

## A Systematic Review: Comparison of Immunocytochemistry, ELISA, and Western Blot Methods in Alkaline phosphatase Measurement at Genistein-induced Osteoblast Cell

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Osteoporosis is a bone disorder characterized by the decrease of bone mass along with bone micro-architecture damage and has a risk become a fracture. One of the causes of osteoporosis is estrogen deficiency. Genistein is a phytoestrogen compound in the isoflavone group containing a similar structure compared to 17 $\beta$ -estradiol, thus it can bind to estrogen receptors and produce an estrogenic effect. Genistein induction can stimulate bone formation and promote the increase of alkaline phosphate (ALP) activities in osteoblast cells which can be observed by immunocytochemistry or Enzyme-linked Immunosorbent Assay (ELISA) or Western blot method. Using the PRISMA guideline technique, choose and strategize article searches by reading the title, abstract, and then the whole text of the article. Articles with the keywords "genistein or osteoblast cells or alkaline phosphate or immunocytochemistry or immunofluorescence or ELISA or western blot" were retrieved from databases including Google Scholar, PubMed, Researchgate, and Scencedirect. 24 relevant research articles were uncovered as a result of this systematic review. Comparison of immunocytochemistry and ELISA methods in order to analyze the activities of ALP in osteoblast induced by genistein includes selectivity, sensitivity, processing time, and cost efficiency parameters. The immunocytochemistry method has a higher level of sensitivity and a faster processing time, whereas the ELISA method has a higher level of selectivity and less cost efficiency. The western blot method has selectivity for detecting complex-level protein expression.

**Keywords:** Cost Efficiency; ELISA; Immunocytochemistry; Selectivity; Sensitivity, Processing Time; Western Blot.

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Osteoporosis prevalence increases with increasing age in women. Over 30% of women aged 60-70 years suffer from osteoporosis, this data can increase to 70% at 80 years.<sup>1</sup> Elderly woman will face the postmenopausal phase due

to estrogen deficiency, which is one of the factors causing osteoporosis.<sup>2-5</sup> Osteoporosis is a bone disorder characterized by a decrease in bone mass and bone micro-architecture damage, leading to an increased risk of fractures.<sup>6</sup>

Treating estrogen deficiency in postmenopausal women can be treated using hormone replacement therapy (HRT).<sup>7</sup> Long-term use of HRT can cause side effects such as endometrium cancer, breast cancer, and stroke.<sup>8,9</sup> Research on the use of natural ingredients to replace estrogen function with minimal side effects has been done and the results have led to the use of phytoestrogen compound.<sup>10-12</sup>

Genistein is a phytoestrogen compound in the isoflavone group, it has estrogenic activities because of the presence of phenolic rings that bind to estrogen receptors to treat target cells.<sup>13,14</sup> In vitro, genistein can suppress osteoclast function and bone resorption, furthermore, induce the apoptosis of mature osteoclast cells. In vivo, genistein can prevent trabecular bone loss in the male rats' model.<sup>15-19</sup> In silico, a proof that genistein has an active compound identity range like 17 $\beta$ -estradiol.<sup>20,21</sup>

Administration of genistein causes a direct effect on the growth and the differentiation of osteoblast and expresses a protein marker for bone formation, one of them is ALP.<sup>2,22-24</sup> Immunocytochemistry and enzyme-linked immunosorbent assay (ELISA) methods can do the measurement of ALP expression.<sup>25-28</sup> These methods have their advantages, several parameters to analyze both methods are selectivity, sensitivity, processing time, and cost efficiency parameters.

Important parameters to be used in analyzing an observational method, when doing observation must pay attention to how much the ability of a method to recognize and measure a protein in a sample affects the success of a study. Also, short observational duration and less cost efficiency are parameters that must be considered in research, thus producing effective and efficient research. This systematic review is expected to summarize the decision of the selection of an appropriate method for in vitro observation, especially to observe bone formation activity in vitro.

## MATERIALS AND METHODS

### Materials

#### Criteria for collecting data

The inclusion criteria in this review article were (i) studies related to the effect of genistein

on increasing ALP expression in osteoblast with immunocytochemistry or ELISA or western blot method; (ii) studies related to genistein compound; (iii) English articles; (iv) full text and open access articles that published in 2010-2020.

