

An Experimental Study of the Effects of Combined Exposure to Microwave and Heat on Gene Expression and Sperm Parameters in Mice

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ABSTRACT

Objectives: Separate exposure to microwaves (MWs) or heat had effects on expression levels of Bax and Bcl-2 and sperm parameters in studied group. **Aims:** The objectives of this research were to determine the effects of separate and combined exposure to 900-MHz MW (as representative of cell phone radiation) and heat on gene expression and spermogram of male mice. **Settings and Design:** This experimental animal study was conducted in the school of public health. **Materials and Methods:** The study was done on 12 male mice randomly divided into four groups (21–23 g): control, test group 1 with separate exposure to 900-MHz MW, test group 2 with separate exposure to hot and sultry climate, and test group 3 with simultaneous whole body exposures to 900-MHz MW and hot and sultry climate. In all studied groups, gene expression and sperm parameters were measured. **Results:** Tissue samples in all test groups showed integrity of the seminiferous tubule followed by all types of germ line cells. Significant increases in the number of dead sperms in mice with separate exposure to heat were observed in comparison with the other studied groups ($P < 0.05$). The ratio of Bax expression was elevated to 0.015 ± 0.006 in mice after combined exposures to 900-MHz MW and heat. **Conclusion:** Separate and combined exposure to 900-MHz MW and heat may induce adverse effects on sperm parameters and gene expression of studied male mice.

KEYWORDS: Combined exposure, gene expression, heat, microwave, sperm parameters

INTRODUCTION

Microwaves (MWs), as a part of electromagnetic field (EMF), have frequencies ranging from 300 MHz to 300 GHz and wavelength between 1 mm and 1 m. Recent evidence suggests that MWs have effects on biosystems.^[1] Surveys, such as that conducted by Yao *et al.*,^[2] have shown that MW exposures had thermal and nonthermal effects on rabbit lens epithelial cells after 8 h of exposures. Their results showed that surface temperature of cell was increased after radiation treatment. No significant differences were found

between protein expression and messenger ribonucleic acid (mRNA) levels in the control and exposed groups after radiation treatment. The relationship between

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How to cite this article: Gohari FA, Saranjam B, Asgari M, Omid L, Ekrami H, Moussavi-Najarkola SA. An Experimental Study of the Effects of Combined Exposure to Microwave and Heat on Gene Expression and Sperm Parameters in Mice. *J Hum Reprod Sci* 2017;10:128-34.

Access this article online

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10.4103/jhrs.JHRS_136_16

exposure to 915-MHz MWs and its biological effects has been investigated in Salford *et al.*^[3] study. It has conclusively been shown that exposure to continuous and pulsed 915-MHz MWs had effects on permeability of the blood–brain barrier to albumin. Several studies have revealed that exposure to electromagnetic radiation may cause changes in the levels of expression of genes.^[4]

Apoptosis has been defined as programmed cell death and is a natural and active process to control the populations of cell during development or aging of cell. Electromagnetic waves, neurodegenerative diseases, and cancer may be the possible reasons for dysregulation apoptosis. Cell shrinkage, membrane damage, chromatin condensation, and deoxyribonucleic acid (DNA) fragmentation are the main features of apoptosis.^[5,6] Apoptosis as a very complex process is regulated by Bcl-2 and Bax proteins. Bcl-2 family proteins were identified as inhibitors of apoptosis. Bax protein promotes apoptosis. Bcl-2 can bind to the mitochondrial outer membrane channel and maintain the integrity of membrane.^[7,8] After exposure of cell to apoptosis-inducing factor, Bax protein is transferred from cytoplasm to the mitochondrial membrane and made changes in the permeability of the outer membrane. These changes cause the release of cytochrome c and other apoptosis-inducing factors from mitochondria and lead to DNA fragmentation.^[9] Whole-body exposure to 900-MHz radiation emitted by a mobile phone in two groups of rats (case and control groups) failed to show any changes in the levels of Bcl-2 proteins in the brain and testes of studied rats after 20 min of daily exposure during 1 month.^[10] The results of exposure to a 900-MHz EMF showed a significant increase ($P < 0.01$) in the cell apoptosis after 2 h of exposure. The results of Marinelli *et al.*^[11] also indicated a decrease in the proapoptotic effects of radiation after 24 and 48 h of exposure. The levels of Bcl-2 proteins were increased after 4 h of exposure to electromagnetic waves and an overexpression in the proapoptotic gene *Bax* was observed after 2 and 4 h of exposure.^[11]

