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Original Article

Effect of repeated xylazine-ketamine or medetomidine-ketamine administration on selected reproductive parameters in male rats

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Abstract

Background: Sperm retrieval methods and surgical interventions are typically performed with the aid of anesthetic agents. In cases where the depth of anesthesia is insufficient, the procedure is often repeated. **Aims:** This study aimed to investigate the effects of repeated administration of xylazine-ketamine (XK) and medetomidine-ketamine (MK), as commonly used agents in laboratory animals, on sperm parameters, reproductive hormones, and testicular oxidative stress status. **Methods:** A total of 60 rats were used in the study. The animals were divided to two groups of thirty animals each as XK or MK groups, then each group was further divided into five subgroups (control, single dose, 1 repeat, 2 repeats, 3 repeats). **Results:** In the MK application group, the 2 repeats group exhibited a significant decrease in motility ($P<0.001$), an increase in the percent of abnormal sperms ($P<0.05$), and an elevated dead-to-live sperm ratio ($P<0.001$). In the MK 3 repeats group, a decrease in testosterone levels was observed ($P<0.001$). In all application groups (XK and MK), mRNA levels of testicular catalase (*CAT*), superoxide dismutase 1 (*SOD1*), androgen receptor (*AR*), and *caspases* 3/8/9 were significantly reduced ($P<0.001$). **Conclusion:** Despite the adverse effects of repeated anesthesia on certain testicular parameters, this study revealed that XK application is safer than MK in terms of spermatological characteristics.

Key words: Ketamine, Medetomidine, Oxidative stress, Sperm, Xylazine

Introduction

Anesthesia is defined as a reversible state characterized by temporary loss of consciousness, decreased reflex activity, and varying degrees of muscle relaxation, without remarkable alteration in vital functions, achieved through the external administration of pharmacological agents (Kayhan, 1997).

In animal studies, anesthesia is commonly employed in surgical interventions and various sperm retrieval methods. It is particularly necessary for techniques such as electroejaculation, epididymal puncture, and testicular or epididymal sperm aspiration (Abril-Sánchez *et al*., 2019; Hidalgo *et al*., 2023). In the veterinary field, combinations of xylazine/ketamine (XK) and medetomidine/ketamine (MK) are commonly used for anesthesia (Jalanka and Roeken, 1990; Kiliç, 2004; Casas-Diaz *et al*., 2011; Kibar *et al*., 2022).

Ketamine interacts with N-methyl-D-aspartate (NMDA) receptors, opioid receptors, monoaminergic receptors, muscarinic receptors, and voltage-sensitive calcium channel receptors (Tranquilli *et al*., 2007). It is a potent analgesic at subanesthetic concentrations. Unlike other injectable anesthetics, ketamine does not interact with GABA receptors (Katzung, 2001). Medetomidine and xylazine, on the other hand, exert their effects as alpha-2 (α2) adrenergic receptor agonists (Suckow *et al*., 2012). These α2 agonists demonstrate their clinical effects by binding to presynaptic α2 receptors, leading to a decrease in norepinephrine release as a negative feedback (Tranquilli *et al*., 2007). The α2 receptors located in the dorsal spinal cord modulate pain relief and transmission (Katzung, 2001; Seymour and Novakovski, 2007). Medetomidine is more potent than xylazine with its binding affinity for central α2-receptors. Medetomidine differs from other α2 agonists due to its lipophilic nature, rapid elimination, and increased potency (Koç *et al*., 2004). Its selectivity ratio for α2/α1 receptors is 1620:1, whereas for xylazine, it is 160:1. The half-life of both medetomidine and xylazine is

