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# Ganodermasides A and B, two novel anti-aging ergosterols from spores of a medicinal mushroom *Ganoderma lucidum* on yeast via *UTH1* gene

Yufang Weng [a], Lan Xiang [b], Akira Matsuura [c], Yang Zhang [d], Qianming Huang [d], Jianhua Qi [a,*]

[a] College of Pharmaceutical Sciences, Zhejiang University, Yu Hang Tang Road 388, Hangzhou 310058, China
[b] College of Biosystems Engineering and Food Science, Zhejiang University, Hangzhou 310029, China
[c] Department of Biology, Faculty of Science, Chiba University 1-33 Yayoi, Inage-ku, Chiba 263-8522, Japan
[d] College of Biology and Science, Sichuan Agricultural University, Yaan, Sichuan Province 625014, China

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ABSTRACT

Two novel ergosterol derivatives, ganodermasides A and B, hydroxylated at C-15 were isolated from the methanol extract of spores of a medicinal mushroom, *Ganoderma lucidum*, showed to extend the replicative life span of *Saccharomyces cerevisiae*, a yeast of K6001 strain. The stereostructures of ganodermasides A and B were determined based on the spectroscopic analysis and comparison of spectroscopic data. These new sterols have a 4, 6, 8(14), 22-tetraene-3-one unit with a unique hydroxylation at C-15. The anti-aging activity of these compounds on yeast is comparable to a well-known substance, resveratrol. Based on results of the investigation of the mechanism of biological activity, ganodermasides A and B regulated *UTH1* expression in order to extend the replicative life span of yeast.



## 1. Introduction

Worldwide populations are aging. Aging is a natural process that involves the progressive deterioration of biological functions of a mature organism. It is a major risk factor for neurodegenerative disorders such as Alzheimer's disease (AD). Recently, the close relationship between the process of aging and AD, an age-dependent neurodegenerative disorder, has been extensively studied. It was found that aging is an important factor in the pathogenesis of AD.[1–3] Thus, anti-aging substances also could be developed as promising therapeutic drugs for the treatment of AD.

In our previous study on leading compounds for AD treatment, the novel cerebrosides, termitomycesphins A–F, were isolated from the edible Chinese mushroom, *Termitomyces albuminosus*,[4] and in a series of steroid glycosides, linckosides A–Q, from the Okinawan blue starfish, *Linckia laevigata*,[5,6] by using PC12 cells as bioassay system. Recently, we became interested in the search for compounds with anti-aging effects, which also can be used in the treatment of neurological disorders. Due to low throughput screening of the traditional yeast replicative life span assay method, we established a modified bioassay system by employing a yeast, *Saccharomyces cerevisiae*, of K6001 strain to screen the anti-aging effect of the methanol extract of traditional Chinese medicines.[7] The K6001 strain expresses *CDC6*, an essential gene for growth, both from the galactose-dependent promoter *GAL1-10* and a mother-specific *HO* promoter. When K6001 cells are grown in galactose, *GAL1-10*::*CDC6* is expressed both in mother and in daughter cells; however, when the expression of *GAL1-10*::*CDC6* gene is repressed by glucose, only the mother cell-specific expression of *HO*::*CDC6* remains to support growth. Most often, the daughter cells of K6001 are grown in galactose medium because they can be hardly supported by a glucose medium. Therefore, the K6001 strain can be used to establish a bioassay system in screening the anti-aging effect of the samples. This bioassay system has guided the screening and purification of the two novel ergosterol derivatives, ganodermasides A (**1**) and B (**2**) (Fig. 1), isolated from the methanol extract

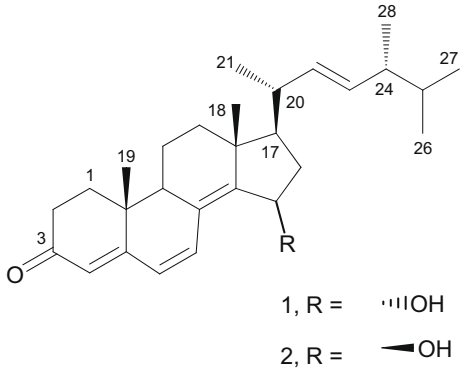


**Figure 1.** Structures of ganodermasides A (**1**) and B (**2**).

* Corresponding author. Tel./fax: +86 571 88208627.
*E-mail address:* qijianhua@zju.edu.cn (J. Qi).

