Alveolar Bone RNA Extraction and Gene Expression in Health and Diabetes

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Abstract

Objectives:-There is a gap in the literature in our understanding of RNA extraction and human alveolar bone gene expression in type 2 diabetes. The overall objective of this unique and novel study is to investigate the gene expression from alveolar bone in health and diabetes. There are two broad objectives; 1. To improve RNA extraction protocol from human alveolar bone and improve RIN (RNA integrity values) 2. Analyze gene expression profiles in health and diabetes RNA sequencing.

Methods:-Healthy and Type 2 diabetic subjects undergoing periodontal surgeries were screened and consented. The subjects were undergoing procedures where alveolar bone removal was indicated like osseous surgery, tori removal, crown lengthening, extraction and alveoplasty. A total of 43 subjects were recruited until now for the study (25 healthy and 18 diabetes). At the surgery appointment, alveolar bone was harvested and immediately placed in a vial containing 500ul of Trizol and immediately frozen in liquid nitrogen. Multiple vials were obtained if there was more bone available. Samples were processed within two months of collection. During the RNA extraction stage, bone samples were homogenized with a bone crusher in liquid nitrogen to uniformly break larger pieces of bone and then were processed in a bullet blender with metal beads in a cold room. The homogenized sample was then extracted and processed by Qiagen mini columns protocol for fibrous tissue as per manufacturer's protocol. RNA was then analyzed on nano-drop spectrophotometer for quality and quantity and a bio-analyzer to obtain RNA integrity number (RIN). Samples that had greater than 3.5 RIN were then processed by a rRNA depletion method to further amplify the purity of the samples and then were submitted for RNA sequencing for differential gene expression.

Results:- Extracting stable and high quality RNA from human bone samples is technique and patient dependent. Our data showed that maintaining low or below freezing temperatures of the bone samples are key to a good RIN values that may influence important gene expression techniques like RT-PCR and RNA sequencing. We recruited until now 25 healthy and 18 diabetics and some of the samples were discarded due to drop outs or low quantity and poor quality of RNA. Our results show that a total of 10 healthy and 8 diabetic samples had RIN Values of 3.5 or more and were submitted for RNA sequencing for differential gene expression. The mean age, HbA1c% and RIN values of healthy vs diabetic subjects is; age (55.3 ± 17.5 vs 63.9 ± 8.7 years), HbA1c% (5.6 ± 0.29 vs 7.3 ± 2.4) and RIN (4.94 ± 1.41 vs 4.43 ± 1.54) respectively. Sequencing analysis shows that overall gene expression was downregulated in diabetes samples.

Conclusions:-Understanding alveolar gene expression in diabetes is very important as a significant percentage of population is affected and its molecular and clinical effects on periodontal regeneration or dental implants are relatively unknown. One of the most important aspect of gene expression experiments from human bone is extracting stable and high quality RNA with good quantity and quality which is a challenge in humans due to clinical and technical factors. Our results here showed that the revised protocol for RNA extraction from human bone samples results in improved RIN values. Higher RIN values (> 6) are preferred for sensitive gene expression techniques like RT-PCR and RNA sequencing, however methods like rRNA depletion techniques are available to purify and amplify the RNA samples. Data from RNA sequencing shows that overall gene expression and specific pathways for inflammation and bone regulation were down regulated in diabetic subjects compared to healthy controls.

Table 1. Patient Demographics

Category	Healthy (n= 10)	Diabetes (n=8)
Gender (Female/Male)	7/3	4/4
Age (Mean and Std Dev)	55.3 ± 17.5	63.9 ± 8.7
Smoking Status (Smoker/Nonsmoker)	3/7	1/7
Distribution of sites of alveolar bone sample collection (maxilla/mandible)	9/1	5/3
HbA1c % (Mean and Std Dev)	5.6 ± 0.3	7.3 ± 2.4
BMI (Mean and Std Dev)	29.9 ± 6.3	29.0 ± 2.8
RIN (Mean and Std Dev)	4.94 ± 1.41	4.43 ± 1.54

