How to Cite:
Abidin, I. Z. Z., Kumar, Y., Ariffin, Z. Z., & Ariffin, S. H. Z. (2022). Human osteoblast and osteoclast stem cell differentiation: A systematic review of biochemical analysis. *International Journal of Health Sciences*, *6*(S7), 5123-5135. https://doi.org/10.53730/ijhs.v6nS7.13100

Human osteoblast and osteoclast stem cell differentiation: A systematic review of biochemical analysis

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***Abstract*---**Stem cells are being better understood in today's research pace of cell biology research and its applications in bone regenerative medicine. However, a vast variety of techniques are available to analyse the differentiation of human osteoblasts and osteoclasts from stem cells. Hence, this review aims to identify and evaluate the biochemical approaches in analysing osteoblast and osteoclast stem cell differentiation using human stem cells. The method used in this study is a systematic review. Four articles were selected from PubMed and Web of Science (WOS) databases based on specific inclusion criteria and following PRISMA guidelines. One main approach was used as the focus point of the review, namely the biochemical (protein and enzyme activity in the cell) approach. This review summarises the findings of all relevant stem cell studies using a biochemical approach in understanding osteoblast and osteoclast differentiation. The results show trends in the usage of biochemical approaches to analyse osteoblast and osteoclast differentiation; the most used techniques for quantitative biochemical analyses are ALP and TRAP assays respectively.

International Journal of Health Sciences ISSN 2550-6978 E-ISSN 2550-696X © 2022.
Manuscript submitted: 9 May 2022, Manuscript revised: 18 July 2022, Accepted for publication: 27 August 2022

***Keywords*---**Biochemical, Osteoblast, Osteoclast, Stem cell, Differentiation.

Introduction

Stem cells are cells that can reproduce on their own through the process of division or duplication and differentiate into various cell types. Stem cells can divide and grow relentlessly to be able to form a perfect human being such as in the uterus, or specialised tissues and organs. These cells are primitive cells that have the potential to produce various other cell types ranging from the more mature ones to forming a complete organism (Intan Zarina et al. 2010).

Stem cells come in many different types, sources, and levels of differentiation. There are five potential levels of stem cell differentiation (Shahrul Hisham et al. 2005): totipotent, pluripotent, multipotent, oligopotent, and unipotent stem cells. Totipotent stem cells are cells formed after the fertilisation of sperm and eggs. These cells can form embryonic as well as extra-embryonic tissue. Totipotent cells can differentiate into any type of cell in the body and ultimately develop into a complete organism. Pluripotent cells can differentiate into any of the germ layers, i.e., ectoderm, mesoderm, and endoderm. On the other hand, multipotent stem cells can only differentiate into a few specific cell types that belong to the stem cell lineage. Oligopotent stem cells are very similar to multipotent cells but have a lesser ability to differentiate into other cells as compared to multipotent cells. Finally, unipotent stem cells have the most limited differentiation potential, i.e., differentiating into only one cell type. This type of cell is also known as a progenitor cell (Shahrul Hisham, 2017).

There are also several types of stem cells inclusive of embryonic stem cells (ESC). ESC cells are pluripotent stem cells that are derived from the inner cell mass (ICM) of an early developing blastocyst. Induced pluripotent stem cells (iPSCs) on the other hand are already differentiated somatic cells that have been reprogrammed to enter an ES-like cell-like state (Christopherson & Nesti 2011).

Adult stem cells consisted of Mesenchymal Stem Cells (MSC) and Haemopoietic Stem Cells (HSC) mainly originate from the bone marrow, i.e., 0.01-0.05% of cells in the bone marrow. However, they can also be isolated from various other sources, such as compact bone, adipose tissue, cord blood, peripheral blood, fallopian tube, and foetal liver and lung. (Muhmmad Dain et al. 2011; Ruzanna et al. 2012). The peripheral blood mononucleated cells were shown to exhibit osteogenic differentiation, i.e., osteoblast and osteoclast cells when induced by specific growth factors. Osteoblast and osteoclast are mature cells originating from MSC and HSC, respectively. This concluded that peripheral blood mononucleated stem cells possess two distinct types of stem cells also known as multipotent stem cells (Intan Zarina et al. 2008).

