The effect of collagenated space filling materials in sinus bone augmentation: a study in rabbits

France Lambert
Angelique Léonard
Pierre Drion
Sophie Sourke
Paul Pilet
Eric Rompen

Abstract

Aim: The inclusion of biomaterial particles used for alveolar bone regeneration in a carrier or in binding agents such as collagen gel or fibers is of interest as a means to help with surgical handling. However, the possible influence of collagen on bone tissue response to biomaterials is poorly studied. The objective of the present study was to investigate, in a sub-sinus bone augmentation model in rabbits, the effect of collagen at different stages of the osteogenesis process. Histologic, histomorphometric and volumetric analyses were performed.

Materials and methods: Rabbits underwent a double sinus lift procedure using bovine hydroxyapatite (BHA), collagenated bovine hydroxyapatite (BHAColl), and prehydrated and collagenated porcine hydroxyapatite (PHAColl). Animals were sacrificed at 1 week, 5 weeks or 6 months. Samples were subjected to X-ray micro-tomography and histology. Qualitative analysis was performed on the non-decalcified sections and quantitative histomorphometric analyses were conducted using scanning electron microscopy (SEM). Volume variations of bone augmentations were calculated at different time points.

Results: The three biomaterials allowed an optimal bone formation and were able to equally withstand sinus reexpansion. A comparable percentage of new bone, as well as 3D volume stability, was found between the groups at each time point. However, the PHAColl resorption rate was significantly higher than the rates in other groups (P = 0.0003), with only 3.6% of the particles remaining at 6 months. At 1 week, both collagenated groups displayed the presence of inflammatory cells although BHA did not show any sign of inflammation. At 5 weeks and 6 months, the inflammatory process had disappeared completely in the BHAColl groups, whereas some inflammatory-like cells could still be observed around the remaining particles of PHAColl.

Conclusions and clinical implications: Within the limitations of this study in rabbits, the findings showed the presence of inflammatory-like cells at the early stage of bone regeneration when collagenated xenogenic biomaterials were used compared to xenogenic granules alone. Nevertheless, similar bone formation occurred and comparable 3D volumes were found at 6 months in the different groups.

Bone augmentation or preservation surgical techniques are often used to preserve or recreate an adequate bone volume for dental implant placement (Esposito et al. 2006; Pi-tursson et al. 2008). Autogenous bone grafting was considered the gold standard for such procedures because of its osteoinductive properties. Nevertheless, autogenous bone grafting has several disadvantages, such as the need for a second surgical step and a variable and unpredictable rate of resorption, which led practitioners to consider alternative biomaterials (Sbordone et al. 2009). The use of biomaterials as an osteoconductive scaffold for bone formation in extraction socket preservation, implant site development [guided bone regeneration] or sinus lift procedures are well documented today and reliably used for several indications (Barone et al. 2008; Chiapasco & Zaniboni 2009).

Most of the biomaterials used in alveolar bone regeneration are available in particle form and can be difficult to apply to the surgical site. Some companies have developed the inclusion of xenogenic particles in a binding collagenated agent to facilitate handling; some have even made them injectable. Nevertheless, the possible influence of collagen...
on the bone tissue response to the biomaterial is poorly investigated in the literature and remains controversial. Busenlechner et al. showed a similar osteoconductivity of bovine hydroxyapatite (both mixed and unmixed) with a carboxymethylcellulose and collagen carrier after 6 and 12 weeks [Busenlechner et al. 2009]. Nannmark et al. showed that the addition of a collagen gel to collagenated porcine hydroxyapatite did not influence the bone tissue response to the material after 2, 4 and 8 weeks post-insertion [Nannmark & Senneryn 2008]. However, Araujo et al. demonstrated that Bio-Oss® Collagen (Geistlich Pharma AG, Wolhusen, Switzerland) obviously delayed the extraction socket wound healing compared to regular socket healing with a simple blood clot and showed that inflammatory cells were present 3 days and 1 week post-insertion [Araujo et al. 2009, 2010]. Nevertheless, in those studies, non-collagenated BHA was not investigated. Comparative studies investigating the effects of collagen at early stages of bone healing as well as over the long term are therefore needed.

