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The effect of low-level laser therapy and stress on wound healing in rats

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ABSTRACT

Background: Many studies have been performed on the effect of low level laser on wound healing which has been associated with different and sometimes contradictory results. On the other hand, considering that stress may affect the immune system the fact that it may delay wound healing has also been addressed. Therefore, the present study aimed to investigate the simultaneous effect of low level laser therapy and stress on wound healing at the three levels of histology (histological changes), biomechanics (stress and strain assessment) and macroscopic (wound size).

Materials and Methods: In this interventional study, 72 male Wistar rats (8-10 weeks old, weight range: 240 to 330g) were randomly divided into three treatment groups and one control group. (18 per group). In all the rats, a 2.5cm full-thickness skin incision was made on the dorsal spine. Intervention was performed from day 1 to day 21 every other day with Kals-DX61 laser (cap s) with wavelength: 660nm, dose 3J/cm^2, 100 sec and power density 30mW/cm^2. Then, wound size was measured weekly until the third week (day 21). Then, tension metric tests were performed to evaluate the stress and strain of the restored tissue. At the end of each week, three animals from each group were sacrificed for histopathological evaluation.

Results: There was a significant difference between the stress/no laser and laser/no stress groups in all stages of evaluation. Mean and standard deviation of stress and strain were not significantly different in the study groups.

Conclusion: Stress can potentially slow the wound healing process, while receiving low level laser therapy speeds up the wound healing process, although in the end there was no significant difference in biomechanical characteristics between the groups.

Keywords: Low level laser therapy; Rat; Stress; Wound healing.

Introduction

The use of low-level laser in skin wound healing is one of the relatively new therapeutic modalities that has been addressed in various studies in vitro [1,2] and in vivo [3,4]. In spite of recounting the positive effects, some articles have also mentioned the ineffectiveness and even the negative effect of laser on wound healing

[4-6]. One of the main reasons for these disagreements is the unknown interaction of laser with the tissue [4-7]. Various studies have suggested various mechanisms, one of which being the increase in collagen synthesis [1,8-10]. Therefore, in many studies, biomechanical parameters at maturation and reorganization stages are examined to assess collagen levels and their positioning [11,12]. In 2007, Yasukawa et al. in Japan compared low level He-Ne laser on complete skin wound healing in 55 rats in 11 groups at the power levels of 17mW and 8.5mW for 15 seconds. On day seven, all the rats were studied in terms of tissue strain and histology. The results showed that the 17mW every other day irradiation group had the highest strain strength and increased collagen synthesis and tissue recovery compared to the control group and no significant difference was observed between the treatment groups [11].

Reddy also investigated the effect of two low level Helium-Neon (He-Ne) and Gallium-Arsenide (Ga-As) lasers at 1J/cm^2 five days a week for three weeks on diabetic rat wounds. He concluded that both types of lasers improved biomechanical parameters. In this study, Reddy concluded that the effect of He-Ne laser on biomechanical parameters is significantly greater than that of Ga-As laser [12]. Rocha et al., in their study results found accelerated wound surface closure in an animal model after four consecutive days of He-Ne laser therapy [13]. Hopkins et al. [14], Simunovic et al. [15], and Al watban et al. [16] reported accelerated wound surface closure after He-Ne laser therapy.

On the other hand, stress has been shown to reduce macrophages in the wound and increase them in tumor cells. Since the secreted substances of these macrophages are highly effective in angiogenesis, they decrease the rate of secretion of angiogenic substances in the wounds [17]. Therefore, it can be stated that stress is likely to slow the wound healing process. Based on previous studies, inflammatory factors that cause heart disease and various infections can be exacerbated by emotional states [18], and therefore wound healing may also be delayed by emotions and stress. Other different mechanisms regarding the effect of stress on the immune system has been studied as well [19,20]. Our major hypothesis in present study was that LLLT results in an accelerated wound repair in under stress rats, therefore, the three specific aim of this study was to:

1. Investigate the effects of a 660-nmlow-level laser on open skin wound healing.

2. Study the effects of immobilization and acoustic stresses on wound healing in rats.

3. Evaluate the simultaneous effects of stress and laser therapy on wound healing in rat model.

Materials and Methods

In this interventional study, 72 male Wistar rats (age range: 8-10 weeks and weight range: 240 to 330 g) were selected to evaluate the simultaneous effect of stress and laser therapy on wound healing. The rats were divided into four groups of 18 as follows:

Group 1: Laser-treated rats under stress.