Osteoblast and osteoclast cells are involved in the bone remodelling process, i.e., bone degradation, resorption, and bone formation. Osteoblasts are the cells responsible for the synthesis and mineralization of bone during bone formation and remodelling. Osteoclasts, on the other hand, are multinuclear

cells responsible for the degradation and resorption of bone (Intan Zarina et al. 2008, Muhammad Dain et al. 2011, Shahrul Hisham 2017). During remodelling, the osteoblasts, which secrete bone matrix, will produce several signalling factors to recruit pre-osteoclasts to the bone surface where the bone needs to be rebuilt. The pre-osteoclasts will differentiate into mature multinuclear osteoclasts and begin the resorption process. During the bone resorption process, the mature osteoclasts secrete hydrogen ions, which form an acidic environment that results in the production of specific bone-degrading enzymes. (Abe et al. 2019).

Biochemical analysis refers to the analysis of cell biochemical activity. The natural biomolecules produced by the cell are analysed such as the presence and action of enzymes, which are functional proteins that carry out anabolic or catabolic functions in the cell. This approach provides an overview of the cell differentiation process as different enzymes are expressed and produced at different stages of cell differentiation. This study aims to systematically identify and compare various biochemical assays and techniques that are commonly and currently used in analysing the differentiation of osteoblast and osteoclast from human stem cells.

2.0 Methodology

This systematic review was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statements (Page et al. 2021). A single reviewer performed the searches and evaluations on the articles to determine their eligibility of the articles for this review. Another reviewer was included to sort out any discrepancies from the stated methods.

2.1 Data Search

The studies included in this systematic review were retrieved from PubMed and Web of Science (WOS) databases. Independent keywords together with their combinations were applied to the search engines of these databases. One search string was used for each database. The search string comprised of the following keywords: Biochemical; Osteoblast; Osteoclast; Human and Differentiation (Figure 1).

2.2 Selection Criteria

Inclusion and exclusion criteria were aligned and followed. Only original articles that were published in the English language from the year 2016 to 2022 were included, while review articles and duplicated articles were excluded from this review. For this review, *in vitro* and *ex vivo* studies involving human stem cells' potential to differentiate into osteoblasts and osteoclasts were included whereas studies using any animal cell lines or primary cultures were excluded. In addition, *in vivo* studies were not considered in this review as well. Studies that had combinations of both animal and human cultures were included but only considered the human section of the studies.

2.3 Data Extraction and Screening Process

Data screening and data extraction were conducted following the PRISMA guidelines (Page et al. 2021). The initial screening was done to sort out any review articles and articles published other than in the English language. Next, articles that did not utilise human stem cells and did not match the parameters of osteoblastic and osteoclastic differentiation were also removed. All the remaining articles were then thoroughly screened for their eligibility.

3. Results

3.1 Systematic selection of Biochemical Approaches for Osteoblast and Osteoclast Detection

The search in online databases, i.e., PubMed (26 articles) and Web of Science (WOS) (32 articles) produced a total of 48 articles. Every potential article was assessed independently based on the inclusion and exclusion criteria. After removing 6 duplicates between the two databases, 42 articles remained. Five review articles were excluded, followed by 16 articles that did not match the parameter of human study and 16 articles which are not relevant to the study. After the thorough and systematic screening, a total of four articles published between 2016 and 2022 were eligible for qualitative analysis for this current systematic review. A flowchart on the article selection process is shown in Figure 2.

In this systematic review, three studies focused on the differentiation of osteoblasts, and one focused on the combination of both osteoblast and osteoclast differentiation. However, no studies focused specifically on osteoclast differentiation. Of the four selected articles, a single article involved was an *ex vivo* study while the other three were i*n vitro* studies. The cultured cells used for the *ex vivo* and *in vitro* studies include human mesenchymal stem cells (hMSC) (Carbonare et al. 2017; Kwon et al. 2016; Heinemann et al. 2019), human blood peripheral mononuclear cells (hBPMC) (Heinemann et al. 2019), and human peripheral blood stem cells (hPBSC) (Tsirkinidis et al. 2018).

