Berkala Ilmu Kedokteran
Vol. 41, No. 4, Desember 2009: 183-189

The effect of zinc supplementation on collagen of periodontitis rat

Praptiwi, Siti Fatimah Muis, Soeharyo Hadisaputro, Suryono
Politeknik Depkes Semarang Jurusan Gizi

ABSTRACT

Praptiwi; Siti Fatimah Muis, Soeharyo Hadisaputro, Suryono - ***The effect of zinc supplementation on collagen of periodontitis rat***

Background: Zinc,cofactors of DNA- and RNA- polymerase, having great role in tissue heal-ing. In periodontitis, collagen type1 the main fiber constituting periodontal structure is des-troyed and be the main cause of lost of teeth among adult in Indonesia. Zn deficiency is still a nutritional problem.
The aim of the study is to obtain the influence of 500 μg Zn supplementation (Zn_1) and 200 μg (Zn_2) per day for 7 days to periodontal collagen of Periodontitis (P) rat through histologic feature.
Method: in the experimental study with factorial design, 29 adult male Wistar rat were used. Subjects were grouped simple randomly into 6 i.e Healthy (H) at the start, H at the end, P, P + Te-tracycline (T), ,P + T + Zn_1, and P + T + Zn_2. Subject other than H groups were induced periodontitis by Porphyromonas gingivalis bactena. Zn concentration was measured by AAS Flame, Periodontal tissue was stained with Mallory,s. T test and ANOVA was used to analyse difference between mean of Zn concentration of groups studies.
Result: There were similarity in the feature of groups H at the start, H at the end, and P + T + Zn_1, in sense of regularity, length, and solidity of collagen. P group had irregular, and short collagen. Groups of P + T with shorter collagen,had similarity to Health at the end. P + T + Zn_2 with shorter collagen similar to P + T + Zn_1. There was significant difference in Zn concentrat-ion between H at the end and P group. No significant differencies among Zn concentrations of Psubject groups. Great variety of Zn concentration found among subjects of groups prob-ably were the cause of absence of difference, although means of the Zn concentration values depicted it.
Conclusion: Zn supplementation dosage 500 μg/day given to periodontitis rat beside Tetra-cycline, gives better effect to collagen structure compared to 200 μg/day.

Key words: Zn supplementation - collagen - periodontitis rat - histologic feature - Zn concentration

ABSTRAK

Praptiwi, Siti Fatimah Muis, Soeharyo Hadisaputro, Suryono - ***Pengaruh suplementasi seng pada serat kolagen tikus periodontitis***

Latar Belakang: Seng (Zn), kofaktor DNA- dan RNA- polimerase,berperan besar dalam pe-nyembuhan. Pada periodontitis terjadi kerusakan kolagen tipe 1, protein struktur komponen utama jaringan periodontal, dan menjadi penyebab utama hilangnya gigi orang dewasa di Indonesia. Defisiensi Zn masih merupakan masalah gizi.
Tujuan: mengetahui pengaruh suplementasi Zn dosis 500 μg (Zn_1) dan 200 μg (Zn_2) per hari selama 7 hari pada kolagen jaringan periodontal tikus yang sakit (Periodontitis = P) melalui gambaran histologiknya.
Metoda: studi eksperimental dengan desain faktorial ini dilakukan pada 29 tikus Wistar jan-tan dewasa. Subyek dikelompokkan menjadi 6, yaitu Sehat awal, Sehat akhir, P, P+ Tetrasi-klin (T), P+ T + Zn_1, dan P+ T+ Zn_2, secara acak sederhana. Subyek studi di luar Kelompok Sehat, diinduksi periodontitis dengan bakteri Porphyromonas gingivalis. Pengukuran kadar Zn dilakukan dengan AAS Flame, pengecatan Mallory untuk jaringan periodontal. Untuk menganalisis beda konsentrasi Zn kelompok-kelompok digunakan uji T dan uji ANOVA.
Hasil: Kolagen kelompok Sehat awal dan akhir serta kelompok P+ T+Zn_1 memperlihatkan kemiripan, teratur, panjang,dan rapat.Kelompok P: kolagen tidak teratur dan pendek. Kola-gen kelompok P + T yang pendek, mirip dengan Sehat akhir. Kelompok P + T + Zn_2 dengan ko-lagen lebih pendek,mirip dengan P+ T+Zn_1 Terdapat beda bermakna kadar Zn kelompok Sehat akhir dengan *P*. Tidak eda beda bermakna kader Zn pada kelompok-kelompok subyek P. Variasi nilai kadar Zn yang besar antara subyek dalam kelompok kemungkinan menjadi penyebab tidak ada beda, walaupun reratanya memperlihatkan beda.
Simpulan: Suplementasi Zn dosis 500 μg pada tikus periodontitis dengan pengobatan Te-trasiklin memberikan pengaruh lebih baik pada struktur kolagen dibanding 200 μg

