

**SUPLEMENTASI ALL-TRANS ASAM RETINOAT (ATRA) DAN ZINK SULFAT PADA PERIODONTITIS**Praptiwi Hanafi<sup>1</sup>✉, Siti Fatimah Muis<sup>2</sup>, Soeharyo Hadisaputro<sup>2</sup>, Suryono<sup>3</sup><sup>1</sup>Fakultas Kedokteran Gigi Universitas Muhammadiyah Semarang<sup>2</sup>Program Pasca Sarjana Universitas Diponegoro Semarang<sup>3</sup>Bagian Periodonsia Fakultas Kedokteran Gigi Universitas Gadjah Mada Jl Denta Sekip Yogyakarta**Info Artikel***Sejarah Artikel:*

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Penelitian ini bertujuan untuk mengetahui pengaruh suplementasi ATRA dan Zinc pada kolagen jaringan periodontium tikus periodontitis yang mendapat tetrasiklin. Studi eksperimental dengan desain faktorial dilakukan pada 54 tikus Wistar jantan dewasa. Periodontitis dihasilkan melalui inokulasi *porphyromonas gingivalis*. Tetrasiklin 90 mg/kg dan suplementasi diberikan selama 7 hari. Sembilan kelompok sampel terpilih secara alokasi acak. Variabel bebas adalah suplementasi ATRA 10; 5 mg/kg; Zn 2,5; 1 mg/kg; dan kombinasinya. Variabel tergantung adalah status kolagen meliputi degradasi dan sintesis, indikator mRNA MMP-2 dan mRNA P1CP, terukur dengan reverse transcriptase polymerizing chain reaction. Kontrol adalah kelompok periodontitis dan mendapat tetrasiklin. Analisis data menggunakan uji t, ANOVA, Post Hoc Duncan, dan LSD. Suplementasi dosis tinggi ATRA 10 mg/kg dan Zn 2,5 mg/kg berturut-turut mendegradasi (0,25 x kontrol) dan meningkatkan sintesis kolagen (4 x kontrol). Kombinasi suplementasi dosis tinggi ATRA dan Zn tidak berefek pada degradasi kolagen, tetapi meningkatkan sintesisnya (3 x kontrol). Suplementasi kombinasi dosis tinggi ATRA dan Zn memberikan sintesis kolagen paling dekat dengan keadaan sehat.

**THE EFFECT OF ALL-TRANS RETINOIC ACID (ATRA) AND ZINC SULPHATE SUPPLEMENTATION ON PERIODONTITIS****Abstract**

*The study executed to know the effect of ATRA and Zn supplementations to periodontitis rat collagen treated with tetracycline. The experimental study with factorial design used 54 Wistar adult male rat. Periodontitis was resulted from porphyromonas gingivalis inoculation. Tetracycline 90 mg/kg and the supplementations were given for 7 days. Nine groups sample were randomly allocated. The independent variables were supplementation of ATRA 10; 5 mg/kg, Zn 2.5; 1 mg/kg, and their combinations. Dependent variable was collagen status i.e degradation and synthesis, consecutively measured by mRNA MMP-2 and mRNA P1CP, using reverse transcriptase polymerase chain reaction. Control was periodontitis group receiving tetracycline. T-test, ANOVA, Post Hoc Duncan and least significant differences (LSD) were used. High dose ATRA 10 mg/kg and Zn 2.5 mg/kg supplementations consecutively degraded (0.25 x of control) and increased collagen synthesis (4 x of control). High dose ATRA and Zn combined supplementation gave no effect to collagen degradation, but increased synthesis (3 x of control). The effect of high dose ATRA and Zn combined supplementation on collagen synthesis was the nearest to healthy condition.*

## Introduction

High amount of periodontitis in Indonesia, becomes a menace for teeth and oral health (Tampubolon NS, 2005; Rosenberg JD, 2010). The disease would rapidly increasing by age through pocket formation (Roeslan BO, 2003). Periodontitis has potentially effect on progress of chronic inflammatory diseases such as DM, arthritis, and also the occurrence of low birth weight baby (Galvao MPA, 2003; Kantarci A, 2005; Siqueira FM, 2007). So, similar to other oral disease i.e caries, periodontitis is becoming crucial public health problem (Widya HC, 2013). The periodontitis risk factors are age, family, smoking, socioeconomic status, DM, stress (Suryono, 2006). Vitamin A metabolite *all-trans* retinoic acid (ATRA), and Zinc (Zn) have great effect on tissue healing (Boyd LD, 2001).