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of spores of a medicinal mushroom, *Ganoderma lucidum*. We report here the isolation, structures, and biological activity of these sterols.

## 2. Results and discussion

### 2.1. Isolation

The methanol extract of spores of *G. lucidum* was partitioned between EtOAc and $H_2O$. The active EtOAc fraction was chromatographed on silica gel and on ODS, which resulted in a mixture of steroids. Then, the mixture was purified by reversed-phase HPLC to produce ganodermaside A (**1**, 0.0014% of dry wt) and an active fraction which retention time was later than that of the **1**. The active fraction was once more subjected to HPLC to yield ganodermaside B (**2**, 0.0005%).

### 2.2. Structure elucidation

Structural analysis was started with ganodermaside B (**2**), which was obtained as a yellowish powder with the molecular formula $C_{28}H_{40}O_2$, determined by means of HR ESI-MS measurement. Ganodermaside B (**2**) showed fluorescence under UV light, which was confirmed by UV and IR absorptions at 331 nm and 1647 $cm^{-1}$, respectively. These UV and IR absorptions correspond to a long conjugated ketone group. The IR absorption band at 3420 $cm^{-1}$ suggested the presence of a hydroxyl group. In particular, the $^1H$ and $^{13}C$ NMR spectra of **2**, as well as the HMQC experiment, revealed the presence of one oxymethine ($\delta_H$ 4.79 and $\delta_C$ 69.4), four double bound ($\delta_H$ 5.18, 5.28, 5.78, 6.15, and 6.82; $\delta_C$ 124.2, 127.0, 129.3, 132.3, 133.2, 134.7, 155.6, and 163.5), one ketone ($\delta_C$ 199.6), and two quaternary $sp^3$ carbons ($\delta_C$ 37.0 and 43.2), as shown in Table 1. The remaining proton and carbon signals were attributed to six methyl groups, including two tertiary methyls (C-18 and C-19), four secondary methyls (C-21, C-26, C-27, and C-28), five methines (C-9, C-17, C-20, C-24, and C-25), and five methylenes (C-1, C-2, C-11, C-12, and C-16). In addition, the analysis of COSY and HOHAHA spectra led to the determination of the partial structures, C-1 to C-2, C-6 to C-7, C-9 to C-11 and to C-12, and C-15 to C-28, depicted by the bold bonds in Figure 2. These partial structures were connected by the long-range H–C correlations, obtained by means of HMBC experiment, giving a gross structure of **2**, as shown in Figure 2. The HMBC correlations that establish the gross structure of **2** were as follows: methyl protons (H-18) to C-12, C-13, C-14, and C-17; methyl protons (H-19) to C-1, C-5, C-9, and C-10; H-2 to C-3; H-4 to C-2, C-6, and C-10; H-6 to C-4, C-5, and C-10; H-7 to C-8, C-9, and C-14; H-9 to C-8, C-10, and C-14; and H-15 to C-13 and C-14. The important HMBC correlations are summarized, with arrows, in Figure 2.

The *R*-configuration at C-20 was determined on the basis of the NOE correlations of H-18/H-20 and H-18/H-21. The large coupling constant, $J_{17-20}$ = 12.3 Hz, suggested the anti-relationship between H-17 and H-20. This coupling constant was obtained through a decoupling measurement, in which H-21 ($\delta_H$ 1.05) was irradiated. Similarly, the 15*R* was determined on the basis of the NOE correlations of H-15/H-17. The 24*R* configuration was assigned on the basis of superimposable $^1H$ and $^{13}C$ chemical shifts (Table 1) of the side chain, C-24 to C-28, on that of reported data.[8] The double bond at C-22 and C-23 was elucidated by COSY correlations, while the *trans*-geometry was determined from the coupling constant, $J_{22-23}$ = 15.0 Hz. Thus, the stereostructure of 15*R*, 20*R*, 24*R*, $\Delta^{4,6,8(14),22}$-tetraene-3-one-ergosterol of **2** was established.

