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Zirconia with laser-modified micro-grooved surface vs. titanium implants covered with melatonin stimulates bone formation. Experimental study in tibia rabbits

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Abstract

Objectives: The aim of the study was to evaluate if zirconia implants with micro-grooved surfaces supplemented with melatonin enhance the bone-to-implant contact (BIC) vs. titanium implants with the same coating.

Materials and methods: Eighty implants divided in four groups were inserted in the tibia of 20 New Zealand rabbits as follows: (group A) 20 titanium implants; (group B) 20 micro-grooved zirconia implants; (group C) 20 titanium implants supplemented with melatonin and (group D) 20 micro-grooved zirconia implants supplemented with melatonin. Histometric and SEM evaluation of BIC were evaluated after 1 and 4 weeks.

Results: At 1 week, group C ($29.7 \pm 2.4\%$) and group D ($28.9 \pm 1.3\%$) implants showed higher BIC % compared with group A and B ($P < 0.05$). After 4 weeks, group D showed higher BIC compared with all the groups ($47.5 \pm 2.2\%$) ($P < 0.05$). Also Connective tissue was higher in groups B ($78.9 \pm 2.1\%$) and D ($88.7 \pm 1.2\%$) related to titanium and zirconia melatonin untreated at 4 weeks ($P < 0.05$).

Conclusions: Within the limitations of this pilot study in rabbits, we can conclude that the local application of melatonin increases the BIC values in titanium and in zirconia implants at 1 week.

Bone tissue undergoes a process of continuous remodelling, mediated mainly by bone cells (osteoblasts, osteoclasts and osteocytes). In this process, growth factors, some systemic hormones including melatonin (MLT), are involved as well (Tresguerres et al. 2014).

Melatonin, or N-acetyl 5-methoxytryptamine, is a hormone synthesized and secreted mainly in the pineal gland (Tan et al. 2007). Melatonin synthesis does not only take place in the pineal gland but also in other areas and cells such as the eyes, gut, bone marrow, skin and gonads where it acts either as a paracrine or an autocrine (Tan et al. 1999). Because of its diverse activity, melatonin is not a hormone in the strictest sense. Melatonin has significant bone protecting properties (Reiter et al. 2007).

MLT inhibits *in vitro* the increased calcium uptake in bone samples of rats treated with pharmacologic amounts of methylprednisolone (Ladizesky et al. 2006) and numerous studies (Koyama et al. 2002; Ostrowska et al. 2003; Suzuki et al. 2008; Calvo-Guirado

et al. 2009, 2010; Ramírez-Fernández et al. 2013) have documented MLT significance as a mediator in bone formation and bone vascularization.

Moreover, it has been observed that MLT, at pharmacological doses, increases bone mass by suppressing resorption through downregulation of the receptor activator of nuclear factor-kappa B ligand (RANKL) mediated osteoclast formation and activation (Koyama et al. 2002; Cutando et al. 2007; Liu et al. 2013). Furthermore, MLT can inhibit bone resorption by suppressing osteoclast activity and can reduce the period of osseointegration (Cengiz & Wang 2012; Clafshenkel et al. 2012; Witt-Endeby et al. 2012).

In addition, it has been proved that MLT applied to the surface of titanium implants or to the recipient bed for titanium implants is able to promote the bone formation at early stages of the bone healing expressed as more bone-to-implant contact (BIC) percentages. However, evidence of the benefits of the MLT application are less evident at late

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stages of the healing process (Cutando et al. 2008; Guardia et al. 2011; Muñoz et al. 2012; Tresguerres et al. 2012).

Therefore, to overcome the limitations of the short availability of the MLT, the surface, the micro-topography and the material characteristics could be modified to enhance the retention of the MLT in the surface, thus enhancing the activity and release of MLT or other bioactive molecules from the implant surface to the surrounding bone during longer time.

Recently, a surface modification was introduced for zirconia implants, a pattern of symmetrical microgrooves of 30 µm width and 40 µm depth, were created using a femtosecond laser along the implant surface, which resulted in decreased surface contaminants, preservation of the tetragonal structure and minimal thermal damage of the treated surfaces (Delgado-Ruiz et al. 2011).

Moreover, animal studies in dogs have demonstrated that microgrooved zirconia implants were able to osseointegrate in a manner comparable to acid-etched and sandblasted titanium implants, demonstrated by similar BIC percentage and similar crestal bone levels (Calvo-Guirado et al. 2013a,b; Delgado-Ruiz et al. 2013).

Hypothetically, a microgrooved surface could serve to carry substances with different biological effects that could be released slowly, favouring the process of osseointegration in later stages of bone regeneration.

Exist a lack of references regarding the relation between laser textured microgrooved zirconia implants and their surface supplementation with MLT for the enhancement of the osseointegration. Hence, the purpose of the present work was to evaluate whether zirconia implants with microgrooved surfaces supplemented with MLT enhances the BIC values after 1 and 4 weeks improving the bone healing at the early stages compared with titanium implants.

Material and methods

Experiment animals

The Ethics Committee for Animal Research at The University of Murcia (Spain) approved the study protocol, which followed the guidelines established by the European Union Council Directive of February 1986 (R.D.53/1986). Clinical examination determined that all animals were in good general health.

Twenty male New Zealand rabbits aged 30–35 weeks and weighing 3900–4500 g were used. The animals were kept in purpose-

designed room and were fed and watered *ad libitum* with standard diet. All the surgeries were performed in an operating room at the University of Murcia Research Support Service.

