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Indonesian Archives of Biomedical Research

Table of Content

Indonesian Archives of Biomedical Research

CORRELATION BETWEEN NEPHRIN EXPRESSION, TUBULAR INJURY, AND SERUM CREATININE LEVEL IN KIDNEY FAILURE MODEL WITH 5/6 SUBTOTAL NEPHRECTOMY IN MICE

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ABSTRACT

Background: *Chronic kidney diseases (CKD) is characterized by glomerulosclerosis, tubular injury, and proteinuria. Neprin is the one of the most important protein involved in glomerular filtration but the mechanism of nephrin expression in chronic kidney failure is not well understood.*
Objective: *We aims to elucidate the correlation between nephrin expression with tubular injury and serum creatinine level.*
Methods: *We performed 5/6 subtotal nephrectomy (SN) in male strain Swiss mice to induce CKD. Sham operation was performed to control group (SO) (n=8). Mice were sacrificed in day 7 (SN7; n=8) and day 28 (SN28; n=8) after operation. We measure creatinine serum level to assess renal function. Tubular injury score was quantified using Periodic Acid Schiff (PAS) staining. Reverse transcriptase PCR (RT-PCR) was carried out to examine Nephrin mRNA expression.*
Results: *5/6 subtotal nephrectomy induced an increased of serum creatinine level in SN7 and SN28 ($p<0,01$ vs SO), followed by an increased of tubular injury score in SN7 and SN28 ($p<0,01$ vs SO). We confirmed reduction of nephrin expression in SN28 ($p<0.01$ vs SO). There was a negative correlation between nephrin and tubular injury ($r=0.719$, $p<0.01$) and the positive correlation between tubular injury and serum creatinine level ($r=0.891$, $p<0.01$). However, we did not find any significant correlation between nephrin expression and serum creatinine level.*
Conclusion:
Nephrin expression downregulation might represent renal function disturbtion in CKD.

Keywords: *nephrin, 5/6 subtotal nephrectomy, remnant kidney, mice, serum creatinine, tubular injury, Sham Operation.*

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INTRODUCTION

Kidney failure enhances the decrement of the renal function and marked by the reduction of Glomerular Filtration Rate (GFR). This alteration was followed by reduction of nephron significantly[1]. Indonesian nephrologist association declared from 2007 until 2012, there is an increase of the new patients who used hemodialyzer, and it reaches 28.782[2].

The 5/6 subtotal nephrectomy (remnant kidney model) has been widely used as a pathogenic mechanism of chronic kidney disease and has been reliance on studies of renal diseases' progressivity. The remnant kidney model is performed by unilateral nephrectomy and followed by contralateral renal ablation. Hypertrophy and hyperplasia are the hallmarks of the remnant kidney model. Shortly, after performing renal contralateral renal ablation various changes develop [3]. Remnant kidney model demonstrates the histopathological features, for example: decreased of podocyte density, segmental proliferation of parietal epithelial cell, tubule cell reflux, and lost of brush border at proximal tubules [4].

Indonesian Archives of Biomedical Research
Volume 1 Issue 1, January-June 2021

Podocyte integrity is maintained by nephrin. Nephrin is located in the slit diaphragm of podocyte specifically [5]. Renal tubules cells destruction is caused by excessive inflammation process that which lead into tissue edema & tubules cells injury, tubule blood circulation distubances, direct contact with toxic agents like drugs, radiocontrast agents, myoglobin, radiation, or tubules obstruction that are caused by *casts*, cellular debris, or crystal sedimentation [6]. Until now, nephrin role toward kidney disease are not well understood. So, this research purpose is to elucidate the correlation between nephrin, creatinine serum level, and renal tubular injury in kidney-failure-model-mice with 5/6 subtotal nephrectomy.

MATERIALS AND METHODS

Preparation of Experimental Animal

We obtained the animals from Unit Pengembangan Hewan Penelitian (UPHP), Universitas Gadjah Mada. We performed quasi-experimental research using 24 male Swiss background mice, 4 months-old (30-40 grams). The mice were divided into 3 groups: (1) SO (*Sham Operation*) group; (2) SN7 (7 days post-subtotal nephrectomy) group; (3) SN28 (28 days post-subtotal nephrectomy) group. Mice were maintained in the Anatomy Laboratorium, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada in the temperature 21°C, 50-60% humidity, and 12 hours light: dark cycle. Mice were fed with standard chows and water *ad libitum*.

5/6 Subtotal Nephrectomy (SN) procedure

We performed the SN procedure in two days. The first day, we anesthetized the mice with intraperitoneal injection of 0,1mL/10grams Body Weight (BW) of sodium pentobarbital. We opened right Flank's region, visualized the kidney, then removed the renal capsule and cut the kidney after renal pedicle ligation (uninephrectomy procedure). In day 2, we did the same procedure for the left kidney, however, we ablated the superior and inferior poles of the left kidney. Bleeding was stopped with electro cauter of the kidney. Mice were kept alive and sacrificed in day7 (SN7 group) and day28 (SN28 group). We committed the same procedure as the SN group without any removing kidney for Sham Operation (SO) group.

Serum Creatinine Level

After the due date, the blood was taken from the retro-orbital vein. Afterwards, we obtained the serum through centrifugation at 10.000 rpm for 10 minutes. The creatinine level was assessed in the clinical pathology laboratory, Faculty of Medicine, Public Health, and Nusing, Universitas Gadjah Mada.

Kidney harvesting

Termination was carried out with euthanasia procedure using lethal dose of ketamine. Then mice were dissected and perfused with NaCl 0.9% through apex cordis. To reduce the intracardiac pressure, we opened the right atrium. The kidney was harvested then put into RNA Later for assessing mRNA expression and fixated the kidney in 4% paraformaldehyde for histological examination.

Histopathological Quantification

Four micrometers of the tissues were cut and stained with Periodic Acid-Schiff (PAS) in Pathological anatomy Laboratory. This staining was performed in order to assess tubular injury. Quantification of tubular injury was performed under microscope using 400x magnification. We captured 10–15 random fields for quantification of tubular injury score.

Indonesian Archives of Biomedical Research
Volume 1 Issue 1, January-June 2021

Tubular injury score was assessed based on histopathological changes such as tubular dilatation, brush border loss, tubular atrophy, nucleus condensation, intraluminal cast, and leukocyte infiltration [7]. Each slide was assessed based on the criteria and divided into score 0 until score 4. Score 0 was given if there is no changing at all (normal), score 1 if there were tubules destruction less than 25%, score 2 if there are tubules destruction between 25 until 50%, score 3 if there are tubules destruction between 50 until 75%, and score 4 if there are tubules destruction more than 75%. Tubular injury score quantification was performed by 3 slide-observers that blinded, then we made average tubular injury score from 3 observers.

Nephrin mRNA Expression

Nephrin mRNA expression was examined using Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). Afterwards, we performed densitometry analysis to analyze nephrin band intensity using ImageJ software with GAPDH as *house-keeping gene*. As much as 3 µL cDNA was used for RT-PCR with addition of these following reagents: (Primers, dNTP, 10×ExTaq buffer, *enzyme*). We prepared RT-PCR mixture for each cDNA that consist of: forward primer Nephrin 0,6 µL; reverse primer Nephrin 0,6 µL; PCR water 8,3 µL; Taq master mix 12,5 µL (last added). The condition for RT-PCR are denaturation step for 10 second at 94^0C, annealing step for 20 second at 60^0C, extension step for 1 minutes at 72^0C with 35 cycles. The amplicon was kept in 4^0C. Nephrin expression intensity was analized using ImageJ software. Analysis score was compared with GADPH expression intensity, so we earned the nephrin expression result.

RESULT

Creatinine serum level in SO group were 0.74 mg/dL; SN7 group 1.28 mg/dL; and SN28 group 1.48 mg/dL respectively. There was significant difference between SO group vs SN7, SO vs SN28, dan SN7 vs SN28 ($p<0.01$). Tubular injury score in SO group was 0.522; SN7 group 2.04; dan SN28 group 3.28 (Fig. 1). There was significant difference between SO vs SN7 and SO vs SN28 ($p< 0.01$). There was significant difference between SN7 vs SN28 ($P< 0.05$). Nephrin mRNA expression was significantly different between SO group and SN28 group. The SN28 group had lower Nephrin mRNA expression ($p<0.01$) compared to SO and SN7 group ($p< 0.05$). There high significant positive correlation between tubular injury and creatinine serum level. There was intermediate no-significant negative correlation between nephrin expression and serum creatinine level. There was high significant negative correlation between nephrin expression and tubular injury score.

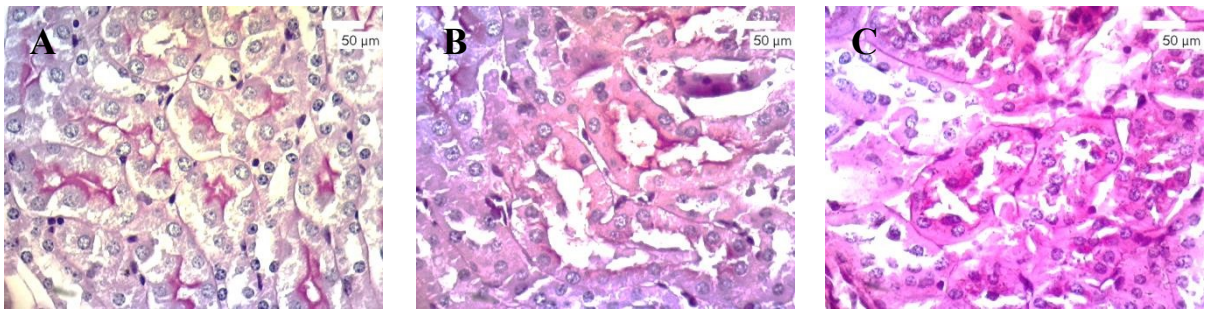

Figure 1. Histopathological tubular injury. **(A)** Tubular injury in SO group**. (B)** Tubular injury in SN7 group. **(C)** Tubular injury in SN28 group

Indonesian Archives of Biomedical Research
Volume 1 Issue 1, January-June 2021

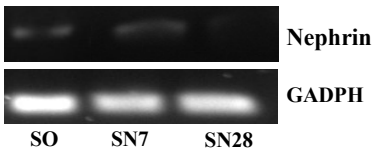

Figure 2. Densitometry result of nephrin expression and GADPH band using electrophoresis.

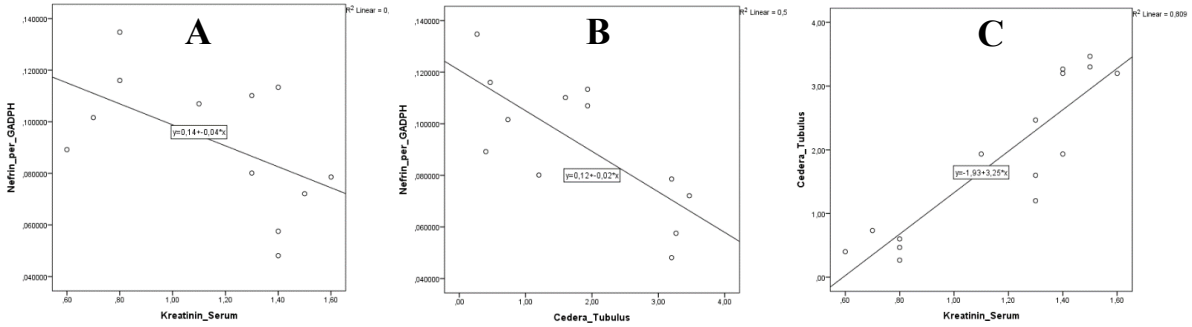

Figure 3. Correlation. **(A)** Tubular Injury with Serum Creatinine. **(B)** Expression of Nephrine with Serum Creatinine. **(C)** Expression of Nephrine with Tubular injury.

Table 1. Average ± standard deviation of serum creatinine level, tubular injury score, and nephrin expression in each treatment group

Group	Average ± Standard Deviation		
	Nephrin expression	Tubular injury score	Serum creatinine level
A (SO)	0.1103 ± 0.019	0.5222 ± 0.1838	0.74 ± 0.089
B (5/6 SN 7 days)	0.1026 ± 0.015	2.0445 ± 0.4305**	1.28 ± 0.109**
C (5/6 SN 28 days)	0.0640 ± 0.0138**	3.2858 ± 0.3074**	1.48 ± 0.083**

** $p<0.01$ vs SO

DISCUSSION

Remnant kidney model induces CKD after 7 days and 28 days with diffrerent degree severity. Chronic kidney failure was confirmed through augmentation of serum creatinine level followed by increasing tubular injury score. Nephrin expression was significantly decreased in SO group with SN28 group and SN7 group with SN28 group. There was significant negative correlation between nephrin expression and tubular injury score. The 5/6 Subtotal nephrectomy stimulates the development of the hypertrophy and hyperplasia of nephron as the compensation of decrement renal function [8,9,10]. It followed by histopathological changes which is shown as glomerulosclerosis, tubulointerstitial injury, renal dysfunction, and the most dangerous is uremic syndrome can occured in mice with 5/6 subtotal nephrectomy [10,11]. There was strong negative correlation between tubulointerstitial destruction with renal function disturbance in some glomerular immune diseases like membranous nephropathy, mesangioproliferative glomerulonephritis, focal segmental glomerulosclerosis (FSGS), type 1 mesangiocapillar glomerulonephritis, lupus nephritis, and other glomerular non-immune diseases like diabetic nephropathy[3]. In this research, 5/6 subtotal nephrectomy in mice can induce the occurence of

kidney failure that confirmed with increasing severity tubular injury and followed by decreasing nephrin expression due to longer treatment.

Creatinine serum level is the gold standard to assess renal function and it affected by tubular secretion and production, and extrarenal elimination.[12]. Creatinine is excreted by kidney in fixed amount based on muscle mass and free in plasma protein, so it can be a marker to evaluate renal function[13]. Increasing serum creatinine level indicated there was reduction of Glomerular Filtration Rate (GFR)[14]. Creatinine serum level that assessed form 24-hours urine is fragile to the error occurence and tend to not practical[15]. Recommendation for creatinine is better using serum creatinine than clearance creatinine[16]. In this study, creatinine level measured using serum creatinine level. There are factors that can affect to GFR like ages, races, genders, and body sizes[16,17].

Creatinine serum used to assess renal function and affected by tubular secretions, production, and extrarenal elimination[12]. We quantified the increment of creatinine serum in order to confirm renal function in chronic kidney disease model, 5/6 subtotal nephrectomy. Quantification of increasing serum creatinine level was performed to confim the occurence of kidney failure[18]. Remnant kidney model leads to damage of histopathological architecture, such as decrease of podocyte density, segmental proliferation of parietal epithelial cell, tubular cell reflux and lost of brush border at proximal tubule [4].

Increase of creatinine was followed by epithelial tubular cells injury which marked by CD24-positive cells. It consists very least of cytoplasm, mitochondria, and lost of brush border [19]. Dilatation of tubules, lost of brush broder, vascular remodeling, detachment of cells from glomerular basement membrane, infiltration of leukocyte, and capillary edema were the characteristic of tubular injury [7] [20]. Tubular injury score was increase significantly in 5/6 subtotal nephrectomy mice. Glomerulosclerosis may lead to tubular injury in kidney failure disease [21].

Prolong chronic kidney disease reduced mRNA expression of Nephrine. Nephrine has an important role in maintain the integrity of the glomerular. Inactivation of the nephrine genes stimulates severe proteinureia. The alteration of the both mRNA and protein of Nephrine have an important role in pathological process of kidney disease. It affects glomerular membrane permeability [22]. Downregulation of the mRNA Nephrine expression followed by proteinuria and increase of creatinine was not significantly statistic ($p>0.01$). Since creatinine serum is the marker of glomerular injury.

From the previous research, downregulation of nephrine expression was correlates to the progressivity of kidney disease. Progressivity of glomerular injury contributes to the development of tubular injury caused by glomerular injury. During glomerular injury, the integrity of glomerular basement membrane was imperfect. Consequently, the level of nephrine and the other podocyte cytoskeleon was altered. Here, we elucidated that the downregulation of nephrine followed by increase of tubular injury, but this alteration does not followed with increase of creatinine serum [24],[25].