Ganodermaside A (**1**) was obtained as a yellowish powder with the molecular formula $C_{28}H_{40}O_2$ determined by means of HR ESI-MS measurement. Ganodermaside A (**1**) is a stereoisomer of **2** at C-15. The $^1H$ and $^{13}C$ NMR of **1** was superimposable on that of **2** (Table 1), except for the signals of C-15 and around the position. Likewise, the 15*S* configuration of **1** was determined by NOESY correlations of H-15/H-18. The assignment of configuration of **1** at C-24 was supposedly the same as that of **2**, while the *trans*-geometry of the double bond at C-22 and C-23 was determined from the coupling constant, $J_{22-23}$ = 15.0 Hz.

**Table 1**
$^1H$ and $^{13}C$ NMR data for ganodermasides A (**1**) and B (**2**) in $CDCl_3$

| Carbon | 1 | | 2 | |
|---|---|---|---|---|
| | $^1H$[a] | $^{13}C$[b] | $^1H$[a] | $^{13}C$[b] |
| 1a | 2.02 m | 34.1 | 2.03 m | 34.3 |
| 1b | 1.83 m | | 1.84 m | |
| 2a | 2.48 m | 34.0 | 2.49 m | 34.3 |
| 2b | 2.54 m | | 2.55 m | |
| 3 | — | 199.4 | — | 199.6 |
| 4 | 5.77 s | 123.8 | 5.78 s | 124.2 |
| 5 | — | 163.6 | — | 163.5 |
| 6 | 6.10 d (9.5) | 125.9 | 6.15 d (9.5) | 127.0 |
| 7 | 7.14 d (9.5) | 134.0 | 6.82 d (9.5) | 132.3 |
| 8 | — | 130.0 | — | 129.3 |
| 9 | 2.29 m | 44.3 | 2.18 m | 44.5 |
| 10 | — | 36.9 | — | 37.0 |
| 11a | 1.68 m | 18.9 | 1.65 m | 19.2 |
| 11b | 1.73 m | | 1.72 m | |
| 12a | 2.12 m | 35.7 | 2.06 m | 36.0 |
| 12b | 1.39 m | | 1.26 m | |
| 13 | — | 44.2 | — | 43.2 |
| 14 | — | 157.1 | — | 155.6 |
| 15 | 4.85 m | 69.8 | 4.79 m | 69.4 |
| 16a | 1.79 m | 40.0 | 2.33 m | 39.2 |
| 16b | 1.85 m | | 1.51 m | |
| 17 | 1.60 m | 52.8 | 1.20 m | 54.0 |
| 18 | 0.93 s | 20.3 | 1.15 s | 20.5 |
| 19 | 1.04 s | 16.9 | 1.05 s | 16.9 |
| 20 | 2.14 m | 38.6 | 2.21 m | 39.6 |
| 21 | 1.08 d (6.5) | 21.4 | 1.05 d (8.0) | 21.4 |
| 22 | 5.21 dd (15.0, 8.0) | 134.8 | 5.18 dd (15.0, 8.5) | 134.7 |
| 23 | 5.26 dd (15.0, 7.5) | 132.9 | 5.28 dd (15.0, 8.0) | 133.2 |
| 24 | 1.89 m | 42.9 | 1.88 m | 43.0 |
| 25 | 1.49 m | 33.1 | 1.48 m | 33.3 |
| 26 | 0.83 d (7.0) | 19.7 | 0.82 d (7.0) | 19.8 |
| 27 | 0.85 d (7.0) | 20.0 | 0.85 d (7.0) | 20.2 |
| 28 | 0.94 d (7.0) | 17.6 | 0.93 d (7.0) | 17.8 |

[a] 600 MHz, coupling constants (*J* in Hz) are in parentheses.
[b] 150 MHz.

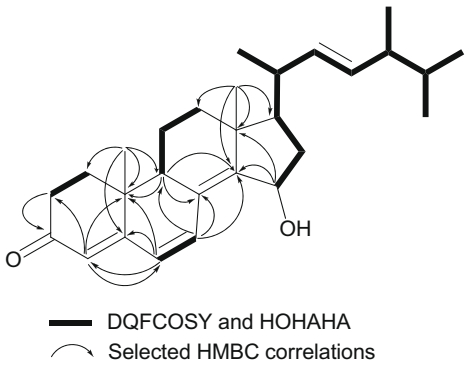


**Figure 2.** Gross structure of ganodermaside B (**2**) with selected HMBC correlations.

### 2.3. Biological activity

The modified bioassay screening method was used as the bioassay system. Resveratol was used as a positive control in the evaluation of the reliability of this bioassay system. Resveratrol,

