Early bone anchorage to micro- and nano-topographically complex implant surfaces in hyperglycemia

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ABSTRACT

The aim of this work was to investigate the effect of implant surface design on early bone anchorage in the presence of hyperglycemia. 108 Wistar rats were separated into euglycemic (EG) controls and STZ-treated hyperglycemic (HG) groups, and received bilateral femoral custom rectangular implants of two surface topographies: grit blasted (GB) and grit-blast with a superimposed calcium phosphate nanotopography (GB-DCD). The peri-implant bone was subjected to a tensile disruption test 5, 7, and 9 days post-operatively (n = 28/time point); the force was measured; and the residual peri-implant bone was observed by scanning electron microscopy (SEM). Disruption forces at 5 days were not significantly different from zero for the GB implants (p = 0.24) in either metabolic group; but were for GB+DCD implants in both metabolic groups (p < 0.001). Contact osteogenesis was greater on GB-DCD than the GB surface. The nano-and micro-surfaced implants showed significantly different disruption forces at all time points (e.g. >15 N and <5 N respectively at 9 days). Such differences were not seen within the GB implants, as all values were very low (<5 N). Even in hyperglycemia the GB-DCD surface outperformed the GB surfaces in both metabolic groups. Significantly, SEM of peri-implant bone showed compromised interfibrillar collagen mineralization in hyperglycemia, while interfibrillar and cement line mineralization remained unaffected. Enhanced bone anchorage to the implant surfaces was observed on the nanotopographically complex surface independent of metabolic group. The compromised interfibrillar mineralization observed provides a mechanism by which early bone mineralization is affected in hyperglycemia.

Statement of Significance

It is generally accepted that the hyperglycemia associated with diabetes mellitus compromises bone quality, although the mechanism by which this occurs is unknown. Uncontrolled hyperglycemia is therefore a contra-indication for bone implant placement. It is also known that nano-topographically complex implant surfaces accelerate early peri-implant healing. In this report we show that, in our experimental model, nano-topographically complex surfaces can mitigate the compromised bone healing seen in hyperglycemia. Importantl, we also provide a mechanistic explanation for compromised bone quality in hyperglycemia. We show that interfibrillar collagen mineralization is compromised in hyperglycemia, but that interfibrillar and cement line mineralization, remain unaffected.
survival rates of endosseous root-form dental implants range from 85% for fixed prosthodontics to 95% and higher for single implants [5], undiagnosed hyperglycemia could be a potential contributor to the remaining 5% of implant failures that occur clinically for unknown reasons.

Despite the controversies in the literature [6], it is generally agreed that diabetic bone is weaker and more fragile than healthy bone [7,8], but the reasons remain unknown. Diabetic individuals have higher rates of osteoporotic fractures [9], decreased skeletal growth during adolescence [10], and delayed or impaired fracture healing [11]. Reports of decreased mechanical retention of implants [12] and bone implant contact (BIC) in type I diabetes [13–15] lend additional support to the notion of compromised peri-implant healing in diabetic subjects. Indeed, reduced osteoid formation, delayed osteoid mineralization, and reduced overall bone volume and maturation in hyperglycemia were reported by Goodman & Hori [16]. But while the effects of hyperglycemia on bone healing have been addressed from a few weeks to several months, little has been done to elucidate its effects on the earliest stages of the bone healing cascade. Yet, it is generally believed that early healing events govern the long-term success of endosseous implants. Indeed, we have recently shown that bone remodeling is delayed in hyperglycemic rats by about 5 days, as compared to a euglycemic population, and there were striking differences in reparative bone volume between the two metabolic groups [17]. Such adverse effects at early stages of healing could negatively impact long-term implant stability, and thus contribute to implant failure.