The exclusion criteria in this review article were (i) studies related to other than the effect of genistein on increasing ALP expression in osteoblast cells with immunocytochemistry or ELISA or western blot method; (ii) studies related to other than genistein; (iii) articles in foreign languages other than English; (iv) articles were not full text and open access that published before 2011.

#### Strategy for article selection and search

The data used in the systematic review was primer data by collecting published articles from Google Scholar, PubMed, Researchgate, and Scencedirect databases with keywords "genistein or osteoblasts or alkaline phosphatase or immunocytochemistry or immunofluorescence or ELISA or western blot." Afterward, those articles were screened and selected based on their title and abstract. Then, a feasibility test was conducted by reading the entire article according to inclusion and exclusion criteria.

#### Methods

The method used in the systematic review uses the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guideline.<sup>29,30</sup>

#### Data analysis

Data analysis according to PRISMA guideline, which describes the results of research articles and provides exposure to relevant results in the form of article summary tables.

## RESULTS

#### Strategy for article selection and search

After selecting a specific and systematic article search strategy using the PRISMA guideline method with four databases, namely Google Scholar, PubMed, Researchgate, and Scencedirect, 24 research articles were relevant to the topic of discussion, including 8 research articles from GoogleScholar, 7 from PubMed, 4 from ResearchGate, and 5 from ScienceDirect (Figure 1). Then every research article found was

published in less than the last 10 years (2010-2020) (Figure 2).

**In Vitro Analysis**

The marker factor observed in this literature study was ALP. ALP protein is produced in the early stage of osteoblast differentiation and is one of the specific marker factors, also plays an important role in bone formation and mineralization.<sup>18,29-33</sup> Type of osteoblast cell can be primary cell cultures from cells, tissues, and organs obtained directly from its origin organism, however, cell lines culture is obtained from the first subculture of primary culture. Primary cell

culture refers to the culture starting primary cell culture has several weaknesses, for instance, needs an experimental animal as a raw material for culture and it most likely occurs virus or microbe contamination. This contamination can infect experimental animals used as culture stocks.<sup>34</sup> The increase of ALP activity as a marker of bone formation can occur through various types of osteoblast cells obtained from primary culture or cell line with the process of bone formation around >10% to >50%.<sup>35</sup>