Group II: Laser-treated rats without stress.

Group III: rats under stress without receiving laser.

Group IV: rats not receiving laser and stress (control group).

The rats were adapted one week before the study and were kept in standard light and heat conditions. The environment was maintained in 12 hours light 12 hours dark condition and during this time the animal's health was monitored. The rats were housed in fiberglass cages, and water and food were completely identical and were provided ad libitum.

Wound formation

The rats were anesthetized by intramuscular injection of ketamine (50mg/kg) and diazepam (5mg/kg). Then, in the paravertebral area, one centimeter from the right side of the spine, a 2.5cm line was drawn. Then, using scalpel No. 11, a full-thickness skin slit was drawn on the line so that the deep fascia of the muscle was not injured. Then, all the rats were wounded (suture type: simple interrupted with nylon thread 4.0). Mosquito forceps was used to prevent bleeding during wound healing. All the groups were injected with an antiseptic agent (gentamicin at a dose of 5mg/kg). Twenty-four hours after wound healing, wound measurement was performed as baseline. All of the above steps were performed from preparing the animal for wound healing to wound formation and closure were performed by a surgeon (Figure 1).

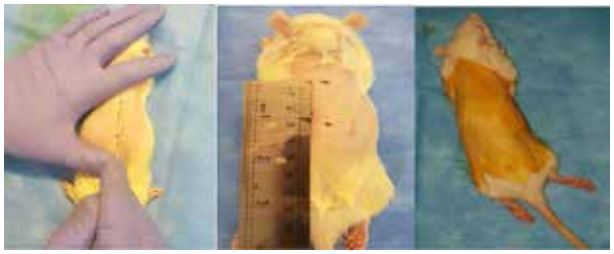

Figure 1. Preparation of the rats and wound formation.

Stress induction

Twenty-four hours after wound formation, immobilization stress (restraint stress) was induced by placing the rats in 50ml Falcone tubes for 2 h (120min) each day. The animal's access to open air was restricted was making a small aperture. The mice were also exposed to acoustic stress by making noise for 2 hours per day while immobilized. The fourth group (control group) was kept in optimum light and dark conditions and water and food were provided equally.

Laser therapy

In this study, a Klas-DX61 laser with a wavelength of 660nm, dose of 3J/cm^2, and density power of 30mW/cm^2 was used (based on the catalog of company manufacturer); in order to irradiate the rats, each animal was placed in prone position and the laser tip (cap S) was placed perpendicular at a 10mm distance from the wound surface. The protocol was applied to the first and second groups. In relation to the third and fourth groups, the device was in contact with the wound site. Each rat's wound was irradiated for 100 seconds. The number of radiation sessions was 10 sessions that were performed every other day 24 hours after the wound formation (Figure-2). In order to evaluate the simultaneous effects of stress and laser therapy on wound size, histopathological changes and finally biomechanical characteristics of the restored tissue were evaluated as follows.

Figure 2. Klas-DX laser apparatus used for laser therpy and the manner of laser irradiation.

Wound measurement

In order to evaluate the therapeutic effect in all the four groups, wound size from day 1 to day 21, was measured weekly using calipers, and the resulting values were recorded in pre-designed tables for further analyses.

Evaluation of histopathologic changes:

On days 7, 14, and 21 after surgery, to evaluate histologic changes, three animals (9 in total) from each group were sacrificed and histologically regenerating tissue specimens (vessels formation, necrosis, collagen, epidermis, giant cell, cell regeneration and infiltration) were evaluated by a pathologist using light microscopy according to the routine evaluation of tissue changes. The findings were descriptively reported and recorded by a pathologist. All the histologic examinations were performed by two pathologists with a comparative approach.

Strain strength measurement of regenerated tissue (tensiometry)

For tensiometric examination, after the wound was completely healed and after day 21, animals of all the four groups (9 from each group) were sacrificed by inhalation of chloroform in desiccator and skin sampling was performed. The skin biopsy was performed in which a 6cm*6cm (3cm from each side to the wound center) part of the skin was cut completely from the deep fascia and placed in 0.9% normal saline solution. Normal conditions of the isolated tissue were maintained to evaluate tissue strain using a tensiometer.

