Analysis of Characteristics of Cancellous Rib Freeze-Dried Bovine Bone as a Substitution Material

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Abstract

Bone substitution materials are widely used in dentistry to replace bone defects. Xenografts offer an alternative to autografts, allografts and alloplast by addressing their limitations. Objective: To analyze characteristics of cancellous rib freeze-dried bovine bone as a potential bone substitute material.

Bovine 4th cancellous rib were cut into 10 mm x 10 mm x 10 mm. Then soak in H_2O_2 for 7 x 24 hours, then cleaned with 95% alcohol in ultrasonic shaker for 6 x 30 minutes, then Freeze-dried for 4x24 hours. Test method using FTIR, XRD, SEM-EDX, ICP-OES.

There are functional groups -OH, PO_4^{3-}, N-H, C-N, CO_3^{2-} and C-O-C, amorphous crystal form, pore size diameter 50.89 µm - 530.7 µm, porosity percentage 95.94%, and bone density 0.094 g/cm3, and there are C(Carbon), N(Nitrogen), O(Oxygen), F(Fluorine), Mg(Magnesium), Al(Aluminum), Si(Silicon), P(Phosporus), Ca(Calcium) content, and heavy metals Pb and Ni below toxicity limit (Pb<30ppm, Ni<25ppm).

Cancellous rib freeze-dried bovine bone have physical and chemical characteristic suitable as a bone substite material.

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Introduction

Reconstructive surgery is extensively performed, with more than 4.5 million procedures conducted worldwide annually [1]. Bone reconstruction can be performed using bone grafts matched to the defect area to facilitate healing and restore the anatomy and functionality of the affected bone [2]. Bonegraft often used in maxillary sinus floor elevation, peri-implant intrabony defects, and periodontal intrabony defects [3].

Xenograft complements the shortcomings of autograft, allograft and alloplast [4]. Bovine femur, commonly processed for bone blocks with the femoral condyle as the retrieval site, is limited to a maximum dimension of 30 mm x 15 mm x 10 mm [5]. This limitation makes bovine rib a promising alternative xenograft source. Bovine rib bone, widely used as a simulation for replacing human mandibular bone due to its resemblance in size, has potential as an ideal bone block material for reconstructing large mandibular defects [6].

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An ideal bone substitute material should exhibit osteoconduction, osteoinduction and osteogenesis properties simultaneously. Bone itself is a complex composite material composed of both inorganic and organic components. The primary inorganic component is hydroxyapatite crystals ($Ca_{10}[PO_4]_6[OH]_2$), while the organic matrix consists of over 30 proteins. Quantitatively, the inorganic composition constitutes approximately 60% of the tissue mass, while the organic component contributes roughly 30%. The remaining 10% is attributed to water [7].

Substitute material must provide adequate mechanical support to the skeletal system and actively stimulate bone regeneration, ultimately aiming for complete bone replacement. This functional integration can be further examined through specific physical properties, such as pore size diameter, porosity percentage, density and bone content [8].

Ideally, the substitute material should possess a matrix closely resembling the natural extracellular matrix structure of the missing tissue. This design aims to facilitate the efficient infiltration, proliferation and differentiation of cells, ultimately promoting tissue development within the substitute material in a manner similar to natural tissue [9].

Bovine bones used as substitute materials can be contaminated with heavy metals if the animal consumed contaminated food or water. Lead (Pb) and nickel (Ni) are of particular concern due to their well-documented carcinogenic and toxic properties [10,11].

This study aims to characterise bovine rib bone blocks based on functional groups, crystal form, heavy metal content, pore size diameter, porosity, density and material content.

Materials and methods

4th rib bone of Madura cow (Slaughterhouse Surabaya), bovine bone cancellous (Dr. Soetomo General Academic Hospital Tissue Bank Surabaya), hydrogen peroxide 3% (Purelizer Pure Indonesia), alcohol 95% (OneMed), and distilated water (Botanica Asri Surabaya).