3.2 Biochemical Analysis Results

Among the methods commonly used to biochemically analyse osteoblast stem cells are Total Alkaline Phosphatase (ALP), Alkaline Bone Phosphatase (B-ALP), Osteocalcin (OC, BGP), Procollagen type I terminal-C propeptide (PICP), and N-terminal propeptide procollagen type I (PINP). On the other hand, the common methods used to biochemically analyse osteoclast stem cells are Hydroxyproline, total and dialyzable (OH-Pro, OHP), Pyridinoline (PYD, Pyr), Deoxypyridinoline (DPD, d-pyr), C-terminal telopeptide cross-linked collagen type I (ICTP), Type I collagen cross-linked C-terminal telopeptides (alpha-CTX, beta-CTX fragments), Type I collagen-type I cross-linked N-terminal Telopeptides (NTX-fragments), Hyl-Glyc hydroxylin-glycoside, Bone sialoprotein (BSP)), Tartrate-resistant phosphatase acid (TRAP) and gamma-free carboxyglutamic acid (GLA). These are

biochemical assays for biochemical bone turnover markers that are known to be used clinically.

This review shows various methods for biochemical analysis that have been used to study the differentiation of osteoblasts and osteoclasts. The biochemical analytical approaches from the four selected articles were ELISA (enzyme-linked immunosorbent assay), ALP (Alkaline Phosphatase) assay, Calcium deposition assay, PTH, CTX, DNA, LDH (Lactate Dehydrogenase) assay (osteoblast), and TRAP5b (Tartrate-resistant acid phosphatase 5b) assay (osteoclast). The summary of the result in Table 1 consists of all the methods involved and the cell sources as well as the findings of the studies.

4. Discussion

Data obtained from the search were focused on the biochemical approach to studying osteoblast and osteoclast stem cell differentiation and extracted only related details from articles from two databases, i.e., PubMed and WOS. There were most likely articles relevant to and within the scope of this study that did not appear in the search results in the databases, therefore they were not reviewed.

4.1 Types of Stem Cells

The cell variant that was most used in this review was human mesenchymal stem cell (hMSC) (3 studies). Human bone marrow-derived mesenchymal stem cells (hBMSC) (1 study) were also used, followed by hMSC cell lines from human peripheral blood (1 study), and primary culture of hMSC from blood peripheral monocytes (1 study).

The properties of hBMSC such as ease of isolation from bone marrow without causing an immunological problem and the ability to expand in a short period make them a great model for *in vitro* osteogenic differentiation studies (Bhat et al. 2021; Ouryazdanpanah et al. 2018). hBMSC have shown high osteogenic differentiation under biochemical and/or mechanical stimuli and also osteogenic differentiation potential at a molecular level in which specific osteoblastic gene expression was increased significantly (Ansari et al. 2021).

Another type of cell variant obtained from the data was hematopoietic stem cells (HSC) (2 studies). The HSC and monocytes cells can be determined in principle by specific surface markers such as CD34 and CD14. In contrast to MSC, the isolation of HSC are more labour-intensive which results in a minute number of isolated HSC. Therefore, HSC isolation approaches require a huge amount of bone marrow or peripheral blood cells as compared to MSC (Ansari et al. 2021).

4.2 Stem Cell Differentiation Analysis

Molecular biomarkers such as enzymes and proteins can detect and identify differentiated cells. Studies involving ELISA (1 study) in which the antibodies from the sample are used to locate and quantify individual protein content by using fluorescent tags. However, this qualitative method has limitations in its accuracy.

Stem cells can fuse with host endogenous cells to appear as differentiated cells, and this type of limitation may demand for gene expression analysis to confirm the presence of stem cell-specific proteins (Nguyen, Nag & Wu 2010).