In this method, a 5cm long and 3cm wide piece of skin is attached to the apparatus clamps; it should be noted that the improved wound effect is located in the middle and perpendicular to the skin length. The movement of the mentioned clamps is controlled by a computer connected to the device. The skin was stretched at a regulated rate and stopped automatically after the skin ruptured. The following parameters are calculated by a tensiometer, and the results are reflected on the computer screen:

- Stress: (the maximum force applied to the skin causing it to tear).

- Strain: (the length of the tissue when the maximum stretch is reached).

The results of tensiometric tests were also recorded in pre-designed tables for future statistical analyses.

Statistical Analysis

At first the normal distribution of the parameters was evaluated by K-S. All the data had normal distribution (P>0.05). ANOVA and Tukey's tests were used to compare the biomechanical parameters of the treatment and control groups. The confidence interval was 95% in all the tests. A confidence level of less than 0.05 was considered significant.

Results

The aim of this study was to evaluate the effects of low-power laser and stress on wound size and biomechanical parameters after 10 sessions of treatment compared to the control group and to evaluate the histopathological changes. The mean and standard deviation of wound size in different groups and at different times are shown in table-1. As shown, the size of the wound in the four groups differed by cm. Levene's test results showed that the variance of wound size in groups was approximately equal (P=0.27). Comparison of the means in different groups with one-way ANOVA showed that the means of at least two groups were different. The post hoc Tukey's test showed that the laser/no stress group had a mean wound size less than the other groups; the results of this comparison are shown in Table-2. As shown in Table-2, pair-wise comparison of wound size on day 7 showed a significant difference between the laser/no stress group and the control (no laser/no stress) (P=0.017), laser/no stress (P=0.001) and the laser/stress groups (P=0.008).

On the 14th day, pair-wise comparison of the groups showed that there was a statistically significant difference between the laser/no stress group and the laser/stress group (P=0.016). No statistically significant difference was observed between the other groups. Also, pair-wise comparison of the groups on day 21 showed that there was no statistical difference between laser/stress group and laser/no stress (P=0.005) and laser/stress groups (P=0.041). No significant differences were observed between the other groups. The mean and standard deviation of stress and strain in the groups under study are shown in Table-3; as can be seen, there was no significant difference between in two variables under study between the groups.

Histopathologic findings

Samples were maintained in formalin for 7, 14, 21 days after sampling from rat skin, and then 4μM slices were prepared and stained with hematoxylin and eosin. The prepared slides were examined for necrosis, inflammation and granulation, fibrosis and epidermal regeneration (wound healing) and scoring was performed as follows:

Necrosis: Absent (0), Low (-1), Moderate (-2), High (-3).

Inflammation and granulation: low (+1), Moderate (+2), High (+3).

Tissue Fibrosis: Low (+1), Moderate (+2), High (+3).

Epidermal regeneration: low (+1), Moderate (+2), High (+3).

The results are shown in Table-4. Images of histological evaluations on different days and magnifications are also shown in Figures 3-6.

In general, it can be stated that:

- The no laser/no stress group recovered significantly earlier (day 14) than the other groups.

- The stress/no laser group had the lowest score on day 14 compared to the other groups and showed less recovery on day 14 than the other groups.

- Three groups of stress/no laser, laser/no stress, and laser/stress showed significant healing on day 21.

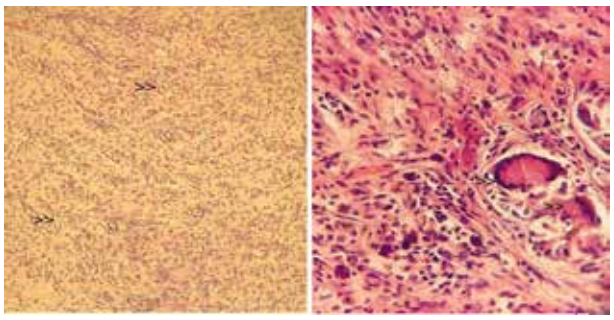

Figure 3. Skin wound after 7 days of healing, Hematoxylin-eosin staining (Left: X100, New capillaries; Right: X400, Giant cells.