Fabrication of Cancellous Bone Blocks from Bovine Rib

Rib bones were separated from the meat and cortical bones, so the cancellous bones were left behind. Cancellous ribs were cut into 10 mm x 10 mm x 10 mm blocks followed by immersion in 3% H_2O_2 for seven days. Each immersion period was followed by rinsing with distilled water. Subsequently, the samples were treated with 95% alcohol in an ultrasonic shaker for six cycles of 30 minutes each. The final step involved freeze-drying for four days.

Functional groups test

The sample is pulverised into a fine. This powder is carefully placed in the centre of sample pan. Placed the infrared beam accurately targets the centre of the mixture. The software program starts running. The results will appear on the monitor screen.

Crystal forms test

The sample is sanded and coated, then placed on a holder in the form of a circular plate with a cover with a diameter of 10-15 mm. The holder filled with samples is inserted into the XRD tool. The monitor was rotated around the sample and set at an angle of 2θ. The monitoring tool is aligned so that the axis matches the sample rotation axis. The XRD results will be printed on paper with a copper radiation source with a nickel filter.

Pore size diameter and material content test

Sample were cut into small pieces with a size of 3 mm so that it could be placed on the SEM holder. The SEM holder is coated with 2-sided carbon tape. The sample that has been placed on the holder is put into a sputter coater for coating, then vacuumed for 30 minutes. The bone block sample is a conducting material so plasma coating using gold (Au) is required for 3 seconds. The sample is inserted into the specimen chamber and irradiated with an electron beam. The electron beam is then converted into an electrical signal, amplified and transmitted to the Cathode Ray Tube (CRT). Selecting and enlarging certain objects is carried out on the sample and photography is carried out to obtain morphological photos of the sample. The magnification used for the SEM test is 100x and 500x. SEM and EDX software are connected and data is collected from the sample.

Porosity percentage and bone density test

Formulas (1) and (2) were used to calculate the porosity and density of the bone block, respectively[12]:

$$\text{Relative density (\%)} = \frac{\text{Bulk density}}{\text{Theoretical density}} \times 100\% \quad (1)$$

$$\text{Total porosity (\%)} = 100\% - \text{Relative density (\%)} \quad (2)$$

Description:

Relative density=The density ratio of a material

Bulk density=Sample weight divided by sample volume

Theoretical density=The maximum density of a material that can be reached

The theoretical density for Hydroxyapatite (HA) is 3.16 g/cm^3 [13].

Heavy metals test

The sample was weighed at 0.7 grams then burned at a temperature of 8000C for 1 hour. The sample that had become ash was added with 5ml nitric acid and heated until the ash was completely dissolved. The sample was filtered using Whatman filter paper into a volumetric flask and filled with distilled water up to 50ml. The sample is placed in the autosampler. The software program starts to run. The results will appear on the monitor screen.

Results

Bone block fabrication results

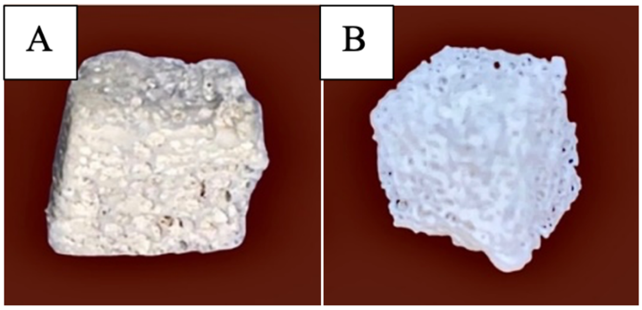


Figure 1. Cancellous rib freeze-dried bovine bone (A); Bovine bone cancellous Dr. Soetomo General Academic Hospital Tissue Bank (B).

Macroscopically, both samples exhibit a porous structure and are cube-shaped (block). Bovine bone cancellous Dr. Soetomo General Academic Hospital Tissue Bank is whiter in color compared to the sample produced in this study.