approximately 30 min (Koç *et al*., 2004; Seymour *et al*., 2007; Tranquilli *et al*., 2007; Suckow *et al*., 2012; Clarke *et al*., 2014; Dugdale *et al*., 2020). Xylazine, ketamine, and medetomidine are known to affect the reproductive system, including the testes and spermatogenesis, through various mechanisms. Both xylazine and medetomidine, as α2 adrenergic receptor agonists, disrupt hormonal balance by decreasing luteinizing hormone (LH) and testosterone secretion, thereby affecting spermatogenesis (Jager *et al*., 1998; Qi *et al*., 2017). Previous studies have shown that ketamine, a dissociative anesthetic, negatively impacts testosterone synthesis and spermatogenic activity by altering the hypothalamic-pituitary-gonadal axis (Ding *et al*., 2015; Cao *et al*., 2021). Moreover, these agents may increase oxidative stress in testicular tissue, leading to apoptosis of germ cells and impaired sperm production (Allaouchiche *et al*., 2001; Lee *et al*., 2015). Therefore, repeated or prolonged use of these anesthetic agents poses potential risks to male reproductive parameters. The aim of this study is to investigate the effects of repeated doses of XK or MK combinations on testicular oxidative stress, serum FSH, LH, and testosterone levels, as well as spermatological parameters of male rats.

Materials and Methods

The study was conducted in accordance with agreement No. 65202830-050.04.04-726, approved by the Animal Experiments Local Ethics Committee of Sivas Cumhuriyet University on 12/05/2023.

Animals

Adult male Albino pathogen-free Wistar rats, aged 3-4 months and weighing between 200 and 250 g, were obtained from Sivas Cumhuriyet University. The animals were housed under a 12-hour light/12-hour dark cycle with *ad libitum* access to food and water. The ambient temperature was maintained at 26°C, and the relative humidity was kept at 60%.

Experimental method

A total of sixty rats were included in the study, divided into two primary groups based on the anesthetic combination administered: group 1 (XK) and group 2 (MK). Each group was further subdivided according to the frequency of dose administration.

Group 1 received a combination of 90 mg/kg, intramuscular (i.m) ketamine hydrochloride (Ketasol® %10, Richter Pharma, İnterhas, Ankara) and 3 mg/kg (i.m) xylazine hydrochloride (Xylazinbio® %2, Bioveta, Czech Republic) (Jalanka and Roeken, 1990; Kiliç, 2004). Group 2 was administered 90 mg/kg (i.m) ketamine hydrochloride and 0.15 mg/kg (i.m) medetomidine (Domitor®, Orion Pharma, Zoetis, Istanbul) (Clarke *et al*., 2014). To ensure optimal anesthetic efficacy, drug dosages and administration methods were carefully selected, with subsequent doses at half of the initial dose administered to each subgroup.

Group 1 (XK group)

A combination of xylazine and ketamine was administered to thirty rats to induce general anesthesia. The anesthetic procedures for the rats are as follows:

Thirty rats were randomly selected and divided into five subgroups of six rats each.

Subgroup a (control group): A single dose of the XK combination was administered, and the rats were sacrificed immediately upon reaching anesthesia (2-4 min). Rats were sacrificed by exsanguination under anesthesia. Following sacrifice, testis tissue samples and epididymal sperm samples were collected.

Subgroup b (single-dose application group): The rats were sacrificed 30 min after receiving XK anesthesia. Rats were sacrificed by exsanguination under anesthesia. Following sacrifice, testis tissue samples and epididymal sperm samples were collected.

Subgroup c (1 repeat application group): This group received an initial normal dose of XK, similar to subgroup b. After a 30-minute waiting period, a half-dose was administered. The rats were sacrificed 30 min after the repeated dose while still under anesthesia. Rats were sacrificed by exsanguination under anesthesia. Following sacrifice, testis tissue samples and epididymal sperm samples were collected.

Subgroup d (2 repeats application group): Following the initial normal dose of XK (similar to subgroup b), a 30-minute waiting period was observed. Two repeated doses were then administered at 30-minute intervals. Rats were sacrificed by exsanguination under anesthesia. Following sacrifice, testis tissue samples and epididymal sperm samples were collected after the second repeated dose.

Subgroup e (3 repeats application group): After the initial normal dose of XK (similar to subgroup b), a 30-minute waiting period was observed. Subsequently, three repeated doses were administered at 30-minute intervals. Rats were sacrificed by exsanguination under anesthesia. Following sacrifice, testis tissue samples and epididymal sperm samples were collected.

Group 2 (MK group)

Thirty rats were administered MK anesthesia. The rats were divided into five subgroups, and the same experimental procedures as in group 1 were applied, using the MK combination. The study design is outlined in Table 1.

