RESEARCH ARTICLE

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Received: 27.01.2020
Acceptance: 05.09.2020
DOI: 10.18521/ktd.680703

Konuralp Medical Journal
e-ISSN1309–3878
konuralptipdergi@duzce.edu.tr
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Investigation of Protective Effects of Dehydroepiandrosterone (DHEA) Against Toxic Damage Caused by Doxorubicin in Rat Ovaries

ABSTRACT

Objective: Our aim is to evaluate whether dehydroepiandrosterone has a protective effect on doxorubicin-induced ovarian damage.

Methods: The rats were divided into three groups. Group 1 (the control Group): no treatment was administered. Intact ovarian tissue was removed, and blood samples were taken for the anti-Mullerian hormone (AMH) test. Group 2 (the doxorubicin Group): Rats received doxorubicin intraperitoneally at a single dose of 3 mg/kg. Group 3 (the doxorubicin + DHEA Group): Rats received doxorubicin intraperitoneally at a single dose of 3 mg/kg at baseline and DHEA subcutaneously for 10 days at a dose of 60 mg/kg daily. Rats in groups 2 and 3 were sacrificed at the end of 10 days, ovarian tissues were removed and blood samples were taken for AMH test.

Results: While normal ovarian tissue damage scores were zero except hemorrhage, doxorubicin showed significant damage and histopathological changes in all rats. Doxorubicin and Doxorubicin + DHEA groups had higher edema, vascular congestion, cellular degeneration, and total damage scores than the normal ovarian group. The number of antral follicles and ovarian volume decreased in the doxorubicin group compared to the normal ovarian group ($p = 0.011$ and 0.002, respectively). In the doxorubicin + DHEA group, ovarian volume was similar to the normal ovary ($p = 0.091$), but the number of antral follicles was significantly lower in this group ($p = 0.002$). AMH values did not differ between the normal ovarian group and the other groups.

Conclusions: It was concluded that DHEA was not effective in preventing ovarian damage caused by doxorubicin.

Keywords: Doxorubicin, Dehydroepiandrosterone, Anti-Mullerian Hormone, Ovary, Rat

Rat Overlerinde Doksorubisinin Neden Olduğu Toksik Hasara Karşı Dehidroepiandrosteronun (DHEA) Koruyucu Etkilerinin Araştırılması

ÖZET

Amaç: Amacımız, dehidroepiandrosteronun (DHEA) doksorubisine bağlı over hasarı üzerinde koruyucu bir etkisinin olup olmadığını değerlendirmektir.

Gereç ve Yöntem: Ratlar üç gruba ayrıldı. Grup 1 (kontrol grubu), tedavi uygulanmadı. Sağlam over dokusu çıkarıldı ve Anti-Mulleran Hormon (AMH) testi için kan örnekleri alındı. Grup 2 (doksorubisin grubu), ratlara 3 mg/kg'lık tek bir dozda intraperitonal yoldan doksorubisin verildi. Grup 3 (doksorubisin + DHEA grubu), ratlara intraperitonal yolla 3 mg/kg'lık tek bir dozda doksorubisin ve günde 60 mg/kg'lık bir dozda subkutan olarak DHEA verildi. Grup 2 ve 3'teki ratların onuncu günün sonunda yumurtalık dokuları alındı ve AMH testi için kan örnekleri alındı.

Bulgular: Normal over doku hasarı skorları kanama dışında sıfır olmakla birlikte, doksorubisin tüm deneklerde anlamlı hasar ve histopatolojik değişiklikler gösterdi. Doksorubisin ve Doksorubisin + DHEA gruplarında normal over grubundan daha yüksek ödem, vasküler konjesyon, hücresel dejenerasyon ve toplam hasar skorları vardı. Antral folikül sayısı ve yumurtalık hacmi doksorubisin grubunda normal over grubuna göre azaldı (sırasıyla $p = 0.011$ ve 0.002). Doksorubisin + DHEA grubundaki over hacmi, normal over hacmine benzerdi ($p = 0.091$), ancak antral folikül sayısı bu grupta anlamlı olarak daha düşüktü ($p = 0.002$). AMH değerleri normal over grubu ile diğer gruplar arasında farklılık göstermedi.

Sonuç: DHEA'nın doksorubisinin neden olduğu over hasarını önlemede etkili olmadığı sonucuna varıldı.