The process of osteoblast cell differentiation can be divided into several

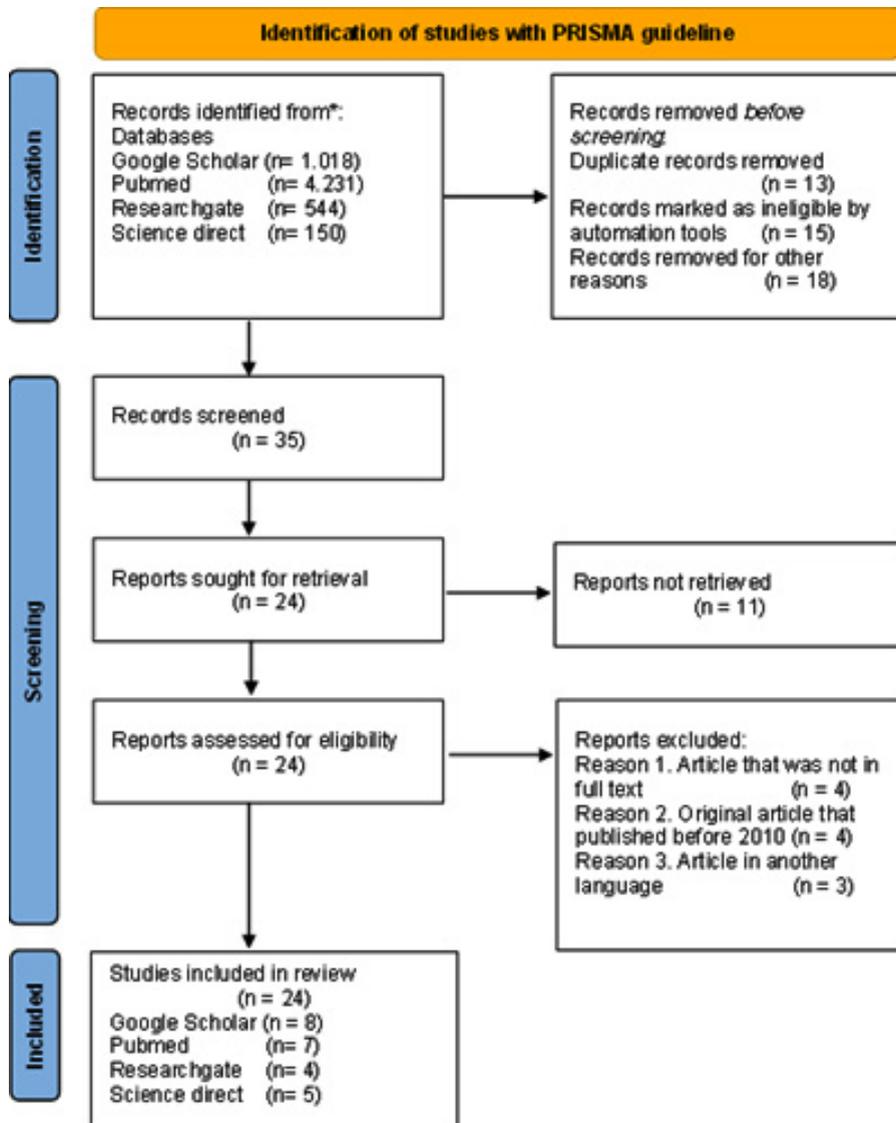


Fig. 1. PRISMA Guideline diagram

stages, namely proliferation, matrix maturation (early differentiation), and mineralization (late differentiation). The main marker protein in osteoblast cells differentiation is ALP, collagen type 1, osteopontin (OPN), and osteocalcin (OSC), with runt-related transcription factor 2 (Runx2) and osterix (OSX) as transcription factors.<sup>18,36-39</sup> Besides, differentiated osteoblasts will express receptor activators of nuclear factor  $\kappa$ -ligand (RANKL) and osteoprotegerin (OPG). RANKL and OPG can be used as markers to observe osteoclast cell function.<sup>37</sup>

Measurement of ALP marker factor shows osteoblastic activity from genistein compound administration. Genistein is an isoflavone group and has structural similarities with 17 $\beta$ -estradiol (Figure 3). Genistein can bind to ER, thus it shows as a phytoestrogen that produces the estrogenic

effect.<sup>18,37-43</sup> Administration of genistein causes a direct effect on the growth and differentiation of osteoblast cells and the release a protein.<sup>18,31,41,43</sup> Therefore, increasing the production of mRNA from several markers of osteoblast cell differentiation, namely ALP.<sup>36,44</sup>

Genistein can affect several molecular pathways that stimulate bone formation and inhibit bone resorption. Administration of genistein can increase the production of ALP, OPN, and OSC and activate the Runx2 transcription factor, hence causing osteogenesis. Genistein can stimulate the occurrence of proliferation and differentiation through nitric oxide (NO) or cyclic guanosine monophosphate (cGMP) pathway and can inhibit osteoclastic activity. Also, genistein can affect the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) pathway, it stimulates