a red wine polyphenol, is well known to promote anti-aging effects in numerous organisms, such as yeasts, nematodes, fruit flies, mice, and rats.[9–12] There is evidence that resveratrol could be used to cure some animals suffering from different types of neurological disorders.[13,14] Figure 3a shows the dose response of the replicative life span on various concentrations of resveratrol. As such, the most effective concentration of resveratrol for extending replicative life span was 10 μM. The biological activity of resveratrol has remarkably decreased at 1 μM and 100 μM concentrations. These data are similar to that of the traditional method on *S. cerevisiae*.[9] The results further indicate that the modified bioassay system is suitable for the screening of anti-aging substances.

The anti-aging effects of **1** and **2** on replicative life span were evaluated using the K6001 yeast strain. Figure 3b and c shows

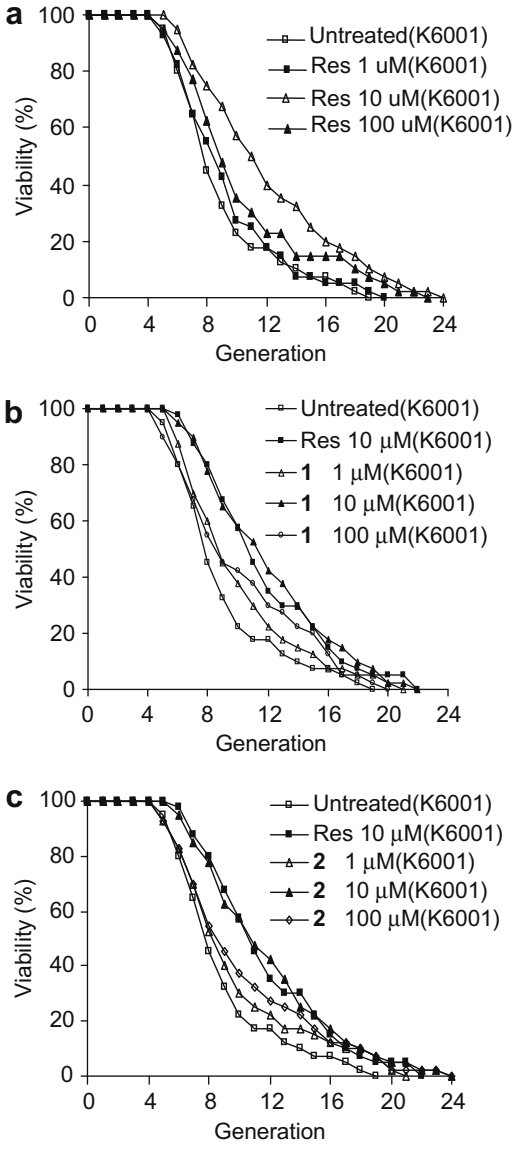


**Figure 3.** The dose response of anti-aging effects of resveratrol (Res, positive control) (a), ganodermasides A (**1**) (b) and B (**2**) (c) on the replicative life span of *S. cerevisiae* K6001 yeast strain. The average life span was, (a): untreated was 8.2, resveratrol at 1 μM was 8.6, resveratrol at 10 μM was 11.4**, resveratrol at 100 μM was 9.7; (b): untreated 8.2, resveratrol at 10 μM was 11.0**, **1** at 1 μM was 8.9, **1** at 10 μM was 11.4**, **1** at 100 μM was 9.4; (c): untreated was 8.2, resveratrol at 10 μM was 11.0**, **2** at 1 μM was 9.1, **2** at 10 μM was 11.1**, **2** at 100 μM was 9.6 (*$p$ <0.05,**$p$ <0.01).

the replicative life span change after administering **1** (Fig. 3b) and **2** (Fig. 3c), in various concentrations, in comparison with the most effective resveratrol concentration, 10 μM. In particular, significant differences were observed at 10 μM concentration for both **1** and **2** with $p$ <0.01 level of significance. These observations were similar to that of resveratrol at 10 μM concentration. In addition, the anti-aging effects between **1** and **2** on the replicative life span of yeast have no significant difference. This implicates that **1** and **2** have identical anti-aging effects on the replicative life span of yeast. Furthermore, the stereochemistry difference at C-15 of **1** and **2** did not cause a substantial difference in terms of biological activity on yeast.