Anahtar Kelimeler: Doksorubisin, Dehidroepiandrosteron, Anti-Mulleran Hormonu, Over, Rat

INTRODUCTION

Recent developments in cancer management and early diagnosis of cancer have led to an improvement in the quality of life and overall survival of pediatric and young females with cancer.(1) All these advancements have increased the life expectancy of patients diagnosed with cancer.(2) Currently, the survival rate has increased to 80%–90% for certain types of cancer such as breast cancer and childhood leukemia.(3, 4) In fact, the 5-year survival rate for pediatric cancer, which was 58% in the mid-1970s, has now increased to 83%.(5) With the increase in the survival rate for cancer, the side effects of cancer treatments are drawing more concern.(6)

In oncology, a conventional approach is defined as an untargeted and non-selective therapy as the initially developed therapeutic agents rapidly affect cell division, thereby impacting normal and cancerous cells.(7) Cytotoxic chemotherapy is the basis for the treatment of various childhood malignancies; however, these treatment methods are known to have several side effects.(8) Doxorubicin is a prototype agent of anthracycline antibiotics.(9) Anthracycline antibiotics are used for the treatment of several human malignant neoplasms such as various solid tumors (ovarian, breast, lung and liver cancer), Hodgkin's disease, Kaposi sarcoma, leukemia, lymphoma and childhood cancer.(10-12) Despite being a commonly used medication with high efficacy, the clinical use of doxorubicin has been limited due to its side effects.(13-16) Ovarian toxicity due to chemotherapy is a critical problem for children (0–15 years) and patients of reproductive age (15–44 years).(17) Chemotherapeutic chemicals are known to cause reproductive toxicity, damage ovarian follicles and increase the risk of premature ovarian failure and premature menopause.(18-20)

Dehydroepiandrosterone (DHEA) is a weak androgenic steroid primarily secreted from the adrenal glands as well as the ovaries and peripheral feedback.(21) Many tissues can intake DHEA and its sulfated metabolite (DHEA-S) after they are metabolized into active androgenic and estrogenic sterol compounds, which are required for growth and development.(22) DHEA has drawn the interest of specialists in recent years for increasing fertility.(23, 24) An international survey showed that in 26% cases of in vitro fertilization (IVF), clinicians used DHEA as an auxiliary agent for such women.(25)

In a meta-analysis, Ji et al. showed that DHEA supplementation before IVF could improve pregnancy rate and increase the number of oocytes collected, although it did not affect miscarriage rate and the total gonadotropin dose used.(26)

The present study aimed to investigate whether DHEA had protective effects on doxorubicin-induced ovarian damage.

MATERIAL AND METHODS

This study was conducted at the Animal Testing Laboratory of University in July 2019 after obtaining the approval of the Ethics Committee.

Laboratory Animals and the Care of Animals in Research: Ten-twelve week-old female Wistar Albino (Rattus Norvegicus species) rats weighing 180 to 220 grams were used in this study. Rats received light exposure 12 hours a day (from 08:00 to 20:00) and had access to food (standard rodent pellet) and drinking water (tap water) without restriction and were kept at a room temperature of 21 to 23ºC with a humidity of 40 to 50%. Rats were housed 4 or 5 per cage. The number of rats was chosen in line with previous studies. Rats were randomly assigned to four groups of 8. Considering bowel transit time, rats were not fed within 6 hours before laparotomy to empty the gut and allow surgery but had access to drinking water.

Study Groups: Group 1 (the control Group): These rats underwent a laparotomy procedure at baseline, and their ovaries were removed. Blood was drawn from the inferior vena cava for AMH testing.

Group 2 (the doxorubicin Group): Rats received doxorubicin intraperitoneally at a dose of 3 mg/kg at baseline(27) and underwent an oophorectomy procedure at the end of day 10 of the study. After sacrificing the rats, at least 2-3 ml of blood was collected for AMH testing. Then, laparotomy was performed, and both ovaries were excised for histopathological examination.