There was no particular trend of biochemical techniques used in the biochemical approach, except ALP assay (2 articles) and TRAP (2 articles) and this could be due to limited articles that fulfilled the inclusion criteria for the biochemical approach. On the other hand, the expression of *RUNX2*, *SPARC*, BMD, BALP, RANKL/OPG, ANGP-1/ANGP-2, and OCN genes were involved in osteoblast and/or osteoclast cell differentiation.

A) Biochemical Analysis of Osteoblastic Differentiation

ALP is an ectoenzyme that is bound to the membrane through phosphatidylinositol glycophospholipid (GPI) linkage. ALP active site involved through the formation of serine phosphate and in alkaline environments it will react with water molecules to release inorganic phosphate. Increased ALP activity represented increased bone formation, therefore represented as a bone formation biomarker (Jafary et al. 2017).

On the other hand, increases in BALP (bone alkaline phosphatase) expression are exhibited by the expansion of bone formation (Kwon et al. 2016). In addition, the presence of E-2, 5-DHB can also stimulate osteoblast differentiation (Heinemann et al. 2019). While the increase in RANKL/OPG (receptor activator of Nf-κB ligand/osteoprotegerin) ratio signifies an increase in osteoclast cell differentiation. In osteoblast, the androgen receptor is present at a low concentration but firmly associated with bone mineral density (BMD) and is not involved during osteoclast activity (Sowers et al. 2009).

Osteonectin that can be found in the bone matrix and blood platelets is the adherent extracellular matrix-binding glycoprotein. However, the isolated osteonectin from both bone matrix and blood platelets differ in the complexity of the N-linked oligosaccharide constituent (Kelm et al. 1992). Furthermore, BSP II also known as bone sialoprotein (BSP) with the estimated size of 70,000 kDa is the major non-collagenous bone matrix protein expressed in osteogenic cells (i.e., osteoblast, osteocytes, and osteoclast), hypertrophic chondrocytes, trophoblast and odontoblast. BSP II was also involved during *in vitro* osteoclast binding and immunolocalization with no accumulation on the bone surface where this osteoclast has attached (Zhou et al. 1994).

Ang-1 is found mostly in bone cells (osteoblast and osteoclast) and some parts of bone marrow, whereas Ang-2 is expressed by osteoblast and osteoclast at various levels during bone modelling and remodelling processes. Therefore Ang-1 and Ang-2 can be used as a biomarker for bone modelling and remodelling process (Horner et al. 2001). Unfortunately, there was no evidence showing that the decrease in the Angiopoietin-1/Angiopoietin-2 ratio (ANGP-1/ANGP-2) would trigger an increase in osteoclastic activity (Kwon et al. 2016).

Parathyroid hormone (PTH) assays can be categorized into three approaches; the 1st generation of competitive immunoassays, the second generation of

immunometric assays, and the current generation assay using mass spectrometry (3rd generation). The first generation involved a single polyclonal antibody binding to PTH followed by a second-generation assay which involved two distinct monoclonal antibodies that were directed against different epitopes, to bind the PTH that is present in the sample where one antibody bound to a solid phase and the other is labelled. The final and current type of approach is based on the mass spectrometry principle that involved serum samples which previously purified through applying the first-generation approach. This current approach/assay was found to be more rapid and sensitive as compared to the previous two (Vieira 2012).

Routine serum creatinine assays that have been used currently were modified from Jaffe, M. in 1886. Jaffe's assay was modified with various versions over the years mainly due to increased specificity. Creatine was isolated from other substances through absorption by aluminium silicate in Lloyd's reagent followed by elution into alkaline picrate. The current approach uses a Jaffe assay at alkaline pH and is followed by acidification to finally form a neutral pH (Peake & Whiting 2006).

B) Biochemical Analysis of Osteoclastic Differentiation

The TRAP 5b is designated due to isoform 5b and is known to be a specific and sensitive enzyme marker for osteoclastic activity. TRAP 5b can be detected in serum and its concentration can be correlated with bone resorption index. TRAP 5b is secreted by osteoclasts that endocytose bone matrix products after digestion with lysosomal protease and acids. The transcytosis vesicles containing bone matrix products will be released into the gap between the cell membrane and the bone matrix and then will be fused to the vesicle containing TRAP. The bone matrix products in the fused vesicles will then be destroyed by the TRAP enzymatic activity through reactive oxygen species generated by TRAP (Habermann et al. 2007).

