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Low Level Laser Irradiation Effects on Proliferation and Apoptosis in Bone Marrow Mesenchymal Stem Cells

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Abstract

Low level laser irradiationis an effective method in treating some diseases and it could improve regeneration and wound healing. Some studies have reported that low level laser irradiation could enhance proliferation by increasing ATP level in the cells mitochondria.Mesenchymal stem cells (MSCs) are a kind of stem cells which are capable of differentiating to other cells. Nowadays, MSCs are an appropriate option for regenerative medicine.

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In this study we want to investigate the optimum protocol for using low level laser irradiation to improve proliferation and make less apoptosis in Mesenchymal stem cells. The Mesenchymal stem cells were isolated from femur and tibia of a male rat and cultured in culture media. The isolated cells were differentiated to adipocyte and osteocyte to confirm their multi potency. The cells were irradiated with different exposure protocols of 808 nm diode laser. After exposure the stem cells markers (CD90, CD44 and CD45), doubling time, colony forming frequency and Caspase 3 activity (for apoptosis evaluation) were assessed.Both 640 mW (8^{th} group) and 830 mW (14^{th} group) have best result in colony forming and doubling time; however the 8^{th} group had the least Caspase 3 activity. The patterns of stem cells markers expression have not changed after laser irradiation.The protocol used in 8^{th} group could improve Mesenchymal stem cells proliferation more than the rest protocols and also it might make the apoptosis less than the other group. This irradiation protocol could be useful in regenerative medicine due to its effects on Mesenchymal stem cells.

Keywords: diode laser; bone marrow stem cell; proliferation; Apoptosis.

1. Introduction

Since low level laser irradiation(LLLI) is a beneficial and effective method in treatment of various disease such as dermatitis, skin ulcers, mucosal healing and mucositis [1,2], numerous investigators have started to study the effects of laser irradiation on biological responses to shed light on its functional effects of different tissues such as bone [3] nerve[4] skin[5] and skeletal muscle[6]. Furthermore, due to notable lack of any effective therapies in some diseases, researchers have motivated to apply novel techniques like laser light as a real method for treatment of incurable diseases. According to the literature, LLLI can affect the mitochondrial respiratory chain, enhance the ATP level and eventually it will promote proliferation of cells [7-9].various parameters such as laser wavelength, power density, time of irradiation, cell type and physiological characteristics of cells irradiated [10-13], are important points to affect cells in vitro and cause laser bio stimulation effects which could be stimulatory or inhibitory outcomes. Although LLLI is a promising method to increase cell proliferation and differentiation [1, 10, 11, 14], however, due to different mentioned factors in this process, contradictory results have been observed and precise mechanisms of this process are not completely understood yet [2].

At the turn of 20th century, researchers provided cell culture as a practical and beneficial techniques in experimental studies [15], Recently, stem cells culture applied in regenerative medicine to achieve an improvement in generate of cells in vitro and vivo. Mesenchymal stem cells (MSCs) are multi-potent and undifferentiated cells which could be harvested from different tissues and they are capable to differentiated into osteocytes, adipocytes, chondrocytes, myocytes and cardiomyocytes[16-19]. Moreover, they may cause increased secretion of vascular endothelial growth factors induced anti-apoptotic effects [20]. As MSCs proliferation is ordinarily slow and the yield of these cells is low after first harvest, so a therapeutic tool like laser light is required to improve proliferation cells without molecular damage. Cell proliferation, is one of the most important physiological effects of LLLI which is used in vitro cell culture methods [2]. Some investigators have revealed that 804 nm wavelength laser irradiation on mesenchymal and cardiac stem cells[21], 809 nm wavelength on human gingival fibroblasts[10], 810 nm wavelength on olfactory ensheathing cells[22]promoted

proliferation of cells in comparison with the control groups.Oron*et al*. showed that Ga-As 808 nm laser irradiation was enhanced ATP level in human neural cells in vitro[23].However, these effects were evaluated by using a wide diversity of laser devices in non-comparable models which is caused contradictory results.

Therefore, in the present study, we compared the effects of various exposure protocols of 808 nm diode laser on proliferation and apoptotic factors (caspase3) in mesenchymal stem cells.