The expression levels of Bax and Bcl-2 in spermatogenic cells may change due to exposure to heat. The expression levels of Bax in experimental groups of rats increased in Xu *et al.*^[12] study. Spermatid and sperm cells showed higher levels of Bax expression after exposure to heat. Bcl-2 expression decreased after heat exposure. Exposure to heat led to increased apoptosis in spermatogenic cells. Previous research findings have revealed that 7-day exposure to 43°C heat for 15 min could change the levels of Bcl-2 expression in testicular germ cell. Increased apoptosis of testicular germ cell in male mice was observed after a day of heat exposure.^[13] Rockett *et al.*^[14] draw their attention to investigate the effects of hyperthermia on apoptosis and gene expression in spermatocytes in adult male mice. The expression levels of Bax in the testis of adult

male mice decreased after exposure to 43°C heat for 20 min. Apoptotic spermatocytes increased after exposure to heat.^[14] The results of examination of relationship between peripheral-blood lymphocyte apoptosis and different temperature–humidity index indicated that the peripheral-blood lymphocyte apoptosis significantly increased at temperature–humidity index of 83.58.^[15]

Some workers in some areas of Iran, such as Asaloyeh, have combined exposure to heat and MW in their workplaces. Mobile phone radiation exposure is the major source of exposure to radiation in these workers.

However, far too little attention has been paid to examine the effects of combined exposure to heat and MW on gene expression and many studies report only the effects of local testicular heat exposure on gene expression levels.

Aims and objectives

The objectives of this research were to determine the effects of separate and combined exposure to 900-MHz MW (as representative of cell phone radiation) and heat on gene expression and spermogram of male mice. We have simulated the exposure of workers who have exposure to 900-MHz MW and hot and sultry climate.

MATERIALS AND METHODS

Preparing the control and test groups

This study was conducted on 12 male mice (6-week old) randomly divided into four groups including: control group ($n = 3$), kept under experimental conditions with no exposure to MW or sultry climate; and three test groups, including 900-MHz MW-exposed group ($n = 3$), heat-exposed group ($n = 3$), and combined-exposed group ($n = 3$) that simultaneously exposed to 900-MHz MW and hot and sultry climate. The weight of the mice was in the range of 21 to 23 g. The mice were obtained from department of physiology and neuroscience and kept in the animal house at 21 to 23 °C temperature and lighting with 12 h dark/light schedule. Ventilation parameters, food, and water supply were considered. All regulations on animal experiments, including ethical issues regarding the use of animals were considered. The exposure of mice was categorized as: test group 1, separate exposure (whole body) to 900-MHz MW with 200-kHz modulation and 6 W power for 8 h a day and 5 days a week; test group 2, separate exposure (whole body) to hot and sultry climate (60°C of ambient temperature and 50% of relative humidity) for 8 h a day and 5 days a week; and test group 3, combined exposure(whole body) to 900-MHz MW frequency and hot climate (60°C of ambient temperature and 50% of relative humidity) for 8 h a day and 5 days a week. The mice were free to move.

Exposure chamber

The 50 × 50 × 50 cm plexi-glass chamber was used as an exposure chamber. MW signal generator was used to

generate 900-MHz MW.^[16] To prevent the reflection of 900-MHz MW generated by device, the exposure chamber was surrounded by pyramid-shaped polyurethane walls. An antenna was used to uniform exposure of male mice to radiation.^[16] Heating of the room was done using portable electric room heater and relative humidity was controlled by spraying water into the room. For controlling the temperature (60°C of ambient temperature) and relative humidity (50%) in the room, digital wet-bulb globe temperature measuring instrument (Questemp 10, Oconomowoc, Wisconsin, USA) with an accuracy of $\pm 0.1^\circ\text{C}$ was used.

