<u>List of Corrections :</u>

Title of Report : "Evaluation of early wound healing events in a rat wound model treated with "active" topical dressings.

All changes highlighted in red font

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1. <u>Page ii - Abstract</u> : Methods Section
   A chronic wound model was created in 128 Sprague-Dawley rats, modifying previously described methods by combining burn and excisional wounds. Wounds were inoculated with a bacterial broth (*Pseudomonas aeruginosa* and *Staphylococcus aureus*) on POD 4. The wounds were then assigned to the following treatment groups on POD 7; Flavonix®, Acticoat® and a negative control. An additional non-inoculated control group (no bacterial or other broth) was included. Eight animals were assigned to each group at each time point. The study was conducted over 21 days and the categorical variables assessed were epithelial gap, cellular proliferation at the wound edge at Days 10,14 and 21 and semi-quantitative culture for bacterial load at Days 10 and 21.

2. Page 14-15 - Porcine Pilot Study (unpublished data) : all paragraphs omitted

3. Page 17 - Wound Creation and Batching Protocol :

   A total of 128 female Sprague-Dawley rats (±300g) were used. The rats were divided into 6 batches of 16 rats/batch. Within each batch of 16 rats, 12 rats were inoculated with a bacterial broth and 4 were not inoculated at all (no broth). All animals were treated with the same secondary covering dressing protocol, consisting of Postop Opsite®

(Smith and Nephew, UK, London), and three other reinforcing layers. Negative control animals in each batch were of two types. Inoculated negative controls that were inoculated with broth but not treated with any “active” agent and non-inoculated negative controls where no inoculum was placed and no “active” treatments were placed either. Of the eight remaining animals per batch, four were treated with 1ml of Flavonix® Gel and four with 2cm water moistened discs of Acticoat®.

4. Page 18 – Burn Protocol at Day 0, Paragraph 1

   Animals were anaesthetized as described, and placed on a drape. All animals were shaved around the area to be burnt.

5. Page 18 – Burn Protocol at Day 0, Paragraph 1

   The block was allowed to stand on the skin surface for 10 seconds and then removed. The weight of the block ensured a uniform force on each burn wound.

6. Page 19 – Burn Eschar Excision, Splinting and Inoculation at Day 4 Paragraph

   The wounds were left for a further 3 days till POD 7 before treatment was commenced with the “active” topical products. This period of 72 hours allowed a biofilm to establish within the wound.

7. Page 20-21 – Dressing Protocol Paragraph 1

   Dressings were changed on days 10, 14, 17/18 and 21. In those animals that required “active” topical dressings, an Acticoat® 2cm diameter disc presoaked in sterile water was placed in the Acticoat® cohort and 1ml of Flavonix® gel was injected onto the wound using a 5ml syringe in the Flavonix® cohort.

8. Page 21 – Dressing Protocol end of paragraph 1

   Four additional rats in one of the Day 14 cohorts had Flavonix® and Acticoat® applied onto the wounds at POD 7, and 10. The moist Acticoat® was applied over the 1ml of Flavonix® gel that was placed first onto the wound. The rest of the dressings for these additional rats remained exactly the same.

9. Page 21 – Dressing Protocol Paragraph 2

   The rats were then treated as described in the "Burn Protocol at Day 0" and returned to individual cages.

10. Page 22 - Termination of Animals at Determined Time Intervals and Treatment Group Assignment within Batches Paragraph 1

    Within each batch of 16 animals, 4 were assigned to each of the treatment groups (n=8 at each time interval); Flavonix®, Acticoat®, Negative Control (NC) and a Non-inoculated Control (NIC) Group. On Day 14, four additional rats were terminated. These rats had been treated with a combination Acticoat® and Flavonix® gel as described in the "dressing protocol". None of these animals were included in the results due to the insufficient sample size of this group (n = 4). The outcomes in these animals is discussed briefly in the "discussion" section of this report.

11. Page 24 – Wound site preparation, and biopsy allocation for histology and semi-quantitative culture Paragraph 3

    The tissue was then halved down the centre, creating 2 equal semi-circular specimens. One half was then inked on its undersurface (Rotring Drawing Ink™ and a histological tissue fixative (Cancer Diagnostics Inc™)) for identification of the deep surface and the specimen placed on a

solid surface for preparation of biopsy samples for histology, immunohistochemistry.