The CTx assay utilizing ELISA (Enzyme-linked immunosorbent Assay) is specific for the beta-aspartate-isomerized form of the epitope KAHDGGR. This epitope originated from the carboxyterminal region of the type I collagen alpha (1) chain. This collagen type I fragment is reactive in the Serum CrossLaps (CTx) assay that is eventually released by osteoclast and can be used to quantify bone resorption activity (Christgau et al. 2000).

Conclusion

This study summarises the findings of all relevant individual studies to provide an improvement in the understanding of stem cell differentiation into osteoblasts and osteoclasts using a biochemical analysis approach. The results showed a pattern in the use of biochemical approaches to analyse the differentiation of osteoblasts and osteoclasts; the most widely used techniques for quantitative biochemical analysis for osteoblasts and osteoclasts are the ALP and TRAP assays respectively.

Acknowledgements

We would like to thank the Ministry of Higher Education, Malaysia (FRGS/1/2018/STG05/CUCMS/02/1), University of Cyberjaya (CRG/01/01/2018) and Universiti Kebangsaan Malaysia (GUP-2017-054) for funding this study.

Figure 1. Keywords used in the search string

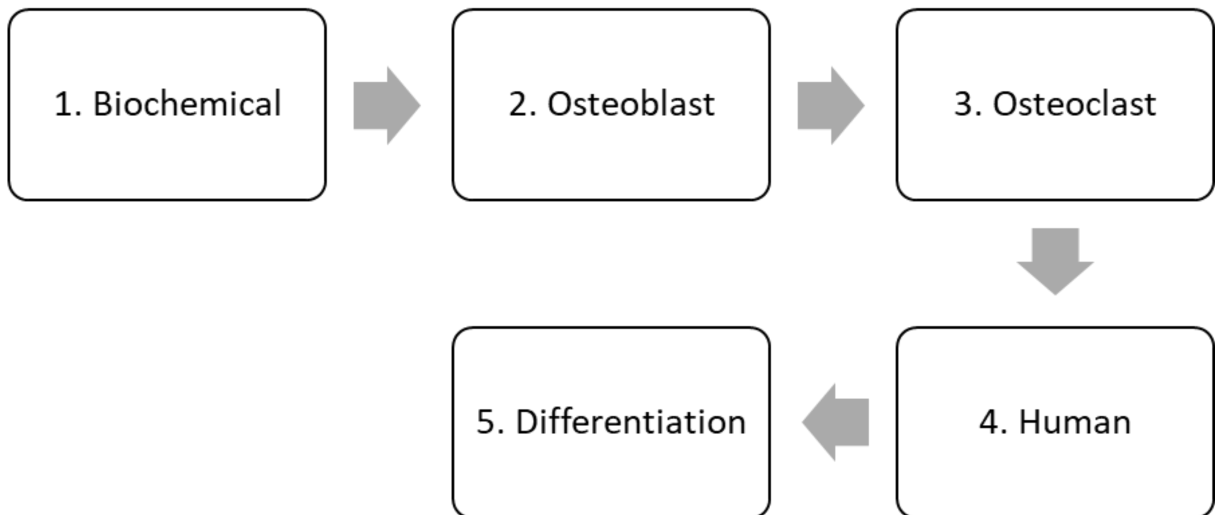

Figure 2. Article selection process

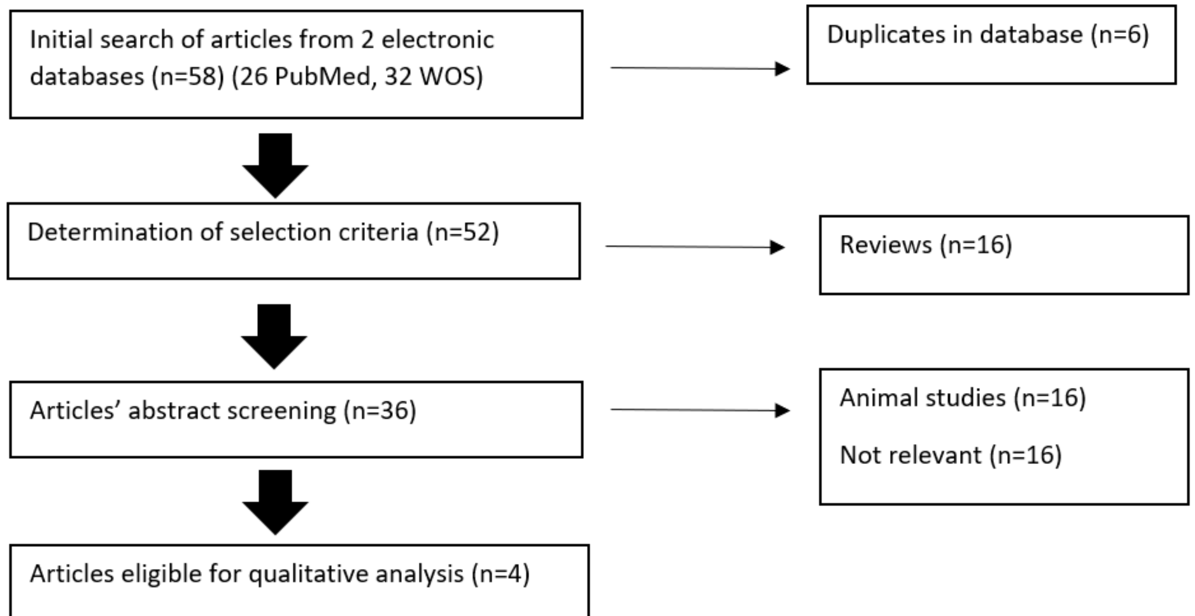

Table 1. The biochemical approach in detecting osteoblast and osteoclast cell differentiation using human stem cells.

No.	Techniques	Cell Variants	Results	References
