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An evaluation of different chemical processing methods on human bone and the effects thereof

Rhyleen Chauke, Cleo Ndhlovu

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Introduction

This study took place at the Centre for Tissue Engineering (CTE), the only multi-tissue bank in South Africa.

Since there aren't any comparative data in terms of bone allograft processing within the SA context, some examples of processing facilities in the US and Europe were looked at.

Seemingly, a number of them have a common thread of utilising hydrogen peroxide in their processing protocols and even in sterilization techniques.

The interest and positive outcomes found, motivated this study's focus on the use of hydrogen peroxide (H_2O_2) in removing bone marrow, blood elements, and lipids from bone.

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Introduction

The current bone processing method at the CTE is time consuming, taking up to 72 hours to complete which, poses a problem for deadlines and distribution.

The method is also costly and the fumes released from the high concentrations of methanol and chloroform used have resulted in the lab technicians filing complaints about discomfort experienced during processing.

Methanol (99%) is mixed in equal portions with Chloroform (99%) making up a 50:50 solution used during delipidation of cortical and cancellous bone tissues.

The study was conducted with the intent of replacing the standard method with the new method (using low concentrations of H2O2) following a thorough evaluation of the efficacy of the improved method as compared to the standard method.

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Objectives

The objective of the study is to evaluate a new bone allograft processing method with the intent of implementing it for future processing of bone allografts within the tissue bank.

Evaluation was based on:

- cleanliness of the bone (visual inspection)
- Histology reports
- Microbiology reports indicating removal of bioburden
- Residual fat content
- Residual chemical content in bone after processing
- Cost benefits
- Time factor comparing both methods
- Opinion of lab technicians in terms of fume inhalation and smell

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Working Hypothesis

Hydrogen peroxide will effectively eradicate endogenous material on human bone allografts compared to methanol and chloroform.

Hydrogen peroxide will be able to eliminate all microorganisms that can be infectious to the recipient as effectively as the methanol and chloroform method.

Hydrogen peroxide will leave no chemical residue as compared to methanol and chloroform.

The use of hydrogen peroxide will be more effective in cleaning bone allograft and safer for laboratory technicians, since there are less chances of lung damage through inhalation.

The cost associated with processing using hydrogen peroxide will be less including the cost of chemical waste generation in line municipality requirements.

The new method is aimed to be shorter and easier to perform.

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Ethical considerations

Ethics approval from the TUT Research Ethics Committee was received (FCRE 2019/01/001(02)(SCI).

All tissue utilised in the study was obtained following the normal legal consent process as documented in the tissue bank's standard operating procedures.

The next-of-kin was contacted for research consent either via telephone or a personal meeting at which time the retrieval procedure was explained. Once verbal consent was obtained, the tissue coordinator ensured that the family completed and signed the following:

- Consent form
- Confidential Donor Information form
- Medical and social risk assessment questionnaire

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Methodology

Standard method

- Selection of cortical bone material from femurs, ulnae, radiuses, fibulae
- Cutting of shafts into 5-10mm pieces
- Placing of bone pieces in glass bottles, weighing and labelling
- Preparation of 50:50 solution by mixing equal amounts of 99% Chloroform and 99% Methanol
- Adding the 1:3 v/v 50:50 solution into the bone pieces
- Deffatting for 16-24 hours at room temperature (no sonication)
- Decanting of solution and rinsing with distilled water
- Dehydrating with 100% ethanol for 16-24 hours
- Decanting of ethanol and wind drying over 12-16 hours at room temperature

New method

- Selection of cortical bone material from femurs, ulnae, radiuses, fibulae
- Cutting of shafts into 5-10mm pieces
- Washing of bone pieces in distilled water
- Preparation of H_2O_2
- Processing in H_2O_2 for 3-4 hours
- Decanting of solution
- Rinsing off with distilled water
- Dehydrating with 100% ethanol for 16-18 hours
- Decanting of ethanol and wind drying over 12-16 hours at room temperature

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Methodology

Histology: Histological analysis was done to ensure that the structure of bone is intact and to compare the outcome of removal of blood and bone marrow from the different methods.

Residual chemical content analysis: A Gas chromatography–mass spectrometry (GC-MS) system was set up in order to detect both methanol and chloroform at these levels, and perform a Solid Phase Microextraction (SPME) analysis.

A Redox titration method to determine the residual hydrogen peroxide content.

Residual fat content analysis: Soxhlet extraction was performed since it is one of the most commonly used methods for determination of total fat. This is mainly because it is fairly simple to use and is the officially recognized method for a wide range of fat content determinations.

Microbiology testing: Anaerobic and aerobic cultures were performed to test for the inoculated bacterial growth.