Praptiwi, Politeknik Depkes Semarang Jurusan Gizi

INTRODUCTION

Zinc (Zn) is important mineral in protein synthesis for growth, recovery from injury, etc.[1]

Zn deficiency would cause a.o impaired chemotaxis and neutrophil function,[2] and also DNA damage.[3] The impact is the pressure of the immune function, infection will frequently occurred,[2] and further,there will be disturbance of tissue repair.[4] As cofactors of DNA- and RNA- polymerase, there will be decreased of collagen synthesis in Zn deficiency.[5]

Periodontitis is the continuation of gingival inflammation as a response to odontophatic bacteria infection.[6] Althogh the calculated incidence rate for periodontitis in Indonesia was 2,07%,[7] with its rapid formation of periodontal pocket, 10% at 20 years old and 40% at fifties directing to loss of teeth, the desease is a vast health problem in community.[8] *Porphyromonas gingivalis (P. gingivalis)* is the most aggressive periodontophatic bacteria.[9] Protease and toxin of the bacteria would cause collagen degradation,[10] and according to Hoaq and Pawlak (1990) would direct to fast formation of gingival pocket.[8] This pocket makes periodontitis as the main cause of rapid loosing teeth among adult.[10]

Effort to overwhelm bacterial infection could be done a.o by using Tetracycline. The broad spectrum antibiotic slowing the growth of bacteria by interfering with the production of pro-teins needed by the bacteria to grow. This action of the antibiotic gives time to body's defense mechanism to destroy the bacteria.[11]

Inflammation as the body response to infection, proceeds in 3 basic stages, in forms of va-sodilation and increased leakage from capillaries, migration of phagocytes to the site of in-fection, and tissue repair, respectively. The second stage resulted extra-vasation, that is exude of blood cells out of the vessel to enter surrounding tissue to conduct body defense functions. Tissue repair indicated by new cells that are produced by mitosis replace damaged fibers and other tissue structures.[12]

Healing is homeostasis and integrated functioning. Many systems interact to protect the body from damage and maintain stable life-supporting functions.[13] In the traumatized or septic tissue, the cytokine Interleukin-1 (Il-1) is increasing, which enhance the expression of metallothionein (MT). This change would increase Zn uptake through – and transport to – the gut.[14] The increase of Il-1 would give proliferative effect on endothel vascularization and fi-broblast.[15]

The function of fibroblast in periodontal ligament is to synthesize collagen.[16] The fibrillar collagen is the main component of periodontal tissue, which provides the tensile strength.[17] In each fibril there are tropocollagen molecules constructed longitudinally. The molecules are connected one to each other through intermolecular bound with increasing number according to advancing age. Older fibril collagen will become rigid and brittle.[18]

Beside Zn, protein is nutrient extremely needed for body defense and healing. Zn requirement for rat is 0,0012% of its daily food consumed[19] and 40µg / day for its growth. The need of protein for growth is 25 – 30% of daily food consumed. Rat in average consume food 5 g / 100 g of body weight per day.[20]

Zn deficiency, also high dose of Zn 1000µg / g food consumed / day for 2 weeks, would lengthen recovery time by the change of inflammatory response. The rate of wound closure was significantly slower in mice fed Zn 1000µg / g compared with mice fed the 500µg / g , despite the fact that there was no significant difference in skin and serum Zn levels between the 2 groups.High Zn intake may decrease Copper (Cu) absorption, leading to Cu deficiency and anemia, and may play an important role in the immunodepression.[1] For human, 50 mg Zn / day per oral in the form of $ZnSO_4$ was reported to fasten wound closure,[21] as for rat describ-ed by Laurence and Bacharah (1964) it would be 50 x 0.018 mg or 900µg.[22]