Functional Group

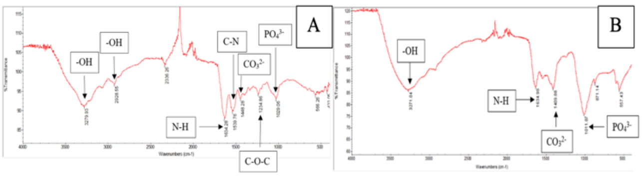
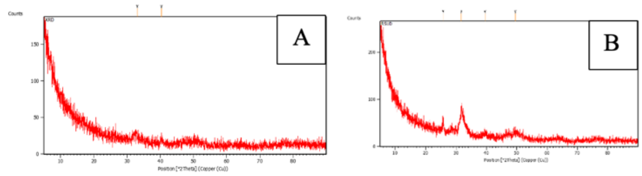


Figure 2. Functional groups analysis results, cancellous rib freeze-dried bovine bone (A); Bovine bone cancellous Bank Jaringan RS Dr. Soetomo (B).

Functional groups identification of bovine bone cancellous block Dr. Soetomo General Academic Hospital Tissue Bank contains OH (3271.04 cm^{-1}), NH (1634.98 cm^{-1}), CO_3^{2-} (1409.88 cm^{-1}), dan PO_4^{3-} (1011.07 cm^{-1}). Whereas block cancellous rib freeze-dried bovine bone contains OH (3279.93 cm^{-1}, 2928.55 cm^{-1}), NH (1634.28 cm^{-1}), CN (1539.76 cm^{-1}), CO_3^{2-} (1448.28 cm^{-1}), COC (1234.86 cm^{-1}), dan PO_4^{3-} (1029.06 cm^{-1}).

Crystal Form



Peak List: (Bookmark 3)

Pos. [°2Th.]	Height [cts]	FWHM Left [°2Th.]	d-spacing [Å]	Rel. Int. [%]
33.0269	27.13	0.0900	2.71003	67.34
40.2851	40.29	0.0900	2.23692	100.00

Peak List: (Bookmark 3)

Pos. [°2Th.]	Height [cts]	FWHM Left [°2Th.]	d-spacing [Å]	Rel. Int. [%]
25.7813	18.44	0.5353	3.45570	14.69
31.7136	35.77	0.5353	2.82153	28.48
39.5407	125.58	0.0900	2.27730	100.00
49.5906	55.84	0.0900	1.83677	44.46

Figure 3. Crystal form analysis results, cancellous rib freeze-dried bovine bone (A); Bovine bone cancellous Dr. Soetomo General Academic Hospital Tissue Bank (B).

Test result on block cancellous rib freeze-dried bovine bone have 2 peaks, 27.13 cts at 33.0269°, and 40.29 cts at 40.2851°, also the degree of crystallinity 6.9%. Whereas test result on bovine bone cancellous Dr. Soetomo General Academic Hospital Tissue Bank have 4 peaks, 18.44 cts at 25.7813°, 35.77 cts at 31.7136°, 125.58 cts at 39.5407°, and 55.84 cts at 49.5906°, also the degree of crystallinity 17.3%.

Pore Size Diameter

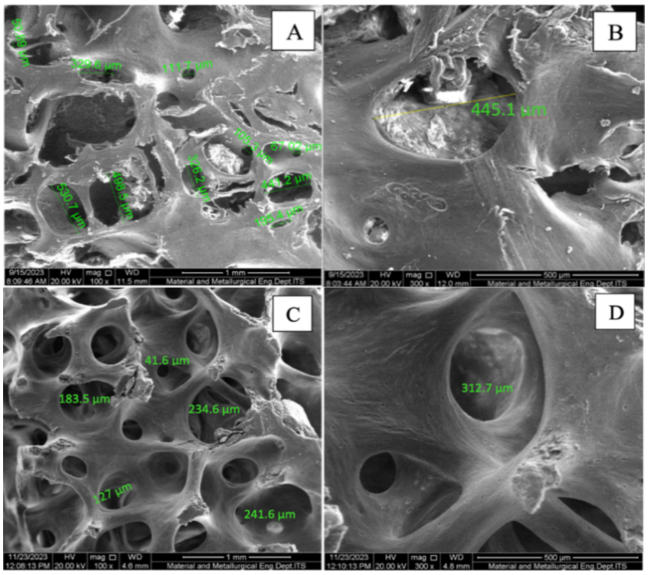


Figure 4. Pore size diameter of block cancellous rib freeze-dried bovine bone with 100× magnification (A), and 300× magnification (B),