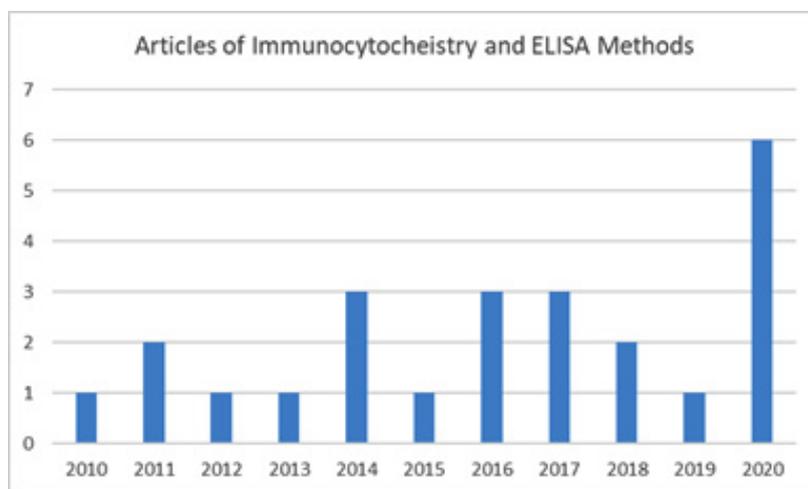


Fig. 2. The year of publication of the article found

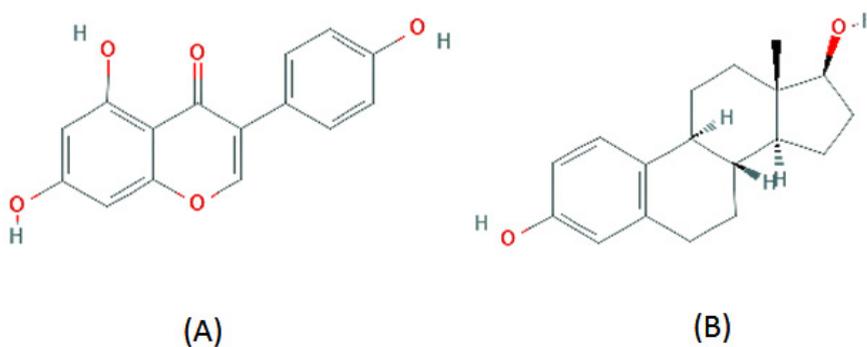


Fig. 3. Structure of (A) Genistein; (B) 17 $\beta$ -estradiol (pubchem.ncbi.nlm.nih.gov)

the occurrence of osteoclastogenesis through RANKL and RANK bonds. RANKL is expressed by osteoblast cell surface and RANK is expressed by osteoclast cell surface.<sup>45</sup> Osteoclastogenesis can be inhibited by OPG, as a result, RANKL binds to OPG and it will maintain the balance of bone formation and bone absorption in the process of bone remodeling. RANKL is a cytokine and plays a role in recruitment, differentiation, and osteoclast cell activation, meanwhile, OPG is a cytokine and plays a role in the inhibition of osteoclastogenesis through its bonds to RANKL.<sup>15,31,36,37,46,47</sup>

Genistein compound induced in osteoblast cells will enter the cell membrane and then translocate into the nucleus and binds to ER or activated estrogen receptor (ER\*). The bond

between genistein with ER will cause transcription by transcription factor Runx2 and encourages osteogenesis through osteoblast cell differentiation and produce ALP.<sup>2,3,38,44,48,49</sup> Measurement of ALP by induction of genistein compound in osteoblast cells was performed by immunocytochemistry or ELISA method.

Characteristics regarding the potential genistein increasing or decreasing ALP activities in various types of osteoblasts by measuring immunocytochemistry or ELISA methods and the mechanism of osteogenesis and osteoclastogenesis was summarized in (Table 1 and Figure 4).

**Comparative Analysis Method**

The most used method in ALP analysis as a bone formation biomarker is immunocytochemistry

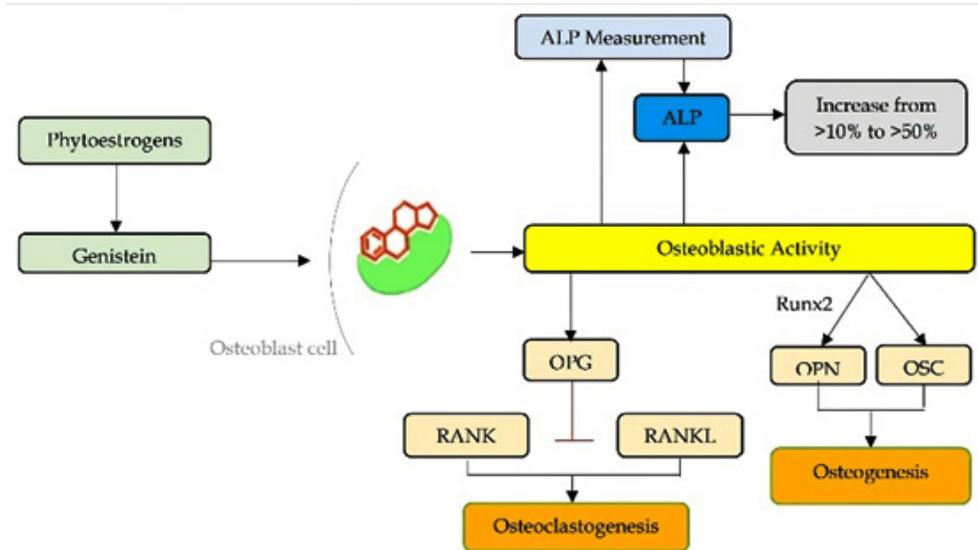


Fig. 4. Mechanism osteogenesis and osteoclastogenesis after genistein induced osteoblast cell

