	**0.009**	2.619	**0.009**
S	1.567	0.117	-	-	2.611	**0.009**	2.611	**0.009**	2.611	**0.009**	2.611	**0.009**
ETD	2.611	**0.009**	2.611	**0.009**	-	-	2.611	**0.009**	2.611	**0.009**	2.611	**0.009**
ETD+R	2.611	**0.009**	2.611	**0.009**	2.611	**0.009**	-	-	2.611	**0.009**	1.567	0.117
LTD	2.611	**0.009**	2.611	**0.009**	2.611	**0.009**	2.611	**0.009**	-	-	2.611	**0.009**
LTD+R	2.619	**0.009**	2.611	**0.009**	2.611	**0.009**	1.567	0.117	2.611	**0.009**	-	-

C: Control; **S:**Sham; **ETD:** early torsion detorsion; ETD**+R:** early torsion detorsion+rapamycin treatment; **LTD:** late torsion detorsion; **LTD+R:** late torsion detorsion+rapamycin treatment; **TAA/TNA:** Total AgNOR area/total nuclear area

When the antioxidant enzymes to be considered, statistically significant differences were detected among the groups for SOD ($\chi2$=21.124, p=0.001), TBARS ($\chi2$=24.892, p=0.000) and GPX ($\chi2$=26.471, p=0.000), respectively (Table 4).

Table 4. The comparison of antioxidant enzyme levels (SOD, TBARS and GPX) in all groups.

Groups	SOD	Mean±SD/Median (Range) for SOD	TBARS	Mean±SD/Median(Range) for TBARS	GPX	Mean±SD/Median(Range) For GPX	χ2	p
C1	23.15702		13.40782		17.67081			
C2	22.45405	23.573±1.047/23.157	11.30503	12.341±0.919/12.260	15.86335	16.520±1.117/16.433		
C3	25.04380	(2.590)	12.25978	(2.103)	16.43298	(2.608)		
C4	24.23702		13.12782		17.57081			
C5	22.97405		11.60503		15.06335			
S1	27.18595		14.21458		17.89745			
S2	29.34033	27.401±1.607/27.186	13.02455	13.561±0.430/13.525	19.99379	18.163±1.403/17.898		
S3	25.75207	(3.588)	13.42468	(1.190)	16.49068	(3.503)		
S4	25.98595		13.61458		17.30745			
S5	28.74234		13.52455		19.12379			
ETD1	63.570		32.869		45.023			
ETD2	56.786	55.141±6.095/56.786	28.779	28.787±2.702/28.779	44.826	40.973±4.396/42.287	21.124*	0.001*
ETD3	50.375	(15.445)	27.938	(7.513)	37.324	(9.618)		
ETD4	48.125		28.992		35.405			
ETD5	56.846		25.357		42.287		24.592&	0.000&
ETD+R1	49.68595		26.05028		36.18012			
ETD+R2	43.28926	39.221±8.922/42.067	25.58101	23.560±3.768/25.581(8.478)	28.51553	29.168±4.723/28.516	26.471®	0.000®
ETD+R3	42.06694	(23.234)	26.28492		30.44497	(12.932)		
ETD+R4	26.45248		17.80670		23.24857			
ETD+R5	34.61157		21.07486		27.45298			
LTD1	50.035		30.070		40.133			
LTD2	47.020		25.366	24.395±3.622/23.783	31.584	32.989±4.202/31.584		
LTD3	37.621	43.949±5.251/45.681(12.413)	21.984	(9.300)	30.769	(10.704)		
LTD4	39.389		20.770		29.428			
LTD5	45.681		23.783		33.033			
LTD+R1	52.59372		21.23017		20.43478			
LTD+R2	37.14876	36.341±11.478/	18.85642	19.394±1.823/18.856	26.54658	23.169±2.961/22.951		
LTD+R3	29.10992	37.149(30.156)	17.71508	(3.704)	20.12422	(6.422)		
LTD+R4	22.43802		17.74860		22.95093			
LTD+R5	40.41322		21.41899		25.78882			

C: Control; **S:**Sham; **ETD:** early torsion detorsion; **ETD+R:** early torsion detorsion+ rapamycin treatment; **LTD:** late torsion detorsion; **LTD+R:** late torsion detorsion+ rapamycin treatment; **SOD:** Superoxide dismutase; **TBARS:** Thiobarbituric acid reactive substances; **GPX:** Glutathione peroxidase;*:For SOD; &:For TBARS; ®:For GPX

When we performed the polynominal regression analaysis statistically significant relation between mean AgNOR numbers of testes cells and all of antioxidant enzymes (SOD, TBARS and GPX) were detected ($p<0.05$) (Table 5, Figure 3). Also statistically significant relation between TAA/TNA ratio of testes cells and and all of antioxidant enzymes (SOD, TBARS and GPX) were detected ($p<0.05$) (Table 5, Figure 3).