Determination of epididymal sperm motility

Immediately following sacrifice, the cauda epididymis was carefully punctured to obtain a sperm sample. The sample was then placed on a glass slide, which was set on a heating table maintained at a precise temperature of 38°C. A cover slip was placed at a 45° angle to the slide. Sperm motility was assessed under a microscope at ×40 magnification. Spermatozoa exhibiting consistent, forward progressive movement were distinguished from immotile spermatozoa, which demonstrated whirling or trembling motions.

Table 1: Study design and grouping system including group 1 (XK) and group 2 (MK) anesthesia protocols, dosing schedule and time intervals for each application

Time (min)	Anesthesia dose	Group 1 (XK)	Group 2 (MK)	Remained rat
0	1	30 rats	30 rats	30
2-4 (control)		Subgroup a sacrified (n:6)	Subgroup a sacrified (n:6)	24
30		Subgroup b sacrified (n:6)	Subgroup b sacrified (n:6)	18
30	2	18 rats	18 rats	
60		Subgroup c sacrified (n:6)	Subgroup c sacrified (n:6)	12
60	3	12 rats	12 rats	
90		Subgroup d sacrified (n:6)	Subgroup d sacrified (n:6)	6
90	4	6 rats	6 rats	
120		Subgroup e sacrified (n:6)	Subgroup e sacrified (n:6)	-

XK: Xylazine-Ketamine, and MK: Medetomidine-Ketamine

Table 2: Primer sequence of target genes

Gene name	Primer sequences (5´-3´)
Beta actin (*ACTB*)	F: CTCCTCAAGGATGGCACC
	R: GCTCATTGTAGAAAGTGTGGT
CAT	F: GGACGCTCAGCTTTTCATTC
	R: TTGTCCAGAAGAGCCTGGAT
SOD1	F: GCTTCTGTCGTCTCCTTGCT
	R: CATGCTCGCCTTCAGTTAATCC
AR	F: GTGAAATGGGACCTTGGATG
	R: TACTGAATGACCGCCATCTG
CASPASE-3	F: TACCCTGAAATGGGCTTGTGT
	R: GTTAACACGAGTGAGGATGTG
CASPASE-8	F: TAAGACCTTTAAGGAGCTTCATTTTGA
	R: AGGATACTAGAACCTCATGGATTTGAC
CASPASE-9	F: GAGGGAAGCCCAAGCTGTTC
	R: GCCACCTCAAAGCCATGGT

Determination of sperm density

Following cauda epididymis puncture, 0.5 ml of Hayem solution (Norateks, Germany) was mixed with 0.1 ml of the sperm sample in Eppendorf tubes. The sperm concentration was then accurately determined using a Thoma cell counting chamber, allowing for the reliable assessment of sperm count per ml.

Assessment of sperm morphology

The sperms obtained from the epididymis were placed in Eppendorf tubes with 0.5 ml of Hancock solution (Norateks, Germany). A minimum of 400 sperms were analyzed under ×40 magnification to determine the sperm abnormality ratio per sample. A reliable estimate of the percentage of spermatozoa exhibiting abnormalities in the head, tail, and neck regions was then obtained.

Determination of live/dead sperm ratio

The sperm sample was combined with two drops of a 10% nigrosin solution (Sigma, USA) and gently mixed. A drop of this mixture was placed on a clean glass slide, spread evenly, and allowed to air dry. This method provides a reliable and accurate evaluation of sperm viability. The smears were then examined under ×40 magnification. Live sperm cells were identified by their white appearance, while dead sperm cells appeared pink. The proportion of live sperms was calculated by counting at least 400 spermatozoa.

Hormone analysis (FSH, LH, testosterone)

Serum was separated from the blood samples by centrifugation at 1048 g for 15 min using EDTA tubes. The resulting serum samples were then analyzed for follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone levels using the respective ELISA kits (FSH: Cat No.: AD3200Ra, LH: Cat No.: AD1683Ra, and testosterone: Cat No.: AD1386Ra) from Andy Gene (China).

mRNA expression analysis

Collection of tissue samples for RNA isolation and preparation for analysis

Testicular tissue samples were collected and stored sterile at -80°C until the day of the experiment. On the experimental day, the tissues were thawed at room temperature, and approximately 30 mg was transferred into sterile tubes. Sterile phosphate buffer (0.2 ml) was added to the tissues, which were then homogenized and centrifuged. The supernatant was discarded, and RNA isolation was initiated from the pellet.