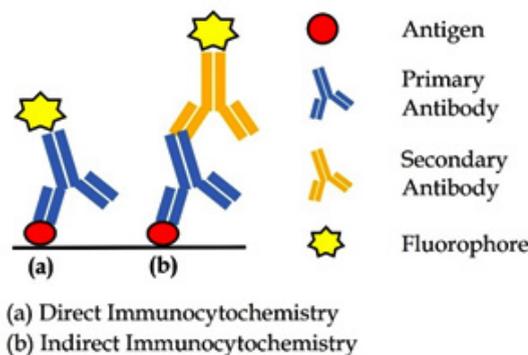


Fig. 5. Immunocytochemistry Method

and ELISA methods. The immunocytochemistry method is a common method used in laboratories (in vitro) to visualize the location or place of a specific protein or antigen in the cells using primary antibodies which specifically bind to these proteins or antigens. Primary antigens allow visualization of protein under fluorescence microscopy after extending binding with secondary antibodies that have conjugated with fluorophore groups.<sup>24</sup> The ELISA method is a serological method based on the specific reaction between antigen and antibody. The method has high selectivity and sensitivity owing

to the change of substrate color (colorimetric) as an indicator for enzyme measurement.<sup>52-54</sup>

**Immunocytochemistry Method**

The immunocytochemistry method is divided into 2 types, direct immunocytochemistry, and indirect immunocytochemistry<sup>55</sup> (Figure 5). Direct immunocytochemistry using primary antibody only, this antibody acts as specific antibodies that have conjugated with the fluorophore group and localize the antigen precisely and specifically, therefore it can be observed on fluorescence microscopy. While indirect immunocytochemistry uses primary and secondary antibodies, the secondary antibody acts as specific antibodies that have conjugated with fluorophore groups and localize antigens precisely

and specifically, as a result, it can be observed under fluorescence microscopy.<sup>56,57</sup>

**ELISA Method**

There are four types of ELISA methods, for instance, direct ELISA, indirect ELISA, sandwich ELISA, and competitive ELISA (Figure 6). Direct ELISA using a conjugated primary antibody, can bind directly to the antigen. Indirect ELISA uses a primary antibody that binds to the antigen and then a conjugated secondary antibody is added. Sandwich ELISA using a suitable pair of antibodies, each specific antibody will bind to a different epitope of the antigen. The first antibody is known as the trapping antibody and the second antibody is known as the detection antibody. Meanwhile, competitive ELISA occurs