Table 5. Model Summary and Parameter Estimates for AgNOR Numbers, TAA/TNA and antioxidant enzymes

		Model Summery					Parameter Estimates			
Variable	***Equation***	***R^2***	***F***	***df1***	***df2***	***sig***	**Constant**	***b1***	***b2***	***b3***
M-AgNOR-N and SOD	Linear	,216	7,723	1	28	,010	18,918	7,554		
	Logarithmic	,241	8,874	1	28	,006	24,543	14,648		
	Cubic	,272	5,032	2	27	,014	-9,915	40,004	-8,368	,000
M-AgNOR-N and TBARS	Linear	,196	6,842	1	28	,014	11,102	3,281		
	Logarithmic	,228	8,261	1	28	,008	13,470	6,496		
	Cubic	,279	5,212	2	27	,012	-4,909	21,300	-4,647	,000
M-AgNOR-N and GPX	Linear	,254	9,530	1	28	,005	13,496	4,915		
	Logarithmic	,288	11,332	1	28	,002	17,104	9,622		
	Cubic	,353	7,373	2	27	,003	-9,693	31,013	-6,730	,000
TAA/TNA and SOD	Linear	,295	11,727	1	28	,002	21,947	146,794		
	Logarithmic	,321	13,266	1	28	,001	57,756	9,106		
	Cubic	,357	7,492	2	27	,003	13,909	395,219	,000	-16254,235
TAA/TNA and TBARS	Linear	,316	12,945	1	28	,001	12,011	69,242		
	Logarithmic	,396	18,353	1	28	,000	29,752	4,606		
	Cubic	,618	21,862	2	27	,000	-,536	561,584	-3630,342	,000
TAA/TNA and GPX	Linear	,355	15,419	1	28	,001	15,381	96,666		
	Logarithmic	,407	19,179	1	28	,000	39,376	6,148		
	Cubic	,487	12,835	2	27	,000	4,445	525,777	-3164,098	,000

M-AgNOR-N: Mean AgNOR Number; **TAA/TNA:** Total AgNOR area/total nuclear area; **SOD:** Superoxide dismutase; **TBARS:** Thiobarbituric acid reactive substances; **GPX:** Glutathione peroxidase

