Surgical Preparation of Bone–Scaffold Interface Is Critical for Bone Regeneration Inside Tissue Engineering Scaffold

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ABSTRACT: The goal of this study was to investigate if the preparation of implantation site affects bone formation inside tissue engineering scaffolds. For this purpose, two different drilling techniques were used to create a hole in distal femurs of rats before the insertion of a bone scaffold: a manually driven wood drill bit and an electrically driven metal drill bit. The size and the position of the hole were identical for the two cases. The bone volume, bone mineral density, and callus formation were assessed noninvasively using micro-CT tomography at several time points after implantation. The formation of bone and soft tissue inside scaffold were evaluated by histology. The bone structure around the holes made by the two techniques was compared ex vivo. The long-term study of bone formation showed that when a wood drill bit was used, the bone formation is accelerated by 3 weeks compared to when a metal drill bit was used. The ex vivo studies suggest that this result is due to the drilling methods differentially affecting the structure of the bone surrounding the generated defects.
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Keywords: bone formation; drilling technique; tissue engineering; micro CT

One of the most important quests in bone tissue engineering is to enhance osteogenesis in scaffolds. Researchers have tried different techniques, like preseeding scaffolds with osteogenic cells,[1–3] utilizing growth factors,[4,5] or applying mechanical stimulation[6,7] to accelerate bone regeneration in scaffolds. While adjacent bone plays a major role in supplying cells, nutrients, and biochemical signals, the importance of the interaction between bone and scaffold has thus far been largely neglected. In a recent study, Chen et al.[8] demonstrated that in the bone marrow stimulation technique for cartilage repair, the preparation technique significantly affected the long-term outcome. They observed that the microfracture technique produced fractured and compacted bone around holes, essentially sealing them off from viable bone marrow. The same issue might be relevant for the bone–scaffold interface.

In order to implant scaffolds or any other kind of bone substitutes, the bone should be cut and shaped accordingly. The common form of cutting is drilling which is indeed the single most frequent procedure performed in orthopedic surgery.[9] There are numerous studies on the various aspects of drilling, like the design of cutting edges,[10–12] drilling speed,[13,14] load,[13] and irrigation.[15] However, the focus of all these studies has been on the corresponding increase in temperature and thermal damage due to drilling the cortical bone.[16–19] The effect of drilling on trabecular bone was not studied, perhaps due to a priori assumption that temperature increase is less significant and hence less crucial in this less dense part of the bone. Indeed, an important effect of drilling trabecular bone might not be the increase in temperature, but the damage to the structure of adjacent trabeculae, which may have serious biological consequences.[8]

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In the present study, two drilling techniques were used to create a hole in rat distal femurs before the insertion of a bone scaffold. The first drilling technique used a manually driven wood drill bit and the second technique used an electrically driven metal drill bit. The goal of this study was to evaluate the effect of drilling and preparation of the implantation site on bone formation inside a scaffold.

METHODS

An in vivo study was designed to evaluate the impact of the drilling technique on bone healing. Holes were made in distal femurs of rats and bone scaffolds were implanted. Bone formation inside the scaffold was quantified using micro-CT imaging and was correlated to the drilling technique. Histological and postmortem studies were done to study the differences in patterns of bone formation and adjacent trabecular structures, respectively.

Drilling Techniques

Two different drilling techniques were used. In group A, a manual wood drill with centering tip and sharp cutting edges was used. In group B, a metal drill was used along with an electric drill (Dremel Stylus, Dremel; Fig. 1). The nominal rotation speed for the electric drill was 9000 RPM. The drilling time was <1 min for group A and less than 5 s for group B. No irrigation was used during the drilling due to the small size of the drill and the hole, as well as the very short duration of drilling. The drill tips were autoclaved before being used in vivo.