RNA extraction and analysis - cDNA synthesis

Total mRNA was extracted from the pellets using the Trizol reagent-chloroform method. The RNA concentration and purity were assessed using a BioDrop nanodrop spectrophotometer. For complementary DNA (cDNA) synthesis, reverse transcription was performed using the WizScript cDNA Synthesis kit (Wizbio, Korea). The reaction was conducted following the manufacturer's protocol with a Rotor-Gene Q thermocycler.

Real-time RT-PCR (RT-qPCR)

The mRNA expression of different genes, including changes in the mRNA levels of *CAT*, *SOD1*, *androgen receptor* (AR), *Caspase 3*, *Caspase 8*, and *Caspase 9*, was examined using the real-time RT-PCR method. Actin Beta (ACTB) served as the reference gene in the expression study, and the SYBR Green Master Mix (ENZO Life Science, cat: ENZ-NUC104-0200) was used for amplification detection. Using the obtained cDNAs, the mRNA transcription levels of the target genes listed in Table 2 were evaluated. The reactions were performed

Table 3: RT-qPCR reaction conditions

Reaction content	For one sample	Reaction cycle
Buffer (2X)	10 µL	95°C 2' denaturation
Primer and control primer (Beta actin)	F: 0.5 µL	95°C 5" 40 cycle
	R: 0.5µL	* 58-60°C 30 s
		Melting curve
		Ramp: 50-99 (1 degree increment)
dH_2O	8.4 µL	90ºC 5 s
cDNA	0.6 µL	
Total	20 µL	

* The binding temperature varied according to the primers; F: Forward, and R: Reverse

Table 4: Hormone analysis results for group 1 (XK) and group 2 (MK) groups. Values are presented as mean ± standard deviation (SD)

Doses	FSH (pg/ml)		LH (ng/L)		Testesterone (ng/L)	
	Group 1	Group 2	Group 1	Group 2	Group 1	Group 2
Control	116.45±3.94	119.90±7.23	44.22±5.86	43.48±1.83	55.62±2.91	46.09±1.56 [a]
Single dose	116.08±6.22	111.26±6.09	44.38±3.02	44.95±6.85	53.57±1.60	43.34±5.25 [a]
1 Repeat	118.09±23.01	110.64±20.50	42.84±2.57	41.05±2.43	52.69±1.72	40.67±3.30 [a]
2 Repeats	95.83±20.84	119.99±19.04	45.36±2.54	47.42±8.44	55.46±4.29	40.67±4.12 [a]
3 Repeats	132.18±9.92	111.68±16.07	44.15±1.76	43.65±4.33	54.79±5.14	33.52±2.86 [b]

The difference between groups with different letters ([a, b]) in the same column is significant (P<0.001)

Table 5: Sperm motility, density, morphology, and viability results for group 1 (XK) and group 2 (MK) groups. Values are presented as mean ± standard deviation (SD)

Doses	Motility (%)		Intensity (M/ml)		Abnormal (%)		Dead-Alive (%)	
	Group 1	Group 2	Group 1	Group 2	Group 1	Group 2	Group 1	Group 2
Control	81.66±7.52	76.66±10.32 [x]	1.94±0.08	1.88±0.14	16.83±1.72	17.16±2.13 [a]	13.66±2.87	14.56±2.13 [x]
Single dose	80.00±6.32	78.33±7.52 [x]	1.88±0.10	1.90±0.09	17.50±1.87	17.83±1.16 [a]	15.02±3.03	15.83±3.16 [x]
1 Repeat	78.33±7.52	68.34±7.11 [x]	1.91±0.10	1.90±0.10	18.50±0.54	18.66±0.51 [a]	17.16±2.31	18.52±4.72 [x]
2 Repeats	75.01±10.48	21.66±74.24 [y]	1.93±0.18	1.95±0.16	18.66±1.63	20.07±1.41 [b]	15.50±3.27	80.33±3.72 [y]
3 Repeats	76.66±8.16	18.33±9.83 [y]	1.92±0.14	1.94±0.15	18.88±1.03	21.18±1.78 [b]	15.83±3.12	81.84±3.32 [y]

The difference between groups with different letters ([a, b]) in the same column is significant (P<0.05), and the difference between groups with different letters ([x, y]) in the same column is significant (P<0.001)

using the ROTOR-GENE Q system (Qiagen, Germany) with the reaction parameters outlined in Table 3, utilizing specifically designed primers for each gene. The cycle threshold (Ct) for each sample was determined at the beginning of the logarithmic phase of amplification. The 2^-ΔΔCt formula was applied to determine gene product levels. Gene expression was presented as the fold change of each gene in different groups relative to the calibrator (control) group.