Measuring of gene expression

Ribonucleic acid (RNA) extraction was performed manually using TRIZOL solution according to the protocol of the manufacturer. Deoxyribonuclease (DNase) I was used to treat RNA and remove genomic contamination using a kit (Fermentas, Vilnius, Lithuania) according to manufacturer's recommendation. Ultra-violet spectrophotometer (Eppendorf, Hamburg, Germany) was applied to measure the concentrations of RNA. The cDNAs synthesis was performed using 500 ng DNase-treated RNA samples with RevertAid First Strand cDNA Synthesis Kit (Fermentas) with oligo (dT) primers. Real-time polymerase chain reaction (PCR) was designed using AlleleID software (Premier Biosoft International, USA). The primer sets were as follows: *Bcl-2*, GAPDH, and Bax gene as housekeeping and normalizer gene [Table 1].

A total volume of 20 μl of reaction was obtained using 1000 ng of cDNA, 1 μl of forward primer, 1 μl reverse primer, 10 μl SYBER Green, and 7 μl ribonuclease (RNase) free water. PCR conditions were determined as 95°C for 15 s, 60°C for 30 s, 72°C for 30 s, followed by 40 cycles. Melting curve program was in the temperature range 60 to 95°C. The standard curve prepared by the logarithmic dilution series of testis. cDNA was used to determine the efficiency for each gene. The expressions of Bax and *Bcl-2* in each group were investigated in the current study. Each experiment was repeated three times. The expression levels of genes were calculated according to the formula presented in the study conducted by Jahromi *et al.*^[17]

Measuring of sperm parameters

Epididymis of each of the mice was removed to analyze sperm then placed in the phosphate-buffered saline (PBS).

The epididymides were minced in PBS. The sperm were allowed to swim-up into the PBS for 30 min at 36°C. A drop of sperm suspension was placed on a slide for light microscopic observation of motility. A light microscope at 400 \times magnification was used to count the motile and immotile sperm cells. A total of 200 spermatozoa were counted for each sample. Neubauer hemocytometer slide was used for sperm count. The sperm smears were stained with Diff-Quick (Baxter; McGaw Park, Waukegan, Illinois, USA) applied for the purpose of determining the number of sperm with normal morphology. A total of 200 cells were counted as the normal and abnormal morphology using the light microscopy (400 \times). Eosin dye was applied to determine the sperm viability and permeability of sperm membrane. Stained cells were considered as dead cells and no stained cells were observed as viable cells. A total of 200 sperm were studied.

Statistical analysis

Numerical data were presented as mean \pm standard error. One-way analysis of variance followed by Tukey's *post hoc* test was performed to analyze data using SPSS software (version 18.0, SPSS Inc., Chicago, Illinois, USA). *P* values less than 0.05 were considered statistically significant.

RESULTS

Tissue samples

The tissue samples in all test groups showed integrity of seminiferous tubule followed by all types of germ line cells. The seminiferous epithelium was characterized by the presence of Sertoli cells, spermatogonia, numerous spermatocytes, spermatids, and spermatozoa. There were significantly more Leydig cells in the control group than in all three test groups. Differences between the types of exposure in the test groups became histologically apparent in the number of sperm in the central lumen and the patency of the seminiferous tubules internal space. The seminiferous tubules were empty and no sperm were apparent; although some vacuolization in germ line cells were present. There were few germ cells in studied mice in the test group 2 in comparison with the other test groups [Figure 1b]. Also, in this group, sperm cells within the deferent duct were rarely observed. The

Table 1: Primers used for real-time PCR

Genes	Primer sequence	Gene bank code
<i>Bcl-2</i>	FOR: 5 -ACCGTCGTGACTTCGCAGAG-3	NM_00177410
	REV: 5 -GGTGTGCAGATGCCGGTTCA-3	
GAPDH	FOR: 5 -GTG GAG TCA TAC TGG AAC ATG TAG-3	NM_008084
	REV: 5 -AAT GGT GAA GGT CGG TGT G-3	
Bax	FOR: 5 -GCTGCAGACATGCTGTGGATC-3	NM_007527
	REV: 5 -TCACAGCCAGGAGAATCGCAC-3	

PCR = polymerase chain reaction, GAPDH = glyceraldehyde 3-phosphate dehydrogenase

lumen of the seminiferous tubules in the test groups 1 and 3 in contrast to the test group 2 were found to contain spermatozoa and spermatogenesis was fully established (including elongated spermatids) [Figure 1c]. However, poor interaction between Sertoli cell and germ cell was seen. Seminiferous tubules and epididymides in mice in the control group were regular. Normal interaction between Sertoli cell and germ cell was seen in mice in the control group. The space between the seminiferous tubules contained some Leydig cells and internal lumen was full of spermatozoa as well as cauda of epididymis [Figure 1a].