Surgery

Sixteen female Wistar rats (4-month old, weight 245–250 g) were randomly separated in two groups of eight based on the drilling method. Left distal femurs were operated (Veterinary Authority from the Canton of Vaud, authorization No. 2140) following a protocol previously used in our laboratory.[3,20] The animals were anesthetized using Isoflorane gas and the left leg was shaved and sterilized. The lateral side of the knee joint was opened and, after exposing the distal femur, the location of the hole was marked on the bone based on the distance from the joint and the insertion point of ligaments. The drill hole, 3 mm in diameter and depth, was made and the scaffold implanted as

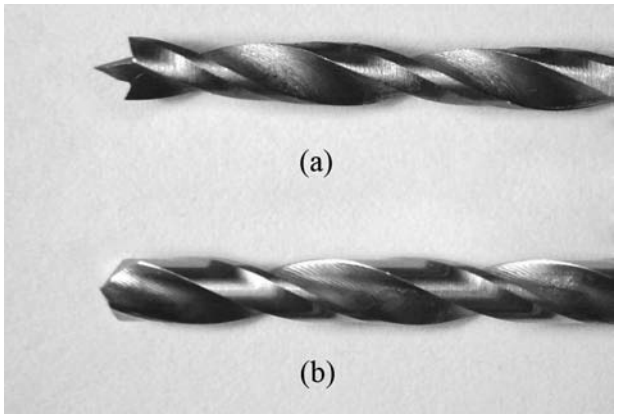

Figure 1. (a) Wood drill bit, (b) metal drill bit. Note the fundamental difference in cutting geometry. The wood drill bit has a pair of sharp slitting edges, which engage in cutting trabeculae immediately, whereas the conical tip of the metal drill bit first crushes trabecular structure and then remove bone chips with cutting edges.

previously described.[7] The scaffold used was a biocomposite made of PLA/β-TCP.[21] Finally, the surrounding tissues were washed with saline, the muscles sutured, and the skin closed using surgical clips (Stoelting, Wood Dale, IL). No antibiotics were given after the surgery. Dafalgan (Bristol-Myers Squibb, New York, NY) was however administered in water for 3 days postsurgery to reduce pain.

Micro-CT Scanning

Prospective micro-CT scanning was done in order to investigate in vivo bone regeneration inside scaffolds (Skyscan 1076, Skyscan, Kontich, Belgium). Group A was scanned at six time points: 2, 4, 6, 9, 13, 18 weeks after the surgery. Group B was scanned at six time points: 2, 4, 7, 10, 14, and 21 weeks after the surgery. Animals were kept under anesthesia by Isoflorane during the scanning. The left leg was stretched, fixed with tape and scanned along with two phantoms (Gloor Instruments, Uster, Switzerland) and a tube of water for later calibration of bone mineral density (BMD) measurements. The scanning parameters were the same for all animals at all time points (18 μm, 80 kV, 124 μA, 1 mm Al filter, 600 ms exposure time, 14 min of scanning duration). The reconstruction and analysis was done using NRecon and CTan software (Skyscan). The volume of the hole was selected as region of interest (ROI). The BMD of each sample was quantified based on the calibrated values of the phantoms. A threshold value of 0.5 g/cm^3 was chosen to segment bone in the scaffold. Bone volume (BV) inside scaffold and BMD of bone tissue were measured accordingly using CTan software (SkyScan). To evaluate the callus at 2 weeks, frontal sections passing through the center of scaffold were reconstructed and the thickness of the callus was measured accordingly.

Ex vivo Investigation of the Cut Trabecular Surface

Three additional female rat cadavers of the same weight range were dissected and the two femoral distal epiphyses were drilled using the two techniques. Afterwards, the bone was cut along its cross-section in plane with the axis of the hole and the distal part was collected. Soft tissue and bone marrow were removed by immersing the samples in hydrogen peroxide overnight. The drilled defect walls were then photographed using a stereo microscope (Leica, Wetzlar, Germany).

Histology

Histological analysis was performed on two animals at 10 and 13 weeks to qualitatively evaluate tissue formation inside the scaffold. Distal femurs were harvested from both groups and fixed with 4% paraformaldehyde, dehydrated in gradually concentrated ethanol baths, cleared in toluene, and embedded in resin. Safranin O staining was used to differentiate bone, cartilage, soft tissue, and scaffold.

Statistics

Linear mixed-effect modeling was used to model the evolution of BV as a function of time according to our previous study.[7] Repeated measures analysis of covariances (ANCOVA) was used to evaluate the differences between the two groups. Differences between means such that $p < 0.05$ were considered statistically significant. All statistical analyses were done in S-PLUS (Tibco, Palo Alto, CA).