To investigate the mechanism of action on the replicative life span of yeast, regulated by **1** and **2**, several important signal transduction pathways were examined. Oxidative stress was considered one of the major causes of damage, affecting the life span of various organisms. Therefore, *SOD1* and *SOD2* gene expressions, after the administration of **1** and **2,** were examined by reverse transcription-polymerase chain reaction (RT-PCR); nevertheless, no significant change was observed.

The sirtuin family is a critical regulator of life span as well. Both the deletion and the over-expression of sirtuins affect the life span of organisms.[15] We used SIRT1/Sir2 deacetylase fluorometric assay kit (CycLex, Ina, Nagano, Japan) to investigate whether Sir2 takes an important role in life span extension by **1** and **2**. In this experiment, no significant increase of SIRT1 activity was found after the treatment of **1** or **2** at various concentrations.

*UTH1* is a yeast-aging gene that has been ascribed to the stress resistance and longer life span of mutants.[16] A *uth1* mutant with K6001 background yeast exhibited longer life span than that of the K6001 alone, as shown in Figure 4a. In this connection, we examined the effects of **1** and **2** on the *uth1* mutant. Figure 4a shows that **1** and **2** did not affect the obvious life span change of *uth1* mutant. This suggested that the **1** and **2** might extend the replicative life span of yeast via *UTH1* gene.

Skn7 is a transcriptional activator. Phosphorylated Skn7 activates the expression of the annosyl transferase Och1 gene, which plays a role in oxidative stress response.[17] The promoter, located at the upstream of *UTH1* gene, was postulated to contain the binding sites for several transcription factors, including Mot3, Yap1, and Skn7, which are involved in response to oxidative stress.[18] We have found that regulation of UTH1 by a transcription factor Skn7 contributes to the regulation of lifespan by plant-derived polyphenols (L. Xiang, manuscript submitted). To obtain more evidence on **1** and **2** target *UTH1* gene, the life span assay of *skn7* mutant yeast with K6001 background was performed. Both **1** and **2** did not affect the life span change of *skn7* mutant yeast (Fig. 4b), revealing that *SKN7* has regulated the effects of **1** and **2** on *UTH1* expression.

## 3. Experimental

### 3.1. General procedures

The preparative HPLC was performed using ELITE P-230 pumps, and the optical rotations were measured on a JASCO P-1030 digital polarimeter. Whereas the IR spectra were recorded on a JASCO FT/IR-4100, the UV spectra were recorded on a PGENERAL TU-1901**.** Still, the high-resolution (HR) ESI-TOF-MS was recorded on a Mariner Biospectrometry Workstation (Applied Biosystems, CA, USA), using background phthalates as internal calibration standards in the positive mode. The NMR spectra were recorded on a Bruker AV III-500 spectrometer, and the NMR chemical shifts in $\delta$ (ppm) were referenced to the solvent peaks of $\delta_C$ 77.0 and $\delta_H$ 7.26 for $CDCl_3$.

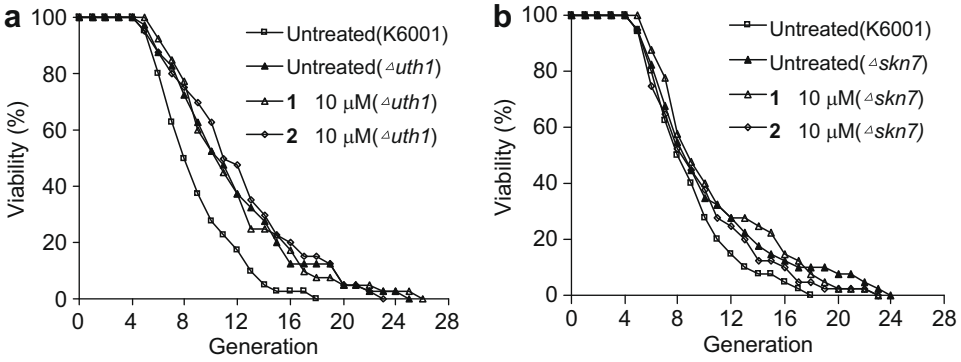


**Figure 4.** Effects of **1** and **2** on the replicative life span of *uth1* and *skn7* mutants with K6001 background yeast. **1** and **2** at 0 and 10 μM on *uth1* (a) and *skn7* (b) mutants in comparison with K6001.