Implants

Eighty implants divided in four groups placing two implants per tibia in twenty rabbits were included as follows:

Group A: 20 titanium implants, sandblasted and acid-etched (Blue SKY®; Bredent Medical GmbH & Co. KG, Senden, Germany) (Fig. 1b);

Group B: 20 zirconia implants and sandblasted (WhiteSky®; Bredent Medical GmbH & Co. KG) (Fig. 1a);

Group C: 20 titanium implants, sandblasted and acid-etched (Blue SKY®; Bredent Medical GmbH & Co. KG) supplemented with MLT 5% in solution (TM-M5250; Sigma-Aldrich Química S.L., Madrid, Spain) (Fig. 2);

Group D: 20 zirconia implants, sandblasted (WhiteSky®; Bredent Medical GmbH & Co. KG) and microgrooved by femtosecond laser (Delgado-Ruiz et al. 2011) supplemented with MLT 5% in solution (TM-M5250; Sigma-Aldrich Química S.L.) (Fig. 3).

The surface treatment was the titanium implants Blue Sky surface made by titanium grade IV with a surface sandblasted with

alumina oxide particles of 350–550 µm and acid-etched with a bath of sulphuric acid at 37% 1 h (Bredent Medical GmbH & Co. KG). White SKY zirconia implants made from



Fig. 2. Titanium dental implant with melatonin doping surface.



Fig. 3. Zirconia microgrooved implant with melatonin inserting in rabbit tibiae.

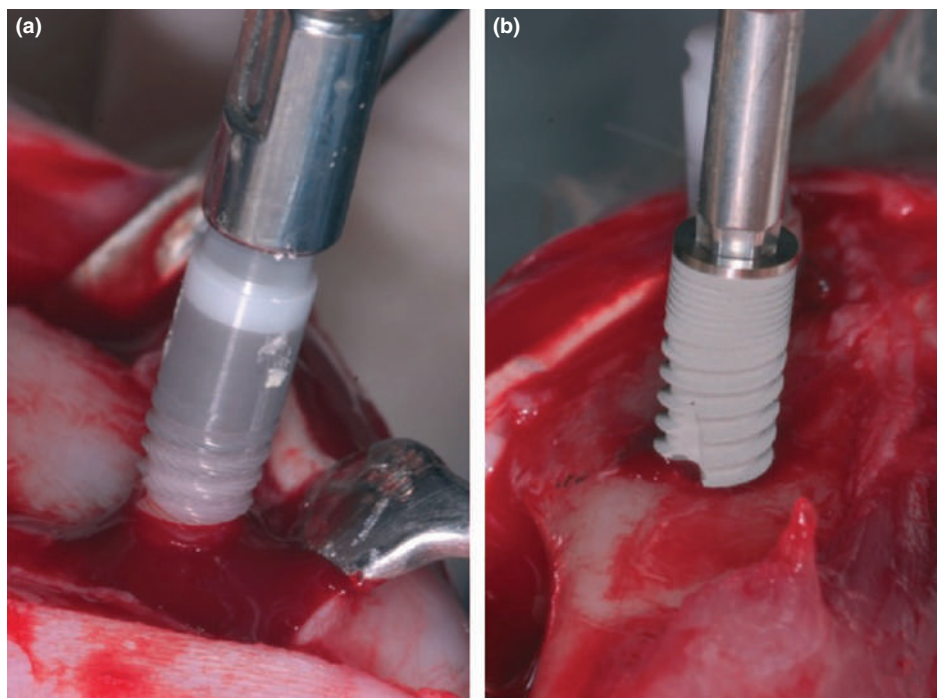


Fig. 1. Dental implants inserted in tibia rabbits: (a) zirconia laser microgrooved implant; (b) titanium implant.

zirconia, with a surface sandblasted with alumina oxide particles of 350–550 nm (Bredent Medical GMBH & Co. KG) treated with femtosecond laser pulses to create 30-mm-wide and 70-mm pitch length microgrooves over the entire intraosseous surface with a method previously described by Delgado-Ruiz et al. (2011). All zirconia implants were treated by commercial Ti:Sapphire oscillator (Tsunami; Spectra Physics, Santa Clara, CA, USA) and a regenerative amplifier system (Spitfire; Spectra Physics) based on chirped pulsed amplification using for microstructuring. The system delivers 120 fs linearly polarized pulses at 795 nm with a repetition rate of 1 kHz. The transverse mode was TEM₀₀, and the beam width was 9 mm (1/e² criterion). Pulse energy can reach a maximum of 1.1 mJ and is reduced by means of neutral filters; and a half wave plate and polarizer system to generate the most suitable fluences on the surface of the material for producing ablation with minimal damage to the surrounding area. The laser pulses travel through air to the focusing system. This consisted of an achromatic doublet lens and an off-axis imaging system (lens, beam splitters and CCD Philips LucaVR cameras) that allow the visualization of the processing area, facilitating implant positioning and beam focusing. Implants were placed on a motorized platform with three-axis motion, X, Y and Z, controlled by Micos ES100VR software (Nanotec Electronic GMBH & Co., Munich, Germany) so that laser pulses impinge perpendicularly to the implant axis. The platform was mounted on an OWISVR rotating motorized base (Nanotec Electronic GMBH & Co.), controlled by software that turned the base at speeds varying between 0 and 30 s, allowing the whole periphery of the implant to be texturized without altering focusing conditions. The outcomes of laser were monitored and controlled using an Axio VisionVR Light Microscopy reflection optical microscope (Carl ZeissVR, Gottingen, Germany), with Axio ImagerVR M1 m software (Carl Zeiss). The lenses used were high-resolution PLAN APO CS: 10×, 20×, 40×, 60× and 100×.