RESULTS

All animals could use their legs immediately after the surgery and all survived the procedure. However, scaffold implantation was not successful in four animals. In group A, one of the scaffolds was loose inside the hole, resulting in almost no bone formation. In the same group, a scaffold did not completely fit inside a hole which resulted in significantly lower bone formation. In group B, the amount of bleeding in holes was remarkably lower than group A (based on visual observations). In two cases, almost no bleeding occurred after drilling which resulted in lower bone formation compared to others. Thus, these four animals were excluded.

Figure 2a,b show the BV and BMD for both groups over time, respectively. The ANCOVA test shows that

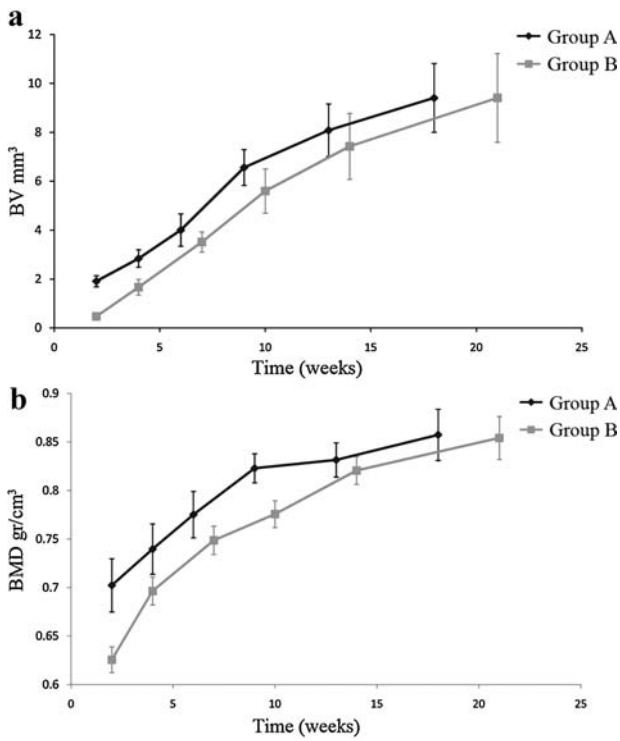

Figure 2. (a) Bone volume (mm^3), (b) BMD (g/cm^3), changes over time for groups A ($n = 6$) and B ($n = 6$).

the group A has significantly higher BV (p-value = 0.0005) and BMD (p-value = 0.0004) compared to group B. However, the rate of change of BV and BMD over time was not significantly different for both groups (p-value = 0.76 and 0.1, respectively). Comparing the two groups, we observed that group A is almost 3 weeks ahead of group B in terms of bone regeneration. The pattern of bone formation was also different between the two groups. At 2 weeks, most of the new bone was formed inside the scaffold for both groups. However, at this early time point, some bone formation was found in the exterior part of scaffold in group A, but not in group B (Fig. 3a,b).

At 2 weeks, we observed the callus formation around the hole (Fig. 3c,d) which was mineralized at 4 weeks and shrunk at later time points. The maximum callus thickness was measured at the coronal plane crossing the middle of the scaffold. The average callus thicknesses for groups A and B were 0.64 ± 0.24 and 0.33 ± 0.11 mm, respectively, and were significantly different (p-value = 0.0002).

Figure 4 shows the magnified view of distal hole walls after drilling using both techniques. The structure of the bone at the two surfaces is clearly different. The metal drill (group B) crushed and sheared the bone, packed the interface, and partly clogged the trabeculae. On the other hand, the wood drill (group A) resulted in a clear cut interface and preserved the open pore structure at the surface.

Comparing Figure 5a with Figure 5b, we did not observe bone formation in pores devoid of soft tissue (blood clot, granulation tissue, or bone marrow). This demonstrates the importance of permeation of pores with blood during implantation.

DISCUSSION

A successful tissue engineering strategy requires early and rapid bone formation inside scaffold. To achieve this, scaffold osteoconductivity is certainly essential, but so is the quality of interface between bone and scaffold. For instance, the preparation of the interface (i.e., the tissue excision) should allow the scaffold access to bone marrow.[8] In this work, we studied the effect of two drilling techniques for preparation of the scaffold implantation site on the long-term bone regeneration.