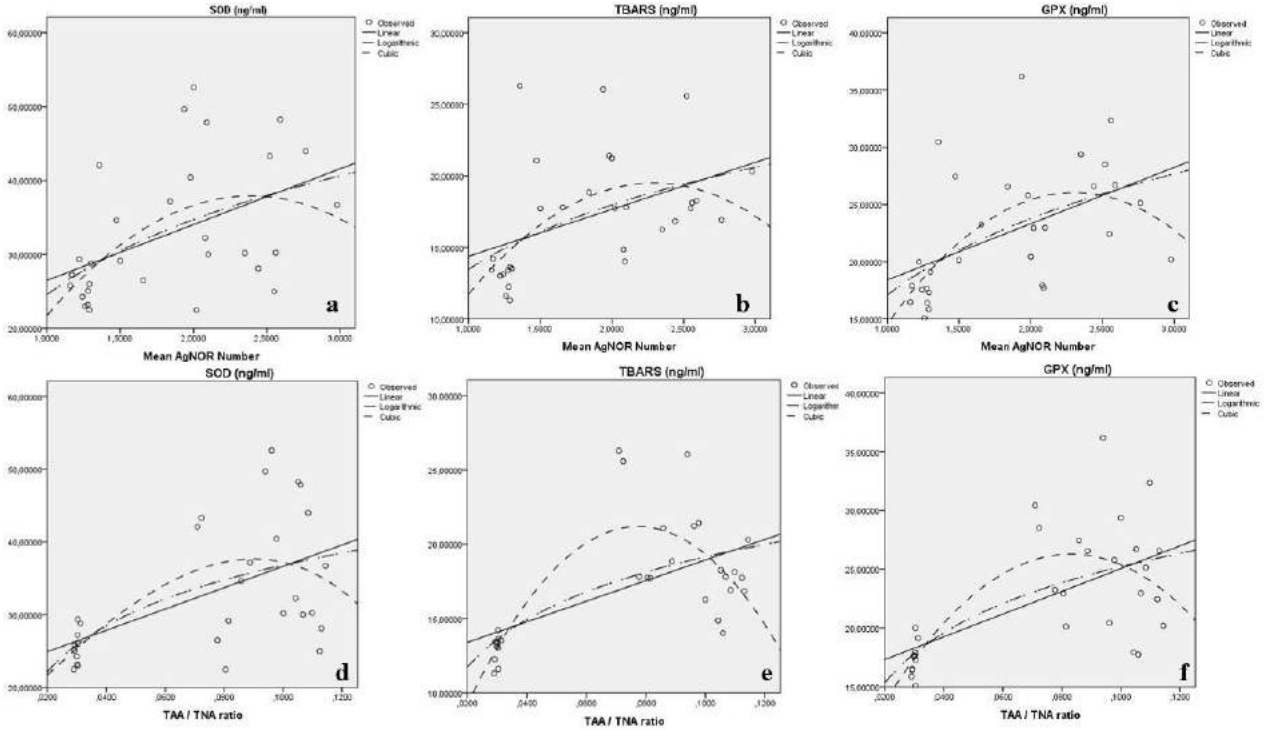


Figure 3. Relation between mean AgNOR numbers of testes cells and SOD (a), between mean AgNOR numbers of testes cells and TBARS (b), and mean AgNOR numbers of testes cells and GPX (c). Relation between mean TAA/TNA ratio of testes cells and SOD (d), between TAA/TNA ratio of testes cells and TBARS (e) and TAA/TNA ratio of testes cells and GPX (f).

DISCUSSION

In case of torsion caused by the rotation of the testicle with its'pedicle, it is a kind of race against time for surgeons dealing with pediatric emergencies to correct before necrosis. If this ischemic condition persists more than a few hours, depending on the degree of torsional and personal characteristics, testicular severely damaged and may even be necessary to remove the affected testicle. However, Ischemia-reperfusion injury (IRI) that occurs after surgical or manual correction is another problem and it is an important cause of fertility dysfunction (34). Thus, preventing or minimizing torsion-induced IRI can improve the long-term results of testicular function. For this purpose, many agents/drugs, one of which is Rapamycin (35), have been studied in the literatüre (7,8).

Rapamycin and its' analogue, everolimus, have been approved by the FDA for use as immunosuppressive in humans, and there have been publications on this subject for decades. At the beginning of the millennium, with an almost 3-fold prolongation of the life span of a mouse strain, rapamycin (called Rapamune or Sirolimus in clinical use) could be used to slow aging in humans and delay the progression of many age-related diseases, which could become a "miracle drug that delays aging today" started writing (36,37). However, it may be more appropriate to evaluate rapamycin in an immunomodulator or anti-inflammatory drug group instead of labeling it as an immunosuppressive agent. The mechanism of slowing down aging of Rapamycin is that it prevents excess, or more accurately, rejuvenates immunity rather than suppressing immunity (38). This means that, on the one hand, it prevents rejection as an immunosuppressor in organ transplant and acts as an immunostimulant on the other hand (39,40), enhancing immunity in cancer patients (41) and the elderly (42,43).

We can obtained the knowledge about the metabolic activities and protein synthesis capacity of the cells using AgNOR staining technique. Various studies are performed on the different cells including human hair root cells (11,12), buccal epithelial cells of down syndrome infants and healthy individuals (13,14), lung cells exposed to carbon monoxide (CO) (15), heart cells exposed to acute and chronic CO intoxication (16,17), femoral and skeletal muscle cells exposed CO gas (18,19), normal, benign and malign thyrocyte cells (20-23), peripheral lymphocytes of patients with chronic obstructive pulmonary disease exacerbation (24), kidney cells exposed to Ischemia/reperfusion (I/R) injury (25), capsaicin exposed human colon adenocarcinoma cells (26,27), rhamnetin exposed ehrlich's ascites carcinoma cells (28) and in oncocytology (29).