Fifteen minutes before general anaesthesia, the animals received an intramuscular injection of an anxiolytic (0.5–1 mg/kg acepromazine maleate). The rabbits were anaesthetized with an intramuscular injection of tiletamine/zolazepam 15 mg/kg (Zoletil 50, Virbac, Madrid, Spain) and xylazine 5 mg/kg (Rompun, Bayer, Leverkusen, Germany). Before surgery, the shaved skin over the area of the proximal tibia was washed with (Betadine®; Meda Manufacturing, Burdeos,

France). Ketamine hydrochloride (Ketalar®; Pfizer, Madrid, Spain) was administered as an anaesthetic at 50 mg/kg IM. A pre-operative antibiotic (Amoxicillin, Pfizer, Barcelona, Spain) was administered intramuscularly and 3 ml lidocaine at 2% was also administered intramuscularly in the surgical area of each leg, with 0.01 mg/ml adrenaline.

Surgical procedure

The internal approach was performed in the proximal metaphyseal–diaphyseal area of each tibia, several millimetres below the anterior tibial tuberosity.

An incision was performed at the proximal metaphyseal area of the tibiae, a total thickness flap was raised, and the periosteum was gently lifted to expose the surgical area taking the tibial plateau as a reference point. Subsequently, two points were marked with a graphite separated by 15 mm distance. Spherical surgical drills at low speed with constant irrigation were used to remove bone tissue to form two concave defects approximately 2 mm in diameter per tibia. The concave points guided the perforation of the cortical bone; perforations were carried with drill bits 3.5 and 4.0 mm diameter and 8 mm length (Bredent Medical GmbH & Co. KG) under profuse irrigation, each tibia received two implants. Implant diameter and length were the same for all the implant groups (4 mm diameter and 8 mm length) (Fig. 1b).

This was a randomized study. The four implants in each rabbit, one of each group, were distributed in the right and the left tibia, allocating the implant zirconia and titanium types to sites by means of the web site <http://www.randomization.com>, study number 23219.

The implants were fixed bi-cortically to ensure stability. Given that the zirconia implants used were one piece, after the zirconia implant insertion diamond drills were used to cut the most coronal portion of the implant to leave exposed only 1 mm over the cortical bone the titanium implants received a cover screw of 1 mm height (Figs 3 and 4).

The flaps were repositioned and sutured with 3-0 silk (Laboratorios Aragón S.L., Barcelona, Spain) followed by the application of an antiseptic plastic spray dressing (Nobecutan, Laboratorios Inibsa, Llíça de Vall, Spain).

Amoxicillin (0.1 ml/kg intramuscularly) was administered at the end of surgery. The animals were given 0.05 mg/kg buprenorphine subcutaneously every 12 h after the operation for 3 days. Within 2–3 days, the animals resumed normal ambulation and did not show signs of pain or distress.



Fig. 4. Clinical image of rabbit tibia with melatonin-treated implants. In the left side the titanium implant and in the right side the zirconia implant.

Study groups

The animals were divided into two groups ($n = 10$) according to the time between initial surgery and euthanization time (healing period), 1 week were 10 rabbits and 10 weeks the other 10 rabbits. Surgery was performed on two tibiae of each rabbit, placing two implants per tibia, four implants per rabbit and one in each study group. The animals were euthanized at one and four weeks by means of intravenous overdoses of sodium thiopental at 2%, 1 g in 50 ml of physiological saline (NaCl 0.9%) (Pentotal®; Braun Medical, Barcelona, Spain). The clear indication of these two time points of sacrifice was carried out because the melatonin acts in early stages of implant insertion.

The rabbit tibiae were extracted, the soft tissues were removed and the tibiae including the implants were processed according to the processing of undecalcified samples, as follows.

Histological processing

These samples were submerged in 10% formalin solution for 24 h and then washed in running water. Thereafter, the samples were dehydrated in graded ethanol solution from 70–100%. Finally were embedded in resin methacrylate blocks (Technovit 7200; Heraeus Kulzer, Wehrheim, Germany) following the manufacturer's recommendations.

Sawing and grinding of the samples was performed till samples of 200 µm thickness were obtained, later polished to a final thickness of 70 µm. The samples were stained with Levai Laczko and then examined under optical microscope (Leica microscope Q500Mc, Leica DFC320s, 3088 × 2550 pixels; Leica Microsystems, Barcelona, Spain). After 4 weeks, the blocks were cut in half, slicing the implants in two, preparing one half for SEM observation and the other for optical microscope observation. These were made into slides with a 0.5 mm thickness and mounted on

aluminium blocks coated in carbon (using a Polaron sputter coater).

Then they were examined using EDX at a working distance of 19 mm, with 15 kV, acceleration and $\times 159$ magnification using the Oxford Instruments INCA 300 EDX System (Abingdon, Oxfordshire, UK) to obtain SEM images and quantify the elements of peri-implant surrounding bone. The most central areas of the images were analysed using Microm Image Processing Software 4.5 (Consulting Image Digital, Barcelona, Spain) connected to a Sony DXC-151s 2/3-CCD RGB Color Video Camera (Sony Electronics Inc., San Jose, CA, USA), which provided images used to measure the BIC. Mean values of BIC%, new bone (NB), medullar bone (MB) and connective tissue (CT) were calculated by implant and by animal within the limits of tibia bone contour.