Group 3 (the doxorubicin + DHEA Group): Rats received doxorubicin intraperitoneally at a dose of 3 mg/kg at baseline. Also, they received DHEA (Cayman Chemical, Michigan, USA, CAS registry no: 53-43-0, item no:15728) subcutaneously for 10 days at a dose of 60 mg/kg daily as dissolved in 0.1 ml of sesame oil.(28, 29) After sacrificing the rats, at least 2-3 ml of blood was collected for AMH testing. Then, laparotomy was performed, and both ovaries were excised for histopathological examination.

Doxorubicin Dose and Preparation: Doxorubicin was administered intraperitoneally at a dose of 3 mg/kg only at baseline. While preparing the drug, we used the central drug preparation unit of our hospital (with Robotic Chemotherapy Drug Preparation System) in a closed environment where microbiological contamination and employee exposure risks are eliminated under conditions in compliance with national and international standards. Negative pressure indoor air environment complied with ISO 5 and had Class 100 and GMP Class A double HEPA filter air cleaning system, safe waste management system, high capacity laminator current, and dose sensitivity information (gravimetric and volumetric) measurement, and the barcode system was performed.

Surgical Procedures: Sterile, powder-free, latex gloves were used during all surgical procedures. The procedure was performed while the rats were lying in a supine position. The abdominal area was shaved before the procedure, and the surgical site was prepared using a 10% Povidone-iodine solution (Batticon; Adeka Laboratories, Istanbul, Turkey). A 5 cm median (on the line between the xiphoid process and pubis) incision was made to enter into the abdominal cavity, and each surgical procedure lasted 5 to 10 minutes to protect the drying effect of the room air. After the removal of ovaries for histological examination, animals were decapitalized and disposed of in red waste containers (Figure 1).

Histopathological Examinations: Surgically excised ovaries were fixed in 10% formalin. Paraffin blocks were prepared 24 hours after the oophorectomy procedure. Tissue sections of 5 micrometers were taken, and follicular activity was assessed in 5 randomly selected samples from each ovary. Slides were stained with hematoxylin-eosin and examined under a light microscope. The paraffin blocks were sectioned using a microtome blade (Leica, Nussloch, Germany). Every slide was blindly assessed by the same pathologist. A light microscope (Olympus Clinical Microscope, Tokyo, Japan) was used to analyze the sections.

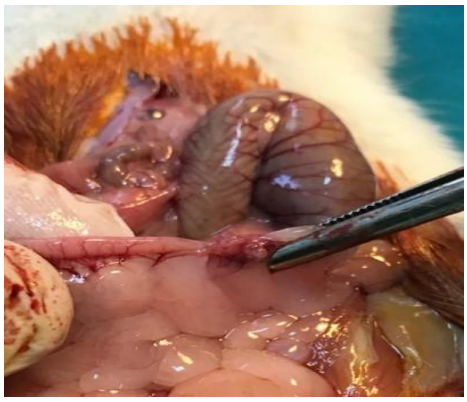

Figure 1. Excision of the ovary

Edema, vascular congestion, inflammation, cellular degeneration, and hemorrhage were examined as histopathological injury scores (figure 1). The scores were evaluated as described by Celik et al.(30). Pathological findings were rated. Grade 0 indicated normal alterations and no abnormal findings; Grade 1 indicated mild edema, mild vascular congestion, absence of hemorrhage or leukocyte infiltration; Grade 2 indicated moderate edema, moderate vascular congestion, absence of hemorrhage or leukocyte infiltration; Grade 3 indicated severe edema, severe vascular occlusion, minimal hemorrhage, and minimal leukocyte infiltration, and Grade 4 indicated severe edema, severe vascular occlusion, hemorrhage, and leukocyte infiltration. (Figure 2)

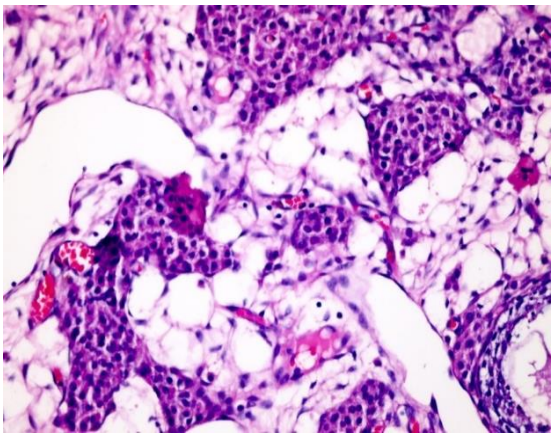

Figure 2. Significant edema x400 hematoxylin-eosin

All follicles were counted to assess ovarian reserve. Primordial, primary, secondary (pre-antral), tertiary (antral), and atretic follicles were counted (Figures 3, 4). Follicles were evaluated as described by Parlakgumus et al.(31).