2. Materials and Methods

2.1 Animals

This investigation was approved by the Animal ethics committee of the Shiraz University of Medical Sciencesfor care and use of animals. All experimental protocols were verified and performed based on theguidelines of Shiraz University of Medical Sciences.

Rat BMSCs were obtained from one adult (7-8 weeks) male Sprague-Dawley rat, weighting 250-300 g, was provided by the Laboratory Animal Center, Shiraz University of Medical Sciences.

2.2 Cell Culture

After anesthesia,cervical dislocation was done under thelaminar-airflow cabinet by maintaining sterile conditions, femurs and tibias were dissected, ends of them were cut.In order to isolate bone marrow stem cells, cavities of bone marrow were flushed out using Dulbecco's modified Eagle's medium (DMEM, Sigma, St Louis, MO,USA) with 10% fetal bovine serum (FBS, Gibco, USA),supplemented with 2mg/L amphotericin (Cultilab, Brazil)and 50mg/L gentamicin sulfate (Cultilab, Brazil). Then, the isolated cells were centrifuged for 10 min at 1200 rpm in medium containing5 ml DMEM,15% FBS and 100U/ml streptomycin (Sigma, USA) supplemented with 100U/ml penicillin (Sigma, USA). Afterwards, cells were cultured in T-25 cm^2plastic flask with culture medium including DMEM, 10% FBS and 1% Pen/Strep,then incubated with humidified 5%CO_2at 37°C.Every three to four days, culture medium was changed to remove non-adherent cells and replaced by fresh one,achieving approximately 80% -85% cell densities.Cells were harvested with 0.25% trypsin (Amresco, Solon, OH) supplemented with1 mMethylenediaminetetraacetic acid (EDTA,Life Technologies, Grand Island, NY) for 5 min at 37°C[24]. Again cells were cultured to achieve next confluence and harvested. Rat BMSCs of third passages were used in this experiment.

2.3 Cells Differentiation Analysis

In order to assess the multipotency nature of the BMSCs, before laser irradiation, cells were seeded in differentiation kits (Invitrogen Corp, Carlsbad, CA, USA)for about 21 days.Cells were fixed with paraformaldehyde 4% , then stained with Alizarin Red S (Nuclear, Sa˜o Paulo SP, Brazil), to evaluate osteogenic differentiation and Oil Red O (Sigma-Aldrich, Rehovot, Israel) was used to assess differentiation[25]. Ultimately, characteristic morphology of BMSCswasanalyzedusing inverted phase microscope (Eclipse TE 2000U, Nikon,Melville, NY, USA)(Figure 5).

2.4 Laser Treatment

Before LLLI, third passages of BMSCs were seeded in 12-well culture plates (Jet-Biofil, Guangzhou, China) with 10000 cells/welldensity.The precision of this method was verified by repeating and analyzing all experiments in triplicate.To prevent the unintentional scattered or overlapping of light, between the cultured wells was left empty, in such a way that only twowellsin the two corners of the plates were filled with cells and hence aluminum foil were used to covered cells from scatters.Furthermore, black backgrounds were used to avoid laser light reflection. BMSCs were randomly divided into 19 groups. Cellsin groups C served as the control; they were without laser irradiation. The otherscaled T served as the tests; theywere accompaniedwith laser irradiation for 1-4 consecutive days, based on exposure protocol (Table 1).Ga-As diode laser (Kondortech Bio Wave,Dual, Brazil) was used in the continuous mode at a power output of 830 mW and wavelength of 808nm. We utilized a lens to achieve different powers density at various distances. Power was 640 mW after Lens.The probe of laser was irradiated perpendicular to each well.Laser power meter was used for measuringpoweroutput after cells and calculating of cells absorb dose (Table 1).Cells were treated with energy density0.05-3 j/cm² during 10-60 seconds laser irradiation. A digital timer was used to confirm laser irradiation timing.After first irradiation,BMSCs were cultured and investigated at 0,24,48,72 hours and 15 days to evaluate the laser irradiation effects.

2.5 ***Colony Forming Assay***

To determine the ability of colony formation of BMSCs, colonyforming unit (CFU-H) assay were used. After laser irradiation, control and test cells were seeded in six-well culture plate with 1000 cells/wellconcentration. Cell aggregations with more than 50 cellswere defined ascolonies. On the 14th day, cells were fixed with Giemsa, Colonies were scored by an inverted phase microscope[26].

