Effect of Nitrate Donor on Nitrate Conversio in *Escherichia coli*.

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Abstract

Nitrate compound are prodrugs and required degradation to nitrite and furthermore bioactivated to nitric oxide (NO), an active beneficial form for physiological purpose e.g. on angina pectoris. Through the use of nitrate donor per oral, it was alleged that *Escherichia coli*, normal bacterial in the gut, decompose and reduce nitrate concentration and shows itself as chemoorganoheterotrophs organism. This research aim to investigate the role of nitrate donor compounds i.e.isosorbide dinitrate (ISDN) and natrium nitrate (NaNO3) on *E.coli*, in terms of concentration of nitrite as catabolism product and bacteria numbers. Six groups of nitrate donors varies in concentration (100, 500 and 1000 ppm) is dissolved in 3000 ppm glucose solution. After incubated for 24 hours Griess method is applied for measurement of nitrite concentration. Using spectrofotometric bacterial counting method, then bacteria number is obtained. The results showed that increasing nitrate concentration does not influence the growth or bacteria numbe of *E. coli*, but correlate with amount of nitrite formed.
Keywords: ISDN, nitrate, nitrite, *E.coli*

Introduction

Nitrate compounds is widely used as anti--ischemic therapy on human cardiovascular diseases e.g. angina pectoris, acute cardiovascular infarct, and heart failure (Jawad and Arora, 2008). Cardiovascular diseases causes 17 million deaths worldwide in 2008 is the highest number of death among non communicable diseases (45%), followed by cancer (21%), chronic respiratory disease (12%), and diabetes (3.5%) (WHO, 2012).

The use of nitrate as therapeutic agent in the treatment of angina pectoris was initially reported in 1847, that is when nitrogliserin is placed under the tongue will cause a tremendous headache. In the year of 1879, it was reported that sublingual administration of nitrogliserin can, relieve angina pectoris and prevent further attacks of the disease. But, if the treatment is done over a long period, the dose should be increase in order to demonstrate their use properties are expected. The situation is further known as nitrate tolerance nitrat (Knot,2003).
Although vasorelaxation effects of nitrate class of drugs considered important and useful for the treatment of angina pectoris disease, but the underlying molecular mechanisms of action of this effect is still an unresolved matter described (Ignarro, 2002).

Under certain circumstance, nitrate is converted to nitrite and further decomposition to nitric oxide (NO),the true vasodilator agent. However, due to limitations in aspects of the analysison the measurement of NO, and due to short half---life of NO, then the quantitative measurement of the donor NO compound regarded as a parameter correlated with the work of pharmacological in clinical research (Ignarro et al., 2002).

Isosorbide dinitrate (ISDN) is considered as a classic nitrate, and currently remained clinically used in the treatment of angina pectoris, similar to nitrogliserin (GTN) but with half---life time is higher (around 40 minutes, compared to 4 minutes for GTN). ISDN is classified as moderate acting with low potential on relaxing smooth muscle of blood vessel and dilate peripheral arteries and veins. ISDN has a simple hydrocarbon (or sugar) skeleton with attached nitrooxy functional group (R---ONO_2). ISDN will break down into isosorbide mononitrate (ISMN), which further decomposed to became isosorbide while releasing NO. (Minamiyama et.al.,1999; Thatcher et al.,2004; Münzel et.al.,2007).
Chemical formula for ISDN (figure 1) is $C_6H_8N_2O_8$, with molecular weight 236,14 g/mol. ISDN is as solid which has experimental data of solubility in water about

0,55 mg/ml with logP 1,31 (DrugBank n.d.).

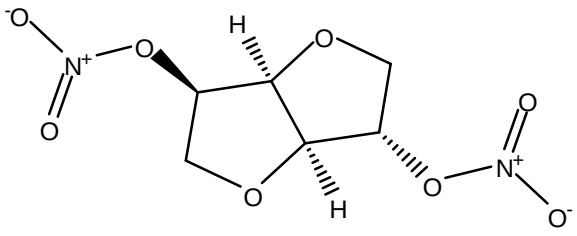

Figure 1Chemical Structure of ISDN

In anaerobic environment, nitrate undergoes reductive dissimilative metabolic process. Nitrate acts as final electron acceptor and gets reduced to products like nitrite (NO_2^{---}), N2 etc. Bacteria like *Escherichia coli* an reduce nitrate to nitrite only, by nitrate reductase enzyme in the process called dissimilatory nitrate reduction (Chawla,2008).