It is also widely accepted, with the emergence over the last decades of topographically complex implant surface designs, that clinical success rates are improved compared to less topographically complex machined surfaced implants, as shown experimentally by Buser et al. [18], particularly in Class III and IV (as defined by Lekholm and Zarb [19]). We have recently described the biological significance of three different scale-ranges of implant surface topography (sub-micron, micron and coarse-micron) in terms of both the “true” and “functional” bone/implant interface [20], and that these mimic the topographic scales ranges of bone at natural remodeling sites [21]. In this context therefore, more topographically complex surfaces will exhibit features at all scale ranges compared to less complex surfaces. Thus, the possibility arises that topographically complex implant surface designs could mitigate the impaired peri-implant bone healing associated with hyperglycemia. We have recently shown that higher bone/implant contact (BIC) values are observed on nanotopographically complex surfaces in hyperglycemic subjects when compared to microtopographically complex implant surfaces in euglycemic subjects [17]. BIC is the histological outcome of the combination of osteoconduction and bone formation. We have defined osteoconduction as the recruitment and migration of osteogenic cells to the implant surface [22] — and shown that this is increased on nanotopographically complex implant surfaces in both euglycemia and hyperglycemia [17,23]. Such contact osteogenesis is an essential pre-requisite for bone anchorage to the implant surface, and while these mechanisms have been explained in more detail elsewhere in healthy animals [24,20], we have not assessed the effects of hyperglycemia on bone/implant anchorage.

Thus we sought, herein, to investigate the temporal effects of hyperglycemia on early bone anchorage to candidate implant surfaces up to 9 days post-implantation in rats. Specifically, we asked whether hyperglycemia would negatively impact bone/implant interfascial stability at such early stages of healing; and whether such impaired bone anchorage could be mitigated by the addition of nanotopographical complexity to a microtopographically complex implant surface. To address this experimentally, we inserted custom implants of two different surface topographies in the femora of either euglycemic or hyperglycemic rats and employed a mechanical disruption methodology which we have described in detail elsewhere [25] to assess bone anchorage. We hypothesized that bone/implant anchorage on the nanotopographic surface in hyperglycemia would outperform that seen on the implant surfaces absent of nano features in euglycemia.

2. Materials and methods

2.1. Candidate implant samples

Custom rectangular implants (4 mm × 2.5 mm × 1.3 mm) were kindly fabricated by BIOMET 3i (FL, USA) from commercially pure Grade 4 titanium. Each implant had a central 0.7 mm hole drilled down the long axis to enable suture fixation at surgery, and, later, to facilitate mechanical testing. Two groups of implants were prepared. All underwent a grit blast treatment to create a microtopographically complex surface (GB). The second group was further treated with calcium phosphate nanoparticles (hydroxyapatite: 10–100 nm) to create a nanotopographically complex surface (GB-DCD). These two surfaces are described as the micro- and nano-surfaces, respectively.

2.2. Surgical procedure

108 male Wistar rats were used in this experiment. All experimental protocols were approved by the Ethics Committee of Animal Research at the University of Toronto. Hyperglycemia was induced via a single intravenous injection of streptozotocin (STZ; 65 mg/kg), 1 week pre-operative. Control animals received an equivalent injection of saline. A blood glucose level of >15 mmol/L in the first 48 h (and maintained thereafter) was considered hyperglycemic for the treated group, and animals underwent surgery 1 week post-induction. To measure blood glucose, following standard tail vein puncture, a drop of blood was collected in a glucose test strip (FreeStyle Lite, Abbott Diabetes Care Inc., Alameda, CA) which was then loaded into a conventional glucometer (Free-Style Lite). In this way only the bone formed during the hyperglycemic period would have been subject to the influence of the new glycemic level – and we have previously shown that such bone can be identified [17]. Rat chow was provided ad libitum and all animals were allowed free access to water throughout the study. Implants were placed bilaterally into the distal femora of rats as described previously [26]. Briefly, the antero-lateral aspect of one hind limb was shaved. Incisions were made in the skin and underlying muscle to expose the femur. A 1.3 mm twist drill (Brasseler, GA, USA) attached to a dental handpiece (ImplantMED DU 900 and WS-75, W&H, Dentalwerk, Austria) was used to create a bicortical hole in the bone, approximately 4 mm (depth) × 1.3 mm (diameter), perpendicular to the long axis of the femur. A pair of dividers was calibrated to guide a second bicortical hole, parallel and approximately 2.5 mm distant from the first one. Following this, a 1.2 mm cylindrical side-cutting bur (Biomet 3i, FL, USA) was used to join the holes, in a proximal–distal direction. Irrigation with saline was used throughout the preparation. An implant was then press-fit into the defect and a suture (4-0 polysorb/PM, Syneture, USA) was used to keep it in position during the post-operative period by threading through the central hole and around the lateral femoral margin. Muscle tissue was sutured with the same biodegradable sutures, and the cutaneous tissues were re-apposed using staples (9 mm wound clips, Becton Dickinson, MD, USA). This process was then repeated on the contralateral femur. Each animal received both a GB and a GB-DCD implant; the side of implantation, right or left femur, was assigned by partial randomization.
2.3. Euthanasia and sample harvesting