2.6 ***Population Doubling Time analysis***

Post laser irradiation,cells in all groups were seededafter third passagesin 12-well plate with 10000 cells/wellconcentration and compared with the control group.Cells of each well were counted daily by hemocytometer. Growth curve were used for determining the population doubling time and growth characters.

2.7 Markers Expression Comparison

Immunocytochemistry was executed and CD44,CD90 andCD45 markersexpression were evaluated in all groups before and after laser irradiation. Paraformaldehyde 4% for 20 minutes was used to fix cells. Using phosphate buffer saline (PBS) for washing cells and incubated at 4° C with appropriate primary antibodies, anti-CD44, anti-CD45 and anti-CD90 then keptfor one hour at room temperature. Finally they washed by PBS three times and conjugated with species-specific secondary antibody.

2.8 Apoptosis Induction in Rat Mesenchymal Stem Cells

For inducing apoptosis, the isolated mesenchymal stem cells were treated with H_2O_2(2000 µ M) under sub-

confluent culture in passage 3.

Table 1: Experimental groups for evaluation of the effects of low level laser irradiation of Ga-As diode laser on bone marrow mesenchaymal stem cells.

Group number	Group	Time irradiation (s)	Number of repeated irradiation	Power density (mW/cm^2)		Power output (mW)	Energy density (J/cm^2)	Total energy (J)	Distance (cm)
1	C	-	-	-	-		-	-	-
2	T10/1	10	1	5	640		0.05	6.4	52
3	T20/1	20	1	5	640		0.1	12.8	52
4	T40/1	40	1	5	640		0.2	25.6	52
5	T60/1	60	1	5	640		0.3	38.4	52
6	T10/1	10	1	12	640		0.12	6.4	34.5
7	T20/1	20	1	12	640		0.24	12.8	34.5
8	T40/1	40	1	12	640		0.48	25.6	34.5
9	T60/1	60	1	12	640		0.72	38.4	34.5
10	T10/1	10	1	35	830		0.35	8.3	72
11	T20/1	20	1	35	830		0.7	16.6	72
12	T40/1	40	1	35	830		1.4	33.2	72
13	T60/1	60	1	35	830		2.1	49.8	72
14	T10/1	10	1	50	830		0.5	8.3	58
15	T20/1	20	1	50	830		1	16.6	58
16	T40/1	40	1	50	830		2	33.2	58
17	T60/1	60	1	50	830		3	13.8	58
18	T10/4	10	4	5	640		0.05	0.2	52
19	T10/4	10	4	12	640		0.12	0.48	34.5

2.9 Apoptosis Evaluation

This assessment was performed based on spectrophtorometric detection of chromophore p-nitroaniline (p-NA) by using Caspase3 assay kit(Colorimetric, abcam,ab39401,UK)at 400-405 nm wavelengths.

3. Statistical analysis

To assess significant differences between group's non-parametric Mann–Whitney and Kruskal–Wallis tests were used. Using SPSS version 11.5 (SPSS Inc, Chicago, Illionois)for all Statistical analysis. GraphPad Prism version 5.01(GraphPad software Inc, San Diego, CA, USA) were used for comparison among the groups. P-value <0.05 was adopted as statistically significant.

4, 6-Diamino-2-phenylindole Dihydrochloride (DAPI) Staining

To detect the cells' nucleus, they were fixed with paraformaldehyde 4% and then 4, 6-diamino-2-phenylindole dihydrochloride (Millipore S7113 1: 1000) was added to the fixed cells and they were kept in room temperature for 30 minutes.

4. Results

4.1 Culture Characteristic

After two days from isolating the Mesenchymal stem cells, they attached to the T-25cm^2 tissue flask with appropriate fusiform shape. They became 80% confluent after 7-10 days. (Figure1)

4.2 Colony Forming Assay

For assessing the isolated single cells colony forming capability, the colonies with more than 50 cells were considered in colony enumeration. The 8^{th} (47.33±4.63) and 14^{th}(51.66±3.82)group had the more colonies among the other groups and there were significant differences between the number of colonies on these two groups and the rest.(Figure 2)