The major finding of this study demonstrated that drilling techniques strongly affect bone formation in scaffolds. Indeed, we found that depending on the technique used, the bone healing process can be accelerated by almost 3 weeks in this in vivo rat study.

The difference between groups A and B was twofold: the type of drill (wood vs. metal drill bit), and the speed of drilling (manual vs. electric). Therefore, the observed effect can be due to either or a combination of both. The speed of drilling can be associated with heat generation and hence, necrosis of surrounding tissue. The effect of heat on bone tissue depends on the temperature and the duration of exposure.[16,17] Lundskog[22] states that if bone is exposed for longer than 30 s at 50°C, cellular necrosis will be induced. Hillery and Shuaib[18] measured the induced temperature in relation with drilling speed and depth for human and bovine bone. They found a significant increase in temperature with increasing drilling depth. They also found that the temperature

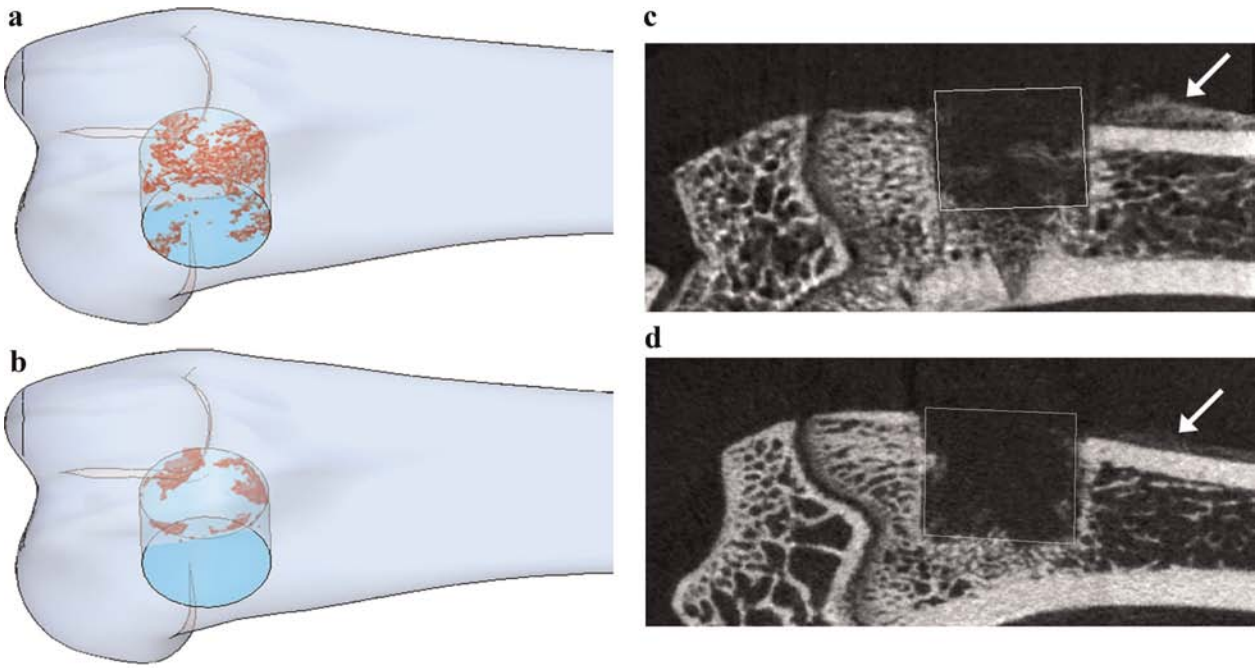

Figure 3. Mineralized bone inside scaffold at 2 weeks in a sample from (a) group A, (b) group B. Cross-sectional view of distal femur at 2 weeks in a sample from (c) group A and (d) group B. The external calluses are shown with white arrows. The ROI shown with white lines, was selected as a cylinder inscribed in the hole. The bottom was always started at 2 and 1 mm from the marking of the tip of drill bit for groups A and B, respectively (the tip of the wood drill bit is longer). With this approach, the same region of interest was always studied.