Recovery process consists of 3 phases, i.e inflammation, proliferation, and *remodeling*.[1]

Proliferation phase is characterized by fast growth of cell surrounding wounded tissue, and new vascularization is produced to repair the decay.[12]

To emphasize the existance of collagen, Mallory's triple stain was employed. Stained with Mallory's, the collagen fibers are identified by the blue color, and red blood cells inside the blood vessels appear red.[23]

MATERIAL AND METHODS

In this experimental study with factorial design, the subject were 29 adult male Wistar rat of the 5th generation of the strain, namely LPPT 5. Subjects were acclimated for 4 days in the individual cage, made of stainless steel, with plaited Zn at the bottom to let the urine and feces go down. The subjects then grouped simple randomly into 6, i.e Healthy (H) at the start, H at the end, Periodontitis (P), P+Tetracycline (T),P+T+ Zn_1, and P+T+ Zn_2. Five rat from group Health at the start sacrified to obtain normal value of periodontal Zn concentration. All four subject of other healthy group were kept healthy until the end of the study; they were Healthy at the end. Four other groups were induced periodontitis through *Porphyromonas gingivalis (P. gingivalis)* ATCC 53977 standard strain bacteria inoculation, 3 times during 4 days.[24] Approximately 10^9 CFU of life bacteria in 100µl of Phosphate Buffered Saline (PBS) [25,24] directly introduced to the stomach and colorectal region, using canulated syringe. The bacteria was also inoculated to gingival ridge of molars region, upper, lower, right and left, using cot-ton bud. Each week after inoculation,[24] a subject was sacrificed. The periodontal tissue separated, then soaked in buffer formalin solution to prepare for histologic examination to find sign of periodontitis. After the finding, a week was needed to make chronic condition of periodontitis. Further,7 days treatment were given to each group according to the groups' name as follows: no treatment given to Periodontitis (P)group; for group of P+Tetracycline (T),18 mg / day of the Tetracycline powder, diluted in sterile water was given; and groups of P+T+ Z_1 and P+T+Z_2 received T 18 mg / day + Zn 500 µg /day and T 18 mg / day + Zn 200 µg /day respectively. The Tetracycline made by Ningxia Qiyuan Pharmaceutical Co, Ltd. Zn used is Zn sulphate ($ZnSO_4$) -7 hydrate. After those 7 days treatment, all subjects sacrificed, and the periodontal tissue of molar regions were separated from maxillar and mandibular bone for histologic examination and measurement of Zn concentration. Zn concentration was measured using Atomic Absorption Spectrometer (AAS) Flame. To prepare the periodontal tissue for histologic examination, the tissue spicemen were soaked in formalin buffer and undergo the Mallory staining procedure. Light microscope with 40x magnification was used in histologic examination, and 80x for group P.Other halves of the specimen were kept in small Eppendorf tube and kept in cold storage before measurement of Zn concentration.

RESULT AND DISCUSSION

The study was approved by 'Komisi Etik Penelitian Kesehatan Fakultas Kedokteran Uni-versitas Diponegoro dan RS dr Kariadi Semarang'. Twenty nine adult male Wistar rat were acclimated in the individual cages for 4 days, with sufficient lighting. Each subject was fed 20g/day Rat Bio 22 containing 73.53 ppm Zn, with free access to tap water containing 9.50 ppm Zn.[26] The rat were 8 weeks old at the start of the study. Food consumed for rat, according to McCoy (1971) in average is 5g / 100 g of body weight / day. Mean body weight of rat stu-died was 364,34 g , which would consume 5 x 3,643 g = 18,215 g food / day.The amount of protein in Rat Bio 22 is 22% minimum.[27] In this study, the rat was sufficiently fed, involving Zn and protein.

Further,the subject were grouped into 6, simple randomly. Unless 2 Healthy groups, 4 gro-ups were induced periodontitis using *P. gingivalis* bacteria 3 times during 4 days.