$NO_3^{---} + 2\bar{e} + 2\ H^+$ à $NO_2^{--} + H_2O$

Escherichia coli is an important member of the microbiota of the large intestine of vertebrates, including humans, thus known as normal inhabitant and its presence is beneficial because it helps produce certain vitamins and breaks down other indigestible foodstuffs (Tortora et al., 2010).

Determination of nitrite content in solution is applied by spectrophotometry nitrite method which first developed by Griess in 1879. Measurement of nitrite amount conducted in two stages that is diazotation stage of sulfanilamide by nitrite under acidic condition, then followed by formation of combined compound with bicyclic amine N---(1--naphtyl)---etilendiamine and formation of colored compound that can be measured at 540 nm (Miranda et al.,2001). Griess reaction can be explained by the schematic in Figure 2.

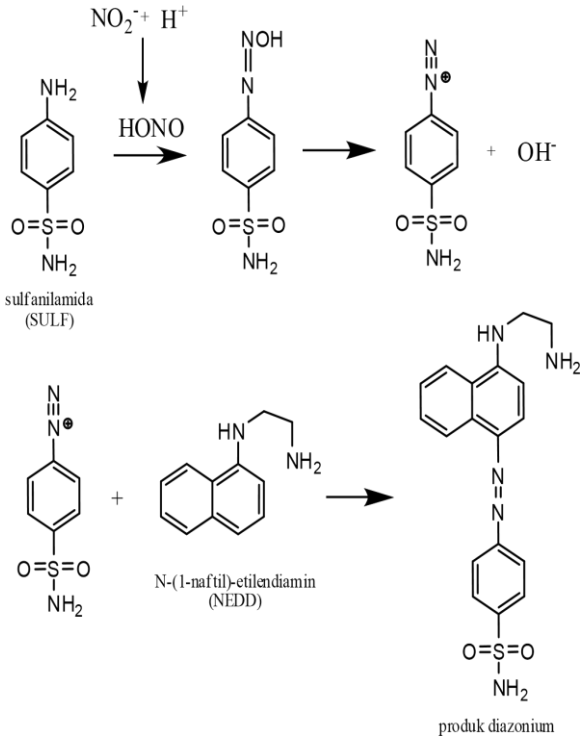

Figure 2. Scheme of Griess Reaction (Tarpey and Fridovich, 2001)

Materials and Methods
Preparation of Griess Reagent

Griess reagent was prepared by dissolving 1.5 g sulfanilic acid in 450 mL of 10% acetic acid. Sulfanilic acid solution then is added to the solution of 0.6 g of alpha naphthylamine in 60ml of boiling distilled water. This clear solution is filtered through Whatman filter paper no.1. This solution will turn red when its activity was tested by adding a few drops of 10% solution of sodium nitrite (Suwitono, 2011).

Solution Preparation

Six groups of solution were prepared by adding nitrate to 3000ppm glucose solution according to types of nitrate donor used with its final concentration, which is 1)100ppm ISDN, 2)500 ppm ISDN, 3)1000 ppm ISDN, 4)100 ppm $NaNO_3$, 5) 500 ppm $NaNO_3$, 6)1000 ppm $NaNO_3$, and 7) with no nitrate added to glucose solution as control solution.

Culture Preparation

E.coli culture was reproduced by using several nutrient agar plate with streak method. Bacteria then transferred to distilled water until it reaches 0.5 McFarland turbidity standard. Each groups of nitrate solution was added to 10% total volume of bacteria

solution, and incubated for 24 hours.

Tubidimetric Bacteria Counting

Viable bacteria afte incubation were measured by spectroscopic methods with the wavelength of 540nm. Turbidimetric methods can be used as long as each individual cell of bacteria blocks or intercepts light. Data then compared with standard curve of bacteria according to several McFarland turbidity level.

Nitrite Measurement

Nitrite in the incubated bacteria solution is measured according to Griess method. With ratio 1:1 with Griess reagent, each bacteria solution were mixed and incubated for 30 min. The sample is then measured with a spectrophotometer at the wavelength of 540 nm and absorbance values (AU) recorded. Nitrite concentration obtained by comparing the absorbance values with standard curve of nitrite previously made by using sodium nitrite ($NaNO_2$).