CONCLUSION

Chronic kidney disease induces downregulation of the mRNA Nephrine expression and negatively correlate with the augmentation of tubular injury score. Meanwhile, the tubular injury was positively correlate with serum creatinine level.

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Indonesian Archives of Biomedical Research

ADMINISTRATION OF *BAJAKAH* (*SPATHOLOBUS LITTORALIS HASSK*) STEM ETHANOL EXTRACT CREAM INHIBITED THE INCREASING OF MMP-1 EXPRESSION AND THE REDUCING OF COLLAGEN IN MALE WISTAR RATS (*RATTUS NORVEGICUS*) EXPOSED TO ULTRAVIOLET B

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ABSTRACT

Background: *Exposure to ultraviolet B (UV-B) rays causes skin aging characterized through increased free radical damaged. To prevent skin aging due to UV-B exposure, it is necessary to additionally provide antioxidants as a measure to inhibit the skin aging process. Bajakah tree (Spatholobus littoralis hassk) is one antioxidants sourche such as phenols, flavonoids, and tannins. The aim was to prove that the administration of Bajakah stem ethanol extract cream inhibits increasing MMP-1 expression and reduction of collagen number in male Wistar rats exposed to UV-B.*

Methods: *A posttest control group study design was coducted in male rats (Rattus norvegicus), Wistar strain, aged 2-3 months, weigh 180-200gram which was divided randomly into two groups. The control group (n = 18) was exposed to UV-B rays and placebo cream (0.2 mg/cm^2), while the treatment group (n = 18) was exposed to UV-B rays and Bajakah stem ethanol extract cream 15 % (0.2 mg/cm^2). Both creams have aplied twice before and after exposure to UV-B rays (3 times a week, with 840 mJ/cm^2) for 4 weeks. Twenty-four hours after the last day intervention, a punch biopsy of skin tissue was prepared for histological examination followed by immunohistochemical assay (for MMP-1) and Sirius red (for collagen) staining.*

Results: *The expression of MMP-1 was significantly higher in the control group (24.3 ± 6.20%) compared to the treatment group (11.5 ± 3.21%), p<0.001. Furthermore, the number of collagen was statistically lower in the control group (63.1 ± 3.94%) compared to the treatment group (82.0 ± 3.02%), p<0.001.*

Conclusion: *The administration of bajakah (Spatholobus littoralis hassk) stem ethanol extract cream inhibited the increasing of MMP-1 expression and the reducing of collagen cells in male Wistar rats (Rattus norvegicus) those were exposed to UV- B.*

Keywords: *Bajakah stem, MMP-1, collagen, ultraviolet B.*

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INTRODUCTION

Indonesia is one of tropical countries with high sunlight intensity that can cause skin aging. Sunlight caused both acute and chronic effects on the skin. In this current study, what was observed was chronic damage, namely photoaging. Inhibiting, preventing, and restoring the skin aging process can be done and is one of the focuses of Anti-Aging Medicine (AAM). Since ultra violet (UV) rays in sunlight exposure causes skin aging, efforts to prevent the development of the aging phenotype is one of the Anti-Aging Medicine.[1]

Sunlight that caused photoaging is UV rays with a wavelength of 10 - 400 nm. Photoaging is characterized by an increase expression of matrix metalloproteinase (MMP).[2] MMP-1 is the main collagenase increased by UV-B exposure. UV radiation induces MMP-1 expression by

Indonesian Archives of Biomedical Research
Volume 1 Issue 1, January-June 2021

dermal fibroblasts, partly stimulated by the formation of excess reactive oxygen species (ROS), and plays an important role in photoaging.[3] MMP-1 together with its inhibitor, tissue inhibitor of metalloproteinases (TIMPs), plays a role in the fragmentation of types I and III collagen. The increasing MMP-1 due to UV-B resulted in the increasing collagen degradation, so that the amount of collagen decreases. The molecular mechanism of MMP-1 degrades collagen is by cutting the collagen fibers in the three alpha chains at one locus which is located around the first quarter of the N-terminal collagen chain and produces TCA and TCB fragments.[4]

An increase of MMP-1 and be followed by a decrease of collagen are the main causes of skin aging due to exposure of UV-B rays; hence, it is necessary to make efforts to inhibit the increase in MMP-1 and the decrease of collagen as part of AAM. Many studies have been carried out using natural creams containing antioxidants and bioactive compounds to prevent photoaging.

Recently, the stem of *Bajakah* tree (*Spatholobus littoralis hassk*), a plant that is growing in forest of Kalimantan island, has come to attention in Indonesia.[5] Traditionally, this plant has been used orally or topically by the local community for curing some health problems.[6] Previously, research has shown qualitatively the presence of flavonoids, saponins, steroids, terpenoids, tannins, and phenols, and has been shown to accelerate the wound healing process.[5]

Scientific research on the Bajakah plant is only limited to its qualitative content and its effect on the wound healing process.[5] The content of active compounds and antioxidants have been widely reported to be able to prevent photoaging. The purpose of the study was to prove that the administration of Bajakah stem ethanol extract cream inhibits the increasing of MMP-1 expression and the reducing of collagen cells in male Wistar rats exposed to UV-B.

METHODS

A post-test only control group design, with subjects male rats (*Rattus norvegicus*), Wistar strain, aged 2-3 months, weighing 180-200gram which were divided randomly into two groups. The control group (n = 18 rats) was exposed to UV-B rays and placebo cream (0.2 mg/cm^2), while the treatment group (n = 18 rats) was exposed to UV-B rays and Bajakah stem ethanol extract cream 15 % (0.2 mg/cm^2). The cream was administered 20 minutes before and 4 hours after exposure to UV-B rays (3 times a week, a total dose of 840 mJ/cm^2), for four weeks. Twenty-four hours after the last day of intervention, a punch biopsy of skin tissue was prepared for histological examination followed by immunohistochemical assay (for MMP-1) and Sirius red (for collagen) staining.

RESULTS

The expression of MMP-1 in the control group was (24.3 ± 6.20)% and the treatment group was (11.5 ± 3.21)%. The comparative analysis using the independent *T*-test showed a *p*-value of <0.001 which indicates that there was a significant difference in MMP-1 expression higher in the control group compared to the treatment groups (pigure 3.(A)). In addition, the amount of collagen in the control group was (63.1 ± 3.94)% and the treatment group was (82.0 ± 3.02)%. Comparative analysis showed a *p*-value of <0.001 which indicates that there was a significant difference in the amount of collagen lower in the control group compared to the treatment groups.

The Visual fiture shown in pigure 1, the experssion MMP-1 (brown color) in the control group (A, B) is higher than that in the treatment group (C, D). So do, the collagen fiture in pigure 2 shown that collagen of the control group (A, B) fragmented and tinner than that the collagen of the treatment group (C,D).

Indonesian Archives of Biomedical Research
Volume 1 Issue 1, January-June 2021

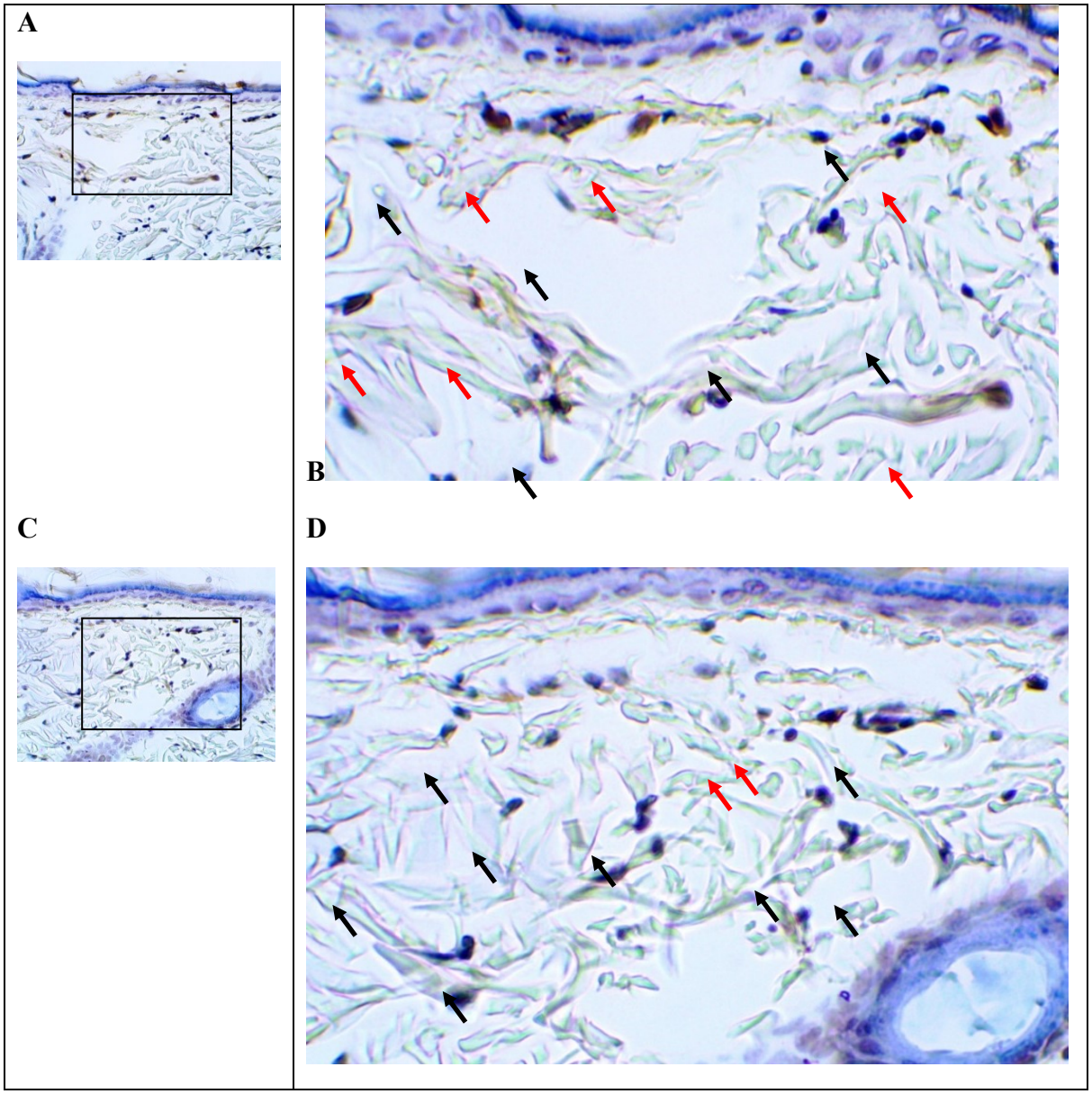

Figure 1. Expression of MMP1 examined by immunohistochemistry in Dermal Tissue

A. Control group (100x magnification).
B. Control group (400x magnification). The expression of MMP-1 (brown color) was higher compared to the treatment groups. Red arrows indicate fibroblast cells expressing MMP-1. Black arrows indicate fibroblast cells that did not express MMP-1
C. Treatment group (100x Magnification)
D. Treatment group (400x magnification). MMP-1 expression (brown color) was less than the control group. Red arrows indicate fibroblast cells expressing MMP-1. Black arrows indicate fibroblast cells that did not express MMP-1.

Indonesian Archives of Biomedical Research
Volume 1 Issue 1, January-June 2021

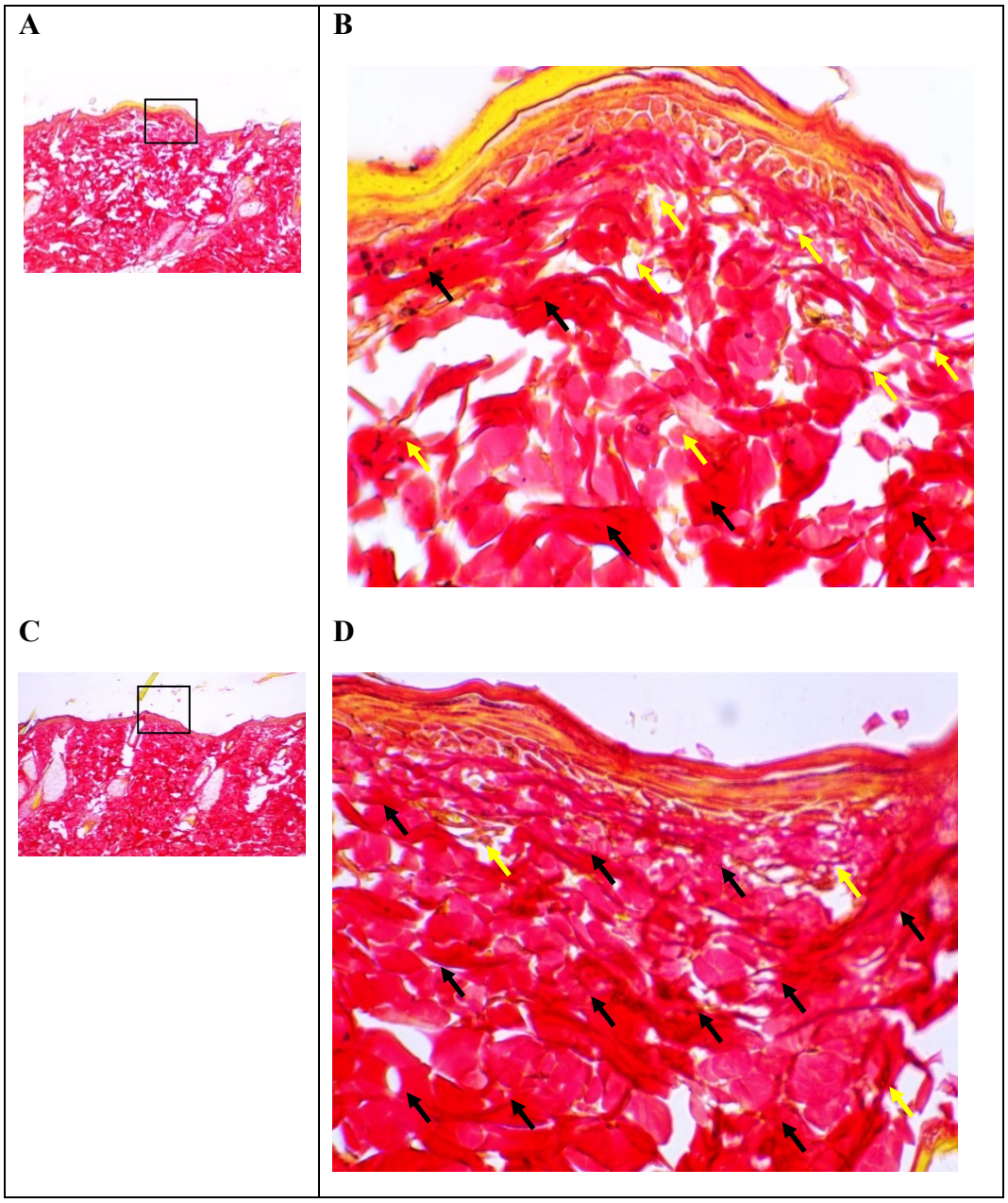

Figure 2. Collagen in Dermal Tissue with Picro-Sirius Red Staining

A. Control group (100x magnification).
B. Control group (400x magnification). The structure of collagen with red collagen fibers appeared to disintegrate and thin. The black arrows indicate thick collagen fibers. Yellow arrows indicate thin collagen fibers.
C. Treatment group (100x Magnification).
D. Treatment group (400x enlargement). The collagen structure was more intact and thick than that of the control group. The black arrows indicate thick collagen fibers. Yellow arrows indicate thin collagen fibers.