Pore size diameter ofbovine bone cancellous Dr. Soetomo General Academic Hospital Tissue Bank with 100× magnification (C), and 300× magnification (D).

The pore size diameter of block cancellous rib freeze-dried bovine bone ranges from 50.89 μm to 530.7 μm. Pore size diameter of bovine bone cancellous Dr. Soetomo General Academic Hospital Tissue Bank in the range of 41.6 μm to 241.6 μm.

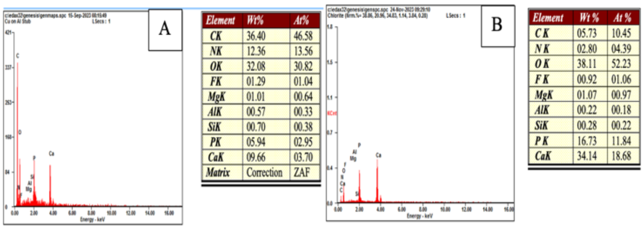
Porosity percentage

The percentage of porosity of the block cancellous rib freeze-dried bovine bone was 89.56%, while the bovine bone cancellous Dr. Soetomo General Academic Hospital Tissue Bank amounted to 88.29%.

Material density

Density analysis of block cancellous rib freeze-dried bovine bone revealed a value of 0.33 g/cm³, whereas bovine bone cancellous Dr. Soetomo General Academic Hospital Tissue Bank exhibited a slightly higher density of 0.37 g/cm³.

Material content



Element	Wt%	At%
CK	36.40	46.58
NK	12.36	13.56
OK	32.08	30.82
FK	01.29	01.04
MgK	01.01	00.64
AlK	00.57	00.33
SiK	00.70	00.38
PK	05.94	02.95
CaK	09.66	03.70
Matrix	Correction	ZAF

Element	Wt %	At %
C K	05.73	10.45
N K	02.80	04.39
O K	38.11	52.23
F K	00.92	01.06
MgK	01.07	00.97
AlK	00.22	00.18
SiK	00.28	00.22
P K	16.73	11.84
CaK	34.14	18.68

Figure 5. Material content of block cancellous rib freeze-dried bovine bone (A), and bovine bone cancellous Dr. Soetomo General Academic Hospital Tissue Bank (B).

Block cancellous rib freeze-dried bovine bone and bovine bone cancellous Dr. Soetomo General Academic Hospital Tissue Bank contain nitrogen (N), oxygen (O),fluorine (F), magnesium (Mg), aluminum (Al), silicon (Si), phosphorus (P) and calcium (Ca).Ca/P ratio block cancellous rib freeze-dried bovine bone with a value of 1.63, while bovine bone cancellous Dr. Soetomo General Academic Hospital Tissue Bank with a score of 2.04.

Heavy metal content

No	Heavy metals	Toxicity limit (ppm)	Blok *cancellous rib freeze-dried bovine bone*	*Bovine bone cancellous* Bank Jaringan RS Dr. Soetomo
1	Plumbum (Pb)	30	14,679	10,581
2	Nikel (Ni)	25	8,083	4,695

Table 1. Heavy metal content of block cancellous rib freeze-dried bovine bone and bovine bone cancellous Dr. Soetomo General Academic Hospital Tissue Bank.