Euthanasia was achieved by exposure to CO₂ followed by cervical dislocation. Samples were harvested at 5-, 7-, and 9-days post-operative for mechanical testing (n = 28 rats per time point). Additional samples were collected for backscattered electron imaging (BSEI) (n = 8 rats per time point). Femora were harvested, cleaned and stored in a 30% sucrose buffer for 3 h to maintain tissue hydration. All specimens were trimmed to the width of the implant using a cylindrical diamond bur (Brasseler, GA, USA) connected to a high speed system (Handpiece: KaVo Dental Corporation, IL, USA; Hand-piece control: DCI International, OR, USA) prior to mechanical testing.

2.4. Scanning electron microscopy

Field emission scanning electron microscopy (FE-SEM) was performed at the Centre for Nanostructure Imaging, University of Toronto. One additional implant in each group was carbon sputter coated and high-resolution photomicrographs were taken of different areas of each implant using an Hitachi S-5200 scanning electron microscope. For imaging the nano-scale undercuts on the GB-DCD surface, the sample stage was tilted up to 16°.

BSEI was conducted at the Advanced Bioimaging Centre at Mount Sinai Hospital (Toronto, Canada) using a Philips XL30 environmental scanning electron microscope (FEI, Hillsboro, OR, USA), without sputter coating. To prepare for BSEI, samples were dehydrated in increasing concentrations of alcohol and embedded in polymethyl methacrylate (Osteobed, Polysciences, PA, USA). Specimens were bisected along the long-axis of the implant using a laser-aligned Exakt 300 CL band saw system (Exakt Technologies, Inc., Oklahoma City, OK, USA). Both faces of the specimen were ground using an Exakt 400 CS micro-grinding system (Exakt Technologies Inc., OK, USA) with grit paper of decreasing roughness (1200, 2000, and 4000 grit; Buehler, USA).

Some implants retrieved following mechanical testing were treated with 4% sodium hypochlorite to remove cells and some organic matter to facilitate examination of the residual peri-implant bone matrix. Following hypochlorite treatment the samples were washed in running water and dehydrated in a series of alcohol concentrations prior to critical point drying. The dried specimens were examined by SEM (using both an Hitachi S-5200 and a QUANTA™ FEG 250 FEI; Centre for Nanostructure Imaging, University of Toronto), with particular attention being paid to the appearance of both the collagen compartment of the residual bone tissue attached to the implant and the cement line interfacial matrix.

2.5. Profilometry

Surface roughness analysis was performed on three samples from each group using the Contour GT-K optical interferometer (Bruker, Billerica, MA). The data was obtained at 2.5× magnification, over a 2,781,650 μm² area of the implant. Following data acquisition, the profilometry data was transformed and analyzed using the vision64 program provided by Bruker to remove the tilt term from the data. The leveled data was used to determine the surface roughness value for each surface. Surface roughness values where compared between groups using a T-Test and p-values less than 0.05 were considered significant.

2.6. Mechanical testing

168 samples (336 arches) comprising 28 arches per surface-type for each metabolic group at each of the three time points were assigned for mechanical testing. The mechanical testing model [25] was adapted from a previous iteration of a model [26] originally developed by Nakamura et al. [27]. All specimens were tested and then observed using a Leica Wild M3Z Stereozoom dissecting microscope (Heerbrugg, Switzerland) to assess residual bone on the implant surface. The height of residual bone on the implant surface was measured using a digital caliper (Fred V. Fowler Company Inc., MA, USA) to assess the effectiveness of the new mechanical testing model in accurately isolating 0.5 mm of the peri-implant region.