Figure 1. Representative Examples of Varying RIN Values

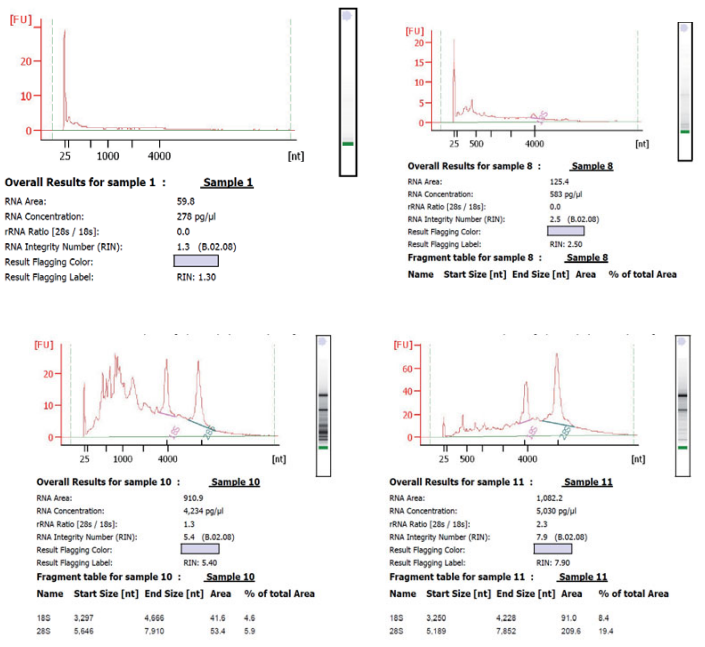


Figure 2. Sequencing Protocol and Bioinformatics Analysis

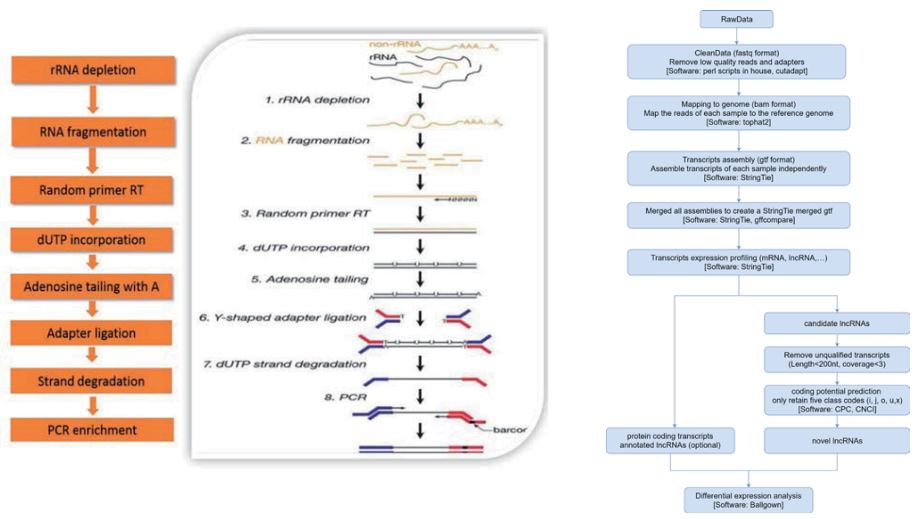


Figure 3. Heat Map of Gene Expression in Healthy and Diabetics

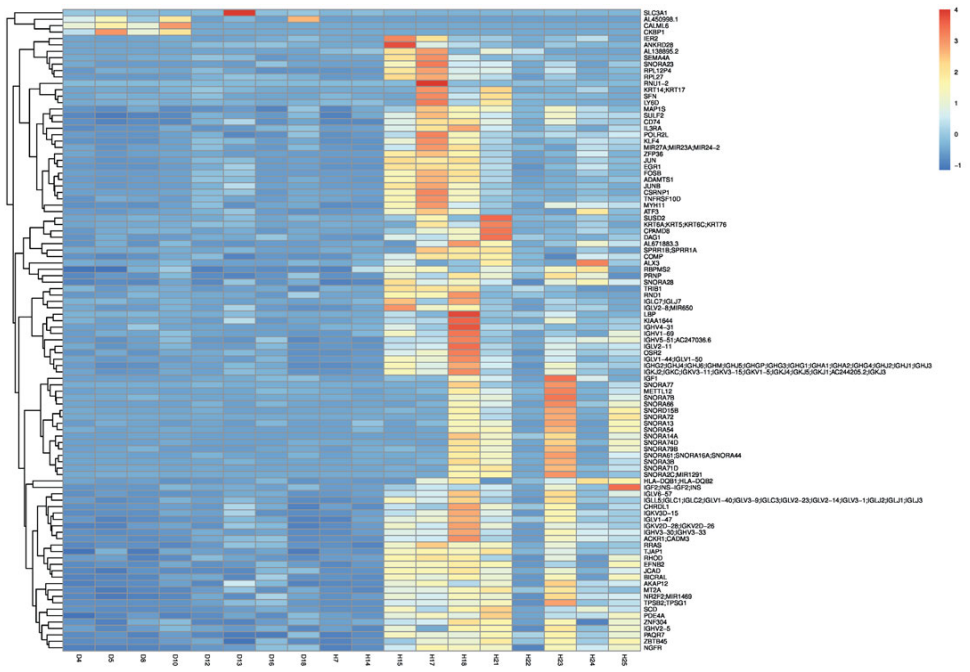
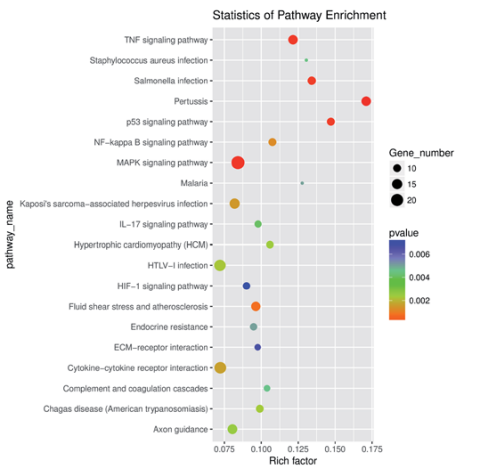
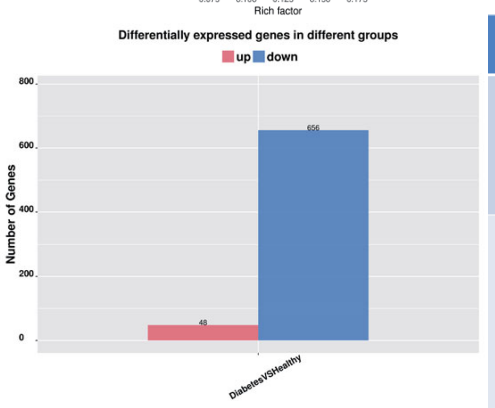


Figure 4. Pathways of Significance and Gene Expression Regulation



Few Significant Pathways

- Osteoclast Differentiation Pathway
- AGE-RAGE pathway
- NF-Kappa B
- IL-17
- Cytokine-cytokine receptor pathway
- Chemokine signaling pathway



Pathways of significance	Genes of interest	Diabetes vs Healthy
Osteoclast Differentiation Pathway	NF-Kb TNF CXCL-1 SOC-3 NFAT	↓
AGE-RAGE	KL4 CCLE TM4SF1 CDK4 JUN RELA	↓

Specific bone markers were evaluated and gene expression of CTSK, ADAMST,TNF,CXCL8, ACP were upregulated in diabetic samples but were not significant. Markers like BMP, RUNX2, CALCR,SMAD were downregulated in diabetes but the difference was not significant.

Conclusions and Future Directions

The results from the study show that extracting good quality RNA from hard tissues like bone is feasible and reproducible and can be utilized to perform advanced genetic sequencing methods. Results show that in this limited sample, many genes were downregulated in type 2 diabetes group suggesting that in well controlled diabetic people, pro-inflammatory genes may be downregulated. Clinical implant studies have suggested success in well controlled diabetes and these molecular findings are in support of them. These results have to be performed in a larger sample size with clinical outcomes to gain more insight.

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