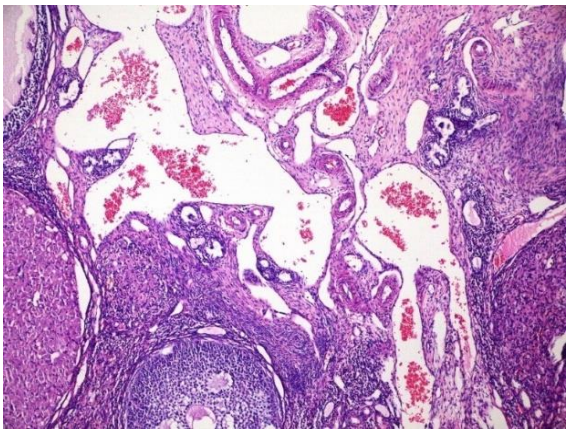

Figure 3. Veins with marked dilatation x200 hematoxylin-eosin

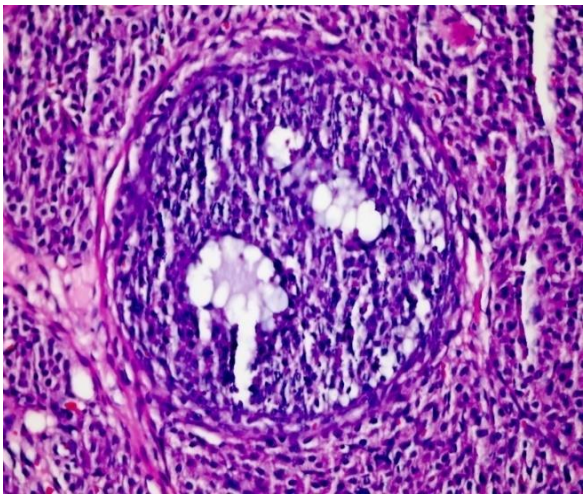

Figure 4. Degenerated follicle x400 hematoxylin-eosin

Primordial, primary, secondary (pre-antral) and tertiary (antral) follicles were counted. Primordial follicle is described as an oocyte with only one surrounding epithelial cell layer, and the primer follicle is surrounded by one or more layers of cuboidal granulosa cells. Secondary/ pre-antral follicle is surrounded by more than two cell layers and consists of antrum follicles and zona pellucida. The tertiary follicle is defined if there are antrum, stratum granulosum and surrounding cumulus oophorus layers. In an atretic follicle, the basement that separated the oocyte from granulosa cells often thickens to become the glassy membrane. Fibrous material replaces the granulosa cells, and loss of cohesion may occur in granulosa cells.

AMH Assays: Blood samples were collected into tubes containing lithium heparin (BD Vacutainer Plasma tubes, Manchester, England). The concentration of the Lithium Heparin additive in these tubes is 17 international units of heparin/ml of blood. The blood samples were centrifuged within 30 minutes of sampling. After 15 minutes of centrifugation at 1000xg, serum was removed, and the remaining plasma was transferred into an Eppendorf tube and stored frozen at -20ºC until the time of analysis. AMH concentrations were measured in "ng/ml" plasma using the ELISA method. The rat AMH kit used in the study had a sensitivity of 0.10 g/mL, a detection range of 0.16 to 10 ng/mL and a coefficient of variation less than 10% (Elabscience, Rat AMH kit; Houston, Texas, ABD). The laboratory technician of the laboratory of the university hospital was blinded to the study groups and unaware of which samples belonged to which rat. All samples were analyzed in the same assay.

Statistical Analysis: SPSS version 17.0 was used for statistical analyses. The normal distribution of variables was evaluated using histograms and Kolmogorov–Smirnov test. Mean, standard deviation, median, and interquartile range are used to present descriptive statistics. Non-normally distributed (non-parametric) variables were compared between two groups using Mann–Whitney *U*-test. Spearman's correlation test was used for the analysis of measurement data. A *p*-value <0.05 was considered statistically significant.