4.3 Population Doubling Time Analysis

T40/1group revealed a mean of 5.07 days as population time and it needed the least time to double between the other groups. There was a significant difference between the T40/1 group and the rest. (P value < 0.05) (Figure 3)

4.4 Markers expression comparison

To identify the markers (CD44, CD45 and CD90) expression in different group after and before laser exposure, their expressions were assessed by immunocytochemistry method as above mentioned. There was no significant difference between markers expression before and after laser exposure. (Table 2)

4.5 Caspase 3 activity assay

According some previous study, low level laser could promote anti-apoptotic gene expression and diminish apoptotic factors such as Caspase 3.In this study the Caspase 3 activity was evaluated for apoptosis inMesenchymal stem cells and 8^{th} group (0.48±0.06) showed the least Caspase activity among the rest of the groups. (Figure 4)

5. Discussion

Our findings revealed that LLLI with 808 nm wavelength in the continuous mode at power output of 830 mW and 640 mW stimulated proliferation and differentiation of Rat- BMSCs as well as enhancement of anti-

apoptotic factors. Various factors such as differences in wavelength, power output, power density, energy density and time of irradiation, affected this process and make stimulatory or inhibitory outcomes. In the present study cells were treated with energy density 0.05-3j/cm² during 10-60 seconds laser irradiation.

Table 2: Mesenchymal stem cells markers expression

Group	**CD44**		**CD45**		**CD90**	
	Before irradiation	After irradiation	Before irradiation	After irradiation	Before irradiation	After irradiation
C	90.43±3.42	90.43±3.42	0.79±0.10	0.79±0.04	94.04±3.62	94.04±3.55
T10/1	92.37±2.49	94.23±2.17	0.70±0.06	0.70±0.02	96.06±2.25	97.92±2.25
T20/1	90.17±4.12	93.28±4.28	0.60±0.13	0.60±0.08	93.77±4.36	97.94±4.49
T40/1	92.56±5.16	92.47±2.42	0.86±0.07	0.86±0.04	96.26±2.51	96.16±2.51
T60/1	94.12±1.28	93.26±1.44	0.72±0.04	0.72±0.03	97.88±1.48	98.85±1.52
T10/1	95.28±3.17	91.30±3.25	0.79±0.09	0.79±0.06	99.09±3.38	94.95±2.49
T20/1	89.23±4.12	92.38±2.36	0.74±0.05	0.74±0.04	86.01±2.27	89.05±3.28
T40/1	88.13±5.23	91.02±3.86	0.87±0.09	0.87±0.07	84.95±3.79	88.65±1.75
T60/1	92.18±3.72	90.18±2.74	0.52±0.06	0.52±0.08	88.86±2.64	86.93±1.46
T10/1	90.15±2.49	93.16±3.15	0.60±0.07	0.60±0.04	86.90±2.97	89.80±3.03
T20/1	91.38±5.29	94.28±2.17	0.80±0.06	0.80±0.03	93.20±2.21	96.16±2.38
T40/1	93.29±1.95	94.28±3.15	0.48±0.09	0.48±0.06	95.15±3.21	98.23±2.27
T60/1	94.28±4.27	92.87±2.74	0.82±0.08	0.82±0.02	96.16±2.80	95.65±1.30
T10/1	95.38±3.18	90.27±1.28	0.83±0.03	0.83±0.04	97.28±1.30	92.07±3.74
T20/1	94.28±2.17	92.10±3.84	0.69±0.09	0.69±0,04	92.39±3.69	89.70±2.21
T40/1	96.76±4.37	94.24±1.49	0.84±0.03	0.84±0.06	94.82±1.46	92.35±1.32
T60/1	93.76±3.96	95.37±2.19	0.79±0.04	0.79±0.05	91.88±2.13	92.89±2.12
T10/4	95.36±3.64	94.28±1.39	0.83±0.06	0.83±0.04	93.45±1.36	92.39±4.94
T10/4	92.86±4.87	95.31±2.17	0.86±0.04	0.81±0.03	91.00±2.09	93.40±3.69