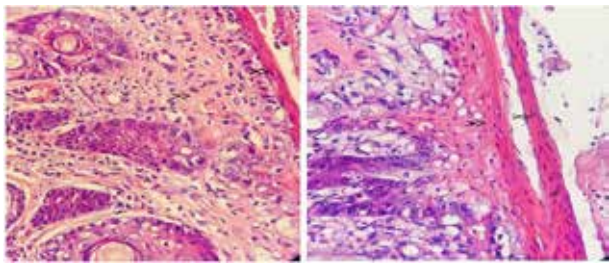

Figure 4. Skin wound after 14 day of healing, Hematoxylin-eosin staining (Left: X400, Epidermal necrosis with acute inflammation; Right: X400, Parakeratosis.

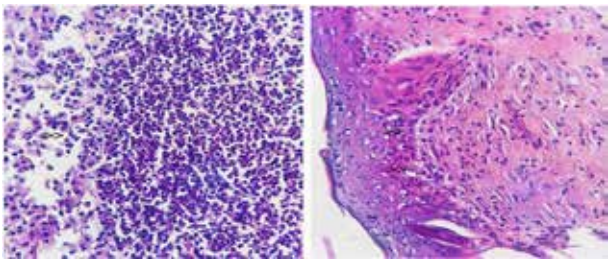

Figure 5. Skin wound after 14 day of healing, Hematoxylin-eosin staining (Left: X400, Neutrophilic collection (Abscess formation); Right: X400, Regenerative change of epidermis.

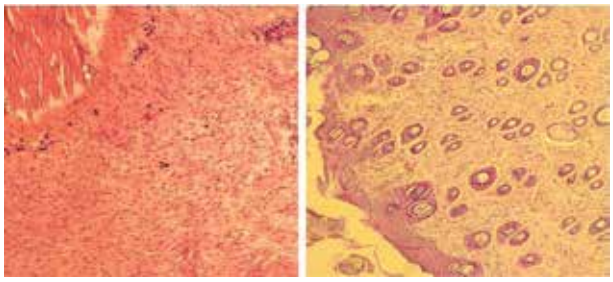

Figure 6. Skin wound after 21 day of healing, Hematoxylin-eosin staining (Left: X100, Fibroblast longitudinally to the incision; Right.

	Laser/stress	*Laser/no stress*	*No laser/stress*	*No laser/no stress*	*P-value*
Day 7	*0.96±0.2*	*0.91±0.13*	*1.27±0.19*	*1.01±0.16*	*0.001*
Day 14	*0.5±0.2*	*0.42±0.13*	*0.66±0.12*	*0.53±0.21*	*0.03*
Day 21	*0.06±0.08*	*0.04±0.07*	*0.22±0.13*	*0.13±0.12*	*0.006*

Table 1. Mean and standard deviation of wound size in the four groups in cm.

	Treatment protocol	*Treatment protocol*	*Mean*	*SD*	*P Value*
Day 7	*Laser/stress*	*Laser/no stress*	*.06500*	*.08504*	*.870*
		No laser/stress	*-.30278**	*.08711*	*.008*
		No laser/no stress	*-.03611*	*.08711*	*.976*
	Laser/no stress	*No laser/stress*	*-.36778**	*.08237*	*.001*
		No laser/no stress	*-.10111*	*.08237*	*.614*
	No laser/stress	*No laser/no stress*	*.26667**	*.08451*	*.017*
Day 14	*Laser/stress*	*Laser/no stress*	*.12750*	*.08334*	*.432*
		No laser/stress	*-.12917*	*.08537*	*.442*
		No laser/no stress	*.00417*	*.08537*	*1.000*
	Laser/no stress	*No laser/stress*	*-.25667**	*.08072*	*.016*
		No laser/no stress	*-.12333*	*.08072*	*.433*
	No laser/stress	*No laser/no stress*	*.13333*	*.08282*	*.388*
Day 21	*Laser/stress*	*Laser/no stress*	*.03500*	*.05132*	*.903*
		No laser/stress	*-.14722**	*.05257*	*.041*
		No laser/no stress	*-.05833*	*.05257*	*.686*
	Laser/no stress	*No laser/stress*	*-.18222**	*.04971*	*.005*
		No laser/no stress	*-.09333*	*.04971*	*.258*
	No laser/stress	*No laser/no stress*	*.08889*	*.05100*	*.319*

Table 2. Comparison of the mean wound size.