After 3 weeks, there was sign of periodontitis, indicated by presence of extravasation. To make a chronic periodontitis, a week is needed after the finding of periodontitis . Then, as be-ing planned, no other treatment given to Periodontitis (P)group. For group of P+Tetracycline (T), 18 mg / day of Tetracycline powder,diluted in sterile water was given. Groups of P+T+ Z_1 and P+T+Z_2 received T 18 mg / day + Zn 500 µg /day and T 18 mg / day + Zn 200 µg /day respectively.

After 7 days treatment, all subjects were sacrificed. Periodontal tissues of each group de-vided into 2. Half of each spicemen were soaked in formalin buffer, then undergo Mallory staining as part of histologic examination. The other halves were kept in Eppendorf small tube and ready for AAS procedure to measure Zn concentration.

a. Histologic feature

FIGURES 1 and 2 demonstrate difference between collagen of the two. Collagen in FIGURE 1 of Healthy tissue at the start of 8 weeks old subject was thinner than collagen in FIGURE 2 of 23 weeks subject. The thicker collagen seen from older subject may be due to the increasing number of tropocollagen intermolecular bound inside the collagen by advancing age.[18]

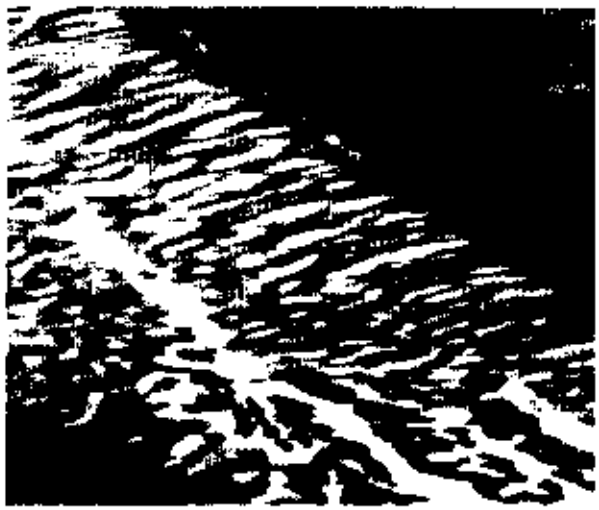

FIGURE 1. Healthy periodontal tissue of Healthy at the start group of 8 weeks old rat. Collagen was regularly constructed, long and straight.

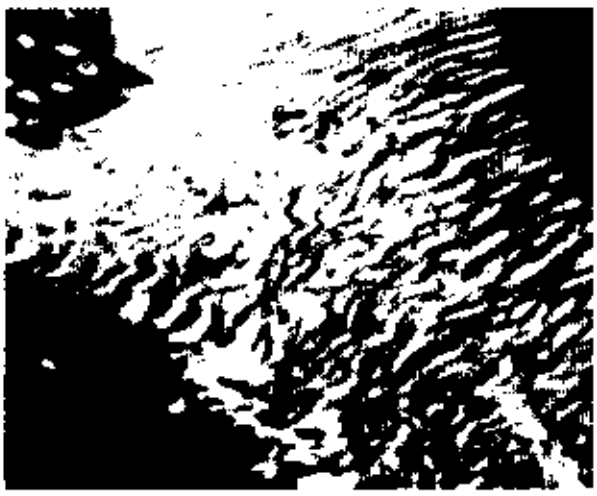

FIGURE 2. Healthy periodontal tissue of Healthy at the end group of 23 weeks old rat. Collagen was less regular and shorter than that seen in Figure 1.

FIGURE 3 denoting chronic inflammation i.e periodontitis. It depicts the degradation of co-llagen by bacteria,[10] at the same time with new collagen to repair.[12] In traumatized tissue, Il-1 is increasing and gives proliferative effect on vascularization and fibroblast.[15] Inflammation as the body response to infection, showed extravasation indicated by the exude of blood cells to surrounding tissue.[12]

FIGURE 3. Periodontitis of Periodontitis group of 23 weeks old rat. Collagen was irregular and short. Many vascularization was detected. Higher magnification than others showed extravasation.

FIGURE 4. Periodontitis treated with Tetracycline (P+T). The antibiotic slowing the growth of bacteria. By doing so, it permits body defence mechanism to destroy the bacteria. Tissue in this chronic periodontitis had more healthy collagen fiber compared to that of Figure 3, but fewer blood vessels compared to a normal one[12] seen in FIGURE 2.