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Results

Visual inspection

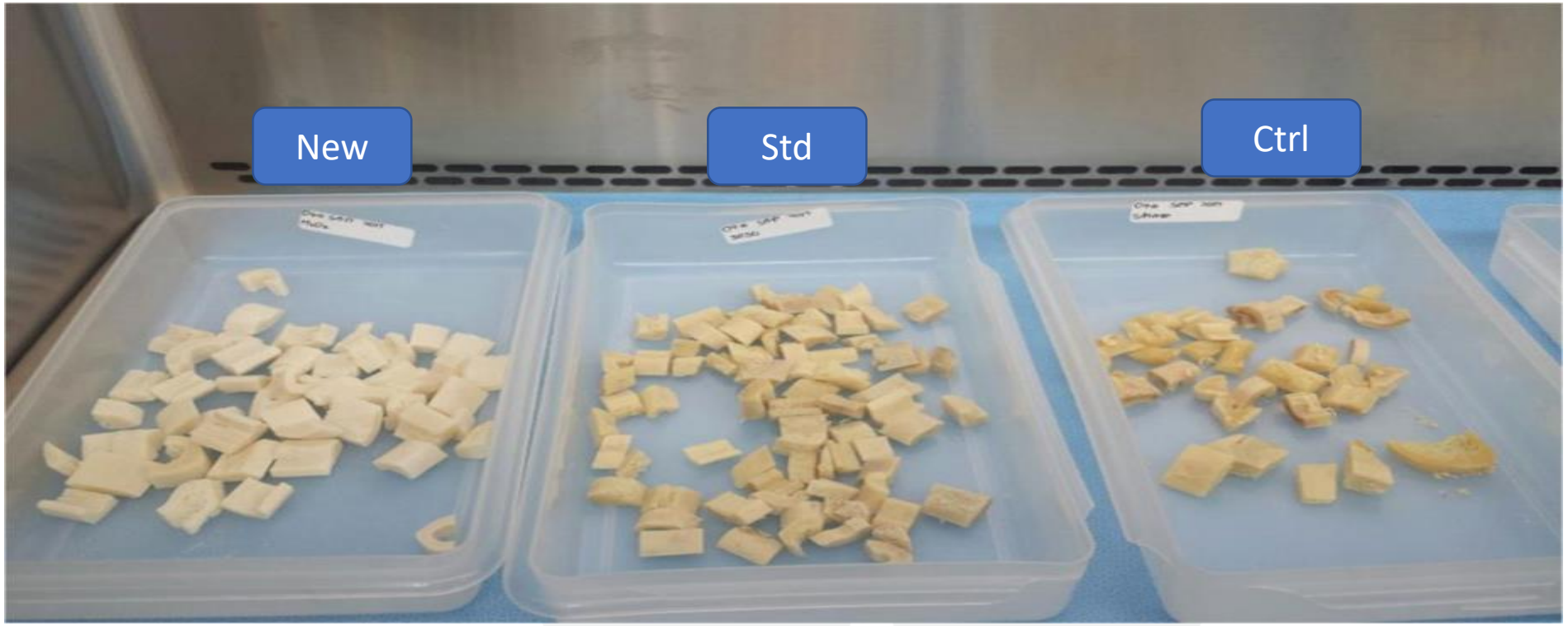

Image 1 above shows the batches of cortical pieces treated with the three methods in the study. By visual inspection, it is clear that the new method utilizing hydrogen peroxide produced a cleaner and more aesthetically pleasing bone.

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Results

Microscopic histology results

When analysing the histology results both the standard method and the new method effectively eradicated the endogenous materials. Only two samples processed with the standard method presented some periosteal fibrous connective tissue & showed incomplete removal of endogenous material. While the other showed the presence of bone marrow and adipose tissue.

For the new method, only two samples showed the presence of osteocytes and none of the samples had any indications of bone marrow or adipose tissue.