Statistical analysis

The four implants inserted in each animal had one of the four test surface modifications belonging to four groups: A, B, C and D, as detailed above.

All data were analysed using SPSS 19 statistical software (SPSS Inc., IBM Corporation, Chicago, IL, USA).

Correlations between subgroups were analysed through nonparametric Friedman test for related samples. Dependent variables included the histomorphometric measurements previously described.

Values were recorded as mean $\pm$ standard deviation. Wilcoxon's test was applied to the comparison of mean averages and to quantify relationships between differences ($P < 0.05$). Equal means were regarded as the null hypothesis, whilst the existence of significant differences between means acted as an alternative hypothesis. Bone-to-implant contact (BIC) percentages were calculated and subjected to a 2-tailed analysis of variance to test for significant differences between the four investigated implants. Statistical testing was carried out at the 5% significance level. A P -value < 0.05 was considered statistically significant.

Results

Histological and histomorphometric analyses

1-week

Figure 5a–d included representative histological images. Microscopically, all 76 implants were well integrated into bone. The implants were in contact with predominantly cortical bone along the upper threads

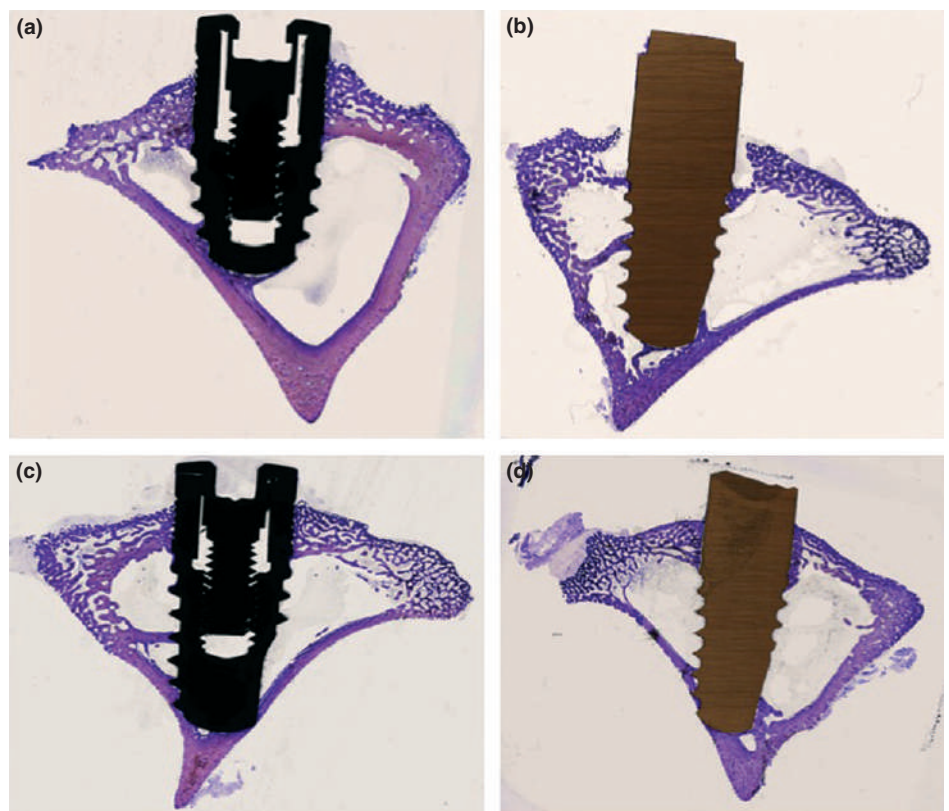


Fig. 5. Histological images of rabbit number. (a) titanium implants without melatonin coating (group A), (b) Zirconia laser microgrooved implants without melatonin coating (group B), (c) Titanium implants covered with melatonin (group C), (d) Zirconia laser microgrooved implants with melatonin coating.

in the cortical region, whilst the threads in the bone marrow were in contact with either newly formed bone or with normal bone marrow. A demarcation line was seen between the newly formed bone and the old bone tissue.

Bone-to-implant contact (BIC) percentage was measured for all the 76 implants in both analysed period, in 1-week time and in 4-week time. Average BIC% evaluation (measuring both cortical and trabecular BIC) of the samples retained for histological analysis produced the following results at 1-week time (Table 1). Four implants failed because of fibrousseointegration, one of each surfaces group in one rabbit. BIC values were significantly higher for groups C and D, compared with the other groups ($P < 0.05$).

The total BIC mean values were as follows: for titanium implants (group A) $25 \pm 1.2\%$; for titanium implants with MLT (group C), the BIC was $29 \pm 2.4\%$; the zirconia implants (group B) had $22.8 \pm 1.5\%$ of total BIC and the zirconia implants with melatonin (group D) had $28.9 \pm 1.3\%$ of total BIC. In the statistical analysis, comparing all the groups, BIC values were significant higher for group C and group D, compared with the other groups ($P < 0.05$).

The results of the percentage of connective tissue are also presented in the Table 2. The total CT mean values were: for titanium implants (group A) $73.6 \pm 1.8\%$, for titanium implants with MLT (group C) the CT value was $74.3 \pm 1.9\%$, the zirconia implants (group B) had a CT value of $78.1 \pm 1.8\%$ and finally, the zirconia implants with MLT (group D) had $81.9 \pm 1.7\%$ of CT. In the statistical analysis, comparing all the groups of CT values, the group D was significantly higher ($P < 0.05$).