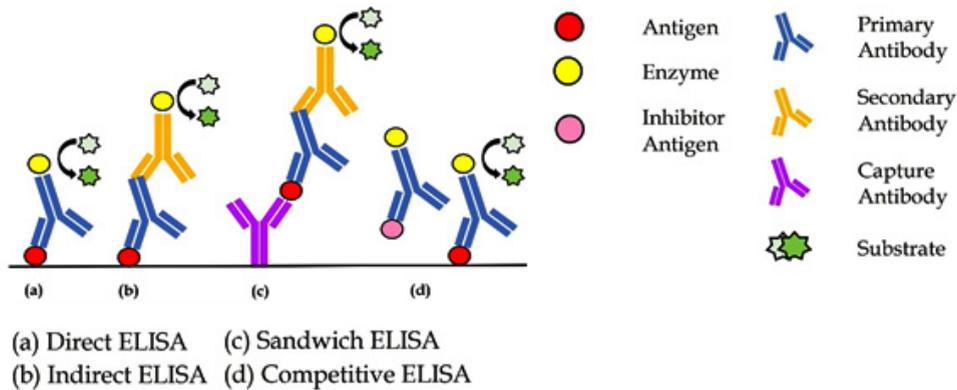


Fig. 6. ELISA Method

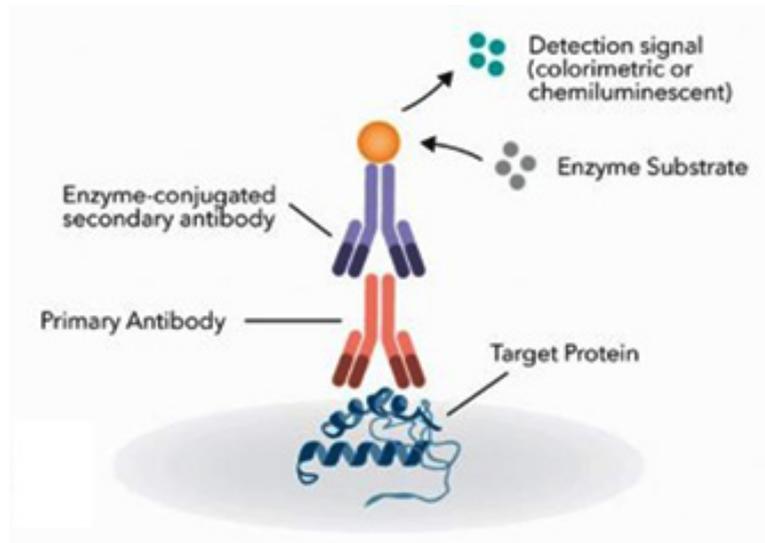


Fig. 7. Western blot Method

Table 1. Result of Genistein-induced

Type of Osteoblast cells	ALP Activity		Condition of Bone Formation Process	Methods
	Increase	Decrease		
Baboon primary cell	✓		Decrease RANKL expression and increase OPG expression	ICC <sup>34</sup>
MC3T3-E1 cell line		✓	Increases production of bone <sup>3</sup> -carboxyglutamate (BGLAP) or OSC	ICC <sup>38</sup>
Calvaria female Wistar rats primary cell	✓		Increase proliferation and differentiation and produce ALP	ICC <sup>39</sup>
MC3T3-E1 cell line	✓		Increase cell proliferation and ALP activity in preosteoblast MC3T3-E1 cells by binding with ER <sup>2</sup>	ICC <sup>17</sup>
MC3T3-E1 cell line	✓	✓	Decrease OPN expression	ICC
Ishikawa cell line	✓		Increases ALP activity by binding to ER	ELISA <sup>48</sup>
Calvaria Wistar rats primary cell	✓		Increase osteoblast cell differentiation by stimulating OSX as a transcription factor	ELISA <sup>15</sup>
Rat osteoblast-like UMR106 cell line	✓		Increases ALP, RANKL, and OPG expressions, but does not increase of Runx2, OSX, and OSC	ELISA <sup>35</sup>
Human bone marrow mesenchymal stem cell cultures (hBMSC) cell line	✓		Increases proliferation and differentiation by binding to ER	ELISA <sup>42</sup>
MC3T3-E1 cell line	✓		Increases differentiation and maturation of MC3T3-E1 osteoblasts and controls osteoclasts by regulating the expression and secretion of OPG and RANKL mediated by ER $\pm$	ELISA <sup>44</sup>
Rat primary cell	✓	✓	Increase MC3T3-E1 osteoblast cell differentiation and ALP activity	ELISA <sup>39</sup>
Sales-2 human cell line	✓		Increase osteoblast cell differentiation and increase ALP activity by binding to ER	ELISA <sup>37</sup>
Bone marrow mesenchymal stem cell (BMSCs) cell line	✓	✓	Enhances Runx2 and OSX expressions and is responsible for controlling RANKL and OPG expressions	ELISA <sup>49</sup>
MC3T3-E1 cell line	✓		Stimulates adipogenic differentiation and stimulates osteogenic by regulating PPAR $\gamma$ expression, thereby decreasing the production of Runx2, ALP, type 1 collagen, and OSC	ELISA <sup>50</sup>
MC3T3-E1 cell line	✓		Increased ALP activity, marked by a decrease in Ereg and Efcab2 and an increase in Lif, Mmp13, and Pde4b	ELISA <sup>36</sup>
Calvaria female Wistar rats primary cell	✓		Increase ALP production through transcription in ER $\pm$	ELISA <sup>46</sup>
Calvaria rat's primary cell	✓		Increase ALP activity, expression of OSC, collagen, and Runx2 as transcription factors in ER $\pm$	ELISA <sup>37</sup>
Calvaria Wistar rats primary cell	✓		Increase osteoblast cell differentiation by increasing ALP and Runx2 expression, and inhibiting osteoclast cell differentiation by inhibiting RANK expression	ELISA <sup>18</sup>
Human osteosarcoma Saos-2 cell line	✓		Stimulate osteogenesis by increasing production of ALP, Runx2, and OSC through transcription in ER $\pm$	ELISA <sup>45</sup>
hBMSC cell line	✓		Increase the expression of RANKL, OPG, and Runx2 as transcription factors, as well as increase ALP activity	ELISA <sup>43</sup>
MC3T3-E1 cell line	✓		Increases ALP activity by binding to ER $\pm$	ELISA <sup>19</sup>
Mesenchymal stem cells (MSCs)	✓		Increasing Runx2 protein	Western blot <sup>68</sup>
Bone marrow macrophage cells	✓		Increasing PPAR $\gamma$ , p PPAR $\gamma$ , Runx2, bone morphogenetic receptor 1A (BMPRI A)	Western blot <sup>69</sup>
MC3T3-E1 osteoblastic cells (Xu et al., 2011)	✓	✓	Reduction of osteoclast differentiation (c-Fos, NFATc1, and TRAP) Increases ALP activity and decreased reactive oxygen species Production	Western blot <sup>71</sup> Western blot <sup>72</sup>