	Laser/stress	*Laser/no stress*	*No laser/stress*	*No laser/no stress*	*P-value*
Stress	*34.46±3.77*	*39.19±3.61*	*35.17±3.41*	*37.27±4.43*	*0.421*
Strain	*46.32±4.37*	*50.19±6.9*	*45.26±5.45*	*48.07±6.12*	*0.238*

Table 3. Mean and standard deviation of wound size in the four groups in cm.

		Laser/stress	Laser/no stress	Stress/no laser	No laser/no stress
Day 7	Necrosis	Absent (0)	Low (-1)	Absent (0)	Absent (0)
	Inflammation and granulation	Moderate (2+)	Moderate (2+)		Moderate (2+)
	Fibrosis	Low (+1)	Low (1+)	Low (1+)	Low (1+)
	Regeneration	Low (+1)	Low (1+)	Low (1+)	Low (1+)
	Final score	4	3	3	4
Day 14	Necrosis	Low (-1)	Absent (0)	Low (-1)	Absent (0)
	Inflammation and granulation	High (+3)	Low (1+)	High (+3)	Low (1+)
	Fibrosis	Moderate (+2)	Moderate (+2)	Low (1+)	Moderate (+2)
	Regeneration	Low (+1)	Moderate (+2)	Low (+1)	High (+3)
	Final score	5	5	4	6
Day 21	Necrosis	Absent (0)	Absent (0)	Absent (0)	Absent (0)
	Inflammation and granulation	Low (+1)	Low (+1)	Low (+1)	Low (+1)
	Fibrosis	High (+3)	High (+3)	High (+3)	High (+3)
	Regeneration	High (+3)	High (+3)	High (+3)	High (+3)
	Final score	7	7	7	7
Descriptions		- Orthokeratosis and parakeratosis coating with the presence of appendices -Mixed inflammation - giant cell formation on days 7 and 14 - Creating neutrophilic abscess on day 14	- Orthokeratosis coating with appendices - Mixed inflammation	-Orthokeratosis coating with appendices - Mixed inflammation - Neutrophilic abscess formation on day 14	- Orthokeratosis coating with appendices - Mixed inflammation

Table 4. Scoring of histopathological findings.

Discussion

Low-level laser therapy (LLLT) was first proposed as a treatment method by Master et al. [21]. This team of researchers showed the positive effects of low-energy ruby laser (dose 1/cm^2) on wound healing. Since conducting this research, an increasing interest in understanding the technology and its applications has been observed. In the present study, we aimed to evaluate the simultaneous effect of stress and laser therapy at the three levels of wound healing, improvement of mechanical parameters and histopathological changes. Various studies that have investigated the effect of laser on wound healing have reported a decrease in the duration of the inflammation phase following laser treatment compared to the control group [22]. This reduction during the inflammatory phase can accelerate the process of proliferation and remodeling which results in faster wound closure and improvement in biomechanical parameters. In the present study, it was observed that within seven days after wound healing, the size of the lesion of the laser-treated group or groups, even in the presence of concurrent stress, showed a statistically significant difference with the group that did not receive the laser. However, the fact that the size of the wound in the control group in the first week was also significantly lower than that of the stress-free group could partially offset the positive effect of laser irradiation, meaning that stress may have more neg-

ative effects than the positive effects of laser therapy. However, the ability to modulate the effect of laser by observing the statistical difference between the laser/stress group compared to the no stress/laser group indicates the potential of the laser to inhibit the negative effects of stress. On the other hand, laser irradiation may push the epithelial cells to the wound site, resulting in a faster closure of the wound surface. The results of this study are consistent with those of Al-watban et al. [16] who suggested that laser therapy, by mildly inducing inflammatory reactions, leads to an earlier onset of proliferation and improved epithelialization. Hopkines et al. [14] and Rocha et al. [13] have similarly reported the positive effects of laser therapy on wound healing. On the other hand, the results of the present study concerning laser efficacy are different from those of the study by Anneroth et al. [23], who have pointed out that laser therapy does not affect wound closure. Also, pair-wise comparison of the two groups at different time intervals showed that on the seventh day after wound healing no laser/stress group was significantly different than the other three groups and wound healing was slower in this group. Overall, these results indicate that the recovery period is longer in the stress/no laser group. The absence of a statistically significant difference between the two groups also shows that at least in the first week after wound formation, stress has a greater effect on the wound healing process than the presence or absence of laser irradiation, although it should have borne in mind that several other factors may delay skin regeneration after skin damage [24,25].