4-week

Bone-to-implant contact data for the implants in the 4-week period are described in the Table 1. We found that the sample size for each group was $n = 10$, except for Group C and D which lost 1 implant each one. BIC values were significantly higher for group D, compared with the other groups ($P < 0.05$). The total BIC% values were as follows: for titanium implants (group A) $38.4 \pm 1.8\%$; for titanium implants with melatonin (group C) was $39.2 \pm 2.5\%$; the zirconia implants (group B) had $37.5 \pm 2.1\%$ and the zirconia implants with MLT (group D) had $47.5 \pm 2.2\%$ of total BIC. In the statistical analysis, comparing all the groups, BIC values were

Table 1. Wilcoxon nonparametric test was applied to evaluate median values (percentage $\pm$ standard deviation) of bone-to-implant contact (BIC)

Time of measurement	Group A (mean $\pm$ SD), %	<i>P</i> value	Group B (mean $\pm$ SD), %	<i>P</i> value	Group C (mean $\pm$ SD), %	<i>P</i> value	Group D (mean $\pm$ SD), %	<i>P</i> value
1 week	25.4 $\pm$ 1.2	0.743	22.8 $\pm$ 1.5	0.853	29.7 $\pm$ 2.4	0.041*	28.9 $\pm$ 1.3	0.023*
4 weeks	38.4 $\pm$ 1.8	0.564	37.5 $\pm$ 2.1	0.732	39.2 $\pm$ 2.5	0.972	47.5 $\pm$ 2.2	0.018*

P value of each group and time of measurements.

*Differences between values achieving statistical significance $P < 0.05$.

Table 2. Wilcoxon's nonparametric test was applied to evaluate median values (percentage $\pm$ standard deviation) of connective tissue attachment (CT)

Time of measurement	Group A (mean $\pm$ SD), %	<i>P</i> value	Group B (mean $\pm$ SD), %	<i>P</i> value	Group C (mean $\pm$ SD), %	<i>P</i> value	Group D (mean $\pm$ SD), %	<i>P</i> value
1 week	73.6 $\pm$ 1.8	0.547	78.1 $\pm$ 3.2	0.034*	74.3 $\pm$ 1.9	0.348	81.9 $\pm$ 1.7	0.023*
4 weeks	74.8 $\pm$ 1.1	0.821	78.9 $\pm$ 2.1	0.579	76.8 $\pm$ 2.5	0.619	88.7 $\pm$ 1.2	0.037*

P value of each group and time of measurements.

*Differences between values achieving statistical significance $P < 0.05$.

significant higher for group D, compared with the other groups ($P < 0.05$).

The results of the percentage of connective tissue are also demonstrated in the Table 2. The total CT mean values were as follows: for titanium implants (group A) $74.8 \pm 1.1\%$; for titanium implants with MLT (group C), the CT value was $76.8 \pm 2.5\%$; the zirconia implants (group B) had a CT value of $78.9 \pm 2.1\%$ and finally, the zirconia implants with MLT (group D) had $88.7 \pm 1.2\%$ of CT. In the statistical analysis, comparing all the groups of CT values, the group D group was significantly higher ($P < 0.05$).

The Figs 6 and 7 showed the histological images of all implants used. Figure 6 is related to zirconia implants. The right side of the image (a) illustrates an untreated zirconia implant (not covered with melatonin). A direct contact between the bone and the implant is presented at the tip of the implant with some bony gaps between new bone and implant. The left side of the image (b) belongs to a zirconia implant covered by melatonin. There is also intimal contact between the implant and the bone, and in this case, the bone is more mature and with higher density. The zirconia implants were in direct contact with predominantly marrow bone along the threads with either newly formed bone raising from the tip of the implant up to the middle implant body.

The other two images in the Figure 7 belong to titanium implants. The right part of the image (a) is a normal titanium implant with good osseointegration. The left part of the image (b), the titanium implant covered with melatonin shows large amount of woven bone (WB) inside the threads and the phenomenon of bone regeneration.