12. Page 25 – Figure 6 – wording changed in text box
13. Page 26 – Histology and Epithelial Gap Measurements Paragraph 4

    Measurements of the epithelial gap were performed at POD 10,14 and 21 for both the treatment and control groups. The use of a measuring graticule on the microscope stage to measure the epithelial gap was not possible due to the inability to fit the entire length of each slide onto the microscope stage at the lowest magnification (10X magnification). The markings were therefore done microscopically and the measurement done macroscopically using a Vernier's caliper off the microscope stage. The inaccuracy of this method should be noted and was mitigated by taking measurements to the nearest millimetre.
14. Page 27-31 – Immunohistochemistry – Ki-67 All Paragraphs

    Order changed and controls explained

    Each slide had an outermost section of tissue composed of a full thickness unwounded skin. This section of skin was used as a "normal" frame of reference, by which to compare the immunolabelling in the wounded skin. The comparison was based on morphological findings and relates to the cellular proliferation index which was determined as explained in the text further. The target antibodies and the secondary antibodies were tested on normal (unwounded) rat skin samples from unwounded animals. Positive controls were assessed using the target antibody (Anti-Rat Ki-67 Antigen MIB-5 Clone, code no M7248) and negative controls using a DakoCytomation Mouse IgG1 (code No X0931). The specificity of

the primary antibody was assessed morphologically comparing staining in the stratum basale to the staining in the progressively superficial layers of the epidermis as well as to other background areas in the tissue section (dermis). Positive staining in the stratum basale was expected morphologically to be greater than in other background areas (dermis and more superficial epidermal layers). The secondary antibody control was tested without the use of a primary antibody and assessed morphologically also in normal rat skin (unwounded animals).

Cellular Proliferation Index Determination

A single observer method was used to assess all slides morphologically. In order to mitigate against the pitfalls of visual assessment the following criteria were followed. Fields were distinctly outlined so that high power field (HPF) allocation was not random and variability hence minimized. Five sequential HPF's on each side of the wound beginning at the epithelial margin were analysed for positively immunolabelled chromogen stained cells. Only cells positively immunolabelled in the stratum basale were counted for the numerator of the ratio . The denominator of the ratio was formed by the sum of the positively immunolabelled cells and the cells stained by haemotoxylin in which nuclei were observed. This is expressed further in this text as the "total number of cells" in the stratum basale (total number of cells = number of positively immunolabelled cells + number of haemotoxylin stained cells in

which nuclei are identified). The observer and cross sectional variability of this method has been noted as a potential flaw of this method due to some nuclei not being included as a result of the cross section. A ratio of stained (positively immunolabelled) over total number of cells in the stratum basale (as described) was calculated for each HPF initially. Corresponding HPF's on either side of the wound were averaged. Cell counts for each HPF were then further divided by a baseline cell count in a normal skin HPF for each section This baseline cell count was taken as the ratio of stained (positively immunolabelled) cells/total number of cells in the HPF most lateral on the slide section. The ratio in this HPF section would most resemble normal unwounded skin. A final relative ratio was then calculated by dividing the ratio of each HPF (1 to 5) over the ratio of the baseline HPF . This ratio is then referred to as the cellular proliferative index. The closer to 1 the index, the closer the resemblance toward normal skin. Each HPF measured 450 μm in length.

15. Page 36 – Epithelial Gap Measurements Paragraph 1

    The variation in "n" indicated in Table 4 accounts for those animals in which the splint had been bitten off.

16. Page 38 – Figure 12 – pictures zoomed in

17. Page 39 – Immunohistochemistry – Ki-67 Staining and Cellular Proliferation Index Paragraph 1

    The variation in "n" is due to only those specimens being included in which 5 HPF's were identified on either side of the wound edge.

18. Page 42 – Microbiology Paragraph 1

The variation in "n" is due to specimens which were not included due to poly-microbial cultures. These were deemed contaminated.

19. Page 49 – Paragraph 2

    Bacterial load or bioburden is known to cause an increase in proteolytic activity and a greater inflammatory response with increase neutrophils in the wound area. This response contributes to the pathophysiology of a chronic wound and the eradication or disruption of biofilms has been shown to improve healing. Biofilms are commonly found in chronic wounds (60% – 80%). No one particular method has been standardized for the purpose of monitoring biofilm formation and degradation. Our study methods used semi-quantitative cultures as a crude indirect method of assessing the bacterial bioburden. Although not directly indicative of biofilm the analysis does quantify bioburden in a manner that is clinically relevant. This method was chosen as it has a 100% positive predictive value and 93,7% negative predictive value of clinical sepsis in a wound and simultaneously avoids the intensive and expensive exercise of counting exact colonies. The assessment of bacterial load does not necessarily translate to a direct assessment of the biofilm. Further work is required in assessing a more accurately quantifiable target within biofilms in order to evaluate the effects of these dressings on biofilm.

20. Page 50 - Potential improvements to this study and future work

    PCR and gene amplification provides a means to better define inflammatory processes by measuring specific markers (IL's, MMP's etc.). Secondly, gene marker identification of the products found within biofilms and those bacteria responsible for the biofilm structures will allow a new dimension in evaluating wounds. Instead of assessing common end pathways, the possibility exists to assess and modulate causative factors at the problematic site. This data can then be compared

to macroscopic outcomes such as epithelial gap reduction to give a more significant and reliable indication of treatments and their effects.

21. Grammar and other errors changed throughout the report with respect to: in vivo changed to *in vivo, ad libitum, in situ* etc.
22. Numbers changed to written if less than 10
23. et al changed to *et al.* throughout the report
24. Page 31 - Figure 8 : Magnification changed to a single value
25. Page 32 - Figure 9 : Magnification provided and dermis (D) inserted into legend