Periodontitis treatment, especially irrigation using antibacterial solution, is not satisfying to treat the pocket. Systemic antibiotic, also its locally application accompanied by subgingival scaling, inconvenient due to the bleeding occurred (Rosenberg JD, 2010).

The study done was the effort to treat periodontitis in rat. Tetracycline was used, to inhibit collagenase activity (Simon H, 2002), besides supplementation of ATRA and Zn to accelerate healing. Recovery state was reached when the value of collagen degradation and synthesis nearest to the healthy group. Messenger Ribonucleic acid (mRNA) Matrix Metalloproteinase-2 (MMP-2) and mRNA carboxy-terminal propeptide of type 1 collagen (PICP) transferring genetic information from the DNA, are highly specific biomolecular indicator of collagen degradation and synthesis subsequently (De Souza AP, 2005).

The deficiency of vitamin A and Zn (Endang Purwaningsih, 2004), which still found high in prevalence among people in Indonesia, also the increasing of periodontitis risk factors, would delay the recovery. *Porphyromonas gingivalis*, the most aggressive periodontopathic bacteria (Kumar PS, 2003), contains toxin leading to production of pro inflammatory cytokines, inhibits phagocytosis, degrades Immunoglobulins and induces Matrix Metalloproteinase (MMP) production. Collagen of periodontal tissue is dominated

by type 1 collagen, which would be destroyed by MMP's activity (De Souza AP, 2005). The increasing of MMP gene expression would be down regulated by ATRA and Transforming Growth Factor (TGF)- $\beta$ . Collagen degradation is increasing in pathologic condition. Matrix metalloproteinase-2 is activated by separation of N-terminal propeptide of procollagen molecule. Carboxy-terminal propeptide of type 1 collagen is procollagen fragment released extracellularly by fibroblast (Gusman H, 2001).

Tetracycline beside its antibacterial property, has potency to minimize inflammation, inhibiting collagenase activity, and delaying bacterial growth. *All-trans* retinoic acid and Zn have important role in collagen synthesis, beside their potency in regulating pathologic tissue growth (Takahashi M, 2007). *All-trans* retinoic acid stimulates the immune system, and through long pathway influence the collagen synthesis. This acid could inhibits MMP gene expression (Brinckerhoff CE, 2003), gives pressure to, and reduces collagen degradation (Brinckerhoff CE, 2003; Frankenberger M, 2001; Lateef H, 2004) beside its potency on decreasing fibroblast extracellular matrix degradation induced by cytokine (Zhu YK, 2001). Zn is part of Retinol Binding Protein (RBP), and is needed to synthesis it (Hakeem A, 2007). This protein is the vehicle of retinol to reach target tissues. Zinc supplementation would increase RBP, further retinol absorption (Hakeem, 2007). Zn as the cofactor of deoxy ribo nucleic acid (DNA) - and ribonucleic acid (RNA)- polymerize enzymes, is crucial for tissue healing (Yanagisawa, 2008).

In connection with ATRA and Zn, Indonesian people accentuate to consume plant product with rice as staple food. Those habits appear to lead to vitamin A and Zn deficiencies. The healing of periodontitis indirectly would be delayed by those deficiencies (Boyd LD, 2001; Ho E, 2003). Supplementations given in this study were ATRA and Zn, single and in combined. The supplementations needed in healing periodontitis were ones that could induce collagen degradation and collagen synthesis to reach healthy condition.

The aim of the study were to explain the effect of supplementations of ATRA and Zn, single and in combination, to collagen

status consisting of degradation and synthesis, using the index of mRNA MMP-2 and mRNA P1CP of periodontitis rat, receiving tetracycline medication. The periodontitis state getting tetracycline as standard medication, supplemented by ATRA and Zn influencing recovery, measured by collagen status including degradation and synthesis using biomolecular indicators mRNA MMP-2 and mRNA P1CP, was never done before. The preclinical study done, would be valuable when continued by clinical study in human suffering periodontitis.