2.7. Statistical analysis

Two different statistical analyses of the data were performed to ensure the accuracy of significant differences observed in comparisons. In the first analysis, a one-way ANOVA was used, with post hoc analysis using Tukey’s HSD test for multiple comparisons, to check for differences between the medial and lateral test data at p < 0.05. To assess differences between the testing groups a non-parametric Kruskal-Wallis test, followed by post hoc Wilcoxon rank sums test for multiple comparisons, was conducted to analyze mechanical testing groups. Statistical significance was set at p < 0.01, with p < 0.0001 considered extremely significant.

For the second analysis, weighted linear modelling was used to analyze the effect that each of the variables (metabolic group, implant type, time point) had on the disruption force data. The model used includes interactions between the variables. Data was weighted by the inverse of the time point to deal with the changes in force variance over time. Individual models were fit for each implant type, due to heteroscedasticity, and a third model was used to interpret the paired implant data. The data were analyzed using the statistical software ‘R’ [28]. A p < 0.05 was considered significant for the Wald test. This analysis returned more conservative values than the Anova, and are those reported in our results herein.

To find the range of differences that could be detected with a power of 90%, we also analyzed a simulated data set, using the same standard deviation and analysis method detailed above.

3. Results

The post-operative period was uneventful for all rats. All survived the operative procedure, recovered uneventfully from the anesthesia, regained normal ambulation and displayed no clinical signs of postoperative complications. Hyperglycemia was successfully induced and maintained in the treated rats, while euglycemia was maintained in the control group (Fig. 1).

3.1. Surface characterization

FE-SEM photomicrographs of the implant surfaces are shown in Fig. 2A and B. Coarse micron scale-range topographical complexity was observed on the GB surface. The surface within the valleys comprised flat areas with few submicron scale-range features (Fig. 2C). The GB-DCD surface exhibited sub-micron scale-range features superimposed on the underlying GB surface, which could clearly be visualized at higher magnifications (Fig. 2D). The microtopography of the underlying substrate was not altered by DCD treatment and could be observed independent of the superimposed crystal topography. The DCD crystals provided the surface with undercuts, which are observed as recessed volumes in Fig. 2E.

Profilometry maps of the GB and GB-DCD surfaces are shown in Figs 3A and B and their X (above) and Y (below) profiles in Figs 3C and D respectively. The GB implants had a mean surface roughness of 3.11 ± 0.03 (standard deviation), with z-plane craters of 20 μm peak to valley size. The GB-DCD implants had a mean...
A surface roughness value of 3.14 ± 0.01. No significant difference was found between implant groups.

3.2. Mechanical testing and statistical analysis

Each bone-apposing surface of each implant (total 336) was photographed using a dissecting microscope following either harvesting (see below) or mechanical testing. This revealed that more bone remained on the nano-surface at all time points compared to the micro-surface (Fig. 4). When the height of the residual bone was measured, as described above, 309 specimens, representing 92% of all test specimens, had residual bone within the 0.5 mm peri-implant region. However, 71 bony arches, with 50 at the 5 day time point, fell off the implant surface during processing and these were given a zero disruption force value in the statistical analysis. The 50 samples at day 5, when subdivided by implant surface, could be grouped as follows: GB, subtotal 43 and GB-DCD, subtotal 7 while the subtotals for each surface for all time points were: GB, 61 and GB-DCD, 10.

The results of the disruption tests are shown in Fig. 5, and clearly demonstrate the differences between the forces registered by nano-surfaced GB-DCD and micro-surfaced GB implants at 9 days (>15 N and <5 N respectively). Even at 5 days the differences between the two surface types was evident (approx. 3.5 N and <1 N respectively). The differences with time and glycemic state were evident with the GB-DCD implants since it was only by 9 days that the HG GB-DCD group had attained values seen in the EG GB-DCD group at 7 days (approx. 12 N).