The objective of this study was to qualitatively and quantitatively assess the early bone formation process and mature bone architectures of two different collagenated xenogenic hydroxyapatite compared to bovine hydroxyapatite alone, in a sinus lift model in rabbits. Cell colonization, bone density, osteoconductivity, resorption rate as well as 3D volume stability of bone augmentation were explored.

Material and methods

Animals
New Zealand White rabbits (adult, males, average body weight of 3 kg) were used in the study. All experimental procedures and protocols used in this investigation were reviewed and approved by the Institutional Animal Care and Use Ethics Committee of the University of Liège, Belgium. The “Guide for the Care and Use of Laboratory Animals”, prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press, was followed carefully.

Study design
This study is part of an overall project where 96 sinus-lift procedures performed on 48 rabbits using 10 different types of space fillers were assessed at three distinct time points, 1 week, 5 weeks and 6 months, respectively. Specifically, the space fillers were allocated to the sinuses by a stratified randomization and 16 rabbits were sacrificed at each time point, so that at least three sinuses were available for each space filler at each time point, yielding a two-factor experimental design [space filler and time] with repeated measurements. The present study focussed on the comparison of three space fillers: a deproteinized Bovine hydroxyapatite (Geistlich Bio-Oss®, Geislich Pharma AG) [BHA], a deproteinized bovine hydroxyapatite incorporated in collagen fibers of porcine origin (Geistlich Bio-Oss® Collagen, Geistlich Pharma AG) [BHAColl], and a porcine hydroxyapatite still containing the original collagen matrix and incorporated a 10% collagen gel [MP3®, Technoss, Italy] [PHAColl]. A total of 27 sinus-lift procedures were analyzed from 26 different rabbits.

Surgical procedure
Anesthesia of the rabbits was induced by administration of a ketamine/xylazine bolus (respectively 65/4 mg/kg, IM), 20 min after a fentanyl/dehydrobenzperidol premedication (0.22 ml/kg of a bolus 25 µg/1.25 mg/ml IM) and 2 h before surgery, animals also received buprenorphin at a dose of 0.05 mg/kg. This was administered twice a day for 2 days. Surgical interventions were performed under strict sterile conditions. The surgical area was shaved and disinfected with iodine, and a straight incision was made to expose the nasal bone and the naso-incipial suture lines. The soft tissues were reflected with the periosteum to access to the upper bone wall of the sinus. Two ovoid windows (approximately 6 x 4 mm) were created bilaterally using a round diamond bur. The membrane was carefully raised from the floor and lateral walls and the space-filling material was inserted into the created compartment [Fig. 1]. The volume of filling material was standardized to 0.4 ml per sinus. The bony windows were covered with a resorbable membrane [Biogide, Geistlich Pharma AG] and the wounds were sutured with 4/0 polyesther thread (Pernashar, Hu Friedy, Rotterdam, The Netherlands). Animals were sacrificed by injection of pentobarbitral [200 mg/kg, IV, after the same premedication as for surgeries]. Samples were dissected and soaked in fixative (6% formal). The surgical procedures were performed by a single operator.

Histological analysis
The samples were processed for non-decalcified histology using polymethylacrylate (PMMA) resin. After fixation for about 1 week, the samples were dehydrated in ascending graded ethanol series (24 h each grade) and then placed in pure acetone for 24 h. Finally, samples were impregnated with methylmethacrylate for 4 days with one refreshment before embedding in PMMA at 4°C for 10 days. Each resulting non-decalcified block was cut sagittally with a circular diamond saw (Leica, SP1600, Germany) at two different levels in the central region. The first cut was in the area of the window and the second 1.5 mm outward. The two slices were then polished using a grinding machine (Metaserv®2000, Buehler) and sputter coated with a thin layer of gold/palladium on both sides. Samples were observed under SEM (Leo 1450 VP). SEM observations were made using back-scattered electron mode (BSE). Moreover, 30-µm sections were cut and polished using the same material from the rest of each block in the close vicinity of the central area and were stained with HTX-eosin and counter-stained with toluidine blue. To allow a better observation of cells, thin 7-µm sections were also created using a hard tissue microtome (Leica

Fig. 1. Insertion of a Bio-Oss® Collagen block.
Polycut SM 2500, Germany) and stained with Goldner trichrome.