**Table 2.** Parameter Analysis Results

No	Parameters	Immunocytochemistry	Method ELISA	Western – Blot	Source
1.	Selectivity		✓	✓	59,64,66
2.	Sensitivity	✓			59,66
3.	Processing Time	✓			62,72
4.	Cost Efficiency		✓		77

Description: sign “–” is a method that has advantages according to each parameter

through a competitive process among antigens in the microplate. After the antibodies are incubated in the sample, next, it is inserted into a micro-plate containing the antigens and then rinsed to remove specific antibodies that do not bind to the antigen. The more antigens in the sample, the more antigens, and antibodies bound are formed, thus fewer antibodies that do not bound will cause those antibodies to bind to the antigens in the micro-plate.<sup>58,59</sup>

#### Western Blot Method

Western blot or immunoblotting is a qualitative technique used to identify specific antibodies from complex protein samples with a certain molecular weight that have been separated. This method can show proteins by reacting antibodies and antigens that are used to detect cross-reactions (Figure 7).<sup>60</sup> Western blot is very effective in detecting antigens that have small sizes in solutions that contain a lot of protein. The stages begin with sample preparation (protein purification), measurement of the concentration and amount of protein used, separation of proteins by polyacrylamide gel electrophoresis, transfer of proteins from gel to a nitrocellulose membrane, membrane blocking, incubation of primary and secondary antibodies, detection of target proteins, and calculation of protein bands. Even though the working technique is simple, specific, and precise in implementation, sometimes there are difficulties with unclear, non-specific, and bubble protein bands.

## DISCUSSION

### Parameter Analysis

Parameters used in the analysis of Immunocytochemistry, ELISA, and Western blot

methods are selectivity, sensitivity, processing time, and cost efficiency parameters. The following is an explanation of each parameter and a summary can be seen in (Table 2).

#### Selectivity

The selectivity parameter is used to describe the ability of a method to observe protein in the complex sample accurately.<sup>58</sup> ELISA method has higher selectivity than immunocytochemistry because the antigen can be detected by using antibodies marked with an enzyme.<sup>59,66</sup> In addition, the western blot method has selectivity in detecting targets at endogenous levels in complex samples and can optimize antibody and assay performance.<sup>64</sup> The results in detecting antigens and antibodies bound to detect autoimmune disorders in humans like a pemphigus foliaceus, bullous pemphigoid, dermatitis herpetiformis, myositis, autoimmune hepatitis, etc, when most studies have used Hep2 cells from Ali *et al.*, 2016, Karumanchi and Oommen, 2018, and Menezes *et al.*, 2020 were performed by immunocytochemistry/immunofluorescence and ELISA methods.<sup>67,73-74</sup> Result of selectivity analysis in immunocytochemistry/immunofluorescence and ELISA methods used kappa analysis and Cohen's kappa coefficient. Showed ELISA method had a higher selectivity around 84-99%, whereas the immunocytochemistry/immunofluorescence method about 40-95%.