These differences were not seen with the micro-surfaced, GB, implants as all disruption values, at all time points, were very low (<5 N). Of the total number of GB implants, 36% of bone arches fell off over the three time points. These were recorded as zero disruption force values. Indeed, at 5 days, 86% of zero values were attributed to GB surfaces, and even by 9 days, the measured disruption forces for GB implants were not different from those of the GB-DCD implants tested at 5 days (approx. 3.5 N). Nevertheless, the disruption forces for GB implants did increase for both EG and HG groups with time and the differences between 5 and 9 days were significant.

Linear modelling of the data showed that the disruption forces at 5 days were not significantly different from zero for the GB implants (p = 0.24) in either metabolic group; but were significant for GB+DCD implants (p < 0.001) in both metabolic groups. Regardless of the implant type or metabolic group, all disruption forces were found to increase over time (p < 0.001). However, the rate at which the disruption force increased over time was dependent on both implant type and metabolic group. The GB+DCD implants were found to have a significantly higher rate of increase in disruption force compared to GB implants (p < 0.001), though the difference was attenuated in hyperglycemic conditions compared to euglycemia (p < 0.01). Similarly, GB+DCD implants had a significantly greater rate of increase in disruption force in euglycemia than hyperglycemia (p < 0.001). In contrast, GB implants showed no significant differences in disruption force between metabolic groups over time (p = 0.77).

Using our simulation power analysis (see above) we determined that, for the GB implants, the difference in the rates of increase between euglycemic and hyperglycemic conditions would have to be at least 0.88 N/day to detect a difference at 90% power. Such a difference in the rate of increase over the experimental period of 9 days would result in an absolute difference of disruption force between the two metabolic groups of 7.9 N – this was not possible since the maximum disruption force measured from any of the GB implants was only 6.8 N. Thus, the detachment force values for the GB surface were too small to detect differences between the two metabolic groups.

3.3. Scanning electron microscopy

Visual assessment of BSEI micrographs clearly demonstrated that peri-implant bone formation had occurred in both the euglycemic and hyperglycemic rats for both implant surfaces (Figs. 6 and 7). In every case, as would be expected, the majority of the cortical bone was of similar appearance as this had been formed, in the HG animals, before STZ injection. However, the reparative
bone, both in contact with the implant and in the medullary cavity in general, exhibited a distinctly different appearance in the two groups. As can be seen when visualizing the whole bony arches, less reparative bone was evident in HG case as evidenced by the larger volume of reparative bone in the EG case where new endosteal bone formation was pronounced and resulted in a change in the endosteal contour of the cortex. At higher magnifications (C, D in Figs. 6 and 7), comparing the EG and HG appearance, the bone was denser, as marked by the lighter grey-scale range in the trabecular bone, and less porous in the EG when compared with the HG samples. Indeed, the porosity of the HG bone was very marked resulting in very thin individual trabeculae and, in many areas, randomly distributed discontinuous linear arrangements of low volumes of mineralized tissue without apparent connections to the trabecular structure within the sampling depth of the probe (Fig. 7D). Differential densities were evident within the HG bone, but were not as obvious as seen in the EG samples.

In both EG and HG, bone could be found in contact with the underlying implant surface, but the BIC was less in the HG group, although we did not quantify this difference from the BSEI data. It was also obvious that the interfacial bone matrix in EG was denser and the trabeculae were thicker than that of the HG samples (see Fig. 7C, D).

The FE-SEM micrographs of the hypochlorite-treated residual bone on the implants following mechanical testing revealed stark differences in the micro-architecture of bone between metabolic groups (Figs. 8 and 9). Euglycemic bone, in the bulk tissue remaining on the implant surface, showed the typical appearance of the woven bone found in the rat with bundles of collagen fibres, containing osteocyte lacunae, weaving around large channels that, prior to the bleaching procedure, would have contained marrow and vascular canals. At higher magnification (Fig. 8B), the collagen fibre bundles were largely intact, although some areas had obviously been fractured as a result of the disruption test. However, in the HG samples, while the vascular channels were still evident, the appearance of the surrounding collagen was quite different, with less linearity to the fibres themselves as a result of less general order in the collagen structure and greater numbers of osteocyte lacunae (Fig. 8C, D). This resulted in a more porous structure as compared to euglycemic bone, and correlates with our BSEI
observations. At higher magnification, this disorder was more easily visualized and there seemed to be more evidence of collagen fibre fracture when compared to the EG group. In addition, while the EG group exhibited a mineralized appearance that was closely aligned to the collagen fibres, in the HG group mineralization was more evident between the collagen fibres, which themselves, especially in the fracture regions, showed little evidence of mineralization (Fig. 8D).