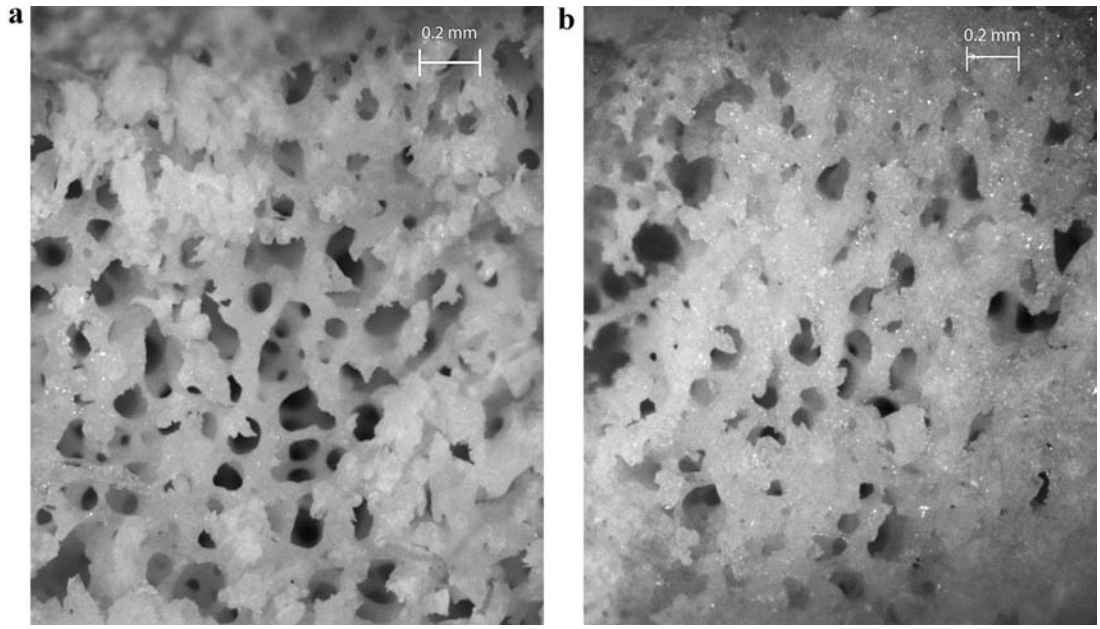

Figure 4. Magnified structures of the bone lining the holes made using (a) a wood drill bit and (b) a metal drill bit.

generated when drilling into bovine bone was higher than that produced when drilling into human bone.[18] According to their findings, the resulting temperature when drilling into cortical human bone down to 3 mm in depth, with a drilling speed of 2000 RPM, is lower than 40°C. Although we have used a higher drilling speed, this difference would not result in significant temperature elevation, as was previously demonstrated.[17–23] The duration of drilling in our experiment was <5 s which is shorter than the 30 s critical exposure reported by Lundskog.[22] Moreover, the thickness of rat cortical bone at distal femoral sites (1 mm) is much thinner than the thickness of human cortical bone. We can then conclude that in our case, the temperature increase is unlikely to cause necrosis of the surrounding tissue. Therefore, the thermal effect of drilling speed can be considered negligible.

The cutting geometry on the wood drill and the metal drill were substantially different. The leading cutting edge of the wood drill was at the periphery of the tip, while the metal drill had a conical protruding point, resulting in a different cutting process. The difference can be seen in Figure 4. The surface lining the holes made by the metal drill was less open and permeable compared