Assessment of sperm parameters

Sperm viability

Significant increases in the number of dead sperms in mice in the test group 2 with separate exposure to heat was observed in comparison with the other studied groups ($P < 0.05$). The sperm viability in the test groups 2 was $36.33 \pm 0.50\%$. However, the sperm viability in the test groups 1 and 3 were the same, but lower than that of the control group [Table 2].

Sperm morphology

Any kind of disorder in sperm appearance, head, tail, and remaining cytoplasmic residues was considered as morphological disorders. The average number of sperms with unusual morphology was reported as percentages. There were increases in the number of sperms with morphological disorders in all studied test groups in comparison with the control group [Table 2]. There was a significant decrease in the average number of sperms with normal morphology in mice in the test group 2 in comparison with the control group ($P < 0.05$).

Sperm motility

The average number of mobile sperms in the test groups 1 and 3 were more similar to each other and no significant difference was observed between the two groups ($P > 0.05$). Statistically significant difference was reported in the average number of mobile sperms in the test group 2 in comparison with the other test groups as well as the control group ($P < 0.05$).

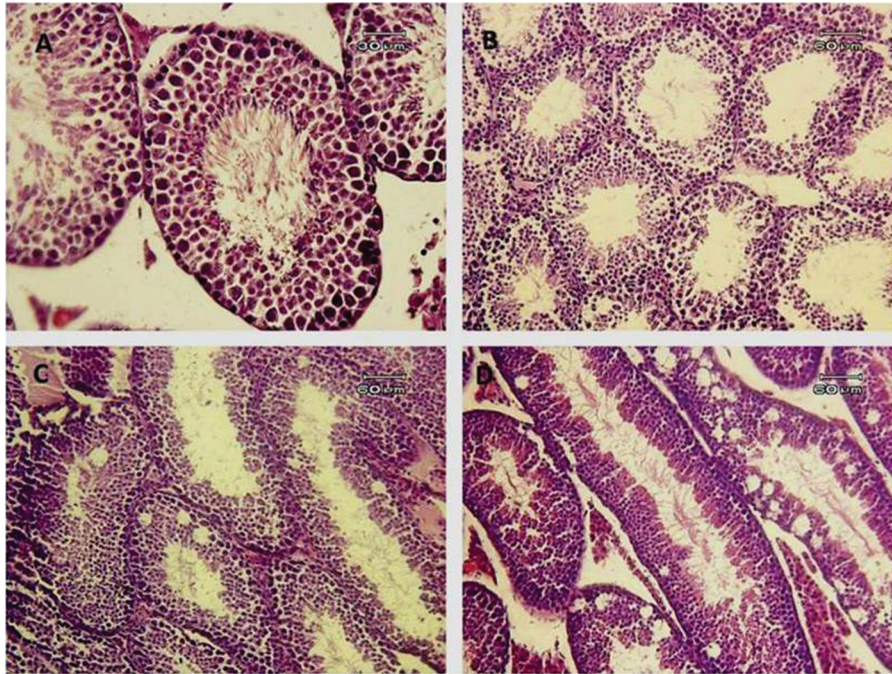


Figure 1: Histological assessment of testicular tissue after exposure to 900-MHz electromagnetic field, warm-humid climate, and electromagnetic field accompany warm-humid climate. Control (a), test group 1 (b), test group 2 (c), and test group 3 (d); H&E staining

Table 2: Comparison of the mean of sperm parameters in the control and experimental groups

Groups	Viability (%) Mean \pm SE	Normal morphology (%) Mean \pm SE	Motility (%) Mean \pm SE	Count ($\times 10^6$) Mean \pm SE
Control	74.35 \pm 1.65	69.70 \pm 2.14	72.40 \pm 1.02	4.51 \pm 0.09
Test group 1	56.14 \pm 0.43	48.80 \pm 1.60*	57.06 \pm 1.70	4.15 \pm 1.17
Test group 2	36.33 \pm 0.50*	37.66 \pm 2.15*	39.33 \pm 1.00*	1.32 \pm 0.009*
Test group 3	58.68 \pm 1.44	45.50 \pm 3.58*	60.70 \pm 0.96	4.11 \pm 1.11

*Significant difference between two groups. $P < 0.05$ considered to be statistically significant difference.