#### Sensitivity

The sensitivity parameter is used to describe the ability of a method to measure a protein in the cells that can be affected by changing situations.<sup>65</sup> The immunocytochemistry method has a higher sensitivity than the ELISA method because the observation is performed by using fluorescence microscopy which can detect an extremely

sensitive antigen.<sup>59,66</sup> The results in the detection of antigen and antibody bound were formed to detect autoimmune disorders in humans like a pemphigus foliaceus, bullous pemphigoid, dermatitis herpetiformis, myositis, autoimmune hepatitis, etc when most studies have used Hep2 cells from Ali *et al.*, 2016, Karumanchi and Oommen, 2018, and Menezes *et al.*, 2020 were performed by immunocytochemistry/immunofluorescence and ELISA methods.<sup>67,73-74</sup> Result of sensitivity analysis in immunocytochemistry/immunofluorescence and ELISA methods used kappa analysis and Cohen's kappa coefficient. It was shown that the immunocytochemistry/immunofluorescence method had a higher sensitivity of about 50-100%, while the ELISA method was only about 30-97%.

Then, from several studies, the western blot method is ideal to be combined with the ELISA or ICC methods. In the study of Marycz *et al.* (2021), Western blot was used in observing to detect protein level expression (increased expression of RUNX-2) protein and ICC identified osteopontin (OPN) and fluorescence imaging (reduced OPN expression) with  $p < 0.05$ .<sup>68</sup> The same thing was also done in the study of Donoso *et al.* (2014), using mesenchymal stem cells (MSCs). The western blot method was used to detect protein content such as transcription factors PPAR $\alpha$ , p PPAR $\alpha$ , Runx2, bone morphogenetic receptor 1A, and the ICC method for cell localization. derived from pSmad1/5/8.<sup>69</sup> Then in the research of Wang *et al.* (2017), the ELISA method was used in the analysis of alkaline phosphatase (ALP) and osteocalcin (OC) as well as the western blot method to see protein expressions such as Sirtuin 1 (SIRT1), nuclear factor (NF)- $\kappa$ B and NF- $\kappa$ B inhibitor (I $\kappa$ B) with  $p < 0.01$ .<sup>70</sup>

#### **Processing Time**

The processing time parameter describes how long it takes to process the ALP observation in each method. The results in the detection of antigen-antibody bound were formed to detect autoimmune disorders in humans like a pemphigus foliaceus, bullous pemphigoid, dermatitis herpetiformis from Ali *et al.*, 2016 and Tayde *et al.*, 2018.<sup>67,76</sup> The research was performed by using immunocytochemistry/immunofluorescence and ELISA methods, explained that the immunocytochemistry/immunofluorescence method could be run in faster processing time than

the ELISA method. The western blot method has a long time starting from preparation to protein calculation, which takes approximately 45 minutes for pretreatment and 1 hour for incubation time.<sup>62,72</sup>

#### **Cost Efficiency**

The cost-efficiency parameter describes the amount of cost used in the observation of each method. Observation using the immunocytochemistry method or fluorescence is more expensive than the ELISA method. It is because in immunocytochemistry method requires fluorescence microscopy that can observe up to the molecular level.<sup>77</sup> In the western blot method, using high-quality antibodies to detect the desired protein such as polyacrylamide SDS, and polyvinylidene difluoride (PVDF).<sup>60</sup> Observation using the ELISA method costs less than immunocytochemistry and western blot method because observation with ELISA methods without fluorescence microscopy and is also easier to apply in the environment, especially in environments that are limited by resources like an operator and equipment.<sup>76,78</sup>

### **CONCLUSION**

The immunocytochemistry and ELISA methods can measure the ALP after genistein induction, which is characterized by an increase or decrease in ALP activities in osteoblast cells. While western blot is used to detect protein expression levels. Both methods have their respective advantages, the immunocytochemistry method has higher sensitivity with faster processing time, meanwhile, the ELISA method has a higher selectivity with less cost efficiency than the western blot has selectivity in detecting targets at the endogenous level of complex samples. This article is expected to be used as a reference by researchers to select suitable methods for the measurement of bone formation markers *in vitro*.

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#### **Conflict of Interest**

There is no conflict of interest.

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