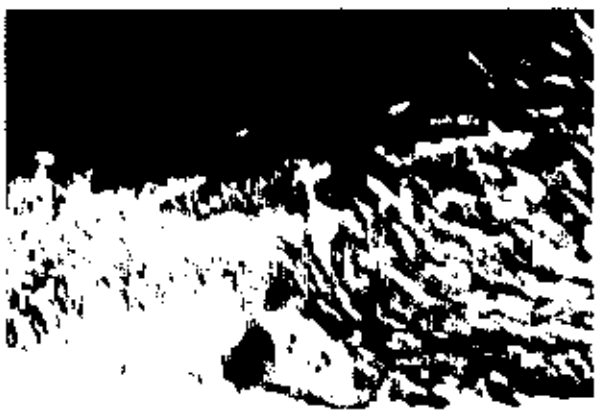

FIGURE 4. Periodontitis treated with Tetracycline of P+T group of 23 weeks old rat. Collagen was irregular and short. Small size blood vessels with red blood cells inside were spread within the tissue.

FIGURE 5. Periodontitis treated with Tetracycline and Zn of 500µg showed the benefit of Tetracycline in the same way as group (P+T) . Accumulated Zn in the area of inflammation as the effect of the increasing Il-1 enhanced protein synthesis including fibroblast.[14,15, 12] Fibro-blast produced sufficient collagen as part of tissue repair.[12]

FIGURE 5. Periodontitis treated with Tetracycline and 500 µg Zn of P+T+Zn_1 group of 23 weeks old rat. Collagen were constructed regular and larger than P+T group.Blood vessels with red blood cells inside, were larger than that seen in FIGURE 4.

FIGURE 6. Subject of Periodontitis treated with Tetracycline and Zn 200µg resulted condit- ion similar to subject treated with Tetracycline and Zn of 500µg. It was seen that higher dose of Zn gave better effect to tissue repair. Collagen as the impact of Zn 200µg supplementation were thinner than of 500µg. The figure made impression that amount of Zn supplemented under its sufficiency influenced in tissue repair through less protein synthesis as showed by thinner collagen compared to higher Zn supplementation. Fifty miligram $ZnSO_4$ / day per oral for human was reported to fasten wound closure.[21] Converted to rat,[22] the $ZnSO_4$ needed to fasten healing is 900µg. In this study, five hundred microgram $ZnSO_4$ which is nearer to 900 µg than 200µg, gave better collagen repair.

FIGURE 6. Periodontitis treated with Tetracycline and 200 µg Zn of P+T+Zn_2 group of 23 weeks old rat. Thin collagen were constructed regular, with smaller blood vessels compared to that in FIGURE 5.

b. Zinc concentration

Data of periodontal Zn concentration using AAS Flame is presented in TABLE 1.

Mean value of Zn concentration between groups of H at the start i.e 52.732 ± 49.60 ppm and H at the end i.e 26.752 ± 8.40 ppm analyzed with t-test gave no significant difference (p=0.341), although there was difference in the mean value seen between the two. Great variation of Zn concentration in H at the start group might be the cause of no significant difference. The variation might be due to 'gnawing-cage' habit of some subjects in group H at the start which gave impact to higher Zn concentration in periodontal tissue. Those habit could be seen by defective plaited Zn at the bottom of some cages.

Using t-test to analyze difference between Zn concentration of groups H at the end i.e 26.752 ± 8.40 ppm and P i.e 10.560 ± 9.31 ppm , there was significant difference (p=0.042) between the two. Zn is used abundantly in defense mechanism [2] in periodontitis, so that the concentration in periodontal tissue was decreased. This situasion was improved by giving Te-tracycline to fight against the bacteria in P+Tgroup, while Zn gave great contribution in the recovery from injury.[1] The synergic effect of Tetracycline and Zn gave the mean value of Zn concentration increasing to 21.877± 9.03 ppm.

Zn concentration of groups P+T+Zn_1 i.e 23.742± 23.31 ppm and P+T+Zn_2 i.e 17.233 ± 22.61 ppm analyzed by t-test gave no significant difference (p=0.711), although there was dif-ferent seen by their mean value. Great variety of Zn concentration value of some subjects in both two groups which could be seen by the SDs might be the cause of the non significancy.