### Method

The design of the experimental study was factorial. The simple random allocation were used to group rats as the experimental animals into 9, each consisted of 6 rats, according to the design. The groups were high dose ATRA ( $A_1$ ) of 10 mg/kg, low dose ATRA ( $A_2$ ) of 5 mg/kg, high dose Zn ( $Z_1$ ) of 2.5 mg/kg, low dose Zn ( $Z_2$ ) of 1 mg/kg, and combined supplementation of ATRA and Zn, i.e  $A_1Z_1$ ,  $A_1Z_2$ ,  $A_2Z_1$ ,  $A_2Z_2$ , and Periodontitis group receiving tetracycline 90 mg/kg without any supplementation, as the control.

The reference population was white male Wistar rat (*Rattus norvegicus L*), the 5<sup>th</sup> generation of the species (LPPT 5), adult, 8 weeks old, weight 200 g. The rats induced periodontitis through bacterial inoculation of *P. gingivalis* ATCC 3277, and get tetracycline medication dose 90 mg/kg. Study population was white male Wistar rat (*Rattus norvegicus L*), LPPT 5, adult, 8 weeks old, weight  $177.2 \pm 27.46$  g, periodontitis through bacterial inoculation of *P. gingivalis* ATCC 3277, get tetracycline medication dose 90 mg/kg, in LPPT UGM Yogyakarta in the year 2007. The sample of the study were part of the study population fulfilling the Inclusive and Exclusive criteria. The inclusive criteria were white male Wistar rat, LPPT 5, adult, 8 weeks old, weight  $177.2 \pm 27.46$  g, periodontitis through bacterial inoculation of *P. gingivalis* ATCC 3277, and received tetracycline medication dose 90 mg/kg. The exclusive criteria consists of loss of appetite and or diarrhoea. Amount of sample according to the design was 54 rats consisting of 9 groups 6 rats each.

The independent variables of the study

were high dose ATRA supplementation ( $A_1$ ), and low dose ( $A_2$ ), high dose Zn supplementation ( $Z_1$ ), and low dose ( $Z_2$ ), single and in combination i.e  $A_1Z_1$ ,  $A_1Z_2$ ,  $A_2Z_1$ , and  $A_2Z_2$  supplementations. The dependent variable is collagen status consisting the degradation and the synthesis, successively measured by indices of mRNA MMP-2 and mRNA P1CP. The intervening variables were RBP and TGF- $\beta$  which were controlled by similar species, age, gender, food consumption, environmental condition, and health.

The unit of the study was periodontal tissue surrounding upper and lower 1<sup>st</sup>, 2<sup>nd</sup>, and 3<sup>rd</sup> molars, left and right. The cut initiated from lateral or buccal side, continued to palatal or lingual with thickness about 2 mm. The cut width was 3 – 5 mm. The study used the term 'periodontal tissue' for the purpose of measurement which means periodontal ligament and gingiva.

The study was agreed by Komisi Etik Penelitian Pemanfaatan Hewan Percobaan untuk Penelitian Universitas Diponegoro. It was executed in Fakultas Kedokteran (FK), LPPT Laboratory (Lab) UGM Yogyakarta and Fakultas Peternakan (FP) UNDIP Semarang, from 2007 to 2009. Microbiology Department (Dept) of FK UGM was the place to take care and to propagate the bacteria. Adult Wistar rat as the sample of the study, got the treatment in Unit 4 LPPT UGM. Their body weight were measured to see the effect of bacterial infection. Body weight give figure of amount of protein, fat, water, and mineral. Unit 2 and 3 LPPT were the place to measure variables of the study. The place to detect the start of periodontitis and to make histopathologic preparation was Pathologic Anatomy (PA) Dept of FK UGM. The Mallory staining was used to outstand the figure of collagen fibre. To make periodontal tissue extraction prepared for measurement of ATRA concentration, Biochemistry Lab of Fakultas Peternakan UNDIP was used.

The samples were accustomed to their individual cages for 4 days. Then, they got the administration of *P. gingivalis* 3 times in 4 days. The sign of periodontitis were found after 3 weeks. The periodontitis becoming chronic after 7 days detection of periodontitis. The treatment with and without ATRA, and, or Zn

supplementation were given for 7 days.