RESULTS

Histopathological damage scores were compared between the groups. While ovarian tissue damage scores were 0, except in cases of hemorrhage, significant damage and histopathological changes were observed in the ovarian tissues of rats that were administered doxorubicin. Edema, vascular congestion, cellular generation, and total damage scores of the doxorubicin and doxorubicin+DHEA groups were found to be higher than those of the normal ovary group. Further, their inflammation and hemorrhage scores showed no increase compared to those of the normal ovary group (Table 1).

Table 1. Comparison of histopathological damage scores of normal ovary vs doxorubicin and doxorubicin + DHEA groups

	Normal ovary	Doxorubicin	*P**	Doxorubicin +DHEA	*P***
Edema					
Mean SD	0,00±0,00	0,75±0,71	**0,010**	1,50±0,53	**<0,001**
Median- IQR	0,00(0,00-0,00)	1,00(0,00-1,00)		1,50(1,00-2,00)	
Vascular congestion					
Mean SD	0,00±0,00	0,88±0,64	**0,003**	1,25±0,71	**0,001**
Median- IQR	0,00(0,00-0,00)	1,00(0,50-1,00)		1,00(1,00-2,00)	
Inflammation					
Mean SD	0,00±0,00	0,13±0,35	0,317	0,00±0,00	1,000
Median- IQR	0,00(0,00-0,00)	0,00(0,00-0,00)		0,00(0,00-0,00)	
Cellular degeneration					
Mean SD	0,00±0,00	0,75±0,89	**0,027**	0,50±0,53	**0,025**
Median- IQR	0,00(0,00-0,00)	0,50(0,00-1,50)		0,50(0,00-1,00)	
Hemorrhage					
Mean SD	0,13±0,35	0,00±0,00	0,317	0,00±0,00	0,317
Median- IQR	0,00(0,00-0,00)	0,00(0,00-0,00)		0,00(0,00-0,00)	
Total score					
Mean SD	0,13±0,35	2,50±1,41	**0,001**	3,25±1,16	**<0,001**
Median- IQR	0,00(0,00-,00)	2,00(1,50-3,50)		3,50(2,00-4,00)	

*, ** Mann-Whitney U Test

Primordial, primary, secondary, tertiary, and atretic follicle counts were compared with ovarian volume. The results revealed that the doxorubicin group had decreased antral follicle count (AFC) and ovarian volume than the normal ovary group (p = 0.011 and 0.002, respectively). The doxorubicin+DHEA and normal ovary groups had similar ovarian volume (p=0.091); however, AFC was significantly lower in the doxorubicin+DHEA group (p=0.002; Table 2).

Table 2. Comparison of normal ovary vs Doxorubicin, Doxorubicin + DHEA groups in terms of follicle count and AMH values

	Normal ovary	Doxorubicin	P*	Doxorubicin +DHEA	P**
Primordial follicle					
Mean SD	12,75±1,91	8,25±5,68	0,091	9,25±5,73	0,339
Median- IQR	12,50(11,50-14,00)	7,00(3,00-13,00)		9,00(5,00-14,00)	
Primer follicle					
Mean SD	10,50±2,33	9,88±5,46	0,833	10,50±6,09	0,792
Median- IQR	11,00(8,50-12,00)	9,50(6,00-14,00)		12,00(4,00-15,50)	
Secondary (pre-antral) follicle					
Mean SD	12,25±1,83	9,63±4,24	0,114	11,13±3,00	0,363
Median- IQR	12,50(10,50-13,50)	8,50(6,50-13,00)		10,50(10,00-13,00)	
Tertiary (antral) follicle					
Mean SD	21,50±3,21	13,88±5,19	**0,011**	14,00±2,27	**0,002**
Median- IQR	22,00(19,50-23,50)	13,00(10,00-16,50)		14,00(12,00-16,00)	
Atretic follicle					
Mean SD	,25±,46	,88±1,13	0,223	,75±1,04	0,268
Median- IQR	,00(,00-,50)	,50(,00-1,50)		,50(,00-1,00)	
AMH (ng/mL)					
Mean SD	3,42±,79	3,02±,94	0,401	2,49±,88	0,074
Median- IQR	3,37(2,64-4,06)	2,96(2,39-3,62)		2,46(1,76-3,26)	
Ovary volume (mm3)					
Mean SD	55,49±9,14	34,49±8,05	**0,002**	47,57±14,04	0,091
Median- IQR	54,12(50,19-55,53)	32,59(28,83-39,38)		46,72(37,64-53,09)	

*, ** Mann Whitney U Test

For both study groups, AMH was evaluated for any correlation with rat weight, ovarian volume, total damage score, atretic follicle count, and preantral+antral follicle count. In the normal ovary group, there was a strong positive correlation between AMH and ovarian volume (Table 3).