Image 2: New method

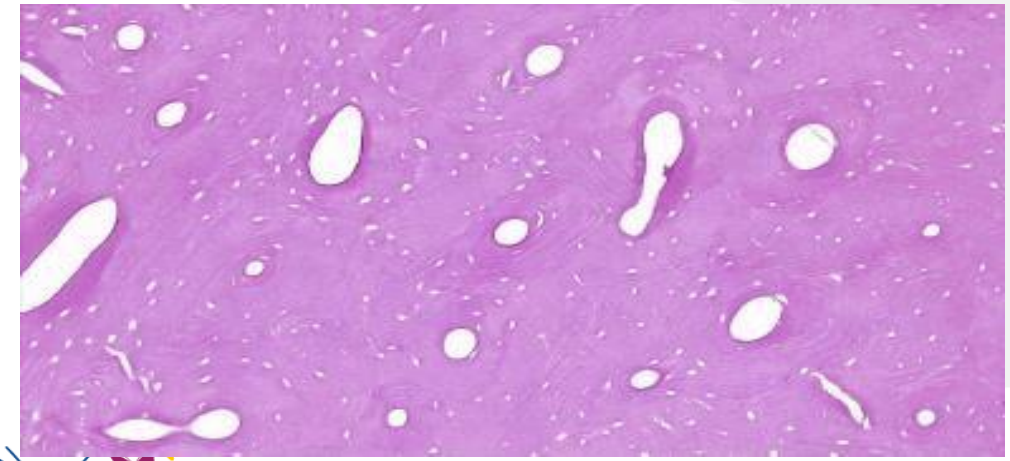
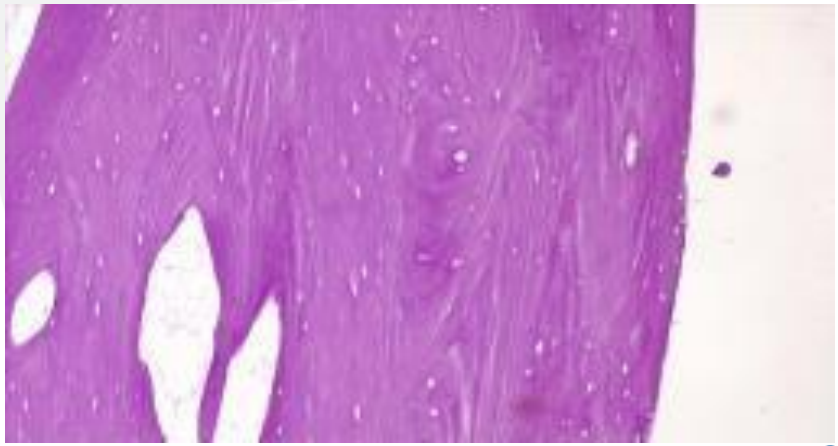


Image 3: STD method



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Results

Microbial test results under aerobic and anaerobic culture

	Under Aerobic conditions		Under Anaerobic conditions	
DONOR NUMBER	STANDARD METHOD	NEW METHOD	STANDARD METHOD	NEW METHOD
028 Aug 2017	No growth	No growth	No growth	No growth
008 Mar 2014	No growth	No growth	E.aerogenes	No growth
001 Oct 2013	No growth	No growth	No growth	No growth
007 Feb 2014	No growth	No growth	No growth	No growth
O26 May 2015	No growth	No growth	No growth	No growth
O65 May 2013	No growth	No growth	No growth	No growth
038 Apr 2014	No growth	No growth	No growth	No growth
002 Jan 2014	No growth	P.aeruginosa	E.aerogenes	No growth
044 Sep 2017	P.aeruginosa	P.aeruginosa	No growth	No growth
072 Sep 2014	No growth	No growth	No growth	No growth

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Results

Percentage Fat Analysis of Samples from Three Different Methods

Sample	M2 (g)	M1 (g)	% Fat	Sample	M2 (g)	M1 (g)	% Fat	Sample	M2 (g)	M1 (g)	% Fat
Saline				**H_2O_2**				**50:50**			
028 Aug 2017	163.68	162.60	7.2	028 Aug 2017	164.55	164.29	1.7	028 Aug 2017	164.58	164.44	0.9
007 Feb 2014	163.98	162.89	7.2	007 Feb 2014	165.73	165.29	3.0	007 Feb 2014	163.92	163.43	3.3
026 May 2015	165.65	164.46	7.9	026 May 2015	162.92	162.56	2.4	026 May 2015	165.44	165.13	2.1
008 Mar 2014	165.01	164.64	2.5	008 Mar 2014	165.09	164.86	1.5	008 Mar 2014	163.41	163.34	0.5
044 Sept 2017	163.27	164.11	5.6	044 Sept 2017	166.02	165.96	0.4	044 Sept 2017	164.74	164.64	0.7
038 Apr 2014	166.12	165.31	5.4	038 Apr 2014	163.81	163.70	0.7	038 Apr 2014	164.34	164.19	1.0
065 May 2013	164.51	162.86	11.0	065 May 2013	166.35	165.92	2.9	065 May 2013	165.13	164.61	3.5
072 Sept 2014	165.91	165.07	5.6	072 Sept 2014	165.00	164.63	2.5	072 Sept 2014	164.95	164.57	2.5
002 Jan 2014	166.81	164.87	13.0	002 Jan 2014	163.56	163.31	1.7	002 Jan 2014	164.07	163.40	4.5
001 Oct 2013	165.62	165.07	6.0	001 Oct 2013	163.43	163.32	0.7	001 Oct 2013	163.70	163.23	3.1

We have followed the Soxhlet extraction method with Petroleum ether.
M2=with extracted oil, M1=without extracted oil.