The concentration of ATRA were measured using High Performance Liquid Chromatography (HPLC), while Zn were measured by Atomic Absorption Spectrophotometry (AAS). Messenger RNA expression were measured by RT-PCR, thermal cycler machine, Gel electrophoresis, Spectrophotometer UV-Vis, and Thin Layer Chromatography.

Analyses of data consist of descriptive analyses of body weight, concentration of ATRA and Zn periodontal tissue, and collagen status i.e degradation and synthesis, in their mean and Standard Deviation. Inferential data analyses were done using t test, and One Way ANOVA, continued by Post Hoc Duncan and Least Significant Differences test.

## Results and Discussion

Using t test, no significant difference were found between sample body weight of Healthy and Periodontitis group in 8,12,16, and 20 weeks old, with p value 0.376, 0.096, 0.093, and 0.058 successively. It was concluded that periodontitis gave no effect on body weight.

The highest ATRA concentration was found in group Periodontitis + Tetracycline supplemented by high dose Zn ( $2.88 \pm 0.847$  ppm), while the lowest was of low dose of ATRA ( $1.71 \pm 0.511$  ppm). The highest Zn concentration was found in group Periodontitis + Tetracycline supplemented by high dose ATRA ( $56.43 \pm 73.596$  ppb), while the lowest was of low dose Zn group ( $17.23 \pm 22.611$  ppb).

The sample of normal (healthy) group had degradation mean  $0.13 \pm 0.061$  ppm,

and synthesis mean  $0.48 \pm 0.211$  ppm. The highest collagen degradation was found in low dose ATRA and high dose Zn combined supplementation ( $0.32 \pm 0.015$  ppm), and the lowest was from high dose ATRA supplementation ( $0.04 \pm 0.019$  ppm). The highest collagen synthesis was found in group of high dose Zn supplementation ( $0.83 \pm 0.283$  ppm), while the lowest was of high dose ATRA supplementation ( $0.17 \pm 0.062$  ppm).

Collagen status data analyzes using 3 samples each taken from 4 groups. Collagen status including degradation and synthesis subsequently indicated by mRNA MMP-2 dan mRNA PICP, in mean and standard deviation seen on Table 1. Treatment 1 was Periodontitis (P)+ Tetracycline (T), Treatment 2 was P+T+ high ATRA high Zn combined supplementation ( $A_1Z_1$ ), Treatment 3 was P + T + high ATRA low Zn combined supplementation ( $A_1Z_2$ ), Treatment 4 was P+T+ $A_2Z_1$ , and Treatment 5 was P+T+ $A_2Z_2$ .

As the comparison to combined supplementation groups, P+ T supplementation group were used. The tetracyclin gave good effect to periodontitis, seen from the lowest degradation compared to other group. It was due to its characteristic on inhibiting collagenase activity. On the other side, collagen synthesis as the effect of the antibiotic is not so prominent. Tetracycline function is to inhibit the production of protein that would be used by bacteria (Simon H, 2002).

The study found that sample of normal (Healthy) group had collagen degradation  $0.13 \pm 0.061$  ppm, and synthesis  $0.48 \pm 0.211$  ppm. Main objective of the study was to gain the lowest collagen degradation and the highest collagen

**Table 1.** Collagen degradation and synthesis of combined supplementation groups

Group	Collagen degradation (ppm)	Collagen synthesis (ppm)
Treatment 1 : P+T	$0.16 \pm 0.047^a$	$0.18 \pm 0.059^1$
Treatment 2 : P+T+ $A_1Z_1$	$0.25 \pm 0.050^b$	$0.60 \pm 0.237^2$
Treatment 3 : P+T+ $A_1Z_2$	$0.18 \pm 0.076^a$	$0.19 \pm 0.080^1$
Treatment 4 : P+T+ $A_2Z_1$	$0.32 \pm 0.015^c$	$0.23 \pm 0.091^3$
Treatment 5 : P+T+ $A_2Z_2$	$0.27 \pm 0.063^b$	$0.22 \pm 0.084^3$

ANOVA test

F=4.472; p=0.025

F=5,817; p=0.011

Post Hoc test : The same letter and number denotes no difference, while different letter and number denotes significant difference.

synthesis, nearest to the normal i.e health. The comparison of collagen status of normal to group receiving tetracyclin and combined supplementation groups were as follows. The lowest collagen degradation was come from group of tetracycline (Treatment 1), while the highest synthesis was from high ATRA and high Zn combined supplementation (Treatment 2). Those seven days all-trans retinoic acid and Zn combined supplementation gave good effect to collagen synthesis. Zn is needed in synthesis of Retinol binding protein (RBP). This protein transfers retinol to target tissues.