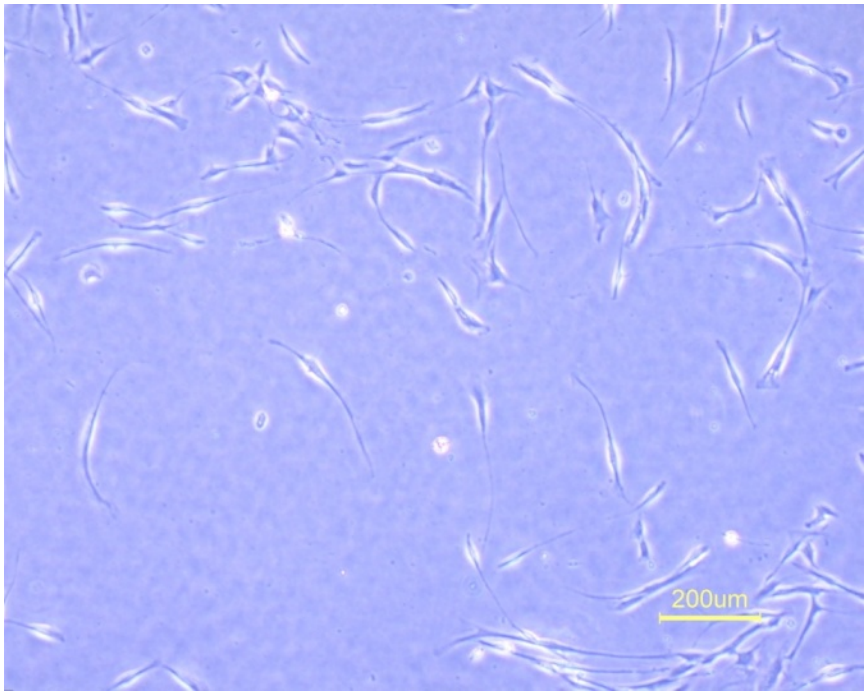

Figure 1: rat bone marrow Mesenchymal stem cells

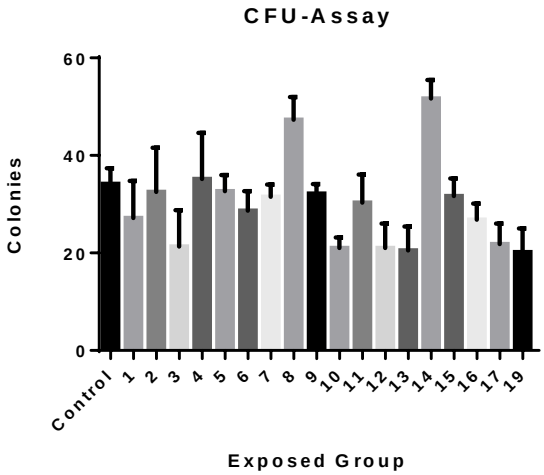

Figure 2: colony forming assay

The best results were achieved with the energy density 0.48j/cm² and 0.5j/cm² with 12 and 50 mW/cm² power densities respectively (8th and 14th groups). Although; Khalid et al. confirmed that 0.5- 4.0 j/cm² energy density was optimized in promoting the proliferation of different cell lines [27] but in our study we didn't find stimulatory effects on Rat-BMSC with other energy densities except 0.48 and 0.5 j/cm² as well as enhancement of anti-apoptotic factors. Our findings are in line with results obtained by Carlos et al. (Carlos Augusto Galvaobarboza et al., 2013), who indicated that a 660 nm diode laser stimulated proliferation of

BMSCs and ADSCs using 0.5 j/cm² energy density with 15 mW/cm² power density. In contrast, some researchers reported that a 660 nm diode laser with 4.8 j/cm² energy density on synovial fibroblasts [28], a 660 nm laser with 3 j/cm² energy density on stem cells of human dental pulp [14], a diode laser with 650 nm wavelength and 2.28 j/cm² energy density on calvarial cells [29] enhanced proliferation of different cells. These contradictory results are attributed with various factors such as differences in wavelength, power output, power density, energy density, time of irradiation and type of cells which affected these processes. However, these stimulatory results are in line with the hormesis theory [30] which defined as a biological dose response, inducing stimulatory effects by low dose of irradiation and not only eliminate the detrimental effects but also induce beneficial outcomes.