In contact osteogenesis the true bone/implant interface is occupied by the implant material surface and the cement line matrix of bone [20]. Thus, we specifically interrogated our samples, using FE-SEM, for evidence of cement line matrix formation. Distinct differences were observed between micro- and nano-surfaces in terms of cement line layer coverage (Fig. 9). In EG, cement line matrix was evident on both GB and GB-DCD implant surfaces, although surface coverage was less on the GB surface such that it was easy
to identify individual cement line globular accretions on the easily identifiable underlying implant surface. In contrast, on the GB-DCD surface in EG, the cement line matrix had been elaborated to a far greater extent resulting in complete implant surface coverage such that the latter was no longer visible. In hyperglycemic conditions, there were no discernable differences in the appearance of the cement line globules on either the GB or GB-DCD surfaces. Even in hyperglycemia the GB-DCD surface was, again, completely covered obliterating the underlying implant surface.

4. Discussion

Clearly, the results obtained with the micro (GB) surfaced implants indicate that the measurement method employed is insufficiently sensitive to detect significant differences between groups when the strength of bone anchorage is minimal. The tissue preparation method, which entails cutting off the femoral bone distal and proximal to the implant can cause disruption to a fragile bone/implant interface and, as we have reported, resulted in many

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**Fig. 5.** The mean disruption force was plotted against time point for each treatment group. The line plotted shows the best fit for the data, with the shaded regions representing the 95% confidence intervals. The error bars shown indicate the standard deviation of each time point. The lines of best fit for the two GB+DCD show the significantly lower rate of increase in disruption force over time in hyperglycemic animals when compared to euglycemic animals. The same trend could not be observed within the GB group due to the significantly lower disruption force values recorded, which makes distinguishing the two lines used to fit the data difficult. A similar comparison between GB and GB+DCD implants within each metabolic group shows that the rate of increase in disruption force over time for GB+DCD implants is significantly greater than GB implants and requires significantly greater forces to disrupt the peri-implant interface at all time points. Lines were fit using the method of least squares and confidence intervals were calculated based on the standard error of the data and sum of squares of the deviations in time.

**Fig. 6.** BS-SEM micrographs of GB specimens from euglycemic (A, C) and hyperglycemic (B, D) animals at 9 days post-operative. There is a considerable decrease in the quality and maturity of peri-implant bone in the hyperglycemic animals. The peri-implant bone in euglycemia is brighter in contrast as compared to that in hyperglycemia, indicating the differences in the maturity of bone between the two metabolic groups. In addition, the peri-implant bone in hyperglycemia appears more porous than euglycemic bone, confirming its compromised quality. Furthermore, there is less peri-implant bone seen on the GB implant surface, indicating poor osteoconduction, regardless of glycemic levels, especially in comparison to the GB-DCD surface (see Figs. 4 and 7). (A, B) scale bar: 1 mm; (C, D) scale bar: 50 μm.
specimens returning a zero detachment value, especially at the earliest time point.