Examination and comparison of wound size on day 14 after wound healing showed that only the two groups of laser/no stress and no laser/stress were significantly different. Finally, pair-wise comparison of the groups on day 21 showed that the stress/no laser group was significantly different than the laser/stress and the laser/no stress groups, which could indicate that the positive effect of laser on wound healing was persistent and it could ultimately mitigate the negative effect of stress on wound healing; in general, based on previous studies when the animal is stressed, for example, being immobilized, stress is increased, in fact, glucocorticoids, which are key mediators of psychological stress, delay skin repair and plasma corticosterone level also increases [26]. On the other hand, previous studies have shown that the expression of vasopressin mRNA and its production in paraventricular nucleus subunits increase during immobilization stress [20]. The expression of corticotropin-releasing factor mRNA in rat paraventricular nuclei also increased significantly after being exposed to acute cold stress (six hours) and chronic cold stress (thirty hours) [27]. Vasopressin has also been shown to be able to elevate corticotropin-releasing hormone when the animal is under different stressors. This hormone enhances the release of adrenocorticotropic. A 2001 study showed that pre-natal immobilization stress significantly induced insomnia in rats, which could be due to increased release of corticotropin-releasing hormone from the hypothalamus. In the mentioned study, rats exposed to antenatal immobilization stress showed increased hypothalamic-pituitary-adrenal axis activity [28]. Given that inflammatory factors can be exacerbated by emotional states and stress [29], it is likely that the stress inflicted on these animals may delay wound healing. Previous studies have reported greater progression of viral infections and tumours in these animals than non-stressed ones [30]. All of these indicate the potential harmful effect of stress on wound healing processes.

On the other hand, according to the findings of the present study, the biomechanical properties of stress and strain were not significantly different between the four groups. It should be noted that with wound closure, tissue should not be expected to return to its normal strength [31]; in fact, wound closure and mechanical properties of the wound are two issues related to wound healing, but they are not necessarily consistent. Wound surface closure requires migration and proliferation of epithelial cells [3], whereas the mechanical properties of the wound tissue are mainly related to the processes of fibroblast proliferation, collagen degradation, collagen fibre maturation, cross-linking between collagen filaments, production of a substrate for connective tissue and the orientation and organization of collagen fibres, which usually occur following normal stresses in the tissue and continue for a longer time after wound healing [1,11].

The biomechanical parameters of the tissue at maturation and Remodelling stages are examined to assess collagen levels and their arrangement. On the other hand, collagen production by fibroblast cells has led to many studies of the effect of different laser treatments on fibroblast cells [11,12]. Collagen production begins with wound formation and continues until complete remodelling [1]. The results of the present study did not show a significant effect regarding the simultaneous effects of laser and stress on the improvement of biomechanical properties in the studied groups. These findings are inconsistent with those Reddy et al. regarding the effect of He-Ne and Ga-As on diabetic surgical wounds. According to the findings of this study,

both types of lasers increase the parameters of stiffness, strain, stress, tissue tensile strength and the area under the curve but they do not affect elastic modulus. Similarly, the results of this study are inconsistent with the findings of Yasukava et al. [11], which showed a significant increase in tissue tensile strength and collagen fiber formation and tissue cross-linking in the 17mW laser group compared to the control group.

It seems that the strength and tensile strength of the skin depends not only on the amount of collagen fibers but also on the cross-linking and organization of collagen fibers and the formation of transverse chains between the filaments. These events occur during a phase called remodelling. Three weeks after injury, between synthesis and collagen degradation homeostasis (equilibrium) state occurs, and the remodelling phase begins 12, so it is likely that better biomechanical parameters compared to wound healing may require longer time. Finally, the results of this study showed that laser treatment can accelerate wound healing in rats and that stress is potentially a decelerating factor in wound healing which has more negative effects especially in the early days of wound healing.

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Conflict of Interest

There is no conflict of interest to declare.

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