Indonesian Archives of Biomedical Research
Volume 1 Issue 1, January-June 2021

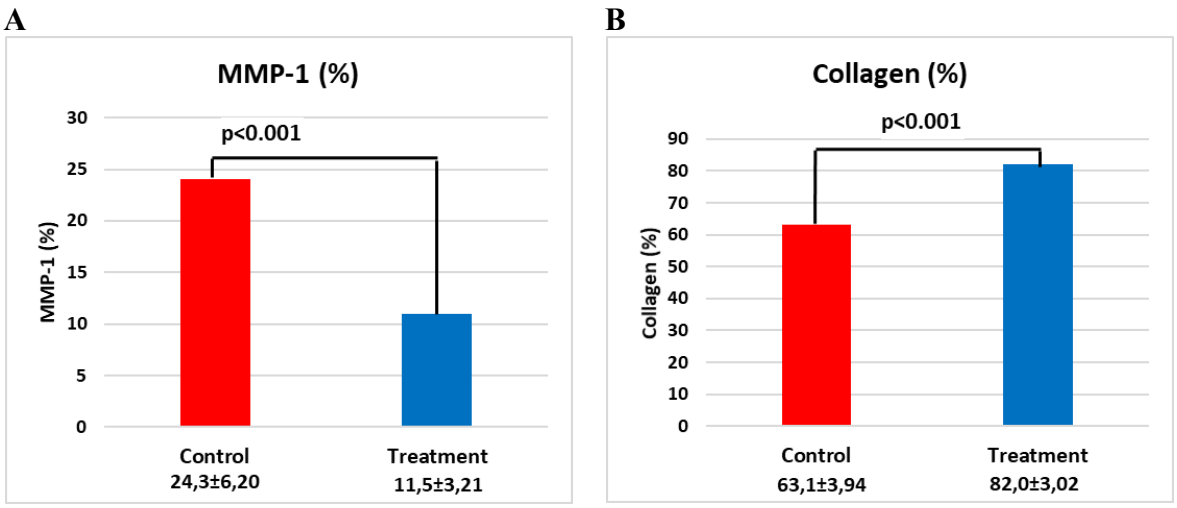

Figure 3. The Comparison of (A) MMP-1 Expressions (%) and (B) Collagen number (%) between Control and Treatment Groups

DISCUSSION

Recently, the Bajakah plant (*Spatholobus littoralis hassk*) receives a lot of attention in Indonesia because of its potential to inhibit the growth of cancer cells.[5] In addition, this plant has been widely used traditionally, for some health problem.[6] However, the current study is the first to demonstrate the potential of the ethanol extract of the stem of bajakah for preventing aging process, particularly for skin aging caused by exposure to UV-B rays.

Anti-Aging Medicine (AAM) aims to maintain health regardless of chronological age i.e. to stay healthy and biologically efficient. AAM aims to treat the causes of aging that underlie the aging process and to reduce all age-related diseases.[1] One aspect of aging that many people focus on is skin aging. By knowing the etiology and pathophysiology of skin aging, efforts can be made to prevent this skin aging process.

Previously, research has shown qualitatively the presence of flavonoids, saponins, steroids, terpenoids, tannins, and phenols, and has been shown to accelerate the wound healing process in mice.[5] These results were then confirmed through the results in this study, showing that the phytochemical content of the Bajakah stem extract were flavonoids, phenols, tannins, and antioxidant capacity. Each of these active compounds contained in the Bajakah stem ethanol extract has their respective contributions and roles in inhibiting the increase in MMP-1 expression and decreasing the amount of collagen.

Polyphenols have a photoprotective effect on oral and topical administration through their antioxidant abilities.[8] As antioxidants, phenolic compounds remain stable and do not experience resonance after donating atoms in radical compounds, thus stopping chain reactions caused by other radicals.[9] Because UV-B radiation increases the production of ROS which then activates MAPK and forms complex with the transcription factor AP-1, which plays an important role in the regulation of MMP-1 transcription which then results in collagen degradation.[10] Hence, polyphenols in the ethanol extract of the Bajakah stem which are antioxidants can neutralize the production of ROS due to UV-B and there is no increase in MMP-1 expression, and collagen degradation is also inhibited.

Research has reported that flavonoids can suppress MMP-1 expression and induce expression of procollagen type I protein in UV-induced cell culture.[11] Flavonoids also inhibit

Indonesian Archives of Biomedical Research
Volume 1 Issue 1, January-June 2021

the activation of nuclear factor kappa B (NFkB), which is a transcription factor for MMP-1 so that MMP-1 levels decrease and collagen degradation does not occur.[12]

Research has shown that tannins can interact with collagen through hydrogen bonds and hydrophobic interactions thereby increasing the thermal stability and enzymatic stability of collagen. Tannins can increase the hydrothermal stability of collagen and inhibit collagen degradation by MMP-1 through the formation of hydrogen bonds and hydrophobic interactions. Tannins also bind to collagen with high affinity because the structural flexibility of collagen compensates for the structural rigidity of phenolics.[13]

To date, many studies have been carried out using natural ingredients creams that contain antioxidants and bioactive compounds to prevent photoaging. This is because Indonesia has vast natural resources that are easily available and relatively cheap. In addition, natural compounds are relatively safer to use (relatively lower toxicity) than synthetic chemicals.

This research was conducted because we believed that the Bajakah stem extract had better potency than other plant extracts that had been studied previously. Bajakah stem extract cream contains phytochemical compounds and antioxidants that are better than other plants. The extract of the Bajakah stem used in this study had flavonoid content (79739.70 mg/100gQE), total phenol (14952.12 mg/100gGAE), tannins (17920.42 mg/100gTAE), antioxidant capacity (63141.06 mg/L) and Inhibitory Concentration (IC) 50% (13.25 mg/L).

Whereas previous research using cherry leaf extract cream (*Muntingia calabura* Linn) contained a total phenol of 2352.77 mg/100gGAE, flavonoids of 1765.34 mg/100gQE, tannins of 289.50 mg/100gTAE, antioxidants of 7563.90 mg/L GAEAC, and IC50% of 53.18 ppm were sufficient to inhibit the increase in MMP-1 expression and decrease the amount of collagen in the skin of male Wistar rats exposed to ultraviolet B rays.[14] The *Lepisanthes amoena* leaf extract containing flavonoids (986.62 mg/100gQE), antioxidant capacity (135627.21 mg/L), and IC50% (101.25 mg / L) can inhibit the increase in MMP-1 and decrease the amount of collagen in male Wistar rats exposed to UV-B rays.[15]

Based on the comparison of these bioactive compounds, it can be concluded that the Bajakah stem extract cream is potentially better for use as an Anti-Aging Medicine, especially skin aging caused by exposure to UV-B rays.

Excessive UV-B exposure is the main etiology of skin aging especially in tropical countries like Indonesia. Meanwhile, an increase in MMP-1 and a decrease in the amount of collagen is pathophysiology of skin aging due to exposure to UV-B rays. So that in relation to AAM it is necessary to make efforts to inhibit the increase in MMP-1 and decrease the amount of collagen. Bajakah stem ethanol extract cream is one of the Anti-Aging Medicine steps because it can prevent and inhibited the pathophysiology of skin aging.

CONCLUSION

The administration of Bajakah (*Spatholobus littoralis hassk*) stem ethanol extract cream inhibited increasing MMP-1 expression and the reduction of collagen in male Wistar rats (*Rattus norvegicus*) exposed to ultraviolet B. Next, it is necessary to perform a comparative study of the Bajakah stem cream with creams of other plant extracts with the same content to prove that natural ingredients are the best anti-aging modality for the skin. However, the toxic potential for long-term topical use ethanol extract of the Bajakah stem has never been reported; thus, further study is necessary. Clinical research in humans is also warranted before it can be used widely in society.

Indonesian Archives of Biomedical Research
Volume 1 Issue 1, January-June 2021

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Indonesian Archives of Biomedical Research

THE ROLE OF ARTIFICIAL INTELLIGENCE IN DESIGNING ANTIBODY-BASED THERAPY FOR COVID-19

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ABSTRACT

For several decades ago, passive immunization has already proven its ability to treat some diseases, especially pandemic diseases. On the contrary, after antibiotics discovery, the usage of passive immunization becomes neglected. Nowadays, we face a pandemic situation, COVID-19, which needs the possible treatment to save patients lives while medicines and vaccines are under development. By learning from history, passive immunization seems to be the best choice to save patient lives. As a kind of passive immunization, antibody-based therapy successfully treats diseases, including infectious diseases. Several antibody-based therapies are developed, as vast as the technology development, especially after the genetic codes announced. This article highlighted the influence of genomics tools, which helps researchers develop various platforms in developing monoclonal antibodies with high safety and efficiency in production and application.

Keywords: artificial intelligence, immunoinformatics, monoclonal antibody, antibody therapy

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INTRODUCTION

Disease prevention and treatment efforts, especially treatment, have developed so rapidly that various treatment models based on herbs, synthetic compounds, vaccines, and immunotherapy. Immunotherapy is conducted by providing an agent that eliminates pathogens without activating the body's immune system. In general, immunotherapy is carried out by delivering immune system components into the body of individuals[1,2]One of the immunotherapy is antibody-based therapy or passive immunization. Passive immunization is an immunotherapy method performed by providing antibody neutralization into the body of the individual[3]

At the time of an outbreak of the disease, passive immunization becomes the most viable treatment method to be applied. At present, when FDA approved one of passive immunization, convalescent plasma, to treat COVID-19 patients with severe symptoms. The approach used to surpress the increasing number of mortality rate caused by the infection of SARS-CoV-2 (FDA)[4]. Treatment with convalescent plasma, which introduced in 1890s by Kitasato and von Behring could eliminate pathogen by neutralizing antibodies provides by convalescent plasma. However, there are several limitations for convalescence plasma, such as difficulties in finding the people who proper to become a donor, the volume of antibodies delivered to the recipient, and antibodies purification to separate antibodies from blood cloths[5].

To overcome the weakness of convalescent plasma, monoclonal antibodies (mAbs) become an option to save patients' lives. At the past, mAbs developed by fusing immortal cells with splenocytes from immunized mice. Nowadays, the big data of sequence information available on the DNA libraries provide a new wave on antibody-based therapy. By using machine learning, antibody therapeutics are developed as humanization and synthetic monoclonal antibody which called engineered antibody[6]. This review will discuss the kinds of

Indonesian Archives of Biomedical Research
Volume 1 Issue 1, January-June 2021

antibody therapies developed and the role of artificial intelligence in developing antibodies for treatment.

METHODS

This article used systematic review according to guidance provided on PRISMA 2009.

Objectives

To find out the role of artificial intelligence in the development of antibody-based therapy for COVID-19.

The Strategy of Literature Searching and Selection

Literatures were searched on January 20th 2021 by using two kinds of electronic database, PubMed and SCOPUS, with keywords "artificial intelligence" AND "antibody", "antibody-based therapy", "immunoinformatics" AND “antibody”, "engineered antibody" and “COVID-19” AND “monoclonal antibodies”. We limit the articles which only articles written in English and published in 2019 until 2021. For collecting the articles, we did not restrict to specific type of articles. We sorted the articles to find out the redundancy possibility by filtering the title. We put several specifications which differ as inclusion and exclusion criteria. All articles for this review were research articles which develop monoclonal antibody using artificial intelligence as part of methods to treat COVID-19. We excluded research articles which related with vaccine development for COVID-19 and avalaible antibody-based therapy on hospitalized patients.

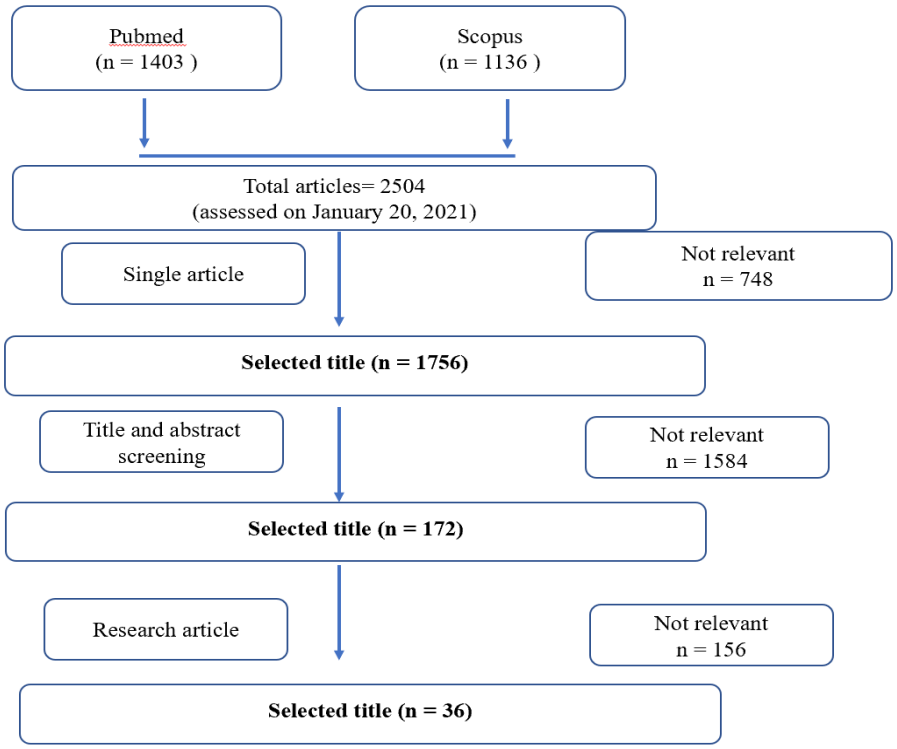

Figure 1. Process of literature searching and selection according PRISMA

Indonesian Archives of Biomedical Research
Volume 1 Issue 1, January-June 2021

RESULTS

A total of 2504 articles obtained from two journal repositories, but only 70% were single articles. After screening the title and abstract only 172 articles related to artificial intelligence in the development of therapeutic antibodies for COVID-19, only 36 articles are research articles. Among the selected articles, only 1 study used only experiments in silico and 17 articles using in vivo experiments to determine the effectiveness of antibodies developed (Table 1). The majority of studies report using cloning strategies and protein expression by mammalian cells, though just two studies use a plant as their expression system, Nicotiana benthamina, to produce antibodies (Table 2).

Table 1. Development humanized antibody for therapy

Ref	Experiment			Animal model for in vivo experiment	Antigen Target	Potential Antibody
	in silico	in vitro	in vivo			
Alsoussi[7]	Y	Y	Y	C57BL/6J mice (for mAb from hybridoma); BALB/cJmice (for SARS-CoV-2 challenge)	RBD	2B04
Hansen[8]	Y	Y	Y	VelocImmune® (VI) mice	RBD	Antibody cocktail (REGN10933+REGN10987)
Brett[9]	Y	Y	N	N	Spike	
Chi[10]	Y	Y	N	N	Extracellular domain of S protein	4A8
Custódio[11]	Y	Y	N	N	RBD	Sb23
Dong[12]	Y	Y	N	N	RBD	9 VHH-Fc
Ejemel[13]	Y	Y	N	N	RBD	mAb362
Fagre[14]	Y	Y	Y	Syrian Hamster	RBD	AvGn-B
Hassan[15]	Y	Y	Y	BALB/c mice; C57BL/6J; DBA2/J	S protein	1B07 and 2F05
Huo[16]	Y	Y	N	N	RBD	CR3022
Kim[17]	Y	Y	Y	Ferret, golden Syrian hamster, and rhesus monkey	RBD	CT-P59
Kreye[18]	Y	Y	Y	C57BL/6J mice; hamster	RBD	CV07-209
Li[19]	Y	Y	Y	BALB/c mice; C3B6 mice	RBD	Ab1
Liu[20]	Y	Y	Y	Hamster	RBD	2-15 mAb
Lv[21]	Y	Y	Y	a humanized hACE2 C57BL/6 mice	RBD	H014 Fab fragment
Miao[22]	Y	Y	N	N	RBD	89C8-ACE2
Noy-Porat[23]	Y	Y	N	N	RBD	MD62 and MD65
Parzych[24]	Y	Y	N	N	RBD; IL-6R	anti-CR3022 dmAb; Anti-IL-6R dmAb
Piccoli[25]	Y	Y	N	N	RBD	None
Pinto[26]	Y	Y	N	N	spike	S309

Indonesian Archives of Biomedical Research

Volume 1 Issue 1, January-June 2021

Ref	Experiment			Animal model for in vivo experiment	Antigen Target	Potential Antibody
	in silico	in vitro	in vivo			
Premkumar[27]	Y	Y	N	N	RBD	
Rattanapisit[28]	Y	Y	N	N	RBD	CR3022
Rogers[29]	Y	Y	Y	Syrian Hamster	Spike	CC12.1 mAb
Schafer[30]	Y	Y	Y	mouse adapted	ACE2; RBD region both surface and within the region	C104 shows Fc effector function
Seydoux[31]	Y	Y	N	N	RBD	CV30
Shah[32]	Y	N	N	N	RBD	in silico study for mutation on RBD influence mAb interaction
Shanmugaraj[33]	N	Y	N	N	RBD	B38; H4
Shi[34]	Y	Y	Y	Macaques	RBD; ACE2	CB6
Sun[35]	Y	Y	N	N	RBD region	2 VH-Fc (ab6 and m397) could bind to RBD
Tai[36]	Y	Y	N	N	RBD	None
Wan[37]	Y	Y	N	N	RBD	11 mAb
Wang[38]	Y	Y	Y	Rhesus Monkeys	RBD	MW05
Wu[39]	Y	Y	Y	hACE2 transgenic mouse	RBD	B38
Zhang[40]	Y	Y	Y	Balb/c	RBD	2H2/3C1
Zost[41]	Y	Y	Y	Balb/c, Macaques	Spike	cov2-2196; cov2-2130
Zost[42]	Y	Y	N	N	RBD	COV2-2130
Zylbermann[43]	Y	N	Y	Horse	RBD	F(ab')2 pAb

Table 2. Utilization on Artificial Intelligence (AI)

Ref	Source of Antibody	AI Utilization		Expression System		Organism for Cloning
		Antigen Design	Antibody Design	Ag	Ab	
Alsoussi[7]	murine lymph node	Y	Y	pFM1.2	pABVec6W Ab expression vector	mammalian cells
Hansen[8]	humanized mice; PBMC from human	Y	N	T7 promoter and helper plasmid expressing VSV and T7 RNA polymerase	expression vectors containing human heavy constant region and light constant region	HEK293T cells (for Ag); CHO cells (for mAb)
Brett[9]	Synthetic antibody	Y	Y	VSV N, P, L and G expression plasmids	pCAGGS	HEK293T cells

Indonesian Archives of Biomedical Research

Volume 1 Issue 1, January-June 2021

Ref	Source of Antibody	AI Utilization		Expression System		Organism for Cloning
		Antigen Design	Antibody Design	Ag	Ab	
Chi[10]	PBMC from human	Y	Y	pCAG vector	pcDNA3.4	HEK 293F (for Ag); Expi293F cells (for mAb)
Custódio[11]	synthetic antibodies	Y	Y	pαH	pCMVExt-Fc	HEK293-F (for Ag); mammalian cells (for Sybody)
Dong[12]	PBMC from Llama	NM	Y	NM	NM	NM
Ejemel[13]	Humanized mice	Y	Y	pcDNA 3.1 Myc/His	Immunoglobulin G1 (IgG) expression vector	Expi293 cells
Fagre[14]	PBMC from human	N	Y	N	various expression vector carrying the constant regions of human IgG1 heavy chain and the kappa chain	Expi293 cells
Hassan[15]	C57BL/6J mouse	N	Y	adenovirus vector	pABVec6W vectors	HEK293T cells (for Ag); Expi293F cells (For mAB)
Huo[16]	Synthetic human antibody	N	Y		pOPING-ET	ExpiCHO cells
Kim[17]	PBMC from human	N	Y		Fc fusion vector	CHO cells
Kreye[18]	PBMC from human	Y	Y	pFastBac	Human antibody expression vector	Sf9 cells (for Ag); ExpiCHO cells (for mAb)
Li[19]	PBMC from human	N	Y		pDR12 vector	Expi293 cells
Liu[20]	PBMC from human	Y	Y	pCAGGS	gWiz or pcDNA3.4	Expi293 cells
Lv[21]	spleen mRNA of mice immunized	Y	Y	pCAGGS	a phage-display scFv	HEK Expi 293F cells
Miao[22]	PBMC from human	N	Y		pFabVk vector and yeast gap repair	Expi293 cells
Noy-Porat[23]	PBMC from human	N	Y		pcDNA3.1+	ExpiCHO

Indonesian Archives of Biomedical Research

Volume 1 Issue 1, January-June 2021

Ref	Source of Antibody	AI Utilization		Expression System		Organism for Cloning
		Antigen Design	Antibody Design	Ag	Ab	
Parzych[24]	Nucleic acid	Y	N	NM	NM	NM
Piccoli[25]	PBMC from human	Y	Y	pcDNA3.1	human Igγ1, Igκ and Igλ expression vectors (for mAb)[26]	Expi293 cells (for Ag and mAb)
Pinto[27]	PBMC from human	Y	Y	phCMV1	human Igγ1, Igκ and Igλ expression vectors	Expi-CHO cells
Premkumar[28]	PBMC from human	Y	N	pαH		Expi293 cells
Rattanapisit[29]	N	Y	Y	pBY2e	pBY2e	Nicotiana benthamiana
Rogers[30]	PBMC from human	y	Y	phCMV3	expression vectors encoding the human IgG1, Ig kappa or Ig lambda constant domains	FreeStyle293F cells (Ag); DH5 alpha (mAB)
Schafer[31]	N	Y	Y	pNL4-3-nanoluc and lentiviral	Ig heavy and light chain expression vectors	FreeStyle293-6E cells (mAb)
Seydoux[32]	PBMC from human	Y	Y	pαH	pTT3 or pT4-341 HC	FreeStyle293-6E cells (Ag)
Shah[33]	N	Y	Y	N	N	n
Shanmugaraj[34]	N	N	Y	N	pBYR2eK2Md	Nicotiana benthamina
Shi[35]	PBMC from human	Y	N	pFasbac1 (ACE2) pCAGGS (RBD);	N	Hi5 cells
Sun[36]	N	Y	N	pFUSE1-Fc2	pFUSE1-Fc2 (Ag)	293T cells
Tai[37]	PBMC from human	N	Y	N	pComb3x vector	Expi293 cell
Wan[38]	PBMC from human	Y	Y	N	pcDNA3.4 mAb)	HEK293E
Wang[39]	PBMC from human	Y	Y	pKN293E	pKN293E	HEK293
Wu[40]	PBMC from human	y	y	pEt21 n pFastBac1, pEGFP-N1	pCAGGS	E coli, Baculovirus (Ag); HEK293T cells (mAb)
Zhang[41]	PBMC from mice	Y	Y	N	pcDNA3.4	ExpiCHO cells

Indonesian Archives of Biomedical Research
Volume 1 Issue 1, January-June 2021

Ref	Source of Antibody	AI Utilization		Expression System		Organism for Cloning
		Antigen Design	Antibody Design	Ag	Ab	
Zost[42]	PBMC from human	Y	Y	pαH	pTwist-mCis	ExpiCHO cells
Zost[43]	PBMC from human	y	y	pαH	pTwist-mCis	ExpiCHO cells
Zylbermann[43]	Serum from Horse	Y	N	pCAGGS	N	HEK-293T cells

DISCUSSION

The discovery of antibody utilization for Kitasato and von Behring treatment in 1870 elicits people to employ antibodies to treat disease. The employment of human plasma as the source of antibodies to treat patients was started in 1907. Nowadays, it is known as convalescence plasma therapy[5,44]. The use of convalescent plasma later turned into intravenous immunoglobulin (IvIg) after Cohn's antibody separation method and the team in the 1940s (figure 1).

Due to several requirements must be fulfilled by potential donors and also the number of donors to obtain enough antibodies become the obstacles to apply convalescence plasma as a routine therapy. On the other side, there is a demand for antibodies to treat diseases due to their ability to identify specific pathogens. The discovery of hybridoma technology in the production of antibodies by Kohler and Millstein makes the production of antibodies easier and more efficient [5].

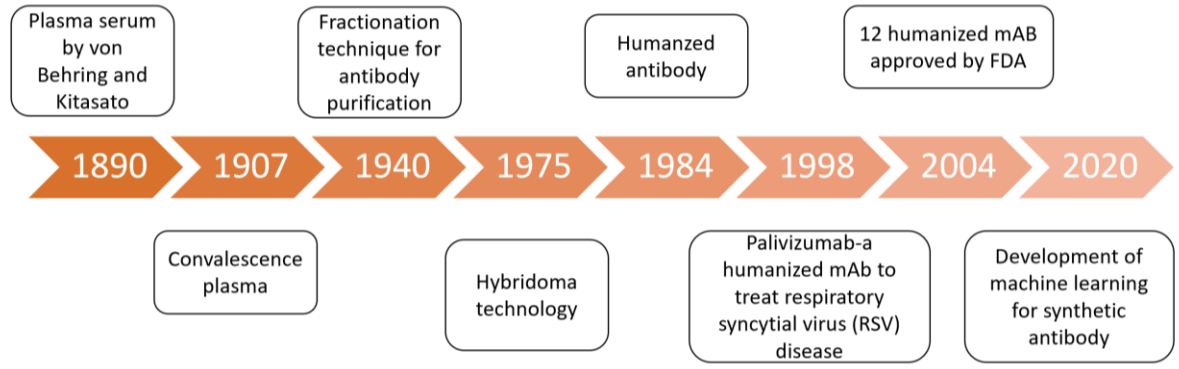

Figure 2. Timeline of antibody-based therapy

The hybridoma technology produces monoclonal antibodies from immortal cells known as hybridoma cells. However, another problem arises due to the patient's immune reaction to the antibodies given, namely the rejection of antibodies which occurs by the formation of HAMA (Human anti-mouse antibody. Two antibody production methods are developed to overcome the problem[45–48]. The first method uses hybridoma technology using human B lymphocyte cells and other methods using engineered antibodies (figure 2).

Indonesian Archives of Biomedical Research
Volume 1 Issue 1, January-June 2021

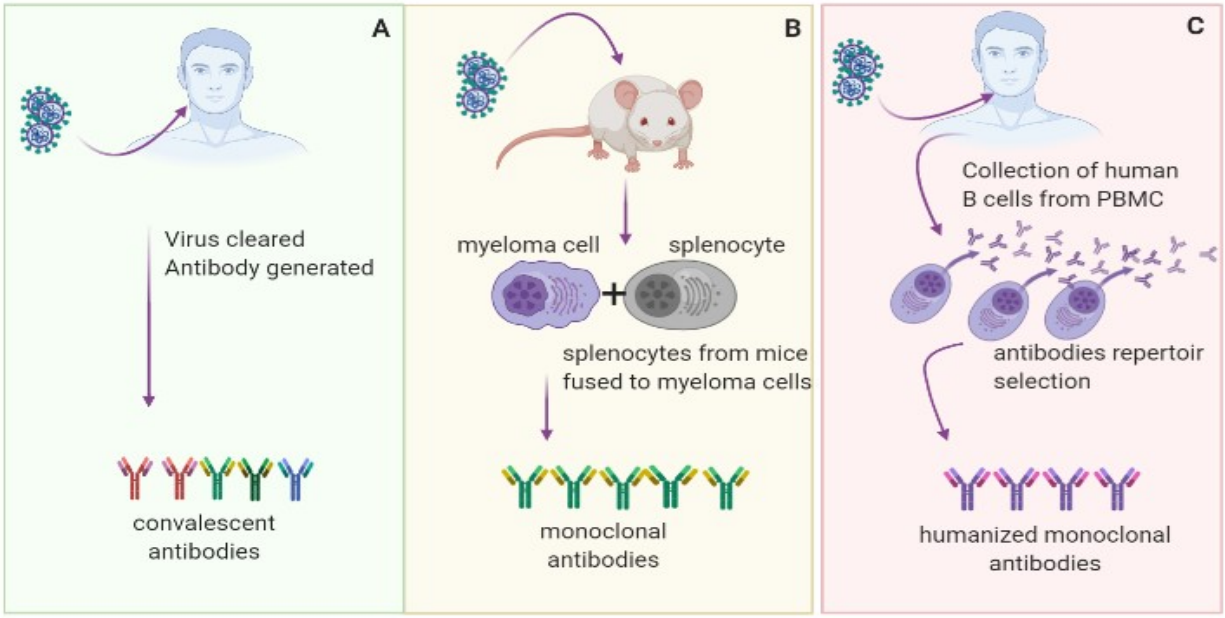

Figure 3. Development of therapy using antibodies. A. Production of antibodies with convalescent plasma method. B. Production of the antibody with the hybridoma method. C. Production of humanized antibody from peripheral blood mononuclear cells. Figure created by biorender.com.

Plasma Therapy for Covid-19

Convalescent plasma therapy has been used as one of medications for some outbreak diseases such as SARS (2003), avian influenza H5N1 (2005-2015), avian influenza H1N1 (2009-2010), and ebola (2013-2015). At present, the therapy becomes the potential therapy to cure COVID-19 patients, although the appliance must be done under supervision from EUA[49] Several studies have reported the effects of convalescent plasma administration in patients who have severe and critical symptoms.

Li et al. (2020) has reported that patients who get plasma therapy show some improvement. In the group of patients who received plasma therapy (n = 52), only 28.6% of patients who died were lower than the other group. Furthermore, the virus clearance is more effective by administering plasma therapy which is confirmed by PCR and shows that it is not detected the presence of viruses that cause COVID-19 from 24 hours to 72 hours after therapy with a 1x24-hour examination interval[50].

Shen (2020) reported his research results on 5 COVID-19 patients who were not smokers and received ventilators during treatment. The study reported that three patients did not use ventilators after plasma administration while the other two patients had been released from ecmo-type ventilators (extra-corporeal membrane oxygenation). In addition, the CT scores of the five patients also reached negative with a range of 1-12 days after convalescent plasma therapy. Similar result also reported by Duan et al who stated that plasma therapy improved the lung condition of patients indicated by the changing in the type of ventilator[51]

The positive effects of plasma administration in patients with severe and critical symptom criteria were also reported by Zeng et al and Duan et al[52,53]. Zeng et al. conducted the research by providing plasma therapy to 6 critical patients. The study reported that 5 out of 6 patients had negative CT scores but died, but the five patients' length of life was longer compared to patients in the group of patients who did not get plasma therapy. This study shows that plasma

Indonesian Archives of Biomedical Research
Volume 1 Issue 1, January-June 2021

therapy can improve the patient's immune system. Research conducted by Duan et al. reported that the administration of plasma convalescence could improve the patient's lung condition, and there is an elimination of the virus characterized by an increase in CT value until it is indicated negatively on the third or sixth day after convalescence plasma administration[52,53].

Human antibodies for therapeutic purposes can be developed with two approaches: hybridoma technology and genetic engineering divided into four platforms: hybrid mouse, phage display, transgenic mouse single B cell (figure 4)[54]. Both approaches require the involvement of artificial intelligence that can process genetic data into a monoclonal antibody. This artificial intelligence is used to design antigens used in hybridoma technology and design antibody structures that will then be produced using recombinant technology.

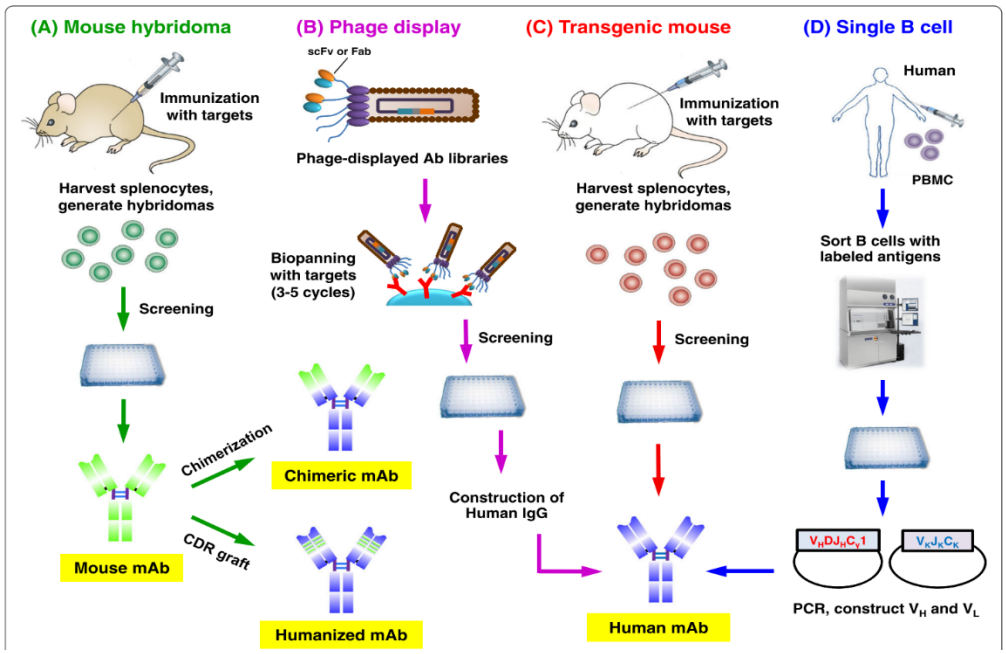

Figure 4. Development of antibody for therapy (cited from Lu et al.[54])

With hybridoma technology, specific antibodies can be obtained by stimulating the immune response to a particular antigen and a particular epitope. Meanwhile, with genetic engineering, antibodies can be produced using genetic information that develops antibodies' structure and is expressed by an expression system. Furthermore, the merging of mice and human antibodies are possible to be produced by engineered antibodies (recombinant antibodies). It is an urge to use bioinformatics tools to develop recombinant antibodies. The bioinformatics also applied to predict the promiscuous epitopes that can be conducted by immunoinformatics [55–57]. By applying bioinformatics, the structure of antibodies, antigen, and antigen-antibody interaction could be visualized by molecuar docking and molecular modelling.

Based on the selected literature, there are two strategies to develop antibodies for therapy in SARS-CoV-2 infection. The first strategy is designing antigens to stimulate the formation of specific antibodies, and the second is by designing human monoclonal antibodies (tables 1 and 2). The in vivo experiments have been conducted to trial the antibodies by using several animal models, namely Llama, horses, and mice[8].

Indonesian Archives of Biomedical Research
Volume 1 Issue 1, January-June 2021

Antigen design for the development of human antibodies with hybridoma technology

Antigen with high functionality is vital in producing antibodies through hybridoma technology. Due to the formation of antibodies requires cooperation between APC cells and T lymphocyte cells to stimulate B cells to release antibodies. With machine learning, the antigen is designed to interact with T and B cells that predicted or determined by various software for mapping epitopes. A wide range of epitope mapping immunoinformatics software can be used either through web servers or installed software.

The development of SARS-CoV-2 antigens to produce specific proteins was carried out using genetically engineered technology to produce both S and RBD proteins from the SARS-CoV-2 virus. Spike and RBD proteins chose because those proteins have an important role in the mechanism of viral infection in the host cell 57. SARS-CoV-2 virus infects humans by binding to human angiotensin-converting enzyme 2 (ACE2) on the cell surface with RBD region on S protein. The binding of RBD to ACE-2 is the main key to the process of viral infection into the cells[58].

In developing antibodies for COVID-19 therapy, the artficial intelligence plays a role in antigen design from protein selection and predicting the interaction between epitopes with B cells and T cells with molecular dynamic and molecular docking 55 (figure 5). This method is commonly used in vaccine development, but it also used to design an antigen for developing antibody therapy[59].

The application of molecular dynamics and molecular docking as a machine learning provides a very beneficial effect for antigen design because it can provide an overview of the structure and model of the designed antigen. Both machine learning will provide good visualization by merging other methods such as electron microscope and small angle X-ray scattering (SAXS)[9]. In a study conducted by Brett et al (2020), the structure of an endogenous glycoprotein G found in a VSV particle can be compared in size with the SARS-CoV-2 virus S protein designed in the same VSV particle 9. Other researchers used machine learning to predict epitope position in RBD SARS-CoV-2 that can interact with ACE2[18].

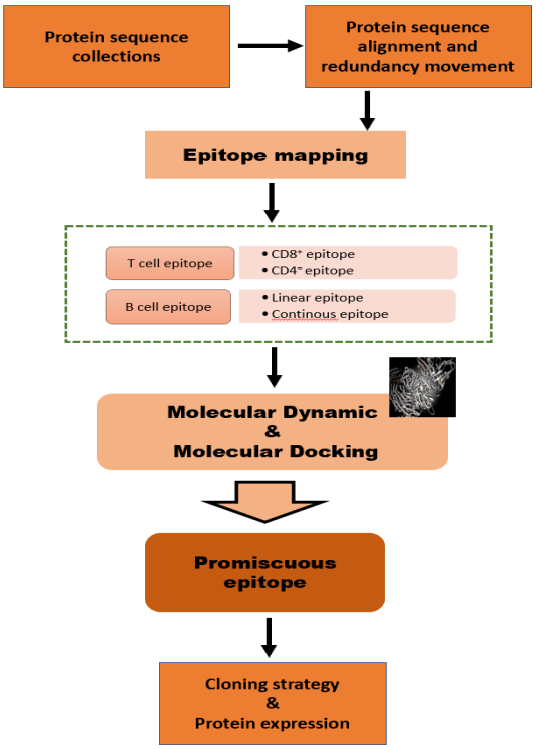

Figure 5. Schematic design of antigen by in silico experiment

Indonesian Archives of Biomedical Research
Volume 1 Issue 1, January-June 2021

Artificial intelligence also provides another benefit to developing antibody therapy, such as designing the antigen and interaction prediction only with a computational-based study. Shah et al. (2020) conducted antigen and antibody design research just using in silico experiment. This study was conducted to determine the interaction of CR3022 and CR3014 antibodies with both the overall spike protein and RBD area with confirmation analysis and analysis of ligand interaction fingerprints (PLIF) proteins. The study also provides an overview of the 3-dimensional structure of spike proteins and RBD regions [32].

Human antibody design with recombinant technology

At the beginning of its development, there are three kinds of recombinant antibodies, namely chimeric antibody, humanized antibody and fully human antibody (Figure 4). Those three antibodies developed to minimize the rejection of patients who receive therapy Antibodies. On chimeric antibody, modifications are made on the constant domain of antibodies with maintains the territory of fragment antigen binding (Fab) derived from animals. Then chimeric antibody developed into humanized antibody conducted by inserting the animal's complementary-determining region (CDR) into the antibody sequence with CDR grafting technology and subsequently developed into a fully human antibody. Fully human antibody provides an auspicious opportunity for treatment with antibodies, but these antibodies can still cause rejection reactions from patients, so two antibody formats are developed, fragment antigen binding (Fab) and single chain fragment variable (ScFv) (Figure 5).[6,47]

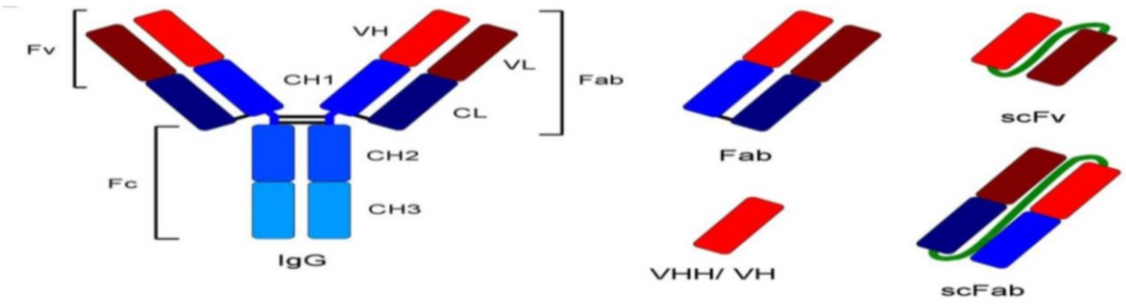

Figure 5. Types of recombinant antibodies (cited from Kuhn et al.[47])

The development of artificial intelligence has a very significant effect on the process of antibody development. In the first beginning, the antibody was employed using recombinant technology and followed by in vitro and in vivo experiments, which were performed by histology and statistics. Currently, the binding position of antibodies designed on antigen targets can be visualized using various machine learning (Figure 6). In developing antibody therapy for COVID-19, scientists not only used viral proteins as the antigen, but receptors (ACE-2) were also possible to be designed as antigens. Scientists used various mammalian cells to express ACE-2, such as CHO (Chinese Hamster Ovarian) and HEK (human embryonic kidney) cells (Table 2).

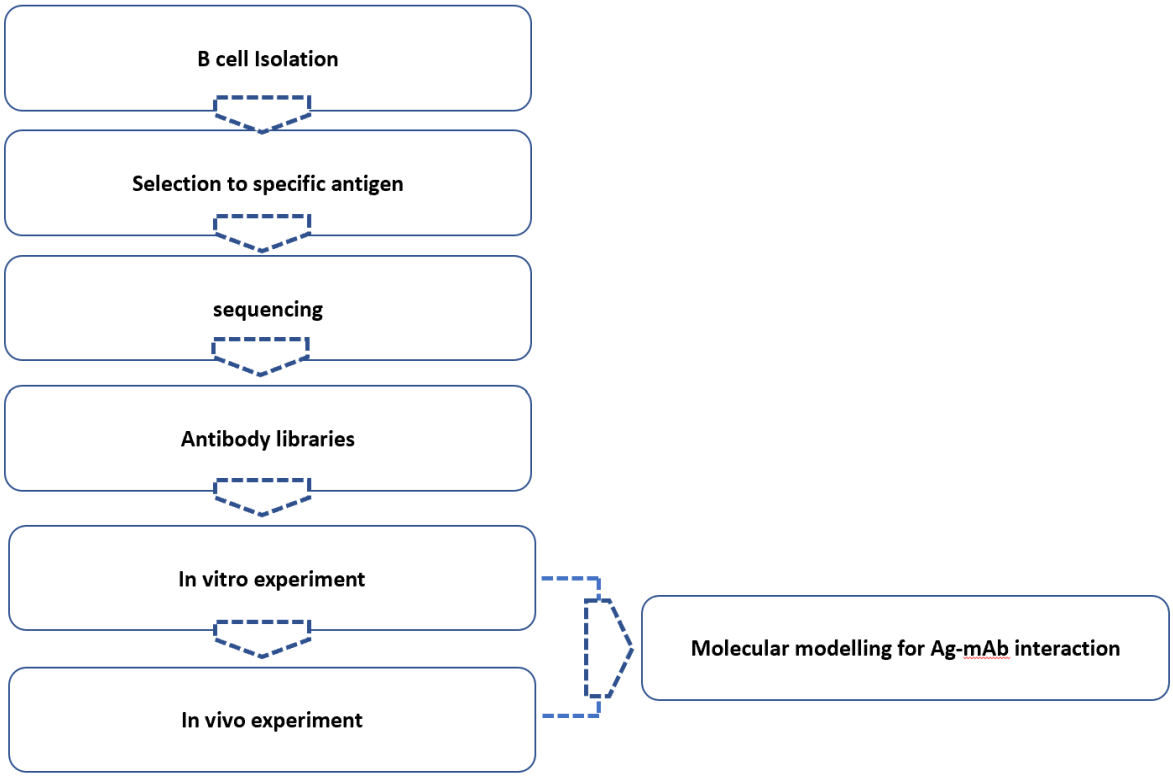

Figure 6. Schematic design of antibody development using artificial intelligence

The employment of machine learning has made it easier for researchers to get accurate information about antigens' interaction with antibodies designed. Besides, the information can be used by researchers to provide an explanation of its effects. Chi et al (2020) use cryo-EM to determine the interaction between the antibodies that have been designed (4A8) to the SARS-CoV-2 virus S-ECD antigen. The results of their study stated that the designed monoclonal antibodies have an excellent neutralization effect which is not caused by interruptions in the binding of RBD with ACE2. The molecullar modelling shows that neutralization is likely to occur as a result of inhibition of changes in confirmation of protein S[10].

CONCLUSION

Antibodies for therapeutic for COVID-19 have been utilized by artificial intelligence. It took parts in antigen and antibody design. Moreover, the visualization of antibody structures is one of the results from data processing using machine learning adopted to predict the neutralization effect by antibodies from research in vitro and in vivo. The utilization of artificial intelligence will enhance the research in developing antibodies as COVID-19 therapy.

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Indonesian Archives of Biomedical Research

GLUT1: STRUCTURE, FUNCTION, AND BIOMEDICAL SIGNIFICANCES

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ABSTRACT

Background; *Glucose is the main energy source for cells. To be utilized by cells, glucose enters the intracellular space facilitated by transporters. GLUT1 is one of the glucose transporters and is the most widely expressed by various tissues in the body. Not only that, cancer cells, which are known to have very high glucose requirements compared to healthy cells, have a high expression of GLUT1 as well.*
Reviews; *This paper reviews the structure, function, and biomedical importance of GLUT1 and specifically describes recent developments regarding GLUT1 inhibition as a novel therapeutic approach in both metabolic diseases and cancers.*
Conclusion; Inhibition of GLUT1 has also been shown to increase cancer cells' sensitivity to chemotherapy agents such as cisplatin and adriamycin. GLUT1 inhibition also increases the sensitivity of cancer cells to radiotherapy.

Keywords: *GLUT1, cancers, metabolic diseases.*

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INTRODUCTION

Glucose is the main energy source for eukaryotic organisms, which plays an important role in metabolism and cellular homeostasis. Catabolism of glucose through glycolysis, the citric acid cycle, and oxidative phosphorylation will produce energy in the form of adenosine-5-triphosphate (ATP). Glucose also functions as an important raw material for synthesizing the main cell biomolecules, such as lipids, non-essential amino acids, and nucleic acids. In humans and animals that are breastfeeding, glucose plays an important role in synthesizing lactose in the mammary glands, which is the main carbohydrate in milk.[1,2]

Glucose is mainly obtained directly from the diet through the enzymatic hydrolysis of disaccharides and polysaccharides in the digestive tract. Under certain physiological conditions, glucose can be synthesized by organs in the body such as the liver through the breakdown of glycogen (glycogenolysis) and the synthesis of glucose from non-carbohydrate substrates, namely pyruvate, lactate, glycerol, and glucogenic amino acids (gluconeogenesis).[3]

Brain and red blood cells require a continuous supply of glucose. Meanwhile, most of the cells in the body need glucose in moderation. An increase in the concentration of glucose in the plasma can cause glucose poisoning (glucotoxicity). Therefore, the concentration of glucose in plasma is maintained in the range of 60-110 mg/dL and involves the role of metabolic hormones, especially insulin and glucagon. Glucose homeostasis in the body is maintained through coordinated regulation of three processes: 1) absorption of glucose as a result of the enzymatic hydrolysis of carbohydrates from food by the small intestine; 2) glucose production by body organs, especially the liver; 3) consumption of glucose by almost all body tissues.[1] The discussion of this paper will further focus on the third regulatory process.

Indonesian Archives of Biomedical Research
Volume 1 Issue 1, January-June 2021

The glucose concentration is maintained over a narrow range by the homeostatic mechanism, as mentioned above, so that most cells obtain glucose from the interstitial fluid passively down the concentration gradient across the plasma membrane.[2] Meanwhile, eukaryotic cells' plasma membrane (lipid bilayer) itself is impermeable to hydrophilic polar molecules such as glucose. Thus, to facilitate the entry and exit of glucose into and from the cell, an integral membrane protein called the glucose transporter is required.[1,4] The exception is the brush border epithelial cells of the small intestine and the proximal renal tubule, where glucose is absorbed against the electrochemical gradient through a pump's secondary active transport mechanism. $Na^+/K^+/ATP$.[2] This also shows that the expression of glucose transporters is tissue-specific, and this expression reflects each tissue's physiological characteristics.[4]

Glucose transporter proteins fall into two distinct groups, structurally and functionally. The first group is the Na^+-dependent glucose cotransporter, an active glucose transport mechanism for glucose absorption in the small intestine and glucose reabsorption in the urinary tract system. As the name implies, sodium-dependent glucose transporter, this protein group is given the SGLT symbol, and its coding genes are included in the solute carrier gene group (SLC) as the solute carrier family 5A (SLC5A). The second group is glucose transporters that are not dependent on sodium (Na^+). This group transports glucose passively across the plasma membrane by a facilitated diffusion mechanism. The protein symbol for this second group is GLUT, which stands for glucose transporter. The gene encodes this protein belongs to the solute carrier family, SLC2A.[2,3,4,5]

The human body has three types of glucose transporter that different in structure and transport mechanism. The first type is the Na^+-dependent glucose cotransporter, an active glucose transport mechanism for glucose absorption in the small intestine and glucose reabsorption in the urinary tract system. As the name implies, sodium-dependent glucose transporter, this protein group is given the SGLT symbol, and its coding gene is included in the solute carrier gene group (SLC) as the solute carrier family 5A (SLC5A). The second type is a glucose transporter that is not dependent on sodium (Na^+). This transporter transports glucose passively across the plasma membrane by a facilitated diffusion mechanism. The protein symbol for this second group is GLUT, which stands for glucose transporter. The gene encoding belongs to the solute carrier family, SLC2A. The third type, SWEETs, is a recently characterized glucose transporter, a uniporter. The SLC50 family codes this transporter.[2,3,4,5,6]

REVIEW

This article will further elaborate on the structure, function, characteristics, and biomedical interests of GLUT1 as a glucose transporter distributed in almost all tissues. Several recent studies regarding GLUT1, especially its prospects as a new drug target for cancer, will be explicitly discussed at the end of this review.

Na^+-independent glucose transporter (GLUT)

Na+-independent glucose transporter (GLUT) or facilitative glucose transporter transports glucose across cell membranes, which is hydrophobic, in a diffusion-facilitated manner without requiring energy but down the concentration gradient between glucose outside and inside the cell or vice versa. To date, fourteen members of the GLUT transporter have been identified based on the similarity in structure and sequence of amino acids in their constituents. The fourteen transporters are then grouped into three main classes based on the similarity of amino acid sequences, namely classes I, II, and III. Class I consists of four well-characterized transporter members, GLUT1, GLUT4, and GLUT14. Class II has a specific feature that is able to facilitate fructose consisting of GLUT5, GLUT7, GLUT9, and GLUT11, also known as the

Indonesian Archives of Biomedical Research
Volume 1 Issue 1, January-June 2021

"odd GLUT" group. Class III is the "even GLUT" group consisting of GLUT6, GLUT8, GLUT10, GLUT12, and HMIT. This last group has a similar feature in that they have an internalization signal that maintains these transporters at the intracellular location under a steady state.[3,4,6]

Based on the amino acid sequence of GLUT, a protein model was created to estimate the orientation of this transporter on the cell membrane. GLUT is estimated to have twelve hydrophobic helical domains. This arrangement shows that the amino acid GLUT sequence forms twelve loops across the plasma membrane with the amino (NH2-) and carboxyl (COOH-) ends located on the cytoplasmic side and a large intracellular loop between the 6 and 7 transmembrane domains. The transmembrane has a high homology between one GLUT and another, while the amino acid sequence of the amino ends, carboxyl ends, and loops vary. The most obvious structural difference between GLUT Class I, II, and III is the long extracellular loop position. The long extracellular loop of class I and II members is between transmembrane domains 1 and 2 and have glycosylation sites that increase the efficiency of transport of these proteins. Whereas class III members do not have a long extracellular loop between transmembrane domains 1 and 2 with potential glycosylated sites between transmembrane domains 9 and 10.[3,4,6]

The GLUT protein model described above explains that the transfer of glucose by GLUT is based on two conformational alternatives. In the first conformation GLUT displays the glucose binding site on the extracellular side of the plasma membrane and in the second conformation GLUT displays this binding site on the intracellular side. The binding of glucose (or other suitable monosaccharide) at one of these sites triggers a conformational change of GLUT. In this process monosaccharides can move across the plasma membrane in two directions (leaving or entering the cell). Despite their similar structure, GLUTs differ in their ability to transport monosaccharides, regulation, and distribution across tissues.

Molecular characteristics of GLUT1

GLUT 1 is a class I glucose transporter isoform firstly isolated from the HepG2 cell line and then successfully cloned and characterized by Mueckler et al. in 1985. The GLUT1 coding gene is a solute carrier family 2 member 1 (SLC2A1). SLC2A1 is an official symbol created by the HUGO Gene Nomenclature Committee (HGNC). HGNC itself is a committee of the Human Genome Organization (HUGO) responsible for approving/assigning unique symbols and names for protein-coding genes, ncRNA genes, and pseudogenes to enable clear scientific communication. SLC2A1 is located on the short arm of chromosome 1 (1p34.2), consisting of 10 exons with the complete reference sequence code in the NCBI gene bank is NC_000001.11 and the reference sequence codes for mRNA and protein are NM_006516.2 (3687 bp) and NP_006507.2. respectively.[7,8]

GLUT1 is an integral membrane protein that plays a role in the glycolysis pathway as a uniporter for glucose with several alternative names: DYT17, DYT18, GTR1, HepG2 glucose transporter, MGC141895, MGC141896, PED, SLC2A1, and solute carrier family 2 (facilitated glucose transporter), member 1.[9, 10] This protein has a reference sequence code from the NCBI gene bank, namely NP_006507.2 and the UniProt reference code is P11166. Table 1 shows the molecular characteristics of GLUT1.

Indonesian Archives of Biomedical Research
Volume 1 Issue 1, January-June 2021

Table 1. The results of the chemical parameter analysis of GLUT1 using ProtParam Expasy

Parameter	Analysis results
Molecular weight	54083.78
Formula	$C_{2503}H_{3916}N_{622}O_{664}S_{23}$
Atomic composition	Carbon (C) 2503 Hydrogen (H) 3916 Nitrogen (N) 622 Oxygen (O) 664 Sulfur (S) 23
Total number of atoms	7728
Theoretical pI	8.93
Number of amino acids	492
Amino acid composition	Ala (A) 34 [6.9%] Arg (R) 21 [4.3%] Asn (N) 14 [2.8%] Asp (D) 7 [1.4%] Cys (C) 6 [1.2%] Gln (Q) 21 [4.3%] Glu (E) 24 [4.9%] Gly (G) 46 [9.3%] His (H) 5 [1.0%] Ile (I) 37 [7.5%] Leu (L) 59 [12.0%] Lys (K) 16 [3.3%} Met (M) 17 [3.5%] Phe (F) 38 [7.7%] Pro (P) 23 [4.7%] Ser (S) 35 [7.1%] Thr (T) 26 [5.3%] Trp (W) 6 [1.2%] Tyr (Y) 13 [2.6%] Val (V) 44 [8.9%]
The estimated half-life	30 hours (mammalian reticulocytes, in vitro) >20 hours (yeast, in vivo) >10 hours (Escherichia coli, in vivo)

GLUT1 is a strong hydrophobic protein consisting of 492 amino acids. Like the class 1 GLUT structure discussed earlier, GLUT1 has a long NH2 end and COOH end facing the cell's cytoplasmic site, a cytoplasmic loop connecting transmembrane domains 6 and 7, and glycosylated extracellular loop between transmembrane domains 1 and 2 10. The GLUT1 activity transport studied in the oocyte of *Xenopus laevis* frogs showed that GLUT1 transports glucose with Km ~3 mM. Under equilibrium exchange conditions, GLUT1 has Km 20 - 21 mM for 3-O-methylglucose and 5 mM for 2-deoxyglucose. Other monosaccharides that can be transported by GLUT1 are galactose, mannose, and glucosamine.[2,11,12] GLUT1 also transports dehydroascorbic acid, the oxidized form of vitamin C, into the brain.11 This transporter is a highly conserved isoform with approximately 74 - 98% identical amino acid sequences between species (humans, cattle, rats, mice, chicken, and fish). The glycosylated part of GLUT1 is the most often part of differences in amino acid sequences between species.[2,11,12]

Indonesian Archives of Biomedical Research
Volume 1 Issue 1, January-June 2021

GLUT1 is expressed in the highest levels in cells actively proliferating, such as in developing embryos, cells forming the blood tissue barrier, erythrocytes, astrocytes, and heart muscle.[9,10] Erythrocytes and brain cells selectively express GLUT1, so it is known as erythrocyte and brain glucose transporter. This protein makes up 3-5% of the erythrocyte membrane protein. However, further research has proven that GLUT1 is the glucose transporter most widely expressed by tissues in the body such as the eyes, peripheral nerves, placenta, and mammary glands. This protein expression is also high in cell lines that are routinely used in the laboratory but not by hepatocytes.[2,10,13,14]

GLUT1 has two forms based on its molecular weight, namely the 45 kDa and 55 kDa forms. These two forms are distinguished only by the length of the glycosylation chain. The 45 kDa form is found in most cells, including astrocytes, and is thought to be responsible for glucose uptake by cells. The 55 kDa form is mainly found in the endothelial cells of the brain micro blood vessels and erythrocytes as the main glucose transporter. If there is a GLUT1 deficiency, the amount of glucose from the blood that enters the brain will decrease. This, in turn, can lead to central nervous system dysfunction.[2,10,13,14]

Biomedical Significances of GLUT1

The biomedical importance of GLUT1 is mainly genetic, caused by mutations of its encoding gene, SCL2A1. The manifestation of this mutation is mainly a deficiency in GLUT1's function as the brain's primary glucose transporter. Some of the syndromes associated with GLUT1 deficiency are GLUT1 deficiency type 1 syndrome (Glut-1 DS1, OMIM 606777), GLUT1 type 2 deficiency syndrome (Glut-1 DS2, OMIM 612126), dystonia, and idiopathic epilepsy. Research data on animals and humans show that the safe limit value for glucose transport across the blood-brain barrier to meet the needs of brain metabolism and cerebral function is very narrow. In the mildest clinical phenotype with intermittent symptoms of epilepsy, dyskinesia, and ataxia, it is predicted that there will be a 25-35% decrease in GLUT1 transporter function, while in the more severe phenotypes, it is estimated that there will be 40-75% decrease in function. Most SCL2A1 mutations are de novo in nature, whereas this mutation is inherited as an autosomal dominant trait in familial cases. A case of autosomal recessive transmission has also been reported. All mutations detected were heterozygous, while homozygous mutations from GLUT1 were thought to be lethal, causing death in utero.[14,15]

Genetic abnormalities in GLUT1 deficiency syndrome or known as De Vivo syndrome, have various clinical manifestations. Patients with the missense mutation generally show mild to moderate symptoms without a clear boundary of phenotype-genotype correlation. The results of other studies showed that mild mental retardation and movement disorders were more common in patients with a missense (type A) mutation than those with a translational initiation mutation (type B) or multiple deletions in exon (type C). It suggests additional mechanisms at work, such as modifying proteins and genes that then influence the phenotype and potentially play a role in these complex states' pathophysiology. It is also possible that secondary genes and proteins are involved in glucose transport. Patients with identical mutations exhibit phenotypic heterogeneity in terms of the range of clinical expression and disease severity.[14]

Cases of GLUT1 deficiency syndrome type 1 have been reported in 27 variations of protein sequences. One of them was reported by Klepper J et al.[16] On the UniProtKB/Swiss-Prot website, P11166 variant is recorded as p.Arg468Trp variant. The position of variation is at position 468 of the wild-type amino acid sequence GLUT1, where the amino acid arginine is replaced by tryptophan caused by a missense mutation of the protein-coding gene. This variant belongs to the type of "disease" variant, which means that the variant found in patients and related diseases has been reported in the literature. Physio-chemically, the amino acid changes that occur are large and alkaline amino acids (arginine / R) into large and aromatic

amino acids (tryptophan/W). This change has a BLOSUM score of -3, which means that the chance of substitution arginine to tryptophan is quite low. The lowest score of BLOSUM was -4, with the lowest probability interpretation of amino acid substitutions and the highest 11 with the highest probability interpretation of substitutions.[17] The secondary structure analysis of GLUT1 using the Psipred program showed quite clear differences in secondary structure between the wild type and the R468W variant even though the amino acid substitution location was not in that part but the range of amino acid sequence 380 - 410.[17] It shows that substitution in one amino acid can causes changes in the secondary structure of GLUT1, which then causes malfunctioning.

GLUT1 expression abnormalities affect a pathway that impacts the pathogenesis of diabetic nephropathy. There are indications that variations in SLC2A1 contribute to the development of microangiopathy in patients with type 2 diabetes mellitus.[18,19] Individuals with the Xbal (-) GLUT1 allele are more likely to develop DM that progresses to diabetic nephropathy.[18] Other studies have shown that GLUT1 regulates cytokines and growth factors that act as pro-sclerotic mediators that induce diabetic glomerulosclerosis.[20] Furthermore, GLUT1 inhibition becomes a further therapeutic approach in diabetes mellitus to prevent serious complications. Studies show GLUT1 is a promising therapeutic target for preventing diabetic retinopathy. Knockdown of GLUT1 by intraocular injection of siRNA directed at SLC2A1 significantly reduced mean retinal glucose levels in diabetic mice. Systemic treatment of diabetic mice with forskolin or genistein, which binds to GLUT1 and inhibits glucose transport, significantly reduced retinal glucose to the same level seen in non-diabetics.[21] Another similar study by Zhi-Peng You et al. (2017) showed similar results.[22]

Research by Yabo Hu et al. demonstrated that 4 mM aspirin administration could inhibit glucose uptake and metabolism in vascular endothelial cells by downregulating GLUT1 expression and suggested that vascular endothelial cell GLUT1 is a potential target for aspirin. This research certainly requires further investigation to be applicable to various new disease treatment strategies through the GLUT1 inhibition approach.[23]

GLUT1 and cancer

Another significance of GLUT1 is its overexpression in cases of malignancy. Cells that undergo malignant transformation experience accelerated metabolism and an increase in glucose demand. In mammalian cells, glucose transport across the plasma membrane is the first step that limits GLUT-mediated glucose metabolism. Increased glucose transport in cancer cells is associated with increased and deregulated expression of glucose transporter proteins, especially in the overexpression of GLUT1 and GLUT3. Oncogenic transformations in mammalian cell cultures lead to increased glucose transport and overexpression of GLUT1 through interactions with the GLUT1 promoter-enhancing elements. Studies in humans have shown that increased expression of GLUT1 in tumors is associated with lower survival. The main regulator of GLUT1, especially HIF, also has increased expression in cancer associated with the extracellular environment of cancer, which tends to be hypoxic.[24,25]

Several studies have shown a relationship between GLUT1 expression and cancer. Among them, Kang SS et al. showed that GLUT1 expression was related to the invasion ability of breast cancer cell lines where cell lines with high GLUT1 expression had a tendency to be more aggressive and potentially malignant than those that did not. Krzeslak et al. reported that GLUT1 and GLUT3 expression was significantly increased in poorly differentiated breast and endometrial tumors compared with well-differentiated ones. Increased mRNA and GLUT1 protein levels have also been reported in colorectal, thyroid, lung, stomach, head and neck, bladder, kidney, and endometrial carcinomas. Carvalho KC et al. demonstrated that GLUT1 was expressed in varying degrees by tumor type. Sarcomas, melanomas, hepatoblastomas, and

Indonesian Archives of Biomedical Research
Volume 1 Issue 1, January-June 2021

lymphomas do not express GLUT1, which means that there are other glucose transport mechanisms that play a role in these tumor types.[26,27,28]

GLUT1 also has biomedical significance in cancer stem cells. Research by Wanandi et al. showed an increase in the expression of the breast CSC GLUT1 gene CD24- / CD44 + in hypoxic conditions, which correlated with the increased expression of HIF1α. This increase in GLUT1 expression was followed by an increase in glucose consumption. Although this study did not measure the activity of the four key enzymes that regulate glycolysis (hexokinase, glucokinase, phosphofructokinase, and pyruvate kinase), it seems that most of the pyruvate formed is converted to lactate as indicated by increased LDH activity accompanied by increased production of lactate by the cells. These results show that under hypoxic conditions, HIF1α regulates the glucose metabolic state of CD24- / CD44 + breast CSCs in the form of increased anaerobic glycolytic activity.[29]

GLUT1 inhibition as a novel cancer therapeutic strategy

Evidence showing increased glucose consumption in cancer cells versus healthy cells implicates the role of GLUT1 and other roles of this protein in oncogenesis, paving the way for new strategies in cancer therapy. Biomedical studies on GLUT1 inhibition in various types of cancer using natural and synthetic compounds have been carried out in the last decade with promising results. Some examples of GLUT1 inhibitor compounds that have been studied on various types of cancer cells and animal models are shown in Table 2.

Tabel 2. Studies of GLUT1 inhibition by synthetic and natural compounds in various types of cancer

Cancer types	Cell lines/model	Synthetic compound	Effect	References
Breast cancer (triple negative)	11 TNBC cell lines and patient-derived samples	BAY-876	BAY-876 impairs the growth of a subset of TNBC cells displaying high glycolytic, lower oxidative phosphorylation (OXPHOS) rates, and high protein level of retinoblastoma tumor suppressor (RB1).	30
Lung cancer	A549/ nude mouse	WZB117	WZB117 inhibited cell growth in cancer cell lines and cancer growth in a nude mouse model.	31
Neuroblastoma	SH-SY5Y	WZB117	WZB117-induced GLUT1 inhibition suppressed tumor cell growth, induced cell cycle	32

Indonesian Archives of Biomedical Research
Volume 1 Issue 1, January-June 2021

Cancer types	Cell lines/model	Synthetic compound	Effect	References
			arrest and reduced glycolysis metabolites.	
Ovarian cancer	A2780 and OVCAR3/NOD-scid IL2Rgamma(null) mice	Ciglitazone	Ciglitazone induces apoptosis in ovarian cancer cells by inhibiting and decreasing expression levels of GLUT-1	33
Colon cancer	HTC-116, SW480		Metformin inhibited Glut1 and SLC1A5 expressions	
Cervical cancer	HeLa	Metformin	leading to reduced influx of glucose and glutamine in cancer cells,	34
Breast cancer	MCF-7		which is associated with reduced tumor growth	
Ovarian cancer	PA-1 (p53 wild type), OVCAR3, MDAH2774 (p53 mutant), and SKOV3 (p53 null)	Resveratrol	RSV induced apoptosis in ovarian cancer cells by impairing glucose uptake, involving Akt-regulated plasma membrane GLUT1 trafficking.	35
Hepatocellular carcinoma	HuH-7		D-allose inhibited cancer growth by reducing both GLUT1 expression and glucose uptake.	
Breast adenocarcinoma	MDA-MB-231	D-Allose		36
Neuroblastoma	SH-SY5Y			
Breast cancer	4T1	Epigallocatechin-3-gallate (EGCG)	EGCG decreased the expression of hypoxia-inducible factor 1α (HIF1α) and glucose transporter 1 (GLUT1),	37
Lung cancer	A549 cells	Curcumin	These results suggested that	38

Indonesian Archives of Biomedical Research
Volume 1 Issue 1, January-June 2021

Cancer types	Cell lines/model	Synthetic compound	Effect	References
			curcumin inhibit lung cancer invasion and metastasis by attenuating GLUT1/MT1-MMP/MMP2 pathway	

SUMMARY

Apart from the direct effect that causes a decrease in GLUT1 expression and a decrease in glucose uptake, which then results in the suppression of tumor growth, inhibition of GLUT1 has also been shown to increase cancer cells' sensitivity to chemotherapy agents such as cisplatin and adriamycin.[39,40,41] GLUT1 inhibition also increases the sensitivity of cancer cells to radiotherapy.[42]

Inhibition of GLUT1 appears to provide new hope for cancer treatment strategies. Various compounds have been shown to be able to inhibit the expression of GLUT1, which then suppress tumor growth both in vitro and in vivo. A more detailed understanding of these compounds' specific inhibitory mechanisms against cancer cells and their delivery mechanisms is needed. Thus, the inhibitory effect does not affect healthy cells because, as stated in the beginning, GLUT1 is the most widely expressed glucose transporter in the body tissues and has a high level of expression in actively proliferating cells.

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Indonesian Archives of Biomedical Research

BIOFILM FORMING POTENTIAL OF *STREPTOCOCCUS SUIS*: FOCUSING ON LUXS/AI-2-MEDIATED QUORUM SENSING SYSTEM

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ABSTRACT

Because of its virulence and ability to survive, the incidence of infection caused by *Streptococcus suis* (*S. suis*), an emerging zoonotic pathogen, is expected to increase significantly. A biofilm-forming process, which is a cornerstone of chronic infection, influences the survival rate of *S. suis*. The mechanism helps bacteria to live longer in host tissues, form colonies, escape immune clearance, and share genetic information. At this moment, the most studied regulatory mechanism of *S. suis* biofilm formation is Quorum Sensing (QS), mainly on LuxS/AI-2-mediated QS system, in which AI-2 is the most closely related molecule to biofilm formation. In this system, LuxS acts as the key player in the process. The understanding of biofilm formation in S. suis, especially the LuxS/AI-2-mediated QS system, is a valuable contribution to future therapeutic research frameworks.

Keywords*: Streptococcus suis, biofilm, Quorum Sensing, LuxS, Auto Inducer-2*

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INTRODUCTION

Streptococcus suis (*S. suis*) is a gram-positive facultative anaerobic bacteria which is well-known as a commensal microorganism at the respiratory system of the swine. On the other hand, in human, this bacteria is a potentially serious threat to induce fatal complications such as meningitis, septic shock, endocarditis, and peritonitis after the onset of infection (1, 2). The majority of *S. suis* infection was found in areas with pig-farming and pork industries. From 2002 until 2013, nearly more than 1.500 cases of *S. suis* infections was reported globally, most of them came from Asian countries (3). South East Asia was considered to be a potentially threatened area with its high amount of pork consumption and swine-related industries. A research conducted in Sanglah Hospital, Bali, from 2014 until 2017 has shown that there were 44 cases of *S. suis* meningitis confirmed by Polymerase Chain Reaction (PCR) of the patients' cerebrospinal fluid sample (4). The incidence of *S. suis* infections in human is predicted to elevate significantly due to unavailability of vaccines as the specific protection and the ability of its virulence factors to induce catastrophic pathological mechanisms (5).

The most prevalent strain of *S. suis* which has isolated in human is serotype 2 (86,5%), followed by serotype 14 (2,3%), and 1 (0,6%). *Streptococcus suis* serotype 2 (SS2) has considered to be the most aggressive subtype among all serotypes (6). The exacerbation of inflammatory pathway is a hallmark of SS2 infection in both human and swine. Biofilm formation by *S. suis* can cause a chronic infection that is especially difficult to treat when the pigs' immunity decreases (7). Biofilm forming ability has known to be a foundation of chronic infection (8) and the most effective mechanism to hinder from host's immunity. By aggregating into a biofilm, microorganisms may escape the dangerous host environment. The activity makes it possible for bacteria to live longer and form colonies in host tissues, as well as prevent immune clearance and share genetic information (9). To date, studies related with the

mechanism of *S. suis* biofilm formation are mainly focused Quorum Sensing (QS), mainly on LuxS/AI-2-mediated QS system. The knowledge about biofilm formation in *S. suis*, particularly on LuxS/AI-2-mediated QS system, is a worthwhile contribution in constructing research frameworks in future therapeutic strategies (8).

The General Concepts of Bacterial Biofilm and its Formation

Biofilm is an architecturally complex communities in which microorganisms are bound to a substratum and trapped in a matrix of polymetric substances that provide them with a high degree of tolerance and resistance (10). Biofilms have a number of effects on humans, the majority of which are harmful (11). In general, all biofilms contain an extracellular matrix that holds cells in a pack. This matrix is frequently made up of a polysaccharide biopolymer as well as proteins and Deoxyribonucleic Acid (DNA) (12). Matrix exopolysaccharide has a wide variety of properties depending on growth conditions, medium, and substrates. Biofilms have adhesive proteins in their extracellular matrix. For example, Biofilm-associated proteins (Bap) are present in the matrix of *Streptococcus aureus* (13). Extracellular DNA, in addition to exopolysaccharides and proteins, provides structural integrity to the biofilm, which was previously believed to be the product of cell lysis and subsequent genomic DNA release. The biofilms are stabilized by the eDNA (14).

There are five stages of bacterial biofilm formation in general: initial/reversible attachment, irreversible attachment, formation of micro-colonies, maturation, and cellular detachment or dispersion. The bacteria make contact with the surface through the cell pole and are transiently bound to the substratum during the reversible attachment stage. There was a reorientation to the longitudinal cell axis, cell cluster growth, nonmotile, and activation during irreversible attachment. During maturation, layered cells form clusters, and at the end of the process, the maximum cell cluster formation was observed, reaching a thickness of 100 m. The majority of cells were also displaced from the substratum at this point. Dispersion, the final stage of biofilm formation, revealed changes in cell cluster composition, the formation of pores and channels, and dispersion (15). Depending on environmental conditions and unique strain characteristics, different bacteria use different mechanisms to form biofilms (16).

Several studies have shown that *S. suis* can form biofilm in vitro using various biological models. A number of factors influence the formation of biofilm in *S. suis*, including the OCT protein, the signaling molecule autoinducer-2 (AI-2), and the collagen-binding 40 (cbp40) (8). There are four categories of bacterial biofilm's regulatory mechanism called Extracytoplasmic Function (ECF) signaling pathway, Two-Component System (TCS), intracellular second messenger cyclic diguanylate (c-di-GMP), and bacterial Quorum Sensing (QS) system (17-20). The most studied regulatory mechanism of *S. suis* biofilm formation at the moment is QS, which is primarily based on the LuxS/AI-2-mediated QS framework, with AI-2 being the most closely related molecule to biofilm formation. In S. suis, the *luxS* gene controls biofilm formation (10). According to one study, inhibiting the expression of *luxS* in *Riemerella anatipestifer* can reduce AI-2 production, which has a direct impact on biofilm formation (21).

The LuxS/AI-2-mediated Quorum Sensing System

In *S. suis*, biofilm formation is largely influenced by bacterial intercellular communication through QS, which is involved in a number of physiological processes including extracellular protein synthesis, biofilm maturation, and virulent factor gene expression. QS is a cell-to-cell communication system that controls the expression of genes in bacteria to promote organized adaptation of various genes (22). When bacteria are faced with a difficult situation or climate, the QS system sends information signals between cells to increase the number of bacteria, biofilm formation, and EPS production, allowing bacteria to

Indonesian Archives of Biomedical Research
Volume 1 Issue 1, January-June 2021

better adapt to their surroundings (8). QS systems can be divided into some classes. They are LuxR-LuxI systems of Gram-negative bacteria, the auto-inducing peptide of Gram-positive bacteria, and the LuxS/AI-2 system found in both Gram-positive and Gram-negative bacteria (10).

The general mechanism of QS is to ensure that a bacteria's capacity to generate and release signaling molecules known as autoinducers (AIs) is not impaired (23, 24). The QS-controlled mechanism, which is a molecular signal network regulated by the *luxS* gene encoding the S-ribosylhomocysteinase (LuxS) enzyme found in virulent SS2, allows *Streptococcus suis* to form biofilms. LuxS has been implicated in enhancing Auto Inducer-2 (AI-2) biosynthesis, adhesions, biofilm formation, cell metabolism, and resistance to host immune responses and antimicrobial therapy in a number of studies (22, 25, 26).

The gene that encodes LuxS; the *luxS* gene, is highly conserved in bacteria. High identity and similarity of *luxS* genes were found in *Streptococcus mutans, Streptococcus pyogenes, Streptococcus pneumoniae, Lactococcus lactis, Clostridium perfringens, Neisseria meningitidis, Escherichia coli,* and *Haemophilus influenza* (27). LuxS is involved not only in the development of the AI-2 signaling molecule, but also in central bacterial metabolism and is a component of the activated methyl cycle (28). LuxS is specifically involved in the conversion of S-adenosine homocysteine to S-adenosylmethionine (SAMe).

SAMe is a typical biomolecule that primarily functions as a methyl donor. The loss of SAMe feature and inhibition of AI-2 synthesis occurs when luxS is mutated or deleted. LuxS is a critical component of the LuxS/AI-2-mediated QS system. AI-2 is a byproduct of bacterial methyl metabolism that helps activated methyl cycles regulate their metabolism (29). The methyl group is extracted from SAMe and then converted into S-Adenocylhomocysteine (SAH), which is a toxic metabolite, in the LuxS/AI-2 system. SAH is then converted to adenine and S-Ribose Homocysteine (SRH) by a 5'-methylthioadenosine/S-adenosyl homocysteine nucleosidase (Pfs). Following that, SRH is converted to 4,5-dihydroxy-2,3-pentanedione (DPD) and homocysteine acid (HCY) by LuxS (30). LuxS then catalyzes the cleavage of SRH's thioether linkage, resulting in HCY and DPD. Self-cyclization creates DPD, which is then transformed to AI-2 (Figure 1). As bacterial density grows, AI-2-mediated QS is activated (31).

When a small amount of AI-2 was applied to the growth medium, the ability of *S. suis* to form biofilm was greatly increased, while high concentrations of AI-2 inhibited the ability to form biofilm. At 24 hours, adding 2 µM AI-2 to the mix significantly improved biofilm formation, but had no effect at 48 hours (32). The capacity of *S. suis* to form biofilm is improved by overexpression of AI-2 and the incubation period, according to these studies (26).

Indonesian Archives of Biomedical Research
Volume 1 Issue 1, January-June 2021

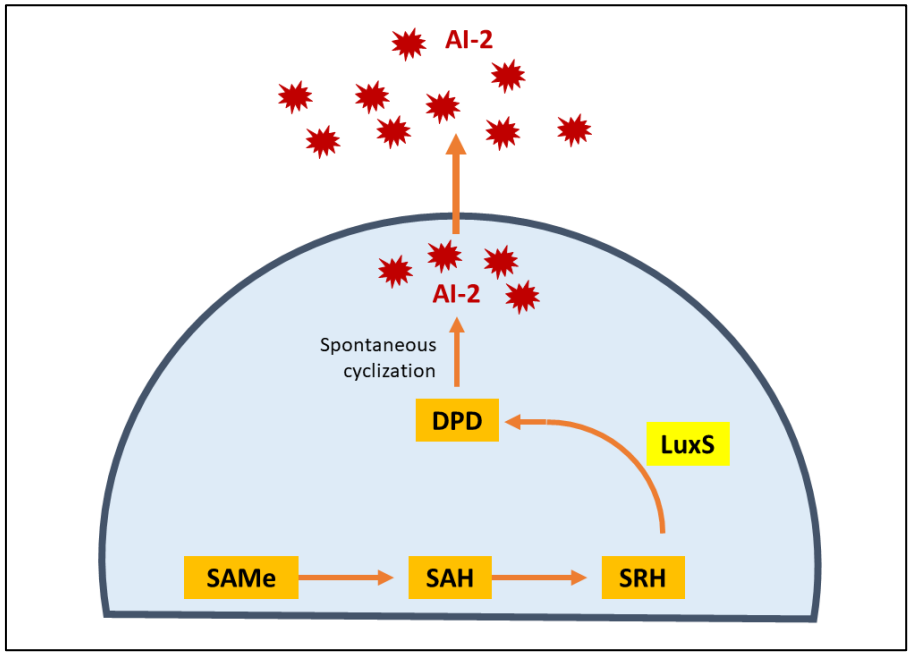

Figure 1. AI-2 Synthesis Pathway in *S. suis*. SAMe, S-adenosylmethionine; SAH, thioglucoside homocysteine; SRH, thioglycoside-type homocysteine; DPD, 4,5-dihydroxy-2,3-pentanedione; AI-2, Auto Inducer-2.

CONCLUSION

The rising number of *S. suis* infections, coupled with the newly discovered importance of the LuxS/AI-2 system in *S. suis* cell-to-cell contact and virulence, necessitates the production of antibacterial strategies that target the LuxS/AI-2-mediated QS system. In order to regulate bacteria by inhibiting pathogen signaling, a better understanding of bacterial regulation and AI-2 uptake regulation, as well as the identification of genes involved in the *S. suis* QS system, is needed.

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Indonesian Archives of Biomedical Research

SEAHORSE (*HIPPOCAMPUS SP*): OPPURTINITIES IN ASSISTED REPRODUCTION WITH NANOTECHNOLOGY APPROACH

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ABSTRACT

Because of its virulence and ability to survive, the incidence of infection caused by *Streptococcus suis* (*S. suis*), an emerging zoonotic pathogen, is expected to increase significantly. A biofilm-forming process, which is a cornerstone of chronic infection, influences the survival rate of *S. suis*. The mechanism helps bacteria to live longer in host tissues, form colonies, escape immune clearance, and share genetic information. At this moment, the most studied regulatory mechanism of *S. suis* biofilm formation is Quorum Sensing (QS), mainly on LuxS/AI-2-mediated QS system, in which AI-2 is the most closely related molecule to biofilm formation. In this system, LuxS acts as the key player in the process. The understanding of biofilm formation in S. suis, especially the LuxS/AI-2-mediated QS system, is a valuable contribution to future therapeutic research frameworks.

Keywords*: Streptococcus suis, biofilm, Quorum Sensing, LuxS, Auto Inducer-2*

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INTRODUCTION

Indonesia is an archipelago country and have more biodiversity, including the seahorse. The marine aquaculture centers have an cultivated this animal, because they have high economic value which is made Indonesia as one of the largest exporting countries in Asia (Nasution et.al, 2019; Wang et.al, 2020).

Seahorses used in traditional medicine which is have an several effects, such as increasing stamina and hematopoesis, anti-fatigue, anti-oxidant, anti-tumor, anti-inflammatory, anti-aging, and important for fertility because have an aphrodisiac effects (Adam et.al, 2014; Kim et.al, 2016; Nasution et.al, 2019; Safryna, 2020; Sanaye et.al, 2014; Wang et.al, 2020; Zhang et.al, 2017).

The function in fertility, it is suggested that bioactive compounds is steroid testosterone, which in animal tissue it is usually used in aphrodisiacs for male. The function of this hormone is to stimulate the spermatogenesis, increasing the development and activity from genital organ (Safryna et.al, 2020). In addition, for pharmacology this hormone can be effect hypothalamus to release Gonadotropin Releasing Hormone (GnRH) in reproductive system (Zitzmann et.al, 2013).

Nowadays, one of the health problems in Indonesia is infertility, which is one of the factors from the male. Male infertility influences from many factors, including from spermatogenesis and testis disorder (Li et.al, 2020; Zitzmann et.al, 2013). Nowadays, the treatment for infertility used GnRH therapy, which is used to influences the maturation of cells, and increasing testosterone levels, and increasing the germ cells (Biers, 2010; Li et.al, 2020).

GnRH therapy can use a analogues GnRH (GnRHa) which is reported that 3100 patients through 50 publications given the GnRHa. It is still controversy between the impact in reproductive system and needed the study (Li et.al, 2020; Schwentnr et.al, 2005). It is caused

Indonesian Archives of Biomedical Research
Volume 1 Issue 1, January-June 2021

by several things, such as a large doses and long periods, and it can make a mutation in gene receptor GnRH when the therapy is failure (Zitzmann et.al. 2013).

Seahorse have an active hormone steroid testosterone from natural product which is suggested an alternative therapy for GnRH. Furthermore, the bioactive compound steroid in seahorse can be optimally to target organs, which is the reproductive system. To evaluate the function of bioactive compound, in this decade it can be using modern science and technology. It can be done through drug delivery systems with several carrier systems, such as a nanoparticle (Schwentner et.al, 2005). The target organ achievements by delivery through nanoparticles are evaluated using transmission electron microscopy (TEM).

Seahorses (Hippocampus sp)

Seahorse is one of the unique fish, because not only about the body's morphology, it is also the male is pregnancy (Blumenfeld, 2019). The female will give the eggs to male individu and spermatids will be included in the pore that fertilization occurs (Zhang et.al, 2017). The Male will incubate the eggs in the anteriomesial /central pore near the abdomen (Koldewey, 2010).

The species of seahorses in Indonesia are Hippocampus barbouri, H. comes, H. histrix, H. kelloggi, H. bargibanti, H. spinosissimus, H. trimaculatus and H. kuda (Meikasari et.al, 2020). Seahorse is a natural product and promising used to be the potential drug for medical use (table 1) (Chen et.al, 2014; Kumaravel et.al, 2012; Meikasari et.al, 2020).

Table 1. Bioactive Compound in Seahorse for Medical Used

No	Bioactive Compound	Medical Used	Ref
1	Amino acids	Anti-inflammatory, inluence the histology vesicular seminalis	1, 9
2	Protein	Increasing Hb	3
3	Alkalase and pepsin	Increasing testosterone and weight testis	6
4	Steroid, taurine	Aphrodisiac	7
5	Amino acid, steroid, fatty acid, microelement	Potential to healthy product	17
6	Steroid, cholesterol	Fertility	19
7	Steroid and fatty acid	Treatment BPH	20
8	Steroid and saponin	Vitality and immune respone	21
9	Trace element	Medical used	22

Indonesian Archives of Biomedical Research
Volume 1 Issue 1, January-June 2021

Gonadotropin Releasing Hormone (GnRH)

GnRH is decapeptide (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2) produced from hypothalamus as the main hormone reproductive in Hypothalamus-Pitutary Gonad axis (HPG Axis) in vertebrate (Gopurappilly et.al, 2013; Jin, 2014; Sakhteman et.al, 2016). GnRH hormones has 30 isoforms, 15 in vertebrates and 15 invertebrates (NCBI, 2020). GnRH influences the spermatogenesis, steroidogenesis, and feedback mechanism in gonads. In mammals, it still consists GnRH1, GnRH2 and GnRH3, but only the GnRH1 and GnRH2 have the same amino acids in vertebrate. In humans, GnRH have a similar name as protein a progonadoliberin. It is located on chromosome 8p.2-p.21 with 276 bp which is translates from 92 amino acids (Jin, 2014; Sakhteman et.al, 2016). This gene encodes to stimulate the release of LH and FSH, and when this gene has a mutation it can be caused a hypogonadotropic hypogonadism in human (Uniport, 2020). GnRH in seahorse has a similar name protein as a gonadoliberin (NCBI, 2020).

GnRH has a G protein receptor which is transmembrane proteins to regulating fertility (Jin, 2014; Sakhteman et.al, 2016). GnRH receptors can be found in humans and the teleost such as seahorse. In vertebrate, HPG Axis will be expressing a various GnRH. In mammals it carried out by GnRH in hypothalamus which binds the G receptors stimulate to release a gonadotropin, FSH and LH in gonad (Zhang et.al, 2018). Nowadays, there is an G receptor from kisspeptin gene as a transcription promote of the GnRH (Jin, 2014; Sakhteman et.al, 2016).

Structure of GnRH in human and seahorse have a different structure from swissmodel.expasy tools (fig.1), but they have the same kisspeptin gene which is regulate the hypothalamus in vertebrate (Zhang et.al, 2018).

The kisspeptin gene is also found in the teleost group of fish, such a seahorse which is a key regulator of reproduction and sensitive with steroid (Gopurappily et.al, 2013; Zhang et.al, 2018) Most teleost fishes have kiss 1 and kiss 2, furthermore in mammals there is only one kisspeptin gene (kiss 1). Kisspeptin gene in seahorse directly regulates GnRH, although it is needed the study. However, the previous study reported the function is the same with mammals as an activator the reproductive axis through the stimulation of GnRH secretion (Zhang et.al, 2018).

The treatment for male with infertility is being developed, for example with testosterone therapy. One of them is with GnRH therapy. But now, several studies were reported have an several problems. There are about the uncomfortable in patients, cost therapy is too high, long period, and influenced from antibody from each individual (Zitzmann et.al, 2013).

Zhang et.al, (2018) reported that in teleost, there is still widely underexplored, because the kisspeptin is still debate, but the previous study suggests that fish kisspeptin have a similar with mammalian kisspeptin, which is the key to activator the reproductive axis. It can be investigated to be an alternative as a substitute therapy for infertility in male. But, the gene kisspeptin between in humans and seahorse have a different structure from pymol tools (fig.2). In this condition, it is needed the study to answer the question is there a seahorse extract as a natural product can be a medical use for male infertility to replace a GnRH therapy, and it can be a novel therapeutic targets and interventions.

The previous study has reported by Zhang et.al (2018) that kisspeptin gene suggest involved the regulation in reproductive function of pubertal onset and gonadal development, and also in seahorse male pregnancy there is regulating testosterone synthesis. In assisted reproduction, study used next generation sequencing was found the Kisspeptin (kiss 1) suggest as a gene which is modulate hormone levels and reproductive outcome and can be a novel

therapeutics targets (Blasco et.al, 2020; Patel, 2020; Trevisan et.al, 2020). Another study by Hestiantoro et.al (2019) reported that kisspeptin concentration was associated with postmenopausal because the serum concentration was lower. In the other study was reported, kisspeptin was reported as a potential biomarker in across pregnancy and can be a future study in reproductive system such as for Polycystic ovarian syndrome (Hu et.al, 2019; Rodrigues et.al, 2019; Talbi, 2019). Khamis et.al (2019) also reported that undergoing kisspeptin treatment as in vitro fertilization and approaches to increasing the reproductive role. Kisspeptin also reported by Oride et.al, (2020) that promising to increasing overcoming diabetic testicular dysfunctions which is enhancing spermatogenesis, as well as reducing the testicular inflammation and apoptosis. The fertility drugs may act on kiss 1 expressing neurons because it was modulating the HPG axis (Chenthamara et.al, 2019).

In this decade, the technology in science have been develop. There is a drug delivery system which is combine between a medicine and high technologies. When we want to know about the bioactive compound in seahorse can be use in medicine as a male infertility, one of them technology we can used the nanotechnology.

Nanotechnology in Reproductive System

Nanotechnology is the design, characterization, production and application of structures, devices and compilation systems between sciences and engineering with a nanometer scale (1-100nm) or one millionth of a meter (Albanese et.al, 2012; Brohi et.al, 2017).

Study about nanotechnology is conducted to determine the interaction of nanoparticles with biological systems is a nano-bio interaction. Nanoparticles (NP) interact with cell surface membranes. Once bound to the receptor, nanoparticles in the cell through endocytes mediated by the receptor (Falchia et.al, 2018).

Nanoparticles is synthesized from various organic or inorganic materials such as lipids, polysaccharides, metals, proteins, and synthetic or natural polymers. Formulation of the drug with nanoparticles are currently developing to increasing efficiency, prolongation of drug in circulation, targeting specific tissues, therapeutic reactions, and side effects or toxicity (Albanese et.al, 2012; Falchi et.al, 2018).

The effects of nanoparticles in cellular and tissue level are not clearly. They are some effect, that used in male and female reproductive systems at the clinical, cellular and molecular levels. In the male reproductive system, it is can be influences fertility with increasing the quality of sperms from in vivo or in vitro (Falchia et.al, 2018; Falchi et.al, 2018).

The previous studies by Safaa et.al, (2016) reported that function of nanoparticle in reproductive system, study about efficiency of NP cerium oxide (CeO2) can protect viability and increasing the motility of sperm. In the other study, they reported with addition of 5 µg/mL of vitamin E combined with 1% Nano-Se can be improve semen quality in chickens (Rezvanfar et.al, 2013). Study about supplementation from SeNPs can protect the quality of spermatozoa (motility, DNA integrity) and spermatogenesis from oxidative damage caused by anticancer agents in the male reproductive system (Saraf, 2010).

Nanoparticles is one of the technologies which is increasing the bioavailability of active substances in seahorses. It can be influences about the smaller size can increasing the delivery of active substances with low solubility because they contain lipids and cholesterol. Nanoparticles will be transferred into cells through endocytosis transitions. Nanonization of natural products provides benefits by increasing active substances, small therapeutic doses, and increasing absorption and bioavailability in the body (Kumar et.al, 2019).

To evaluating the mechanism from nanoparticle in human body, we can use a gold standar for this with a transmission electron microscopy.

Evaluation of Nanoparticles in Reproductive System

Transmission electron microscopy (TEM) is the most technique used to characterize nanoparticles, and to assess the safety and toxicological potential of a nanoparticle. TEM is a gold standard for nanoparticle because such as a reason, there are about the resolution in nanometer sizes in the range 1-100 nm, image display with atomic dimension, it can describe the physical properties quantitatively (size, shape, and surface morphology), and also can determine the agglomeration of a material (Lina et.al, 2013; Masta et.al, 2020).

In addition, TEM also has a limitations, because the analysis process depends on the transfer of representative fractions of samples containing sufficiently large amounts of particles to the specimen carrier (TEM grid) which is affected by the purification process and the concentration of the sample and the specimens must be very thin (Walther, 2017).

TEM produces an electron beam from transmitted to the specimen. The electron interacts with the specimen and transformed into a scattered electron. Electrons will be focused by electromagnetic lenses that are projected the screen to produce diffraction, contrast-amplitude images, phase-contrast images or various image variations (Hu et.al, 2019). Interpretation data from the TEM obtained interactions between electrons and matter, giving rise to a dynamic diffraction effect involving X-rays from high-voltage cathode tubes or synchrotron sources (Walther, 2017).

CONCLUSION

Seahorse (Hippocampus sp) as a natural product with bioactive compound steroid testosterone has the oppurtinities to investigate the further study as a substitute for GnRH therapy for male infertility, it is because the same structure and receptor protein in human and needed the study. The treatment can be evaluation with nanotechnology to know the efficient and efficacy to use in reproductive system.

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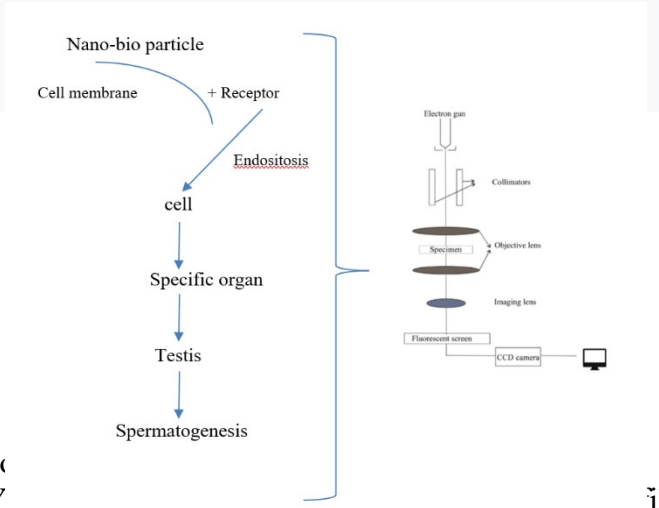
Figure 3: Overview the Nanoengineering Process

Source: Brohi et.al, 2017



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Indonesian Archives of Biomedical Research
Volume 1 Issue 1, January-June 2021

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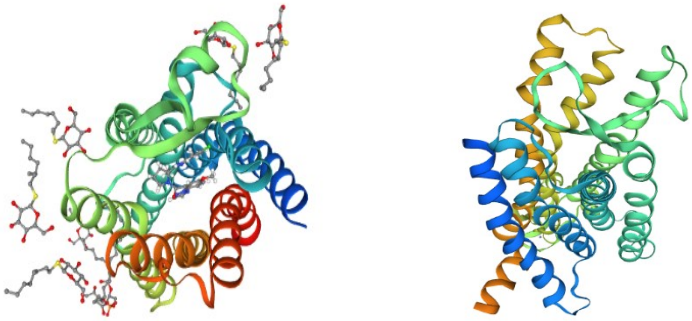
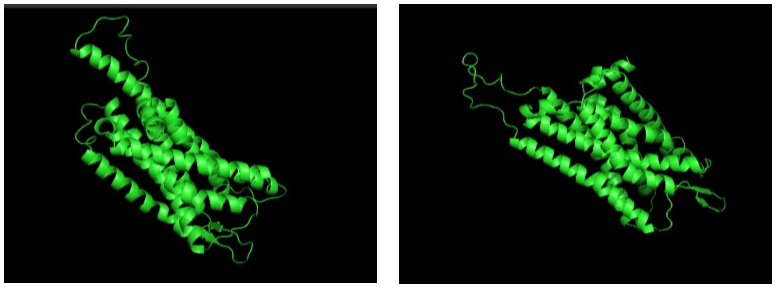
Figure:



A B

Figure 1: A. GnRH in Human, B. GnRH in Seahorse
Source: Swissmodel, 2020



A B

Figure 2: A. Kisspeptin Gene in Human, B. Kisspeptin Gene in Seahorse
Source: Pymol, 2020

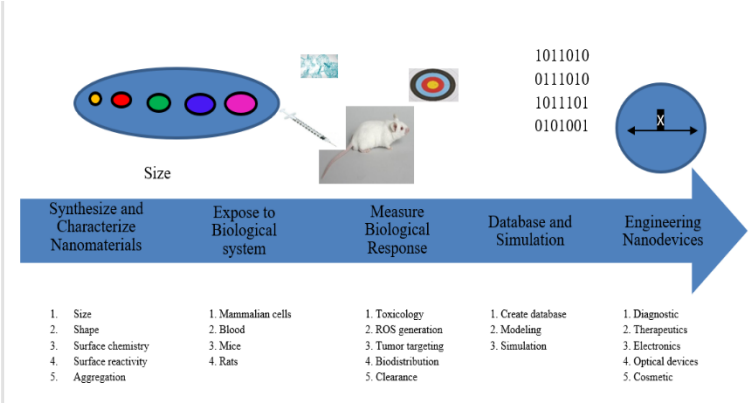


Figure 3: Overview the Nanoengineering Process
Source: Brohi et.al, 2017