Nevertheless, our results show that hyperglycemia does have a negative impact on both the quality of reparative peri-implant bone and bone anchorage to the underlying implant, in this rat model. This corroborates other work we have reported recently that both reparative bone formation and bone remodeling are delayed in hyperglycemia [17]. In the present work, the decreased peri-implant bone anchorage in hyperglycemia seen with the GB-DCD group was evident by the slower increase of disruption force values with time when compared to euglycemic controls and the significantly higher disruption values in euglycemic subjects compared to those in hyperglycemic subjects. These differences could be attributed to either compromised peri-implant bone quality, which was clearly observed in the BSEI micrographs (Figs. 6 and 7), or decreased bone-implant contact (BIC), which we did not measure. In this context we should report that Abrahamsson et al. [29] compared the BIC on two titanium surfaces with, or without, nanometer scaled calcium phosphate crystals similar to those employed herein with the DCD treatment. Measuring BIC they concluded that such a nano-structured surface modification provided no improvement in early tissue integration. However, Wong et al. [30] had previously shown that “percentile surface coverage measurements [BIC] are a less sensitive indicator of
osseointegration than mechanical tests” and thus we chose to measure disruption force, rather than BIC, in the present study. Indeed, following mechanical testing of both euglycemic and hyperglycemic samples, there was a greater amount of residual bone on the GB-DCD, compared to GB, surfaces, which was indicative of greater functional tissue integration.

We have previously reported an increased probability of osteoconduction on nano-topographically complex surfaces compared to those devoid of such features [17]. Therefore, it is reasonable to assume from the present results that it is the poor quality of peri-implant bone that plays the predominant role in the decreased peri-implant bone anchorage in hyperglycemia. Indeed, when we examined the quality of residual peri-implant bone following mechanical testing, striking differences were revealed in the mineral content of the collagen compartment between the metabolic groups.

It is known that the mineralization of the collagen compartment of bone is both inter-fibrillar and intra-fibrillar (also referred to as extra- and intra-collagenous mineralization) [31,32]. Extra-fibrillar mineral is deposited first and, although there are some species differences [33], the structure of mineralized collagen fibrils is highly conserved across species and types of bone [34]. This is an important consideration since our work was with rat bone and we saw evidence of differences in inter- and intra-collagenous mineralization in our SEM photomicrographs. Thus, while collagen fibre mineralization was obvious in the EG group, inter-fibrillar mineralization was predominant in the HG group. Furthermore, cement line mineralization was seemingly not influenced by hyperglycemia, and easily visualized in both HG and EG groups on both implant surfaces, although in each case the amount was considerably greater on the nano-surface. These observations suggest that intra-fibrillar mineralization is compromised in hyperglycemia while inter-fibrillar and cement line mineralization is unaffected.

Conventional wisdom dictates that collagen itself does not nucleate calcium phosphate mineralization; but requires acidic phosphoproteins, of which there are several candidates including non-collagenous proteins and proteoglycans. These are implicated in both inter- and intra-fibrillar mineralization. Such phosphoproteins enable hydroxyapatite crystals to grow from an amorphous phase, in a site-specific manner governed by the collagen structure itself [35]. Interestingly, such intra-fibrillar mineralization has been found to occur only when the collagen fibrils were well organized [36] implying that changes in collagen fibril structure may have a negative impact on the intra-fibrillar mineralization process. It is well known that modulation of both collagen structure and the proteins which interact with type 1 collagen have a profound effect in other bony pathology [37]. Indeed, the lack of intra-fibrillar mineralization has been shown to result in reduced mechanical properties of dentine [38]. Furthermore, it is known that collagen cross-linking, and thus collagen structure, are altered in hyperglycemia [39]. This may in turn compromise intra-fibrillar collagen mineralization and decrease the availability of calcium binding domains that nucleate the mineralization cascade [40]. This diminished intra-fibrillar mineralization may be sufficient to cause the decreased uptake of fluorophores that we have previously observed in this rat model [17], although transmission electron microscopy studies would be required to confirm this assumption. Nevertheless, a decrease in intra-fibrillar mineralization would also explain the reduced mechanical properties of the HG bone as witnessed by our disruption test results herein.

Despite such compromised bone quality and poor anchorage of bone to the implant surface in hyperglycemia, the nano-surface outperformed the micro-surface regardless of healing time or metabolic group, which confirmed that the addition of nano features, with undercuts, improves endosseous integration. We have previously only shown this increase in interfacial stability in euglycemic subjects [26]. The anchorage of bone matrix to the implant surface was corroborated in the present results by the cement line globular accretions that fully covered the GB-DCD surface in both EG and HG, while the GB surface only showed scattered evidence of this interfacial matrix.