Mean sperm count

The results of the study showed that the mean number of sperms in mice in the test group 2 was $1.32 \pm 0.009 (\times 10^6)$. There was a significant decrease in the mean number of sperms in mice in the test group 2 with separate exposure to hot climate in comparison with the other studied groups ($P < 0.05$) [Table 2].

Gene expression

Bax and Bcl-2 expressions in the test and control groups were evaluated using RT-qPCR. Expression of both of genes was normalized relative to the housekeeping gene in each studied group. The mean calibrated genes expression was compared between the study groups [Figure 2]. There were significant differences in the expression of both genes in the test groups in comparison with the control group ($P < 0.05$). The findings indicated that the ratio of Bax expression was elevated to 0.015 ± 0.006 in test group 3 after combined exposures to 900-MHz MW and heat [Figure 2]. The ratio of Bax expression in test group 2 was increased to 0.059 ± 0.039 after separate exposure to heat. Also, the ratio of Bcl-2 expression in the test group 2 was 0.010 ± 0.007 [Figure 2]. The ratio of Bax/Bcl-2 in the control group was 0.00023.

DISCUSSION

The present results prove that exposure to 900-MHz MW had significant effects on the testicular tissue of mice. The results of a study on mice demonstrated that Leydig cells as the most susceptible cells to electromagnetic waves affected by exposure to cell phone radiation. Injuries of Leydig cells affected spermatogenesis.^[18] The lumen of the seminiferous tubules in mice exposed to 900-MHz MW was found to contain spermatozoa and spermatogenesis was fully established (including elongated spermatids). The findings of Ozguner *et al.*^[19] on adult male rats exposed to MW emitted from mobile phones suggested that the germ cells

conglomerate in the lumen of seminiferous tubules in MW exposed group. Degenerative changes in the germinal epithelium of rats were not significant.^[19]

The results of sperm analysis in test group 1 revealed that exposure to 900-MHz MW led to decreases in all sperm parameter means in comparison with the control group, but not significantly ($P > 0.05$). Sperm viability and motility decreased significantly in male rats exposed to cell phone simulated waves in comparison with the control group in Ghanbari *et al.* study.^[20] However, no significant differences were found in sperm morphology and count between the exposed and control groups.^[20]

The results suggested that exposure to 900-MHz MW-induced cell apoptosis and the alteration in the levels of expression of Bax and Bcl-2 genes. There were significant differences in the expression of Bax and Bcl-2 in test group 1 in comparison with the control group ($P < 0.05$). The findings demonstrated that the ratio of Bax expression was elevated to 0.015 in the test group 1 after exposure to 900-MHz MW. The ratio of Bax/Bcl-2 in the test group 1 was 3.87. The expression of the Bcl-2 gene was significantly lower in the exposed group than that in the control group ($P < 0.05$). This finding is in agreement with Liu *et al.* findings which showed a lower level of the expression of Bcl-2 gene in exposed group in comparison with the control group.^[21] The expression level of Bax in exposed mice was significantly higher than in the control group. The findings of the current study are consistent with those of Liu *et al.* who found higher level of expression of Bax gene in the sperm of the rats in exposed group than in control group. Bcl-2 family proteins were identified as inhibitors of apoptosis and these proteins may prevent Bax gene expression. Radical generation is inhibited by Bcl-2. The decrease in Bcl-2 expression level may lead to increase in the level of reactive oxygen species.^[21]

Total numbers of germs cells was lower in the test group 2 with whole body exposure of mice to hot and sultry climate (60°C of ambient temperature and 50% of relative humidity) than in the other test and control groups [Figure 1b]. Sperm cells within the deferent duct were rarely observed in the test group 2. The lumen of the seminiferous tubules in the test group 2 did not contain any spermatozoa. The results of the germinal epithelium of rat testes heat exposure to 43°C for 15 min suggested that heat exposure may damage the specific types of germinal epithelium cells and can have no effect on phases of spermatogenesis, resting spermatocytes stage, spermatogonia and Sertoli cells.^[22] The findings of local testicular heat exposure of rats to 43°C for 15 min revealed that local heat exposure may induce selective damages to specific germ cells, spermatogenesis, and spermatogenic cycle.^[23] Perez-Crespo *et al.*^[24] demonstrated high sensitivity of spermatocytes to heat exposure in a mouse