Mean value of Zn concentration of P+T+Zn_1 group i.e 23.742 ± 23.31ppm was the nearest to Health at the end group i.e 26.752 ± 8.40 ppm compared to others. There was no significant difference (p=0.813) of Zn concentration between the two. Great variety of Zn concentration value of some subjects in P+T+Zn_1 group might be the cause of the non significancy.

Tetracycline slowing the growth of bacteria by interfering with the production of proteins needed by the bacteria to grow. Slowing the bacteria's growth allows the body defense me-chanisms to destroy them.[11] Sufficient amount of Zn gave contribution on strengthening body defense mechanism[2] and all at once accelerate recovery from injury [1] by its proliferative effect on fibroblast [13] as seen in group P+T+ Zn_1.

In general, in Periodontitis groups i.e P; P+T; P+T+Zn_1; P+T+Zn_2, ANOVA test gave no significant difference of Zn concentration among groups (p=0.731), although mean values showed the difference. Great variety of Zn concentration value of some subjects in the groups seen by the SDs might be the cause of the non significancy.

TABLE 1. Zinc concentration of groups with different treatment

No	Group	N	Zn concentration (ppm)	Mean	SD
1	Health at the start	5	72.24		
			131.10		
			15.01	52.732	49.60
			29.28		
			16.03		
2	Health at the end	4	39.29		
			23.26		
			21.37	26.752	8.40
			23.09		
3	Periodontitis	4	5.55		
			0.01		
			17.20	10.560	9.31
			19.48		
4	Periodontitis +T	4	20.83		
			21.74		
			11.45	21.877	9.03
			33.49		
5	Periodontitis +T+Z_1	6	49.64		
			8.57		
			25.21	23.742	23.31
			5.19		
			0.01		
			53.83		
6	Periodontitis +T+Z_2	6	13.95		
			62.66		
			7.07	17.233	22.61
			11.17		
			6.00		
			2.55		

CONCLUSION

In periodontitis rat treated with Tetracycline, dosage of Zn supplementation 500 µg / day for 7 days gave better effect to structure of collagen compared to 200 µg. There were no significant difference of Zn concentration among Periodontitis groups, although the mean values showed it. It is recommended to prevent the subject from gnawing object containing Zn in this case part of the cage, in order not to confuse the result of the study. Cage

made completely of stainless steel would be a good choice.

ACKNOWLEDGEMENT

The author wish to thank Experts, Staff and technicians in Microbiology Department and Pathology Anatomy Department Faculty of Medicine Gadjah Mada University, for the service and patient guidance. The author is grateful for the generousity of Phapros Pharmaceutical Industry to offer the Tetracycline used in this study.