1.	Creatinine, s-Calcium, ALP, CTX, PTH, 25-OH Vitamin D assays	Human mesenchymal stem cells	(1) Significantly increased expression levels of Runt-related transcription factor 2 (*RUNX2*), and Osteonectin (*SPARC*). (2) A decrease in bone turnover markers (CTX, P1NP, and ALP) was observed; the decrease was more pronounced for CTX than for P1NP and bALP. Both turnover marker values rose between 6 and 12 months. (3) SPARC and COL1A1 gene expression showed the same trend of bone formation markers (P1NP and ALP) between 6 and 12 months.	Carbonare *et al.* 2017.
2.	ELISA (TRAP, BALP, RANKL/OPG, ANGP-1/ANGP-2)	Human peripheral blood stem cell (HSC)	(1) Bone alkaline phosphatase (BALP) increased (2) Receptor activator of Nf-κB ligand/osteoprotegerin ratio (RANKL/OPG) increased (3) Angiopoietin-1/Angiopoietin-2 ratio (ANGP-1/ANGP-2) decreased	Tsirkinidis *et al.* 2018.

3.	Alkaline phosphatase (ALP) activity and calcium deposition	Human mesenchymal stem cells (osteoblasts)	(1) E-2, 5-DHB enhanced ALP activity. (2) Histological and immunohistochemical evaluation showed significantly higher calcium deposition in the E-2, 5-DHB group. (3) Osteocalcin (OCN) was highly expressed in cells implanted in the gels containing E-2, 5-DHB.	Kwon *et al.* 2016.
4.	Quantitative biochemical methods (DNA, LDH, ALP, TRAP5b	Human mesenchymal stem cells and human peripheral blood monocytes (monocytes)	(1) OrmoHAP embedded in the gelatine matrix enhanced TRAP 5b activity. (2) ALP activity and gene expression of BSP II of osteoblasts increased.	Heineman *et al.* 2019.

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