Data Analysis

Data for number of bacteria and nitrite concentration were analyzed severally by using Analysis of Variance (ANOVA) statistic, with α=0.05

Results and Discussion

Calibrations

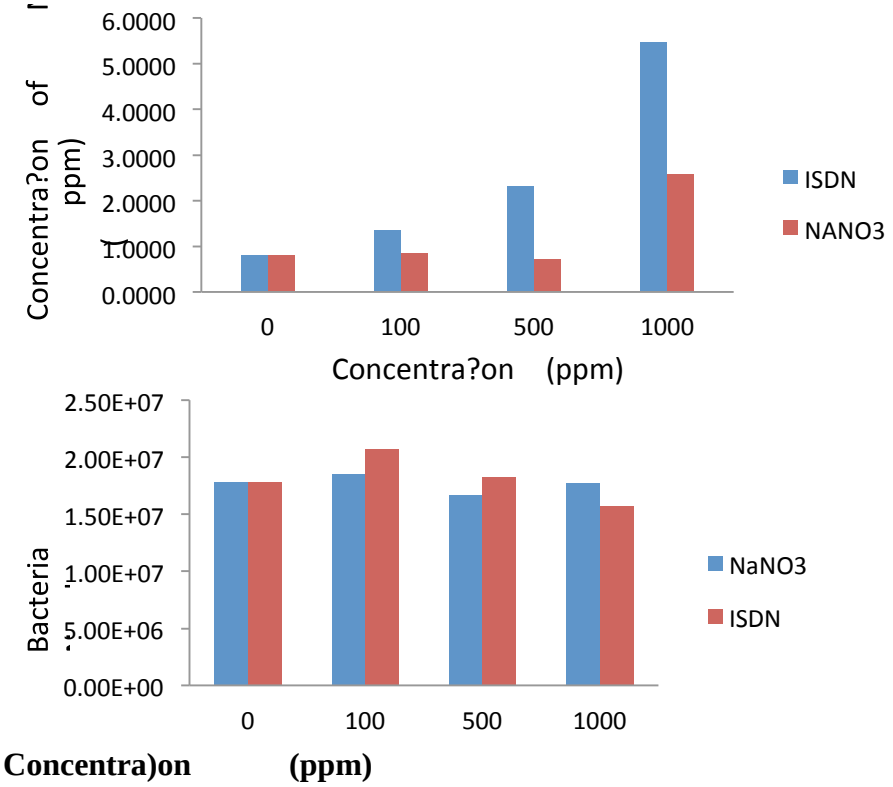

Figure 3. Bacteria Number of Groups Treatment

Calibration for turbidimetric bacteria counting was done by measuring several solution of bacteria according to McFarland turbidimetric level. Calibration equation was obtained, which is Y = 2.782 X, with r = 0.972.
Calibration for Griess method also obtained, by measuring several solution of sodium nitrite. Trend line for nitrite concentration is Y= 0.027 X + 0.053, with r = 0.999.

Number of Bacteria

Number of *E. Coli* was produced for each groups of treatment of nitrateby using turbidimetric bacteria counting, and then calibration equation applied.
Data of bacteria number is shown infigure 3. ANOVA statistic calculation for number of bacteria gives p = 0.043, and result as there is significance difference between each group of nitrate donor and concentration, in terms of bacteria number after incubated for 24 hours. But, its correlation does not show positive correlation, therefore it concluded that no correlation between increasing of concentration of nitrate with number of

bacteria.

Nitrite Concentration

Concentration of nitrite was measured by spectrophotometric Griess method, and followed by calibration equation.

Data of nitrite concentration is shown in figure 4.

Figure 4. Concentration of Nitrite of Groups Treatment

ANOVA statistic calculation for number of bacteria gives $p = 0.000$, and result as there is significance difference between each group of nitrate donor and concentration, in terms of concentration of nitrite produced after incubated for 24 hours. Its correlation shows positive correlation, therefore it concluded that there is correlation between increasing of concentration of nitrate with concentration of nitrite produces by *E. Coli* after incubation period.

Discussion

Although result showed no significant different on number of bacteria after incubated with several concentration of nitrate used, but nitrite concentration showed an increase. *Escherichia coli* is the best understood enteric bacteria that colonizing the human intestine. The E. Coli encodes several distinct nitrate reductase enzymes that is used during anaerobic respiration. In the absent of oxygen, nitrate play a role as electron acceptors in the respiration of *E.coli* which become toxic to the cell upon reaching high intracellular concentrations (Tiso and Schechter,2015).The found of nitrite concentration increased without significant increase in number of *E. Coli* confirm that dissimilatory nitrate reduction is undergoes in *E.coli*, and nitrate decomposition is slowed, if not stopped, on nitrite build up / accumulation.

Conclusion

Through this research, we conclude that there is no correlation between increasing of concentration of nitrate with number of bacteria, in other word, no positive direction of correlation between concentrations of nitrate versus number of bacteria. The result also showed that there is correlation between increasing of concentration of nitrate with concentration of nitrite produces by *E.coli* after incubation period.

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