### 3.2. Extraction and isolation

The spores of *G. lucidum* were bought in 2008 in Shanghai, China. The dried spores (dry wt: 650 g) were extracted with MeOH. The supernatant was separated by filtration and concentrated to give 48.13 g of extract. Then, the extract was partitioned between $H_2O$ and EtOAc. The EtOAc layer was concentrated to give 32.81 g of dried sample, which was chromatographed on silica gel (200–300 mesh, Yantai Chemical Industry Research Institute) eluted with *n*-hexane/EtOAc (10:0, 9:1, 7:3, 5:5, 0:10) in order to afford 28 fractions. The active sample, eluted with *n*-hexane/EtOAc (7:3, 5:5), was separated through ODS (Cosmosil 75 C18-OPN, Nacalai Tesque) and eluted with MeOH/$H_2O$ (80:20, 85:15, 90:10, 95:5), MeOH, and MeOH/$CHCl_3$ (1:1), successively, in order to afford 31 fractions. The active sample (70.0 mg) eluted with MeOH/$H_2O$ (90:10, 95:5) was subjected to HPLC [Develosil ODS-HG-5 ($\varphi$ 10/250 mm), Nomura chemical, flow rate: 3 mL/min, MeCN/$H_2O$ (7:3)], to give pure ganodermaside A (**1**) (9.0 mg, $t_R$ = 102 min) and an active fraction (5.4 mg, $t_R$ = 113 min). The resulting active fraction was purified again by HPLC [Develosil ODS-HG-5 ($\varphi$ 10/250 mm), Nomura chemical, flow rate: 3 mL/min, MeOH/$H_2O$ (87:13)], to yield ganodermaside B (**2**) (3.3 mg, $t_R$ = 45 min) as active component.

Ganodermaside A (**1**): a yellowish powder, $[\alpha]_D^{29}$ = +360 (*c* 0.40, $CHCl_3$), IR (KBr) 3416, 1640 $cm^{-1}$, UV (MeCN) $\lambda_{max}$ (log $\varepsilon$) 333 (3.42), high-resolution ESI-TOF-MS *m/z* 409.3072, calcd for $C_{28}H_{41}O_2$ (M+H) 409.3101, and for $^1H$ and $^{13}C$ NMR (see Table 1).

Ganodermaside B (**2**), a yellowish powder, $[\alpha]_D^{29}$ = +376 (*c* 0.30, $CHCl_3$), IR (KBr) 3420, 1647 $cm^{-1}$, UV (MeCN) $\lambda_{max}$ (log $\varepsilon$) 331 (3.01), high-resolution ESI-TOF-MS *m/z* 409.3065, calcd for $C_{28}H_{41}O_2$ (M+H) 409.3101, and for $^1H$ and $^{13}C$ NMR (see Table 1).

### 3.3. Life span assay

The K6001 yeast strain, *uth1* and *skn7* mutants, with K6001 background, were grown in YPGal medium containing 3% (w/v) galactose, 1% yeast extract powder, and 1% polypeptone, or on YPD that contains 2% (w/v) glucose instead of galactose. Agar plates were prepared by adding 2% (w/v) agar to the medium. For the screening of active samples, the K6001 yeast strain or mutants with K6001 background were inoculated into a 2 mL of galactose medium, and incubated in a shaking incubator, at 28 °C and 160 rpm, for 48 h. One milliliter of yeast culture media was centrifuged at 10,000 *g* for 5 min. The yeast pellet was washed three times and diluted with distilled water. Cells were counted by using a hemocytometer, after which 5000 cells were plated on glucose agar plates that were prepared by mixing agar with various concentrations of the samples in homogeneous phase. The plates were incubated at 28 °C for 2 days, and micro-colonies that formed were observed under a microscope. The daughter cells generated by each of more than 40 mother cells were counted to evaluate the biological activity of the samples.

### 3.4. Statistical analysis

The significant differences between groups were determined by ANOVA, followed by a two-tailed multiple *t*-tests with Student–Newman–Keuls through SPSS, a biostatistics software. Values with $p$ <0.05 were considered significant.

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