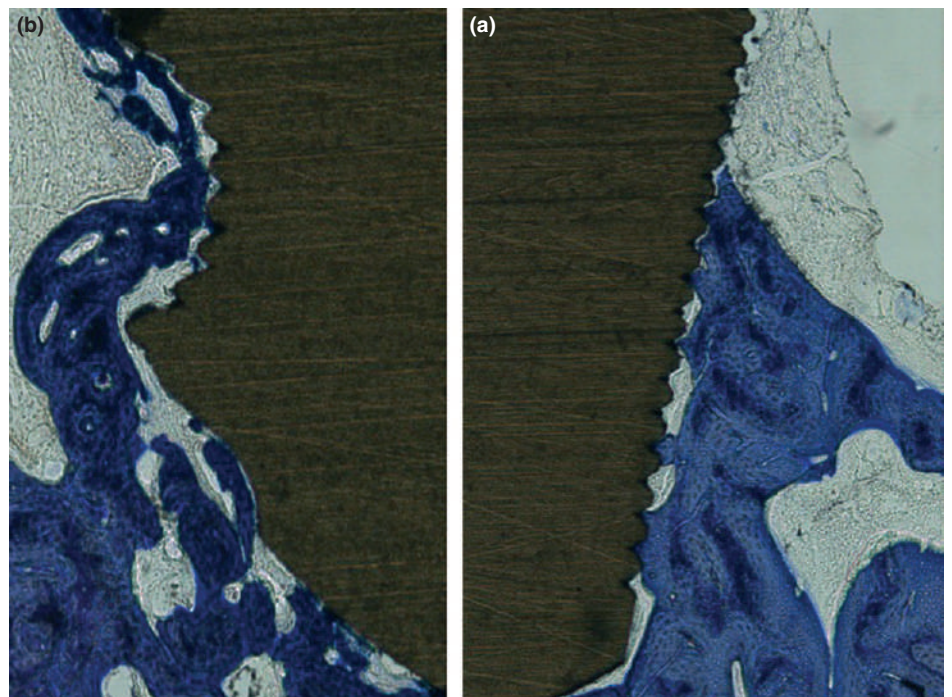


Fig. 6. Histological images of zirconia implants: (a) new formed bone was observed around non-melatonin treated zirconia, (b) greater mature bone formation around zirconia microgrooves melatonin treated from the tip of the implant till implant body.

SEM evaluation of BIC

The method used in our study is based on the high contrast between bone and implant SEM images similar to other authors (Abrahamsen et al. 2009; Santis et al. 2011; Calvo-Guirado et al. 2012; Manresa et al. 2014).

The SEM examination revealed bone formation and bone debris still entrapped at the interface separated from implant threads on surface in group A at 1 week (a). Furthermore, large pores in the cortical bone were evident and the repair process was in the formation stage at 1 week in group C (c); however, morphometric measurements showed

the zirconia surface (group B) most of these grooves around threads, covered by immature bone and surrounded by vascular spaces at 1 week (100 $\times$ magnification) (b). Overall, microgrooved zirconia-treated surface (group D) demonstrated the highest early and late BIC values at 1 week (d) (Fig. 8).

The evaluation at 4th week, the percentage of BIC clearly increased up to the last time point in all groups, but group D was showed a higher level of contact between the implant and bone structure with less vascular spaces and more mature bone formation (Fig. 9).

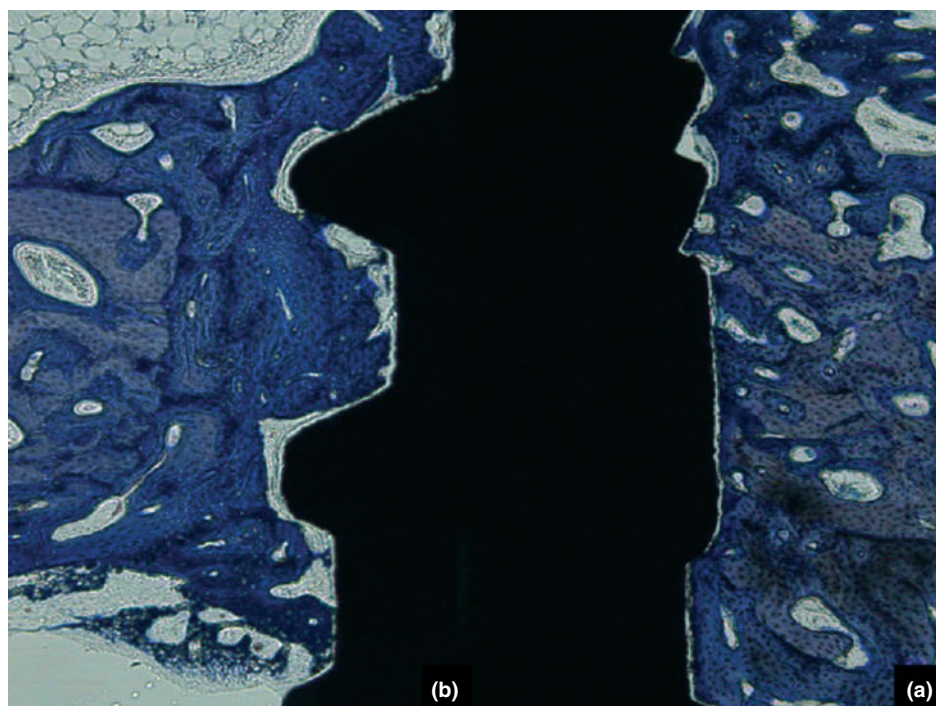


Fig. 7. Histological images of titanium implants: (a) titanium implant without melatonin addition shows new marrow bone surrounding the threads; (b) melatonin-treated titanium implant shows more mature bone surrounding threads.