Finally, the differences in the disruption force values between the GB surface, which comprises only titanium oxide, and the non-collagenous proteins and proteoglycans. These are implicated in both inter- and intra-fibrillar mineralization. Such phosphoproteins enable hydroxyapatite crystals to grow from an amorphous phase, in a site-specific manner governed by the collagen structure itself [35]. Interestingly, such intra-fibrillar mineralization has been found to occur only when the collagen fibrils were well organized [36] implying that changes in collagen fibril structure may have a negative impact on the intra-fibrillar mineralization process. It is well known that modulation of both collagen structure and the proteins which interact with type 1 collagen have a profound effect in other bony pathology [37]. Indeed, the lack of intra-fibrillar mineralization has been shown to result in reduced mechanical properties of dentine [38]. Furthermore, it is known that collagen cross-linking, and thus collagen structure, are altered in hyperglycemia [39]. This may in turn compromise intra-fibrillar collagen mineralization and decrease the availability of calcium binding domains that nucleate the mineralization cascade [40]. This diminished intra-fibrillar mineralization may be sufficient to cause the decreased uptake of fluorophores that we have previously observed in this rat model [17], although transmission electron microscopy studies would be required to confirm this assumption. Nevertheless, a decrease in intra-fibrillar mineralization would also explain the reduced mechanical properties of the HG bone as witnessed by our disruption test results herein.

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**Fig. 9.** FE-SEM images of the (A, C) GB and (B, D) GB-DCD surfaces in (A, B) euglycemia and (C, D) hyperglycemia following the disruption test. Scattered cement line globules are observed on the GB surface in both euglycemia (A) and hyperglycemia (C). However, on the GB-DCD surface, a complete coverage of the surface by the cement line globules is observed following the test regardless of the glycemic state (B, D). Scale bars: 1 µm.
GB-DCD surface, which contains additional calcium phosphate nanocrystals, may be debated to be related to differences in the surface chemistries, and raises the question of whether implant surface topography or surface chemical composition are the driving force for the differences in bone anchorage reported. Indeed, increases in both BIC of approximately 20% [41] and removal torque values 8–20% [42] have been attributed to changes in implant surface chemical composition and/or implant surface hydrophilicity, but such analyses overlooked the changes to the nanotopography of the implant surfaces employed [43]; and thus the role of implant surface chemistry remains to be proven. Indeed, our present results reflect our previously published data, which demonstrated that introducing a nanotopography on an already microtopographically complex surface can increase osteoconduction by 42–55%—for commercially pure titanium (cpTi) and titanium alloy (Ti6Al4V) implants respectively—and disruption force by approximately 7-fold for both Ti6Al4V and cpTi surfaces [26]; and this data mimicked that seen in human clinical data [44,45]. We would add that we included profilometry data in response to reviewer request although, as could be reasonably expected, the two surfaces employed herein showed no statistically different roughness values despite the obviously different nano-structure as witnessed by high magnification SEM (Fig. 2) and the radically different biological responses engendered (Fig. 5).

Thus, there has been much debate on the relative importance of implant surface topography and chemistry in the phenomenon of bone bonding [24], but we have shown that bone bonding can occur on metal oxide surfaces that present a reticulate nanostructured surface with undercuts, devoid of calcium phosphate [46]. Some have also suggested that calcium phosphate released from implant surfaces may contribute to the mineralization of peri-implant bone [47], but we have previously reported that the nanocrystals employed in the DCD process described herein are stable for up to one month in vivo at normal pH and could only be experimentally dissolved at acid pHs [48]. Thus, we consider it unlikely that calcium phosphate was released from the nano-surface, to contribute to the increased maturity of the peri-implant bone tissue observed around the nano-surface.

5. Conclusions

Our study addresses our hypothesis since nanotopographically complex surfaces led to an enhanced anchorage of bone to the implant surfaces independent of metabolic group compared to microtopographically complex surfaces. Specifically, our results indicate that, in hyperglycemia, intra-fibrillar collagen mineralization is compromised—and this provides a mechanism by which hyperglycemia affects early bone mineralization—while inter-fibrillar, and cement line, mineralization remains unaffected.

6. Disclosures

The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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