**Histomorphometry**
Scanning electron micrographs (SEM, Leo VP 1450) were taken using the back-scattered electron (BSE) mode at 30× magnification and assembled to visualize the entire sinus. These contiguous BSE pictures allowed a quantitative evaluation of the mineralized bone, the remaining biomaterial, and the soft tissue areas based on their respective gray levels using a semi-automatic image analyzer (Leica Qwin, Germany). The regions of interest were manually defined, and the different areas were automatically calculated. The following measurements were made: bone formation, space filler area, and non-calcified tissues, all expressed as percentages of the augmented area;

**X-ray microtomography analysis**
All collected samples were first submitted to x-ray microtomography. Before scanning, the samples were transferred to an Eppendorf® tube containing fixative. The tube was affixed to the brass stub and examined using a Skyscan 1172 high-resolution desk-top micro-CT system (Skyscan®, Kontich, Belgium). The cone-beam source operated at 100 kV and 100 μA. The detector was a 2D, 1048 × 2000 pixel, 16-bit X-ray camera. The sample was rotated through 180° with a rotation step of 0.49°, giving an acquisition time of 30 min per sample. Taking into account the camera definition and the source-object-camera distance, 2D images with a pixel size of 17.28 μm were obtained, using a cone-beam reconstruction algorithm. The corresponding 3D images were produced by stacking all the 2D cross sections.

Analysis of the 3D images allowed the calculation of the total volume of the regenerated space at baseline, at 5 weeks and at 6 months. The 3D measurements were carried out using the CTscan software (release 2.5, Skyscan®, Kontich, Belgium).

**Statistical analyses**
Among the 26 rabbits, 23 (88%) had one sinus included in the experimental design and only three had their two sinuses included, yielding a total of 29 sinus-lift procedures. Hence, there was little loss in

---

Fig. 2. Histologic data observed with light microscopy for each studied space filler at 1 week. (a) Early steps of osteogenesis in a region close to the bone wall, 2×. (b) higher magnification: note the presence of inflammatory cells in the BHAColl and PHAColl groups. (c) high magnification, 40×: inflammatory cells were observed in the BHAColl and PHAColl groups while mostly mesenchymal cells and fibroblasts were seen in the BHA group. (d) Images in the center of the sub-sinus created space, 20×: collagen structures are distinguished in light green. (7 μm non-decalcified section, Goldner Trichrome staining).
efficiency by considering the 29 sinus as independent statistical units. Results were expressed as mean, standard deviation (SD), minimum and maximum. The experimental data were analyzed by two-way analysis of variance (ANOVA) with repeated measurements, allowing a test for interaction between the two factors (time and space filler). When the interaction term was significant, space fillers were subsequently compared at each time point by one-way ANOVA. Otherwise, the overall time and space filler effects were tested. Results were considered to be significant at the 5% level ($P < 0.05$). A Bonferroni correction was applied to account for multiple comparisons. Statistical analyses were done using SAS version 9.1 [SAS Institute, Cary, NC, USA].

Results

Descriptive analysis

One week after implantation (Fig. 2)

In the BHA group, no evidence of inflammation was found, whereas elongated mesenchymal and fibroblastic cells were found everywhere in the cavity. Remnants of the clot were almost invisible. The penetration of connective and vascularized buds into the sub-sinus space was observed along the bone walls.

In the BHAColl group, a rich-cell tissue (inflammatory-like) was observed in the periphery of the cavity, along the bone walls and under the lifted sinus membrane. The center of the cavity was still poorly invaded by the cells, whereas remnants of red blood cells were seen among BHA particles and collagen fibers. In the PHAColl group, rich-cell tissue (inflammatory-like) were present in the inter-particle areas all throughout the cavity. Osteoclasts were observed along the PHA particles.

Five weeks after implantation (Fig. 3)

In the BHA group, newly formed bone bridged the particles of hydroxyapatite together. Most of the particles' surfaces were in tight contact with a layer of new bone. Only the center of the regenerated area was not filled with any new bone. Some osteoclastic cells could be found along the particles, and osteoblastic cells were observed only in the central region.