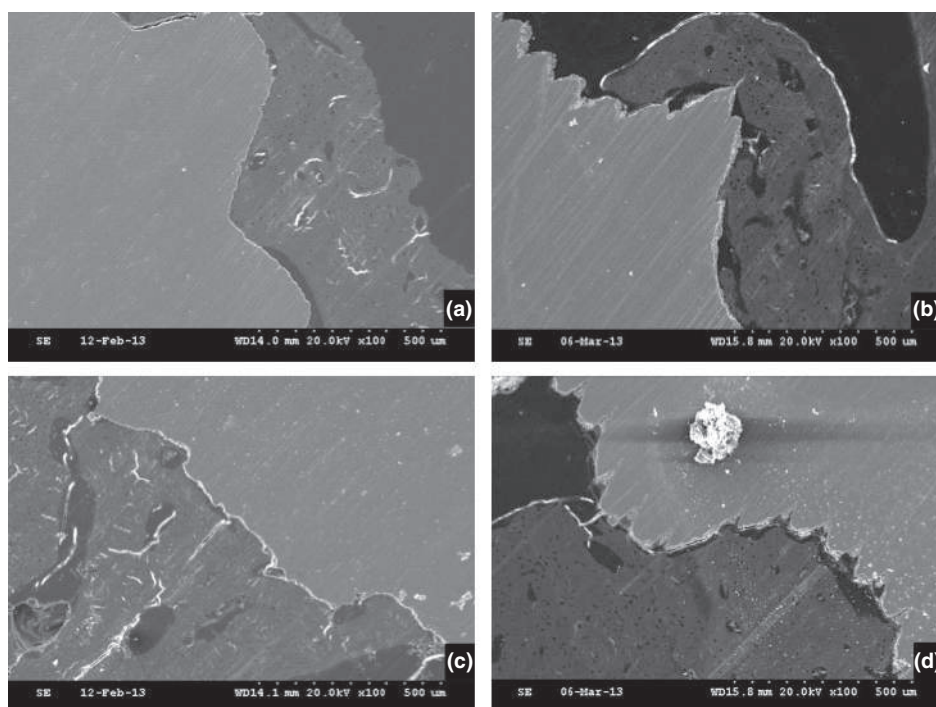


Fig. 8. Scanning electron microscopy of all implant groups at 1 week: (a) group A implants a calcified gray level tissues separated from the implant surface; (b) group B zirconia nontreated melatonin implants showed less bone to implant contact over laser microgrooved surface; (c) group C showed more BIC on the surface than group A and B; (d) group D showed more mature bone around microgrooves and all of them were partially filled with new bone.

In the SEM analysis, several chemical elements were evaluated. The measurement of carbon, titanium, phosphorous, calcium and

oxygen was taken and also the ratio Ca/P was calculated. Results are shown in Table 3. The presence of all the elements analysed

was almost constant in all the surfaces. For the carbon element, we found $36.36 \pm 8.05\%$ for the group A, $35.11 \pm 1.05\%$ for the group B, $36.76 \pm 1.43\%$ for the group C and $35.27 \pm 3.87\%$ for the group D. If we look in to the oxygen measurements, we found $14.22 \pm 3.15\%$ for the group A, $15.15 \pm 1.78\%$ for the group B, $16.7 \pm 1.79\%$ for the group C and increased the value at $19.79 \pm 1.11\%$ for the group D. In the Phosphorous element, the values are $4.27 \pm 1.4\%$ for the group A, $3.99 \pm 1.65\%$ for the group B, $4.32 \pm 1.34\%$ for the group C and $4.91 \pm 0.16\%$ for the group D. For the calcium element, the data are $6.35 \pm 1.9\%$ for the group A, $6.43 \pm 2.2\%$ for the group B, $7.53 \pm 2.19\%$ for the group C and $9.62 \pm 1.13\%$ for the group D. Finally, the titanium elements which the values were elevated in group A $48.56 \pm 8.21\%$ and group C $59.03 \pm 4.67\%$ and less amount in groups B $4.21 \pm 5.32\%$ and group D $4.22 \pm 9.19\%$, respectively.

Finally, the ratio Ca/P was highly obtained in groups C and D, both treated with melatonin, which means that titanium and zirconia surfaces treated with melatonin increase bone turnover.

Table 4 showed a nonparametric test comparing all surfaces BIC and connective tissue (CT) variables that were measured in the study. The mean differences were found statistically significant between groups C vs. D, A vs. C and A vs. D. The data rejected the null hypothesis and described that group D had more BIC percentages than other surfaces evaluated (A, B and C).

Discussion

The effects of MLT in bone metabolism is nowadays well documented and very well demonstrated by various studies. A high number of *in vitro* investigations reported that MLT is an important mediator in bone formation and stimulation, promoting osteoblast differentiation (Piatelli et al. 1999; Nannmark & Sennerby 2008) and bone metabolism (Donath & Breuner 1982; Lynch et al. 1991; Hallman et al. 2001; Satomura et al. 2007).

Also, a high number of publications have confirmed BIC increases around titanium implants when they are submerged in MLT. Calvo-Guirado et al. (2009) concluded that MLT combined with porcine bone on DCD (discrete crystal deposition) implants reveals more BIC at 12 weeks ($84.5 \pm 1.5\%$) compared with MLT treated ($75.1 \pm 1.4\%$) and

non-MLT treated surface implants ($64 \pm 1.4\%$) (Calvo-Guirado et al. 2010). Muñoz et al. (2012) also confirmed in an experimental study with dogs that the presence of MLT and growth hormone enhance new bone formation around titanium implants in early stages of healing (Muñoz et al. 2012). Also

Takechi et al. (2008) combined MLT and fibroblast growth factor-2 (FGF-2) in rats and strongly suggested that MLT and FGF-2 have the potential to promote osseointegration. The present study is in correspondence with the other reported studies. In the 1-week analysis, the titanium with MLT implants

had significantly higher value ($P > 0.05$) than the control group (titanium implants).

The zirconia implants have been defined as a good alternative to titanium implants. In a rabbit study, similar to the present study, published by Shin et al. (2011), the authors compared titanium vs. zirconia implants. They concluded that zirconia implants demonstrated a lower bone remodelling activity especially in areas close to the periosteum. In addition, the data showed that bone at the bone-implant interface presented a significantly lower cortical bone density, a higher trabecular density and trabecular mineral content. Finally, zirconia and titanium implants showed similar bone-implant responses in terms of BIC; therefore, they do not present significant differences with the present study (Shin et al. 2011).