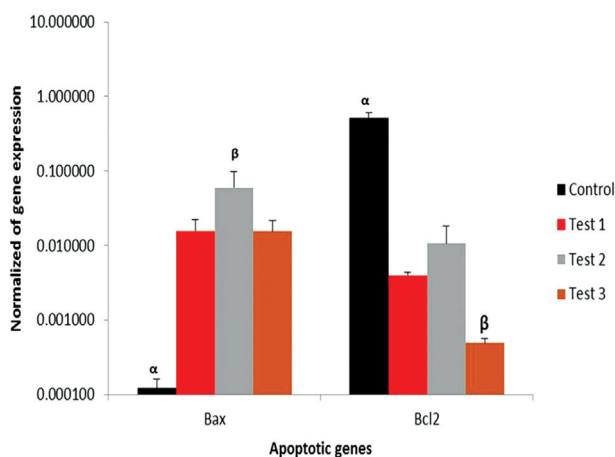


Figure 2: Real time PCR results for assessment of the gene expression in the control and test groups. α and β: significant difference in comparison with other groups in the same gene ($P < 0.05$)

model. High level of DNA damage among spermatozoa during heat stress was reported.

The number of dead sperms of mice in the test group 2 significantly increased in comparison with the other studied groups ($P < 0.05$). The means of all sperm parameters in the test group 2 were significantly lower than in the control group ($P < 0.05$). Testicular sperm numbers in Lue *et al.*^[25] findings significantly decreased in the heat exposed rats (43°C for 15 min) than in control group.

The ratios of Bax and Bcl-2 expression in the test group 2 were increased after exposure to heat. Also, the levels of expression of both genes in this test group were higher than the two other test groups. The results of real time PCR indicated that heat exposure had effects on the expression of apoptosis regulatory genes. The ratio of Bax/Bcl-2 in this group was 5.657. The ratio of Bax/Bcl-2 in the control group was 0.00023. This study produced results which corroborate the findings of a great deal of the previous work in this field. The results of heat-induced apoptosis demonstrated that heat-stress-induced apoptosis in exposed rats (40°C and 60% relative humidity) than in the normal control. The ratio of Bax/Bcl-2 in heat exposed rats was significantly higher than in control group ($P < 0.05$).^[26]

The results demonstrate that the lumen of seminiferous tubules contained spermatozoa and spermatogenesis was fully established. Poor interactions were found between Sertoli cells and germ cells after combined exposure to 900-MHz MW and hot and sultry climate.

The sperm morphology was significantly different in the test group 3 compared to the control group. Sperm viability, motility, and total sperm count was lower in the test group 3 than in the control group.

The findings indicated that the ratio of Bax expression was elevated in the test group 3 after simultaneous exposures to 900-MHz MW and heat. Also, the ratio of Bcl-2 expression in this test group was 0.00488 ± 0.00008 . The statistically significant difference was reported between Bcl-2 expressions as inhibitors of apoptosis in the test group 3 compared to the control group. The level of Bcl-2 expression was lower in the test group 3 than in the control group and test groups 1 and 2. The ratio of Bax/Bcl-2 in this group was 4.51. Although there is a large volume of published studies describing the effects of separate exposure to 900-MHz MW and local testicular heat exposure on gene expression, far too little attention has been paid to assess the effects of combined whole body exposure to 900-MHz MW and heat on gene expression. Further studies, which take these variables into account, will need to be undertaken.

CONCLUSION

This study has shown that separate and combined exposure to 900-MHz MW and hot and sultry climate may have effects on seminiferous tubules of studied mice in test groups. Heat exposure significantly increased the number of dead sperms in mice. The minimum Bcl-2 expressions were found in mice with combined whole body exposure to 900-MHz MW and heat.

Acknowledgement

The authors are grateful to Shahid Beheshti University of Medical Sciences as well as Dr. Safari-Varyani for the generous supports.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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