Mo=15g sample size

Formulation %
M2-M1*100/Mo

- In 50% of the samples, the new method had lower fat percentages.
- Additionally, a closer look into the results as shown in the table, indicates that the new method have significantly lower percentages overall.

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Results

Summary of results for chloroform and methanol residue in bone samples

*max quantifiable level of 50ppm for chloroform & 70ppm for methanol	Concentration (µg/gor ppm)		Sample name	Concentration (µg/g or ppm)		Samplename	Concentration (µg/g or ppm)	
	Chloroform	Methanol		Chloroform	Methanol		Chloroform	Methanol
SOLIDBONE			MILLEDBONE			MILLED BONE SALINE CONTROL		
007 Feb 2014_50/50	33..25	*>70	001 Oct 2013_50/50	15.94	LOD	001Oct2013_Saline	LOD	LOD
026 May2015_50/50	24..53	*>70	002 Jan 2014_50/50	38.43	*>70	001 Oct 2013_Saline	LOD	LOD
028 Aug 2017_50/50	LOD	*>70	007 Jan 2014_50/50	29.39	*>70	002 Jan 2014_Saline	LOD	LOD
038 Apr 2014_50/50	*>50	*>70	008 Mar2014_50/50	LOD	*>70	007 Feb 2014_Saline	LOD	LOD
072 Sep 2014_50/50	22..26	47.26	026 May2015_50/50	LOD	35.87	008 Mar 2014_Saline	LOD	LOD
			**028 Aug 2017_50/50	23.60	*>70	026 May 2015_Saline	LOD	LOD
			**028 Aug 2017_50/50	23.77	*>70	028 Aug 2017_Saline	LOD	LOD
			**028 Aug 2017_50/50	23.44	*>70	038 Apr 2014_Saline	LOD	^23.66
			038 Apr 2014_50/50	14.47	52.89	044 Sep 2017_Saline	LOD	LOD
			044 Sep 2017_50/50	LOD	*>70	065 Feb 2013_Saline	LOD	LOD
			065 May2013_50/50	14.01	LOD	072 Sep 2014_Saline	LOD	LOD
			072 Sep 2014_50/50	42.44	*>70	LOD (limit of detection) suggests that the compound was below confident detection levels.		

Five solid bone samples were tested to determine whether there is any difference in residual content between solid samples and milled samples.

Based on the concentration levels there isn't much of a difference.

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Results

Summary of results for hydrogen peroxide residue in bone samples

Sample name	Concentration (µg/g or ppm)	Sample name	Concentration (µg/g or ppm)	Samplename	Concentration (µ/g or ppm)
	Hydrogen peroxide		Hydrogen peroxide		Control group
SOLIDBONE		MILLEDBONE		MILLED BONE	
007 Feb2014	LOD	001 Oct 2013	LOD	001Oct2013_Saline	LOD
026 May2015	LOD	002 Jan 2014	LOD	002 Jan 2014_Saline	LOD
028 Aug 2017	LOD	007 Jan 2014	LOD	007 Feb 2014_Saline	LOD
038 Apr 2014	LOD	008 Mar2014	LOD	008 Mar2014_Saline	LOD
072 Sep 2014	LOD	026 May2015	LOD	026 May 2015_Saline	LOD
		028 Aug 2017	LOD	028 Aug 2017_Saline	LOD
		038 Apr 2014	LOD	038 Apr 2014_Saline	LOD
		044 Sep 2017	LOD	044 Sep 2017_Saline	LOD
		065 May2013	LOD	065 Feb 2013_Saline	LOD
		072 Sep2014	LOD	072 Sep 2014_Saline	LOD

Five solid bone samples were tested to determine whether there is any difference in residual content between solid samples and milled samples.

LOD (limit of detection) suggests that the compound was below confident detection levels.

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Results

Cost Analysis

Total chemical cost spent for the study:

The table below indicates that the new method has a significantly lower cost of chemicals than that of the standard method which is a good finding since it is important to keep cost of operation as low as possible.

Method	Money spent	Price per quantities used in this study
Standard method	R1 353.20	5L at R707.80
New method	R591.40	5L at R87

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Time spent on chemical processing

The table below shows a significantly longer period of processing that the standard method takes. This means that by implementing the new method, processing time can be decreased by 30%.

Method	Time spent
Standard method	Over 67 hours
New method	Just over 20 hours

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Conclusion

The aim of the study was to evaluate two bone processing methods for efficacy based on a range of analysis. A standard method utilizing chloroform and methanol was compared to a new method using hydrogen peroxide.

Analysis	STD method	NEW method
Visual inspection	√	√√
Histology	√	√√
Microbiology	√	√√
Fat content	√	√√
Residual chemical content	√	√√
Cost analysis	√	√√
Time spent	√	√√

The study concluded that the new method was successful in all the criteria measured.

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