Another study published by Gahlert et al. (2012) studied also titanium and zirconia implants but in minipig model. Thirty-four implants placed in 18 minipigs were compared at 4, 8 and 12 weeks of healing. It was concluded that there was no difference in osseointegration between ZrO_2 implants and Ti-SLA controls regarding peri-implant bone density and BIC%, even it was a tendency to best results in titanium SLA implants in our results (Gahlert et al. 2012). In the present study, all zirconia implants with MLT had better results ($P > 0.05$) in all periods, even compared with titanium plus MLT.

Conclusions

The use of MLT in zirconia implants has not been studied previously. The results of the present study indicate the importance of this molecule to improve new bone formation around implants, especially around zirconia implants better than titanium implants. Further studies are needed to establish this material in implant dentistry in the future improving the aesthetic outcome with low complication rate.

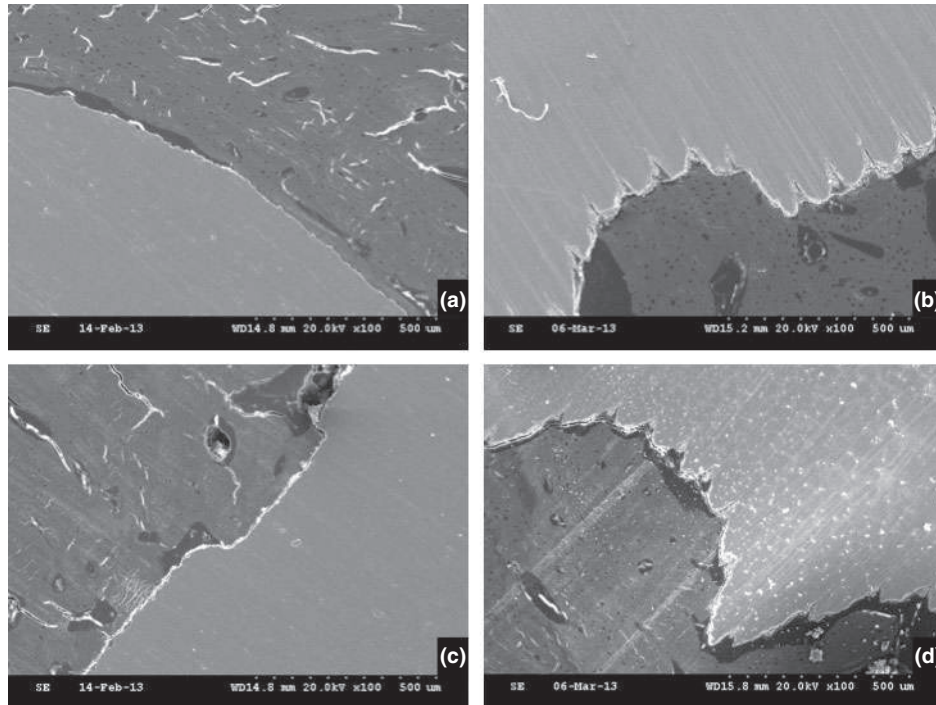


Fig. 9. Scanning electromicroscopy of all implant groups at 4 weeks: (a) group A implants a calcified gray level tissues separated from the implant surface; (b) group B zirconia nontreated melatonin implants showed less bone to implant contact over laser microgrooved surface; (c) group C showed more BIC on the surface than group A and B; (d) group D showed more mature around microgrooves and all of them were partially filled with new bone. (500 $\times$ magnification).

Table 3. Descriptive mean and SD measurements of chemical composition of the surrounding bone of implants with the four different treated surfaces at 4 weeks evaluation time

	Group A (%) Mean $\pm$ SD	Group B (%) Mean $\pm$ SD	Group C (%) Mean $\pm$ SD	Group D (%) Mean $\pm$ SD
Carbon	35.11 $\pm$ 1.05	36.76 $\pm$ 1.43	36.85 $\pm$ 3.45	35.27 $\pm$ 3.87
Oxygen	14.22 $\pm$ 3.15	15.15 $\pm$ 1.78	16.7 $\pm$ 1.79	19.79 $\pm$ 1.11
Phosphorus	4.27 $\pm$ 1.4	3.99 $\pm$ 1.65	4.32 $\pm$ 1.34	4.91 $\pm$ 0.16
Calcium	6.35 $\pm$ 1.9	6.43 $\pm$ 2.2	7.53 $\pm$ 2.19	9.62 $\pm$ 1.13
Titanium	48.56 $\pm$ 8.21	4.21 $\pm$ 5.32	59.03 $\pm$ 4.67	4.22 $\pm$ 9.19
Ca/P	1.812	1.567	1.929	2.178*

The significant level was * $P < 0.05$.

Table 4. Nonparametric test for all Surfaces Comparison of BIC

Null hypothesis	Test	Sig.	Decision
The median of differences between group A and group B	Related Samples Wilcoxon Signed Rank Test	0.016*	Reject the null hypothesis
The median of differences between group A and group C	Related Samples Wilcoxon Signed Rank Test	0.031*	Reject the null hypothesis
The median of differences between group A and group D	Related Samples Wilcoxon Signed Rank Test	0.028*	Reject the null hypothesis
The median of differences between group B and group C	Related Samples Wilcoxon Signed Rank Test	0.578	Reject the null hypothesis
The median of differences between group B and group D	Related Samples Wilcoxon Signed Rank Test	0.573	Reject the null hypothesis
The median of differences between group C and group D	Related Samples Wilcoxon Signed Rank Test	0.856	Reject the null hypothesis

Asymptotic significances are displayed. *The significant level was $P < 0.05$.

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