Table 3. Correlations between rat weights, over volume, total damage score, number of atretic follicles, and AMH levels

	Normal AMH	Doxorubicin AMH	Doxorubicin +DHEA AMH
Ratweight (grams)	0,443	-0,647	-0,467
Ovaryvolume (mm3)	**0,778***	-0,262	0,132
Total damagescore	0,082	0,160	-0,630
Pre-antral+ antralfolliclecount	-0,072	0,452	-0,337

Spearman's Correlation Test *p<0.050

DISCUSSION

The risk of developing amenorrhea following doxorubicin treatment ranges between 40% and 80% depending on age (high incidence at ≥40 years; moderate incidence at 30–39 years).(32, 33) To date, doxorubicin-induced pathomechanisms such as apoptosis, oxidative stress, and inflammation have been extensively studied.(34, 35) The chemical structure of doxorubicin causes cell damage, induces oxidative stress due to the production of free radicals(36), and leads to tissue damage as a result of these effects. The doxorubicin and doxorubicin+DHEA groups had higher edema, vascular congestion, cellular generation, and total damage scores than the normal ovary group. Chemotherapy was found to cause significant histopathological damage to ovarian tissues. However, DHEA appears to have no protective effect on such damage possibly due to the differences between the mechanism by which doxorubicin causes damage and the mechanism of action of DHEA. Doxorubicin has been shown to damage mitotically active granulosa cells, induce follicular apoptosis and eventually disrupt ovarian function and efficiency(2, 32, 37). Suitable agents are required to prevent such damage. Certain studies have shown improvements in AFC, ovarian volume and follicular activity after DHEA supplementation even in women with premature ovarian failure.(38) In the present study, the doxorubicin group had significantly decreased AFC and ovarian volume compared to the normal ovary group. In the doxorubicin+DHEA group, while there was no decrease in the ovarian volume, AFC showed a significant decrease.

DHEA is converted into testosterone in ovarian connective tissues (theca/stroma) and then processed by granulosa cells to be converted into estradiol. Therefore, the prohormone state of an endogenous precursor and a metabolic intermediate product is assumed to be involved in follicular steroidogenesis.(39, 40) This mechanism underlies the effects of DHEA. In the present study, primordial, primary, and preantral follicles were present in similar amounts in the doxorubicin+DHEA and normal ovary groups.

Many studies have verified and

confirmed that AMH is a reliable molecular bioindicator of ovarian reserve.(41, 42) Its decrease to minimal levels can be correlated with decreased follicle counts.(43) AMH expression is seen in the granulosa cells of small growing follicles (preantral and small antral follicles).(44) In the doxorubicin group, the AMH level was similar to that of the normal ovary group. The absence of significant damage to preantral follicles might have led to this result. Besides, a decrease in the AMH level might not have accompanied chemotherapy at an early stage. Evaluation of the long-term results can be beneficial to gain a complete understanding of this subject.

In the present study, there was a strong positive correlation between AMH level and ovarian volume in the normal ovary group. Only the doxorubicin group exhibited a significant decrease in the ovarian volume compared to the normal ovary group. There was no decrease in the ovarian volume in the doxorubicin+DHEA group. AMH is known to have a positive correlation with ovarian volume and peripheral follicular distribution.(45) Preservation of ovarian volume and follicles can affect AMH levels.

Taken together, the results indicated that the additional use of DHEA in rats administered doxorubicin could not decrease ovarian tissue damage scores or prevent follicle loss and did not lead to changes in AMH levels.

CONCLUSION

The use of DHEA was not effective in the prevention of doxorubicin-induced ovarian damage in rats.

Acknowledgment: All authors have acknowledged that they received no technical help or financial support.

Conflict of interest: The authors declare no conflict of interest.

Disclosure: There is no disclosure.

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