Statistical analysis

The statistical analysis was conducted using the SPSS v.20 program. Data were expressed as the mean ± standard deviation. Group comparisons were performed using one-way ANOVA followed by post hoc multiple comparisons (Tukey's test). A P-value of less than 0.05 was considered statistically significant.

Results

The results of hormone analyses indicated a significant decrease in testosterone levels after the administration of the 3 repeats of MK (Table 4) (P<0.001).

Sperm analysis results demonstrated that, following 2 and 3 repeats, motility rate decreased in the MK group (P<0.001), the abnormal sperm percent increased (P<0.05), and the dead-live sperm ratio increased (Table 5) (P<0.001).

After XK administration, a statistically significant decrease was observed in the levels of *CAT*, *SOD1*, *AR*, *Caspase 3*, *Caspase 8*, and *Caspase 9* across all treatment groups compared to the calibrator group (Fig. 1) (P<0.001). Similarly, following MK administration, the levels of *CAT*, *SOD1*, *AR*, *Caspase 3*, *Caspase 8*, and *Caspase 9* decreased significantly in all treatment groups

compared to the calibrator group (Fig. 2) (P<0.001).

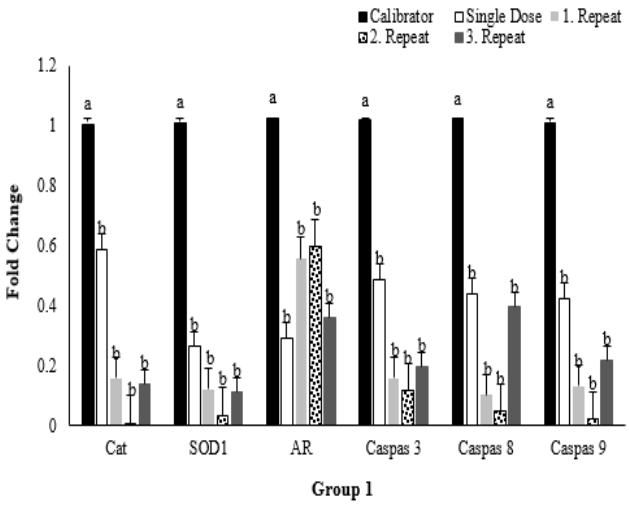

Fig. 1: Group 1 RT-qPCR results. Different letters (a, b) indicate statistically significant differences between groups (P<0.001)

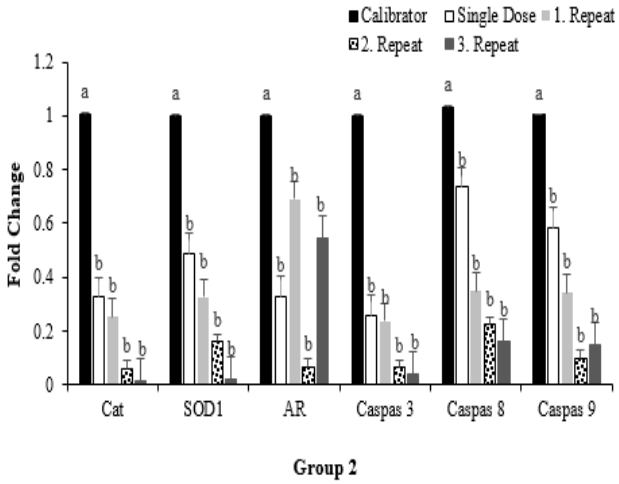

Fig. 2: Group 2 RT-qPCR results. Different letters (a, b) indicate statistically significant differences between groups (P<0.001)