Discussion

Bovine bone cancellous Dr. Soetomo General Academic Hospital Tissue Bank does not have the C-O-C and C-N functional groups, while the block cancellous rib freeze-dried bovine bone have the C-O-C and C-N functional groups. C-O-C and C-N are functional groups of collagen. Collagen can experience denaturation starting at a temperature of 46.85°C. The physico-chemical phenomenon that controls the denaturation process is the shrinkage of bone mesostructure due to loss of water. Bone mesostructural shrinkage is greater in the femur than in the rib [14].

Research by Kuhn et al.,[15] states that bovine cancellous bone has fewer crystals than cortical bone because bone remodeling activity is greater in cancellous bone. The amorphous phase in the resulting crystal form can also be influenced by the organic phase because the organic matrix can interfere with accurate mineral characterization.

The shape of amorphous crystals is also influenced by the freeze-dry or sintering temperature when making substitute materials. Material that is not sintered (heated below the melting point with the aim of forming a new crystal phase) has lower crystallinity than sintered material. Crystallinity is very dependent on the sintering temperature because a high sintering temperature produces more perfect crystals so that the degradation rate is lower [16].

Block freeze-dried bovine bone cancellous rib are similar to amorphous calcium carbonate and it can be interpreted that this material is amorphous with semi-crystalline characteristics because there are more sloping peaks. Amorphous calcium carbonate (ACC) is known as a precursor phase of $CaCO_3$ crystals that plays an important role during biomineralization [17].

The size of the pore diameter of the block cancellous rib freeze-dried bovine bone and cancellous bovine bone Dr. Soetomo General Academic Hospital Tissue Bank meets the standard diameter of porosity for bone substitute materials with a diameter size range of 100 - 500 µm[18].

Percentage of porosity of block cancellous rib freeze-dried bovine bone and cancellous bovine bone Dr. Soetomo General Academic Hospital Tissue Bank meets the requirements for a substitute material pore percentage that has a standard above 80%. The higher the percentage of pores in the bone substitute material, the better the bone cell proliferation and differentiation process will be [19]. The difference in pore diameter size diameter and porosity percentage between the two is probably caused by the difference in the duration of the freeze-drying process which affects the water content removed.

Block density of cancellous rib freezedried bovine bone and cancellous bovine bone Dr. Soetomo General Academic Hospital Tissue Bank is influenced by the size and percentage of pores in the material. The higher the diameter and percentage of pores, the smaller the density value of the bone substitute material. This is because the mechanical strength of hydroxyapatite which has brittle properties will be lower if it has a percentage and size of pore size diameter that tends to be high [20].

The ratio results of Ca/P cancellous rib freeze-dried bovine bone and cancellous bovine bone Dr. Soetomo General Academic Hospital Tissue Bank are nearly at 1.67. Ca/P ratio of 1.67 is the standard for hydroxyapatite because it has good mechanical properties and is strong against fracture. The calcium (Ca) content in bones plays an important role in bone metabolism and remodelling, while phosphorus (P) contributes to the formation of hydroxyapatite crystals which provide strength and stiffness to bones. Too much calcium content can cause the formation of bone tissue that is less mineralized so that the bones become weaker and brittle. Too much phosphorus content can inhibit bone mineralization and disrupt the structural integrity of the bone graft [21].

The metals lead (Pb) and nickel (Ni), require testing because of their potential toxicity at high levels. Pb have carcinogenic properties and therefore pose a risk of cancer, while Ni cause their toxicity through interference with oxidative stress mechanisms [10,11].

The results between the two samples are below toxicity level, it indicates a potential for bone substitute material. Research shows that livestock or livestock living close to human settlements or urban areas are more susceptible to the accumulation of high amounts of heavy metals. This increased vulnerability most likely comes from contaminated inputs such as crops, soil, and water exposed to sources such as industrial waste, vehicle emissions, or agricultural chemicals [22].

Conclusions

Block cancellous rib freeze-dried bovine bone have physical and chemical characteristic suitable for bone substitue material.

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Declaration of Interest

The authors report no conflict of interest.

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