![Fig. 3. Histologic data observed with light microscopy for each studied space filler at 5 weeks. (a) Newly formed bone was found in the periphery of the created space in the three groups. However, in the BHA groups, bone colonization to the center areas seemed more advanced, 2×. (b) Higher magnification: Osteoclasts were seen in the three groups but more predominantly in the PHAColl group. Note the presence of inflammatory cells localized in around the PHAColl granules. (c) Images in the center of the sub-sinusal created space. (7 μm non-decalcified section, Goldner Trichrome staining).](image-url)
In the BHAColl group, newly formed bone combined with a large number of capillaries was observed along the bone walls, which was nevertheless less extended to the center than with BHA. Signs of inflammation and collagen fibers were no longer observed. In the PHAColl group, newly formed bone also was observed along the bone walls. The presence of small round cells as well as a substantial amount of osteoclasts, was still observed around the PHA.

Six months after implantation (Figs 4 and 5)

In the BHA group, bone marrow and adipocytes were observed much more frequently than at 5 weeks, while multinucleated cells were not visible. Lamellar bone was found solely in intimate contact with the particles and was seen to bridge them together. Osteoblastic activity was very low, with neither osteoid tissue nor osteoblasts being visible. Bone trabeculae were covered with a unicellular flat layer of cells.

In the BHAColl group, the anterior part of the section mostly surrounded by the preexisting bony walls displayed a similar architecture as the BHA samples. However, the posterior part, under the sinusale membrane, displayed a less mature tissue, with a dense connective tissue surrounding the bone particle network.

Samples treated with PHAColl also displayed a gradient of maturity from the anterior to the posterior part of the sample, similar to samples treated with BHAColl. In the mature areas, PHA particles were completely resorbed and were replaced by rarefied lamellar bone trabeculae displaying remodeling activity; the marrow spaces were occupied by adipocytes. In the less mature areas, remnants of PHA granules surrounded by small rounds cells and multinucleated osteoclasts were still observed and the non-calcified tissue was of a dense fibrous tissue type.

MicroCT analysis: 3D volume variation

After 1 week, the mean volume of the augmented tissue reached 344, 327, and 377 mm³ for BHA, BHAColl, and PHAColl, respectively. These were considered as the baseline values. Two-way ANOVA applied to the 3D volumes did not reveal any significant interaction \( P = 0.47 \) between space filler and time. An overall negative time trend of 3D volume variation was observed \( P = 0.0041 \) mainly between 1 week and 5 weeks, although there was no effect of the space filler \( P = 0.34 \) [Table 1, Fig. 6].

Histomorphometric analysis

By applying a two-way ANOVA to the histomorphometrical data [bone formation, space filler area and non-calcified tissues], bone formation increased significantly with time \( P < 0.0001 \), although no difference was seen among the three biomaterials \( P = 0.84 \) [Table 2]. For the space filler area, a significant interaction was observed between space filler and time \( P < 0.0001 \) [Table 2]. Although this parameter remained fairly stable for BHA and BHAColl, a marked drop was observed for PHAColl. After 6 months, the results were significantly lower for PHAColl than those for BHA and BHAColl \( P = 0.0003 \). When considering the percentage of soft tissue, a significant interaction was found between space filler and time \( P = 0.0002 \). Specifically, although values between space fillers at 1 week were comparable \( P = 0.23 \), they significantly differed at 5 weeks \( P = 0.0039 \) and at 6 months \( P = 0.0006 \). A decrease was observed for BHA and BHAColl as opposed to an increase for PHAColl.

Figure 6 shows the correlation between the volume change over time as well as the percentage of new bone, percentage of space filler, and percentage of non-calcified tissues.

Discussion

The goal of the present study was to assess the effect of collagenated xenogenic space fillers on bone regeneration compared to the use of...
BHA granules alone. Indeed, adding collagen fibers or gel to biomaterial granules is of interest as a means to help with surgical handling.

Araujo et al. have described a slower bone healing process when Bio-Oss® collagen (Geistlich Pharma AG) was placed into dog extraction sockets compared to a simple socket healing with a blood clot. More recently, using the same model, they showed the presence of inflammatory cells including PMNs and monocytes/macrophages in the presence of Bio-Oss® Collagen (Geistlich Pharma AG) at 3 and 7 days. Inflammatory cells were no longer visible 2 weeks after implantation of the collagenated biomaterial and did not impair new bone formation. Busserlechner et al. did not find any signs of inflammation when bovine hydroxyapatite was either mixed or not mixed with a carboxymethylcellulose and collagen gel as a carrier. However, only 6 and 12 weeks of results were assessed; a possible inflammatory process could have occurred in the first few days and subsequently disappeared once the binding gel was resorbed.