We previously reported that the AgNOR proteins amounts increased depending on the CO exposure (cause of hypoxic condition) in lung (15), heart cells (16,17), femoral muscle cells (18,19) and chronic obstructive pulmonary disease (COPD) exacerbation (24). According to our results, statistically significant differences were detected among the groups for both mean AgNOR number and TAA/TNA, respectively. To understand these differences causes from which groups, double comparison was performed. For mean AgNOR number, statistically significant differences were found between control and ETD, between control and ETD+R, between control and LTD, between control and LTD+R, between sham and ETD, between sham and ETD+R, between sham and LTD, between sham and LTD+R, between ETD and LTD. When the TAA/TNA ratio to be considered, the statistically significant differences were found between control and ETD, between control and ETD+R, between control and LTD, between control and LTD+R, between sham and ETD, between sham and ETD+R, between sham and LTD, between sham and LTD+R, between ETD and LTD, between ETD+R and LTD. According to our results, it may be said that the AgNOR proteins amount increase depending on hypoxia injuring caused from torsion/detorsion conditon, too. Considering our results; we think that both AgNOR protein amounts (mean AgNOR number and TAA/TNA ratio) may give information about the levels of the testes injury after T/D process. One of the most known certain reality is the obligation of themselves protection of all living cells to external and internal dangerous agents such T/D injury for keep alive. So, the increasing of these proteins may caused from the continuity of this process or these proteins have functions on the occuring various gene products that protective effect on the regulation of gene expression and/or signaling transduction pathways in the T/D injury. May these proteins be used the developing of the new and more effective therapeutic approaches to prevent the negative effects of the T/D injury? Additional studies including large series are needed to elucidate these topics as more clearly.

A positive correlation between mean AgNOR number and the pCO2 levels in patients with chronic obstructive pulmonary disease (COPD) exacerbation (24), between histopathological injury score and both of mean AgNOR number and TAA/TNA ratio in renal ischemia/reperfusion (I/R) injury (25), between histopathological injury scores and TAA/NA ratio (17), between AgNOR values and both cardiomyopathy and carboxyhemoglobin levels (16), between both the AgNOR values and histopathological scoring methods (15) were reported. In the current study, the level of antioxidant enzymes to be taken into consideration, statistically significant differences were detected among the groups for SOD, TBARS and GPX,

respectively. According to the polynominal regression analaysis, we detected statistically significant relation between antioxidant enzymes (SOD, TBARS and GPX) and both of mean AgNOR numbers and TAA/TNA ratio of testes, too. So it may be said that, we can also obtained information about the antioxidant enzymes and injury levels of testes tissue caused by torsion/detorsion injury.

Rapamycin is the most effective anti-cancer drug known to increase life expectancy in cancer-prone mouse strains (44-47). In fact, it was thought that rapamycin prolonged life expectancy because of cancer prevention. With these properties, how does rapamycin play a role in ischemic events? When determining the dose of rapamycin, it was necessary to consider the short-term effects that limited us. Since the animals were sacrificed after 3 days in the early period groups (groups 3 and 4), drug use was restricted for this period, although it was a safe drug that could be administered for a longer time. While a higher dose range could be given as a dose, the daily routine dose was chosen, whereas recorded fatal cases of acute rapamycin overdose were not detected (48). The LD50 of rapamycin, a measure of drug lethality, could not be measured in rats as it was higher than 2500 mg/kg. A single dose of rapamycin is safe, but sufficient to prolong life and reduce obesity in various rodent models (36,49). Moreover, temporary treatment with rapamycin can be long lasting, prolong life and prevent obesity long after drug withdrawal (50-54). Another restriction in dose selection is the negative effects of rapamycin and its analogs on spermatogenesis. However, research has shown that these effects are temporary and no more important than saving organ life (55). Long-term use and intermittent treatment options will be evaluated as researches conducted to prolong organ life in ischemic events become widespread.

Additionally it was reported that both AgNOR amounts may give information about the most reliable therapeutic dose selection of Curcumin (33), capsaicin (27) and Rhamnetin (28). In our results, statistically significant differences were found between ETD and ETD+R, between ETD and LTD+R, between LTD and LTD+R for mean AgNOR number. For TAA/TNA, statistically significant differences were found between ETD and ETD+R, between ETD and LTD+R and between LTD and LTD+R, too. According to our results, Rapamycin has protective effect for torsion/detorsion injury on testes tissue.

As a results, because the AgNOR proteins amount increase depending on hypoxia injuring caused from torsion/detorsion conditon, we may obtained information about the levels and duration of the testes injury after T/D process considering the levels of these proteins. According to our results, rapamycin has protective effect for torsion/detorsion injury on testes tissue. We can also obtained information about the antioxidant enzymes and injury levels of testes tissue caused by torsion/detorsion injury considering these proteins. So it can be said that, these proteins may be used the developing of the new and more effective therapeutic approaches to prevent the negative effects of the T/D injury.

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