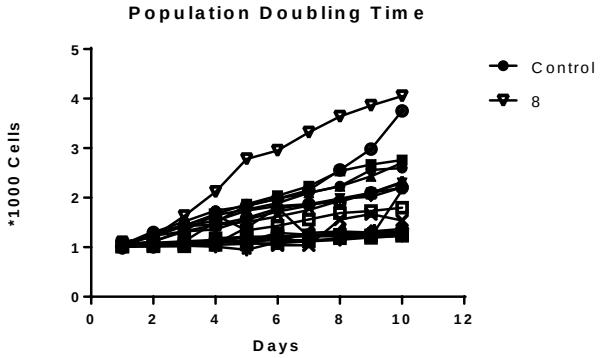

Figure 3: Doubling time of Mesenchymal stem cells

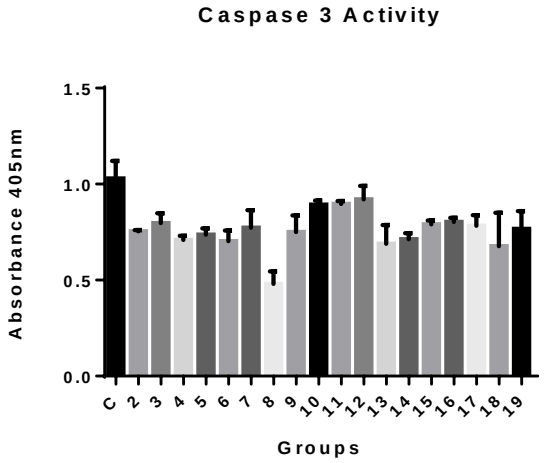

Figure 4: Caspase 3 activity

Different mechanisms are able to induce hormetic effects but the exact mechanism is unknown. It seems that LLLI can stimulate some biological processes which results in activation of mitochondrial respiratory chain, enhancing supply of adenosine triphosphate (ATP), leading to increase of intracellular reactive oxygen species (ROS) within the mitochondria, may cause reduction of oxidative stress and eventually result resistance

to stress, inducing radiation hormesis[31]. In this light, Oron et al. have demonstrated that Ga-As laser treatment with 808 nm wavelength and power density 50mW/cm², increased ATP Production normal human neural progenitor (NHNP) cells in vitro cell culture after 10 min laser irradiation [23]. Extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K), both are signal transduction pathways regulating cell proliferation [32-34]. Extracellular ATP binding to its receptor, activates ERK1/ERK2 and PI3K pathways, stimulates cell proliferation and reduces apoptosis [35]. On the other hand, some anti-apoptotic factors such as serine/threonine kinase (AKt) and phosphorylated AKt (pKAt) inhibit caspase 3 activity and caspase 9 expression which are apoptotic inducer [36, 37]. In addition, enhancement of the level of intracellular ROS production can mediate the activation of AKt induced proliferation by ROS/AKt signaling pathway[38].

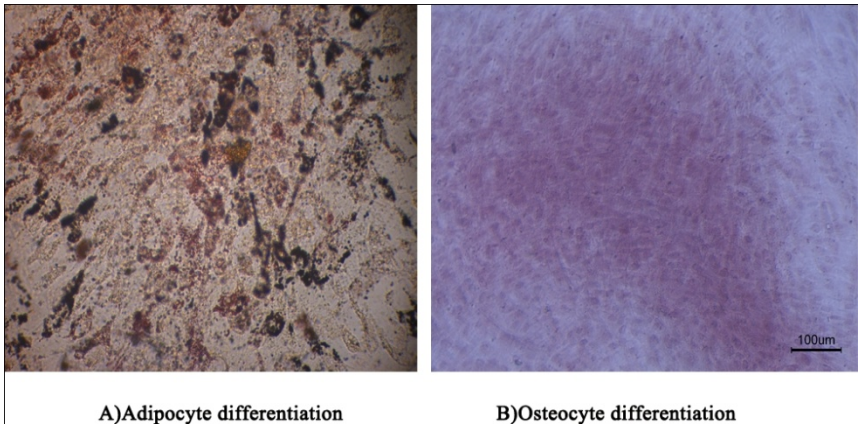

Figure 5: Mesenchymal stem cells differentiation.

6. Conclusion

In general, our findings of this study showed that low level laser irradiation is able to stimulate the anti-apoptotic factors and prohibit apoptosis and also lead to increase of cell proliferation.

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Conflict of interest

None Declared

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