In the present research, collagen-free bovine hydroxyapatite (BHA) was compared to collagenated xenograft (BHAColl/PHAColl). The descriptive analyses emphasized different findings between the groups: A notable proportion of inflammatory-like cells were observed in both the collagenated groups at 1 week although inflammatory cells were not observed with BHA. In the BHAColl group, the inflammatory-like cells were no longer found at 5 weeks and 6 months, whereas small round cells which might be indicative of a mild local inflammatory reaction were still present in some localized areas close to the remaining particles of PHAColl even though the bound collagenated gel was most likely fully resorbed at these time points. The manufacturing process of PHAColl does not calcinate the xenogenic bone particles at high temperatures, thus preserving the natural collagen of the porcine bone in the granules and possibly explaining the persisting inflammation localized around the residual particles. However, Nannmark et al., also using a rabbit model, did not find any signs of inflammation at any time point. Inflammation is the very first stage of healing and might not be harmful for bone regeneration. Indeed, the presence of inflammatory cells is also widely described in studies assessing the efficacy and biodegradation of collagen membranes in guided bone regeneration (Rothamel et al. 2005). Further investigations, including immunohistology and human histology, should be performed to clarify these findings.
Table 2. Augmented area and histomorphometric data–Mean (SD), (Min–Max)

<table>
<thead>
<tr>
<th>Time</th>
<th>Space filler</th>
<th>BHA</th>
<th>BHAColl</th>
<th>PHAColl</th>
</tr>
</thead>
<tbody>
<tr>
<td>% bone</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 week</td>
<td>0.1 (0.1)</td>
<td>0.05 (0.01)</td>
<td>0.04–0.06</td>
<td>0.1 (0.1)</td>
</tr>
<tr>
<td>5 weeks</td>
<td>14.8 (2.1)</td>
<td>10.8 (6.9)</td>
<td>3.2–16.8</td>
<td>9.7 (5.9)</td>
</tr>
<tr>
<td>6 months</td>
<td>16 (3.7)</td>
<td>20.8 (1.9)</td>
<td>18.7–22.3</td>
<td>18.9 (3)</td>
</tr>
<tr>
<td>% filler</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 week</td>
<td>42.7 (3.9)</td>
<td>34.2 (1.1)</td>
<td>33.3–35.4</td>
<td>41.8 (9.9)</td>
</tr>
<tr>
<td>5 weeks</td>
<td>40.3 (3.1)</td>
<td>26.1 (2.8)</td>
<td>23.3–28.8</td>
<td>24.7 (4)</td>
</tr>
<tr>
<td>6 months</td>
<td>34.9 (5.1)</td>
<td>25.7 (4)</td>
<td>21.1–28.4</td>
<td>3.1 (4.3)</td>
</tr>
<tr>
<td>% soft tissues</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 week</td>
<td>57.2 (3.9)</td>
<td>65.7 (1.1)</td>
<td>64.5–66.7</td>
<td>58.1 (9.9)</td>
</tr>
<tr>
<td>5 weeks</td>
<td>45.7 (2.1)</td>
<td>63.9 (6.3)</td>
<td>58.9–70.9</td>
<td>66.6 (3)</td>
</tr>
<tr>
<td>6 months</td>
<td>49.2 (4.5)</td>
<td>53.5 (3.6)</td>
<td>50.2–57.3</td>
<td>78.2 (5.8)</td>
</tr>
</tbody>
</table>

*Time effect (P < 0.0001); space filler effect (P = 0.84); interaction (P = 0.25).†
†Time effect (P < 0.0001); space filler effect (P < 0.0001); interaction (P = 0.0001).‡
‡Time effect (P = 0.71); space filler effect (P < 0.0001); interaction (P = 0.0002).