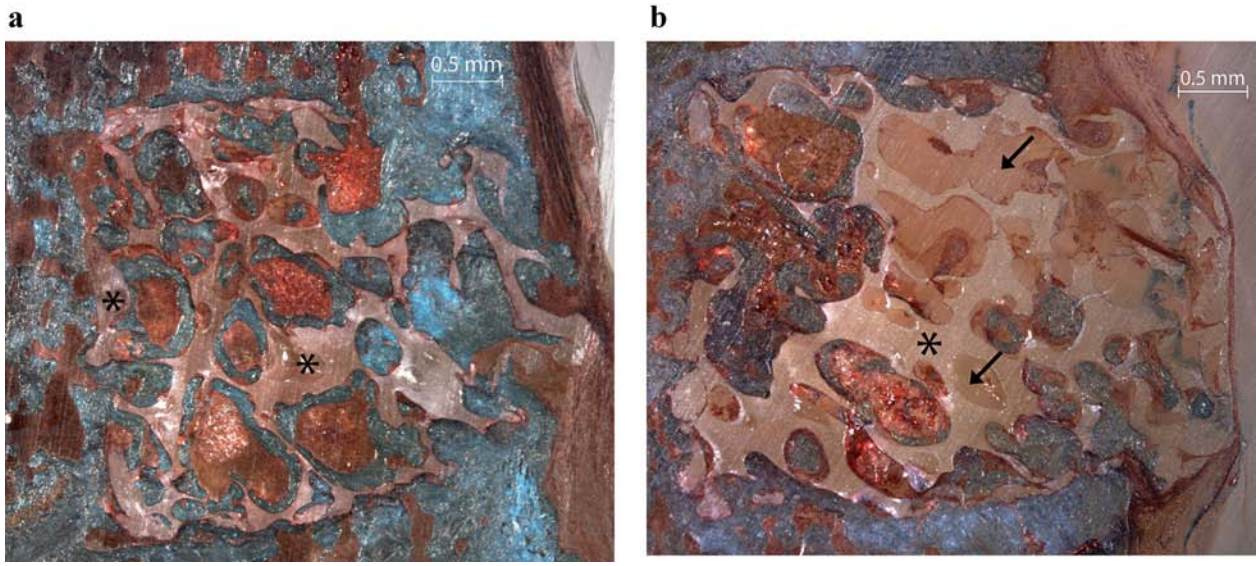

Figure 5. Histological photomicrographs of scaffold cross-sections showing (a) bone and soft tissue present in all pores (at 13 weeks from group A) and (b) some pore devoid of any tissue (at 10 weeks from group B). Bone is in green/blue, soft tissue is in red, scaffold and voids are in different shades of pink. Scaffold and voids are shown with asterisks and arrows, respectively. The empty pores were not flooded with blood during implantation. Cells were therefore unable to later penetrate and produce bone.

to those made by the wood drill. This might be the reason why, during surgery, less blood was extravasated while making the holes using the metal drill.

As the scaffold was not seeded with cells nor contained growth factors prior to implantation, the only source of stimulating cellular activity was the hematoma formed in the scaffold. A hematoma releases a large number of signaling molecules and growth factors (PDGF, TGF-β, and FGF, to name a few)[24] that attracts fibroblastic and osteogenic cells and therefore plays an important role in the bone formation process. The blood clot is then transformed into granulation tissue, which serves as the matrix on which bone cells reside and produce bone. As the drilling in group B resulted in less blood extravasation, a smaller amount of blood would then enter into the scaffolds. Some pores were indeed not permeated and left empty (Fig. 5b).

Another important difference between the wood and metal drill groups was the size of the callus formed around the holes. The external callus is formed following hematoma formation under the periosteum. This external hematoma also releases numerous signaling molecules and growth factors into the scaffold. Moreover, the periosteum is an important source of osteogenic cells which can migrate into the scaffold, proliferate, and differentiate into osteoblasts.[24] As the callus is adjacent to the scaffold, osteogenic cells from the former can migrate into the scaffold along with diffusing growth factors and other biochemical signals. If the differences in BV were mainly due to the difference in callus size, bone formation would have predominantly occurred at the exterior side of the scaffold. This however did not occur, thereby discounting a direct correlation between callus size and BV. This was further demonstrated when no relevant changes were observed at 4 weeks following callus shrinkage. We therefore believe that the resulting difference in size of external calluses does not play a key role in bone formation and is simply a side-effect of drilling.

The metal drill used is similar to commonly used orthopedic drill bits in terms of cutting edge geometry and design. Previous studies on the effect of drilling on bone mostly focused on the effect of heat generation and its biological implications in the surrounding bone. This indeed is a very relevant concern for cortical bone. However, for trabecular bone, the quality of the cut and the damage induced to the interface are arguably of greater importance. To the best of our knowledge, this aspect has been largely neglected so far. This study suggests that the resulting structure of trabecular bone induced by the drilling process plays a central role in bone regeneration. In particular, drill bits which induce a sharp and clear cut of the trabeculae should be favored as this will maintain open pores in trabecular bone, thereby facilitating the influx of bioactive agents. A translation of these results could result in clinical application by designing a bone drill presenting characteristics similar to the ones of wood drill.

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