Discussion

Many methods used in both animal and human health practices require anesthesia. Combinations of xylazine, medetomidine, and ketamine are commonly used for sperm collection in techniques such as electroejaculation, rectal massage, testicular sperm extraction (TESE), testicular sperm aspiration (TESA), percutaneous epididymal sperm aspiration (PESA), and microdissection epididymal sperm aspiration (MESA) (Soyalp and Kosal, 2020). Anesthesia is administered in repeated doses, as prolonged procedures lead to decreased anesthetic effectiveness. As the procedure duration increases, the drug's effect diminishes due to factors like metabolism and clearance from the body, requiring additional doses to maintain adequate anesthesia levels (Jalanka and Roeken, 1990; Kiliç, 2004). This study investigates the effects of repeated doses of XK or MK combinations on the reproductive system of male rats.

Similar to the effects of sevoflurane on testicular tissue and reproductive hormones observed by Kaya *et al*. (2013), repeated doses of ketamine and medetomidine in our study also demonstrated adverse effects on male reproductive parameters. In the sperm analysis, no statistical differences were found in motility, density, abnormal sperm percentage, and dead/live ratios in the XK group. However, in the MK group, a decrease in motility rate was observed after the second application, and an increase in the dead/live sperm ratio was found after the second application. Although many researchers recommend medetomidine for electroejaculation studies across various animal species, our findings indicate negative effects on sperm parameters following the second application (Abril-Sanchez *et al*., 2018; Silva *et al*., 2021). The parallel relationship between sperm parameters and testosterone levels is well-known. In the MK group, a decrease in testosterone levels was observed after the third application. No significant differences were found in FSH and LH hormone levels. The unchanged levels of FSH and LH are thought to be due to their pulsatile release and the fact that the hypothalamus-pituitary gland is not immediately affected by the administration. Studies investigating the effects of repeated doses of anesthesia on the male reproductive system are limited. Only studies using isoflurane and sevoflurane for 20-30 days have been conducted (Campion *et al*., 2012; Qingzhen *et al*., 2023; Zanin *et al*., 2023). These studies have reported a decrease in motility levels. Anesthetic agents may affect testosterone mobilization by disrupting the hypothalamic-pituitary-gonadal axis through the *AR-Kisspeptin-GPR54* pathway (Ding *et al*., 2015).

In studies where sperm collection under anesthesia was conducted across different animal species (Zambelli *et al*., 2007; Santiago-Moreno *et al*., 2011; Kirschner and Rodenkirch, 2017), no differences were reported between the effects of xylazine and medetomidine. In this study, similar results were obtained with single doses of medetomidine and xylazine, but changes in sperm parameters were observed following the second application. Xylazine, ketamine, and medetomidine have been reported to suppress androgen receptors by many researchers (Jager *et al*., 1998; Qi *et al*., 2017; Cao *et al*., 2021). In this study, suppression of *AR* was also observed in the treatment groups (P<0.001). The presence of *AR* is essential for the effect on sertoli cells and the continuity of spermatogenesis (Chemes *et al*., 2008).

In the present study, a decrease in oxidative stress parameters was observed in all treatment groups, regardless of the anesthetic agent and the number of doses. It is well-known that anesthetic agents increase oxidative stress (Allaouchiche *et al*., 2001; Wang *et al*., 2010; Lee *et al*., 2015; Ottolenghi *et al*., 2020; Senoner *et al*., 2021). Oxidative stress results from an imbalance

between reactive oxygen species (ROS) and antioxidants. While basal ROS levels are necessary for normal cellular functions, excessive ROS can damage cellular macromolecules such as DNA, lipids, and proteins, ultimately leading to mitochondrial DNA damage (Slater *et al*., 1995; Chandra *et al*., 2000; Kannan and Jain, 2000).

In this study, it was observed that after the third dose of MK administrations, the motility rate began to decrease, the rate of dead spermatozoa increased, and testosterone levels decreased. Regardless of the anesthesia group, it was found that *CAT* and *SOD1* levels decreased in all treatment groups, leading to an increase in oxidative stress, while *AR* levels also decreased. It was determined that the repeated doses of the XK anesthesia protocol were more reliable in terms of male reproductive parameters compared to the MK anesthesia protocol. However, further studies are needed to evaluate the effects of MK and XK anesthesia on reproductive parameters in other animal species.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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