Similar percentages of newly formed bone and regenerated areas were statistically observed at 5 weeks and 6 months. Within the limitations of the present study and even though the differences in these histomorphometric results were not significant, Fig. 6 and 11 show that the time evolution of the percentage of new bone was stable from 5 weeks to 6 months for BHA, whereas it kept increasing in the collagenated groups to reach similar results at 6 months. Moreover, in most of the sections at 5 weeks, the newly formed bone was still localized at the periphery of the cavity for BHAColl and PHAColl, whereas in the BHA group, concentric osteogenesis reached the deeper regions of the cavity. In addition, at 6 months, only the collagenated groups were found with immature [10] (woven) bone areas (Fig. 8). Therefore, the overall feeling is that the collagenated biomaterials slightly delayed the osteogenic process. Similar investigations on larger numbers of subjects would be necessary to confirm this hypothesis.

Some authors have suggested that a non-resorbable biomaterial would be more suitable to withstand against reexpansion of the sinuses [Asai et al., 2002, Lambert et al., ????, ??]. The percentage of PHA particles progressively decreased from 1 week to 6 months. The particles were almost completely resorbed after 6 months, with only 3.6% remaining. These results confirm the resorption properties of PHA, already described by Nannmark et al.; they found only 9.3% of remaining particles surfaces 8 weeks after implantation in artificial sockets in rabbits. Nevertheless, after 6 months, similar regenerated 3D volume stability was observed within the groups in this study. Longer time points would be necessary to demonstrate if non-resorbable biomaterials are really necessary.

Considering the above findings, from a clinical point of view, the use of biomaterials containing binding agents such as collagen, to ease surgical handling, might have a biological effect on the alveolar bone healing process. However, furthermore clinical investigations should be considered before raising any clinical recommendations concerning the use of collagen in alveolar bone regeneration and preservation procedures.

Conclusion

Within the limits of this study in rabbits, these findings showed the presence of inflammatory cells at the early stages of bone regeneration when collagenated xenogenic biomaterials were used compared to xenogenic granules alone. Nevertheless, similar bone formation occurred similarly and comparable 3D volume stabilities were found in the different groups. Furthermore comparative histologic studies in human are needed.

Acknowledgement: The authors would like to thank Laurence Seidel for her statistical assistance. The authors declare that there is no conflict of interest in this study. The study was self-funded by the authors and their institutions.

References


Dear Author,

During the copy-editing of your paper, the following queries arose. Please respond to these by marking up your proofs with the necessary changes/additions. Please write your answers on the query sheet if there is insufficient space on the page proofs. Please write clearly and follow the conventions shown on the attached corrections sheet. If returning the proof by fax do not write too close to the paper’s edge. Please remember that illegible mark-ups may delay publication.

Many thanks for your assistance.

<table>
<thead>
<tr>
<th>Query reference</th>
<th>Query</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AUTHOR: A running head short title was not supplied; please check if this one is suitable and, if not, please supply a short title of up to 40 characters that can be used instead.</td>
<td>OK</td>
</tr>
<tr>
<td>2</td>
<td>AUTHOR: Please provide manufacturer information for Leica, SP1600: company name, town.</td>
<td>Leica Microsystems GmbH Ernst-Leitz-Straße 17-37 Wetzlar, 35578, Germany</td>
</tr>
<tr>
<td>3</td>
<td>AUTHOR: Please provide address information for Buehler: town, state (if applicable), and country.</td>
<td>Buehler GmbH, In der Steele 2 40599 Düsseldorf, Germany</td>
</tr>
<tr>
<td>4</td>
<td>AUTHOR: Please provide manufacturer information for Leo 1450 VP: company name, town, state (if USA), and country.</td>
<td>(LEO 1450 VP, Carl Zeiss AG, Oberkochen, Germany)</td>
</tr>
<tr>
<td>5</td>
<td>AUTHOR: Please provide manufacturer information for Leica Polycut SM 2500: company name and town.</td>
<td>Leica Microsystems GmbH Ernst-Leitz-Straße 17-37 Wetzlar, 35578, Germany</td>
</tr>
<tr>
<td>6</td>
<td>AUTHOR: Please provide manufacturer information for Leica Qwin: company name and town.</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>AUTHOR: Please provide manufacturer information for Eppendorf®: company name, town, state (if USA), and country.</td>
<td>Was removed</td>
</tr>
<tr>
<td>8</td>
<td>AUTHOR: Please provide manufacturer information for X-ray camera: company name, town, state (if USA), and country.</td>
<td>Hamamatsu Photonics K.K., Hamamatsu City, Japan</td>
</tr>
<tr>
<td>9</td>
<td>AUTHOR: Please check the citation figs 5.9 and 5.12.</td>
<td>Fig 6</td>
</tr>
<tr>
<td>10</td>
<td>AUTHOR: There are only six figures in this article, please check the validity of the citation Fig. 8.</td>
<td>Fig 4</td>
</tr>
<tr>
<td>12</td>
<td>AUTHOR: Please provide the year of publication for the reference Lambert et al. (????).</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>AUTHOR: Figure 5 has been saved at a low resolution of 131 dpi. Please resupply at 600 dpi. Check required artwork specifications at <a href="http://authorservices.wiley.com/submit_illus.asp?site=1">http://authorservices.wiley.com/submit_illus.asp?site=1</a></td>
<td></td>
</tr>
</tbody>
</table>
USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