REFERENCES

1. Lim Y, Levy M, Bray TM. Dietary Zinc Alters Early Inflammation Responses during Cutaneous Wound Healing in Weanling CD-1 Mice. Nutritional Immunology 2004;134: 811-16.
2. Prasad AS. Zinc, Infection and Immune Function. In: Calder PC, Field CJ, Gill HS, editors. Nutrition and Immune Function. Wallingford; CAB International, 2002:193-207.
3. Ho E, Courtemanche C, Ames BN. Zinc Deficiency Induces Oxidative DNA Damage and Increases P53 Expression in Human Lung Fibroblasts.The Journal of Nutrition 2003;133: 2543-48.
4. Berdanier CDa, editor. Micronutrients, Human health and well being.In: Advanced Nutrition. Micronutrients. Boca Raton; CRC Press LLC, 1998:1-8.
5. Starcher BC, Hill CH, Madaras JG.Effect of Zinc Deficiency on Bone Collagenase and Collagen Turnover. The Journal of Nutrition 1980; 110:2095-102.
6. Carranza FA a, Camargo PM. The Periodontal Pocket. In: Newman MG, Takei HH, Carranza FA, editors. Clinical Periodontology 9th ed. Sydney; WB Saunders Company, 2002: 337.
7. U.S Census Bureau. Periodontitis in Asia (Extrapolated Statistics). International Data Base. 2004 [cited 2008 May 28]. Available from: URL,:http://www. Cure Research-com.htm.
8. Roesian Boedi Oetomo, M Andi Hidayat, Harris Rahmadi, Bakti Prasetyo, Ermayanti. Kadar IgA dan IgG dalam saliva penderita penyakit periodontal sebelum dan sesudah operasi. Jurnal PDGI 2003; 53(2):25-9.
9. Kido J, Kido R, Suryono, Kataoka M, Fagerhol MK, Nagata T. Induction of Calprotectin released by *Porphyromonas gingivalis* lipopolysaccharide in human neutrophils. Oral Microbiology and Immunology 2004;19:182-7.
10. Haake SK , Newman MG, Nisengard RJ, Sanz M. Periodontal Microbiology. In: Newman MG, Takei HH, Carranza FA, editors. Clinical Periodontology 9th ed. Sydney; WB Saunders Company, 2002: 96-112.
11. Wikipedia. Tetracycline.2009 [cited 2009 July 13];1 [3 screens]. Available from:URL, :http://en.wikipedia.org/wiki/Tetracycline.
12. Benjamin CL a, Garman GR, Funston JH, editors. Body Defenses: The Lymphatic System and Immunity. In: Human Biology. Sydney; The McGraw-Hill Companies, Inc, 1997: 330-53.
13. Benjamin CL b, Garman GR, Funston JH, editors. The Skin. Integrated Functioning and Homeostasis. In: Human Biology. Tokyo. Sydney; The McGraw-Hill Companies, Inc, 1997:110-34.
14. Berdanier CD b, editor. Zinc. Trace mineral. In: Advanced Nutrition. Micronutrients. Boca Raton; CRC Press LLC, 1998; 194-200.
15. Roitt IM, Brostoff J, Male DK, editors. Cell Cooperation in the Antibody Respons. Immunology. 3rd ed. Sydney; Mosby, 1993:7.1-7.6
16. Carranza FA b, Bernard GW. The Tooth-Supporting Structures. In: Newman MG, Takei HH, Carranza FA, editors. Clinical Periodontology 9th ed. Sydney; WB Saunders Company, 2002: 36-57.
17. Sassi ML. Procollagen and collagen synthesis. Oulu; Department of Clinical Chemistry University of Oulu, 2000:1-2.
18. Needleman I. Aging and the Periodontium. In: Newman MG, Takei HH, Carranza FA, editors. Clinical Periodontology 9th ed. Sydney; WB Saunders Company, 2002: 58-62.
19. Boardman A.Rat Nutrition.http://www.boardmanweb.com/rattery/nutrition.htm. 6/16 /2009.
20. McCoy RH. Dietary Requirements of the Rat. In: Farris EJ, Griffith JQ, editors. The Rat in Laboratory Investigation. New York: Hafner Publishing Company, 1971:68-103.
21. Linder MC, editor. Seng (Zn) Dalam: Biokimia nutrisi dan metabolisme. Terjemahan Aminuddin Parakkasi. Jakarta: Penerbit Universitas Indonesia, 1992;279-84.
22. Dharmana E. Model Binatang pada Penelitian Biomedik. Semarang: Laboratorium Parasitologi FK Undip;2005.
23. Kelly CD, Fellers TJ, Davidson MW. Mallory-Stained Human Tongue Section. National High Magnetic Field Laboratory. The Floridan State University. Florida. 2003.
24. Lalla E, Lamster IB, Feit M, Huang L, Schmidt AM. A murine model of periodontal disease in diabetes. J Periodont Res 1998;33:387-99.
25. Baker PJ, Howe L, Garneau J, Roopenian DC. T cell knockout mice have diminished alveolar bone loss after oral infection with *Porphyromonas gingivalis*. FEMS Immunology and Medical Microbiology. 2002; 34:45-50.
26. Laboratorium Penelitian dan Pengujian Terpadu Universitas Gadjah Mada (LPPT UGM) a. Kadar Zn dalam Pakan dan Air.Laporan Hasil Analisis No: 1739/LPPT-UGM/U/V/2008 Air dan Pakan. LPPT UGM. 2008.
27. LPPT UGM b. Kandungan zat makanan. Rat Bio – 22. LPPT UGM. 2008.