Using Mallory staining with 100x magnification, collagen fiber was seen in dark blue, and fibroblast in white. In ATRA supplemented groups, the lowest collagen degradation was seen in high dose ATRA supplementation or group A<sub>1</sub>. The fibroblasts were almost looked like a normal one, due to the lowest collagen degradation.

Of Zn supplemented group, the highest collagen synthesis was given by high dose Zn supplementation or group Z<sub>1</sub>. Fibroblast was hardly seen due to the thickness of collagen surrounding.

It could be seen that ATRA supplementation had its role in collagen degradation, while Zn supplementations role was in collagen synthesis. The highest collagen synthesis of combined supplementation group was given by high dose ATRA and Zn. That combined supplementation had collagen degradation and synthesis similar to normal condition i.e healthy group, with  $p=0.060$  and  $p=0.540$  successively. The best effect on collagen status was found in high dose ATRA and Zn combined supplementation compared to others.

The collagen degradation of high dose ATRA supplementation showed the lowest mRNA MMP-2 expression compared to others. Collagen degradation is increasing in pathologic condition. It means that the supplementation was able to decrease collagen degradation to the lowest level. It may be caused by the ability of ATRA to reduce collagenase produced by fibroblast and monocyte (Brinckerhoff CE, 2003; Frankenberger M, 2001; Lateef H, 2004), beside its potency on decreasing fibroblast extracellular matrix degradation induced by

cytokine (Zhu YK, 2001).

The collagen synthesis produced by high dose Zn supplementation was the highest in synthesizing collagen. It proved the effect of Zn as cofactor of DNA- and RNA- polymerase synthesizing protein including collagen, which is needed to wound tissue healing (Boyd LD, 2001).

The collagen synthesis through high dose ATRA and Zn combined supplementation was the highest among other combined group ( $p=0.011$ ). Compared to high dose Zn supplementation, collagen synthesis of high dose ATRA and Zn combined supplementation was decreased. It is known, that Zn is the part of, and is needed for RBP synthesis (Hakeem A, 2007), vehicle to transport retinol to target tissue. The decrease effect of high dose Zn supplementation in high dose ATRA and Zn combined supplementation may be caused by Zn priority to participate to RBP. This choice was crucial, to think over important role of ATRA in combating degradation which is increasing in pathologic condition (Lateef H, 2004). The Retinol binding protein would be increased by Zn supplementation, and then further retinol absorption (Hakeem A, 2007). Both would reduce the amount of Zn to synthesize protein, which affected low mRNA P1CP produced. As for ATRA, its role in synthesizing collagen could not be seen in the limited time of study. The long pathway of ATRA to give effect to collagen synthesis might be the cause.

## Conclusion

The treatment of periodontitis rat using tetracycline, ATRA and Zn supplementation during 7 days, concluded as follows : High dose ATRA supplementation 10 mg/kg had the role in collagen degradation, while high dose Zn supplementation 2.5 mg/kg in collagen synthesis. High dose ATRA supplementation suppressed collagen degradation, with the expression of mRNA MMP-2  $0.04 \pm 0.019$  or 0.25 x of control. The high dose Zn supplementation increased collagen synthesis, with the expression of mRNA P1CP  $0.83 \pm 0.283$  or 4 x of control. The high dose ATRA and Zn combined supplementation gave no effect to collagen degradation with the expression of mRNA MMP-2  $0.25 \pm 0.050$  or 1.6 x of control,

but increased collagen synthesis to normal condition with the expression of mRNA P1CP  $0.60 \pm 0.237$  or 3 x of control. The high dose ATRA and Zn combined supplementation gave collagen synthesis the nearest to the normal or healthy condition.

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