Required software to e-Annotate PDFs: Adobe Acrobat Professional or Adobe Reader (version 8.0 or above). (Note that this document uses screenshots from Adobe Reader X)

The latest version of Acrobat Reader can be downloaded for free at: [http://get.adobe.com/reader/](http://get.adobe.com/reader/)

Once you have Acrobat Reader open on your computer, click on the Comment tab at the right of the toolbar:

This will open up a panel down the right side of the document. The majority of tools you will use for annotating your proof will be in the Annotations section, pictured opposite. We’ve picked out some of these tools below:

1. **Replace (Ins)** Tool – for replacing text.
   - Strikes a line through text and opens up a text box where replacement text can be entered.
   - **How to use it**
     - Highlight a word or sentence.
     - Click on the Replace (Ins) icon in the Annotations section.
     - Type the replacement text into the blue box that appears.

2. **Strikethrough (Del)** Tool – for deleting text.
   - Strikes a red line through text that is to be deleted.
   - **How to use it**
     - Highlight a word or sentence.
     - Click on the Strikethrough (Del) icon in the Annotations section.

3. **Add note to text** Tool – for highlighting a section to be changed to bold or italic.
   - Highlights text in yellow and opens up a text box where comments can be entered.
   - **How to use it**
     - Highlight the relevant section of text.
     - Click on the Add note to text icon in the Annotations section.
     - Type instruction on what should be changed regarding the text into the yellow box that appears.

4. **Add sticky note** Tool – for making notes at specific points in the text.
   - Marks a point in the proof where a comment needs to be highlighted.
   - **How to use it**
     - Click on the Add sticky note icon in the Annotations section.
     - Click at the point in the proof where the comment should be inserted.
     - Type the comment into the yellow box that appears.
USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

5. Attach File Tool – for inserting large amounts of text or replacement figures.

- Inserts an icon linking to the attached file in the appropriate place in the text.

**How to use it**
- Click on the Attach File icon in the Annotations section.
- Click on the proof to where you’d like the attached file to be linked.
- Select the file to be attached from your computer or network.
- Select the colour and type of icon that will appear in the proof. Click OK.

6. Add stamp Tool – for approving a proof if no corrections are required.

- Inserts a selected stamp onto an appropriate place in the proof.

**How to use it**
- Click on the Add stamp icon in the Annotations section.
- Select the stamp you want to use. (The Approved stamp is usually available directly in the menu that appears).
- Click on the proof where you’d like the stamp to appear. (Where a proof is to be approved as it is, this would normally be on the first page).

7. Drawing Markups Tools – for drawing shapes, lines and freeform annotations on proofs and commenting on these marks.

- Allows shapes, lines and freeform annotations to be drawn on proofs and for comment to be made on these marks.

**How to use it**
- Click on one of the shapes in the Drawing Markups section.
- Click on the proof at the relevant point and draw the selected shape with the cursor.
- To add a comment to the drawn shape, move the cursor over the shape until an arrowhead appears.
- Double click on the shape and type any text in the red box that appears.

For further information on how to annotate proofs, click on the Help menu to reveal a list of further options: