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Effect of +Dalethyne on *Pseudomonas aeruginosa*

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Kayapan Satya Dharshan

PT. Dermozone Pratama, Mayapada Tower, South Jakarta, Indonesia.

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ABSTRACT

Objective: The objective of the research was to find the solution for treatment of the nosocomial infection specially infected due to *Pseudomonas aeruginosa* and to evaluate the effect of +Dalethyne on amount of IL-1β expression in *Pseudomonas aeruginosa* in infected rat. **Methods:** Young healthy male wistar mice (*Rattus norvegicus*) were incised on their back and divided into three groups. Group I and II were inoculated with *Pseudomonas aeruginosa* and Group III was further given 10 ml of +Dalethyne using cotton bud. On day 4, each group of rats was sacrificed to take the skin tissue. The skin tissues were further tested for amount of IL-1β expression in skin tissue by using immunohistochemistry method for detecting antigens. This is a post test control group design and comparison between groups. **Results:** The results showed that the amount of IL-1- β expression in fibroblast cell was decreasing in group III by median value of 31 with treatment by +Dalethyne, followed by groups I and II with wound and wound + infection by mean value of 45 and 33 respectively. **Conclusion:** It has been shown that +Dalethyne reduces the amount of IL-1β. The aldehyde compounds in +Dalethyne inhibits the NF-Kb production and JAK2/STAT1 signaling. Thus the inflammatory phase in the wound area stops quickly and initiates the proliferation and tissue remodeling phase. Therefore this can be concluded that +Dalethyne is an alternative treatment that can be used in patients with post-surgical nosocomial infections or burns.

INTRODUCTION

Nosocomial infections occur worldwide and affect both developed and developing countries and also the major causes of death and increased morbidity among hospitalized patients. A prevalence survey conducted under the auspices of World Health Organization (WHO) in 55 hospitals of 14 countries representing four WHO Regions including Europe, Eastern Mediterranean, South-East Asia and Western Pacific showed that an average of 8.7% of hospital patients had nosocomial infections. The highest frequencies of nosocomial infections were reported from hospitals in the Eastern Mediterranean and South-East Asia Regions (11.8 and 10.0% respectively) with a prevalence of 7.7 and 9.0% respectively in the European and Western Pacific Regions.

LITERATURE REVIEW

Nosocomial Infection

Nosocomial infection is an infection which a person catches during healthcare or health check in hospital within 3 to 24 hours since they are admitted to hospital without signs of prior infection [1]. While in 2013, a research showed that 20% of nosocomial infections happen in ICU room and become the main cause of death of patients being cared there [2]. One of the nosocomial pathogens that infect patients in hospital is *Pseudomonas aeruginosa*. Infection symptoms include pneumonia, eye infection, burns and surgical wounds infection, skin infection, urinary tract infection, bacteremia, and septicemia. The main targets of *P. aeruginosa* are the patients with cystic fibrosis having damp tissue such as mucous membrane or area around injury.

Pseudomonas aeruginosa

Pseudomonas aeruginosa is the cause of nosocomial infection, especially in patients with low immune system and burns, as well as chronic infection to cystic fibrosis patients and also increases the resistance against various antibiotics in hospitalized patients. The antibiotics include β-lactam, ciprofloxacin, tobramycin, and colistin. Low permeability of antibiotic and chromosomal gene mutation in bacteria is some of the factors that induce antibiotic resistance. Therefore, it is crucial to have a compound which can enhance the immune system plays an important role in destroying bacteria and repairing injured skin tissue [3]. As *P. aeruginosa* is an intracellular bacteria which triggers pro-inflammation cytokine production,

such as IL- 1β, in high amount that prolongs inflammation phase, causing longer time for wounds to heal. Therefore, it is needed to have compound which can act as immuno-regulator in balancing the high amount of cytokine IL-1β and stimulating new skin tissue formation.

Skin Structure

Skin is the first defense against microorganism in the surrounding environment. Skin consists of three layers of tissue with different functionality and characteristic. The three layers are epidermis, dermis, and subcutaneous [4].

a) Epidermis layer: This layer is the thinnest and outer most layer of skin. It's crucial for cosmetic as the layer gives texture, moisture, and color of skin. The main cell component of epidermis layer is keratinocyte. Epidermis layer is divided into four layers, namely as Basal layer (stratum basal); Prickle cell layer (stratum spinosum); Granular cell layer (stratum granulosum) and Stratum corneum.

b) Dermis layer: It is the layer between epidermis and subcutaneous. It's thicker than epidermis. Thickness of epidermis layer varies between ages. As it gets older, the thickness and moisture will decrease. Nerves, blood vessel, and sweat glands are located in this layer. The main cell component of dermis is fibroblast which synthesizes collagen, elastin, and glycosaminoglycan. This layer functions as adhesive between epidermis and dermis, defense against outer damage, and skin integrity protection.

c) Subcutaneous layer/hypodermis: The layer is located under the dermis layer. It consists of loose connective tissue and lipid. The main cell component is adipocyte, which is a mesenchymal cell to store lipid, important as source of energy for body. Hypodermis often contains adipocytes in various amounts according to location in the body and in various sizes according to nutritional status of the person.

Wound Healing

Wound is a functional and anatomical damage to normal organ. Clinically, wound can be classified into two categories which are acute wound and chronic wound. Time is a crucial factor in wound management and wound repair. In acute wound, organ/tissue can normally repair itself by repairing its anatomical and functional structure. Time needed for acute wound is 5-10 days or within 30 days. Chronic wound takes longer time wound in wound

healing process. Several factors that cause delay in wound healing are infection, tissue hypoxia, necrosis, and high production of inflammatory cytokine. This can happen in hemostatic, inflammation, and proliferation or remodeling phase. Chronic wound can also be caused by naturopathic, pressure, arterial venous insufficiency and burns vasculitis [5].

Wound healing is a series of complex process which involves interaction and coordination between various immunological and biological system. In acute wound, there are several phases of healing process. They are coagulation and hemostatic which are early phase of injury, inflammation which is second phase, proliferation which is third phase and also the main process in wound healing process and tissue granules formation, then remodeling when new tissue will form in the wound which can take a year or more [5].

Mode of Action of *+Dalethyne*

+Dalethyne is a fraction of olive oil extract which turns into new aldehyde compounds. One of the primary components in +Dalethyne is aldehyde apart from the palmitic acid, stearic acid, oleic acid, linoleic acid, and myristicine. As evidenced in a research conducted in the year 2010 aldehyde can function well as anti-inflammation by suppressing the expression ICAM-1, vascular cell adhesion molecule (VCAM-1), TNF-α, IL-6 and CD40 in endothelial cell [6]. Besides, aldehyde also inhibits formation of NFkB by inhibiting IkB protein. Another research in the year 2016 expressed that aldehyde significantly acts as both *in vivo* and *in vitro* anti- inflammation by decreasing the production of nitric oxide (NO), and inducible nitric oxide synthase (iNOS) expressed by macrophage. Aldehyde can decrease phosphorylation of Janus kinase 2 (JAK2) and signal Transducers and Activators of Transcription 1 (STAT1) [7]. Accordingly, a research to test the effect of *+Dalethyne* in regulating immune cells and helping wound healing process by checking the IL-1β expression was conducted.

METHODOLOGY OF THE EXPERIMENTAL WORK

The experiment was done by using post-test only control group design and comparison between groups. For experiments, male wistar mice (*Rattus norvegicus*), aged 2-3 months, were used. Total 36 mice were used which were divided into three groups of 2 sets each. Comparison between groups include group (I) wound+ bacterial infection, (II) wound, (3) wound+ bacterial infection and +Dalethyne.

Strain of bacteria used in the experiment was *Pseudomonas aeruginosa* in 1.510^8 cell/mL suspension from Microbiology laboratory of Dr. Soetomo Hospital, Surabaya.

Application in experimental mice

The mice were depilated on the back and cutaneous circular wound of 2 cm deep were inflicted on the pre-shaved sterile dorsal surface of the animal by cutting. Each animal were given single wound. The wound was left undressed to the open environment.

Rats of Group I and Group III were inoculated with 50 µL *P. aeruginosa*. Further, the animals of Group III were given 10ml of +Dalethyne using cotton bud.

On day 4, each group of mice was killed to take the skin tissue. The skin tissues were further tested for amount of IL-1β expression in skin tissue by using immunohistochemistry method. Primary antibody used in the experiment was bioss bs-6319 conjugated HRP and secondary antibody Thermo.

Immunohistochemistry Staining IL-1β

Skin tissues were incised by microtome and placed in object glass. Then, deparaffinization was done with xylol, dehydration with ascending grads of ethanol 70, 80, 90, 95 and 100%. Each step has been given 1 min to eliminate the paraffin in the tissues. Tissues were then washed with water for 5 minutes and 3% peroxide was added for 30 minutes to eliminate peroxidase endogenous. It was then washed with water for purification. Phosphate buffered saline (PBS) was added for 2 minutes. Incised tissues were then put in trypsin (0.25%) in PBS (pH 7.4) for 6 minutes at 37^0C and tissues were further washed with PBS for 2 minutes. Then, primary monoclonal antibody (mouse anti – Rat IL-1β) has been added and incubated for 30 minutes. It was further washed with PBS thrice for 2 minutes each and added with secondary monoclonal antibody (Rabbit anti mouse biotinylation label) for 30 minutes. Then, it was kept in streptavidin HRP for 30 minutes and washed with PBS twice for 2 minutes each. It was then kept in chromogen substrate and further added with DAB solution for 5 minutes. It was then further washed with PBS and distilled water twice for 2 minutes each. Then, it was kept in Mayer Hematoxylin for 6 minutes and washed in running water. Further, the mounting was done and the results were observed under microscope using 400 x magnifications in 10 scope of view.

RESULTS AND DISCUSSION

Table 1. Mean of IL-1β Observation

	Group I	Group II	Group III
Mean ± SD	42±18	33±16	29±5
Median	45	33	31

Fig. 1 shows that the amount of IL-1- β expression in fibroblast cell is decreasing in group with wound + infection and +Dalethyne (Group III), followed by group with wound (Group II) and lastly group with wound + infection (Group I).

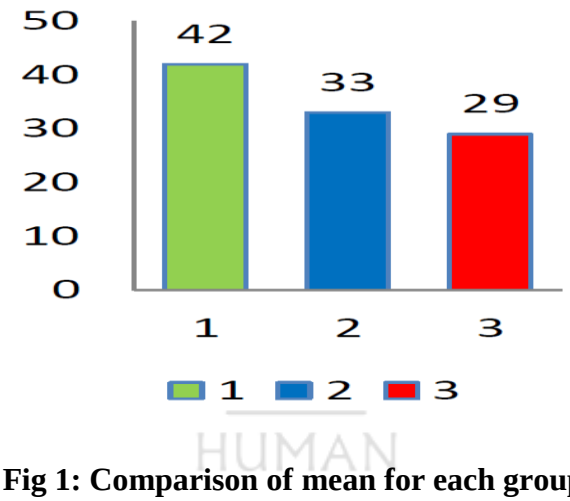
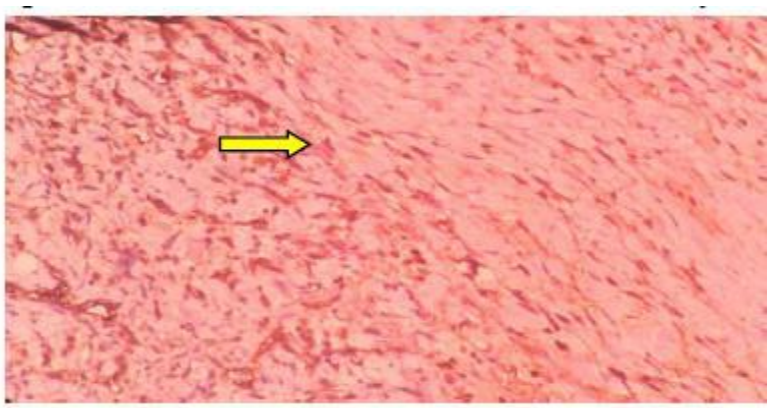
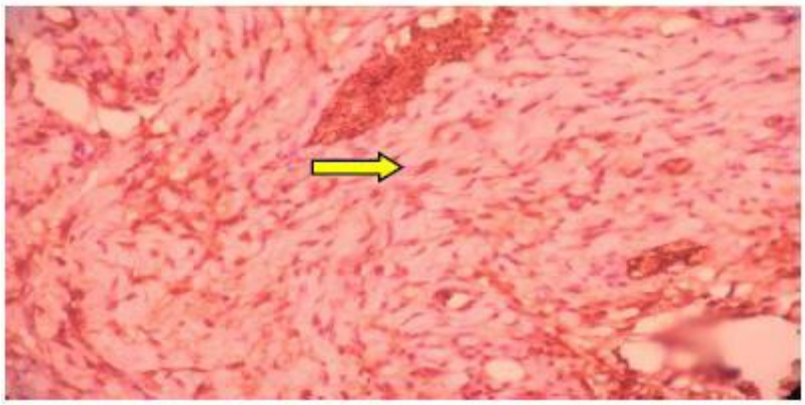


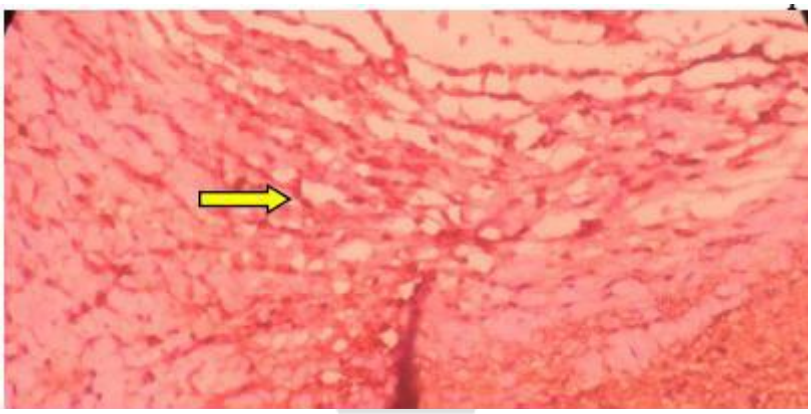
Fig 1: Comparison of mean for each group



Wound + Infection with 400x magnification (Group I)



Wound with 400x magnification (Group II)



Wound + Infection + D*alethyne* with 400x magnification (Group III)

DISCUSSION

If determined physiologically the inflammation process in the wound happens for at least 3 days and then enters the pro-inflammation phase. In case of infection due to *Pseudomonas aeruginosa* inflammation process lasts longer than usual. *P. aeruginosa* is a gram negative bacteria having superantigen such as exotoxin causing longer time to destroy. Host cell infected by *P. aeruginosa* recognizes it by several ligands, LPS and flagella. After injury, skin tissue induces the increase of intracellular Ca^{2+} and formation of H_2O_2 or increase of extracellular ATP. ATP produced in this way is called DAMPs (Damage-associated molecular patterns), which then binds purine receptor P2Y in healthy cell and releases cytoplasmic signal that activates intracellular Ca^{2+} and metalloproteinase. This releases EGFR ligand, which is HB-EGF (*heparin- binding EGF-like growth factor*), ATP and Ca^{2+} release can activate PI3K-AKT and ERK- MAPK signal in epithelial cell. Further, wound area also suffered with hypoxia which stimulates fibroblast activation. After given incision on

the back, mice was infected with *P. aeruginosa* and applied with *+Dalthyne* as antibiotic. Fibroblast also expresses TLR which activates several pathways including (1) cpG DNA recognition by TLR 9, (2) bacterial ligand, LPS, recognition by TLR 4, (3) flagella recognition by TLR 5, and (4) flagella recognition by NLRP3 in cytosol.

Pathway (1), (2), and (3) will activate MyD88TIR domain which interacts with TLR TIR domain activating 2 serine threonine protein kinase IRAK4 (IL1-receptor associated kinase 4) and IRAK 1. IRAK complex recruits TRAF 6 (TNF receptor associated factor 6) which is an E3 ubiquitous ligase. E2 ligase TRICA1 along with TRAF 6 produce polyubiquitin chain scaffold in TRAF 6 and activate serine threonine kinase TAK 1 (Transforming growth factor beta-activated kinase 1) and MAP (Mitogen-activated protein) kinase. TAK 1 phosphorylates and activates IκB kinase (IKK) complex that consists of 3 proteins: IKKα, IKKβ, and IKKγ or known as NEMO. IKK activation phosphorylates IκB which degrades and releases NF-κB. NF-κB then enters the nucleus and activates and pro-inflammatory cytokine especially IL-1β. Then, in pathway (4) a binding between flagella and NLRP3 occurs. This bound forms oligomer and binds protein adaptor called ASC. Binding between ASC and NLRP3 sensor activates incaspase-1; bacterial flagellum (rod) will enter cytosol through T3SS and activates pro-IL-1β. Pro-IL-1β will interact with inscaspase-1 to activate IL-1β.

TRAF 6 also activates MAP kinase that stimulates JNK (c-Jun N-terminal kinase) and fos activation. JNK and fos are two components building AP-1 (Activator protein-1). Both NFkB and AP-1 stimulate pro-inflammation cytokine, such as IL-12, IL-1β, TNF-α, IL-6, activation as well as ROS (reactive oxygen species) activation. Fibroblast can also stimulate IL-1β with autocrine effect.

Therefore, production of IL-1β will increase and prolong inflammation period. By applying +Dalethyne to group with wound + infection, wound healing process is faster compared to group with wound + infection only. It is because *+Dalethyne* contains aldehyde as well as palmitic acid, stearic acid, oleic acid, linoleic acid, and myristicine. This compound are claimed to help bactericidal effect and reduces the wound healing process. Aldehyde which acts as anti- inflammatory agent can lower and inhibit NFkB by suppressing ICAM-1, VCAM-1, CD40, TNF-α, and IL-6. Aldehyde also inhibits JAK2/STAT1 pathway and iNOS expression. While palmitic acid, stearic acid, oleic acid, and linoleic acid play role in killing bacteria by increasing the release of extracellular and intracellular Ca^{2+} as well as H_2O_2 that induces ROS (reactive oxygen species).

CONCLUSION

The study reveals that the application of +Dalethyne is very efficacious and effective in lowering the amount of IL- 1β expression by fibroblast cell on day 4 when inflammation phase is supposed to be still increasing and effective against the nosocomial infection caused by *P. aeruginosa.*

CONFLICTS OF INTERESTS

The author has none to declare.

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