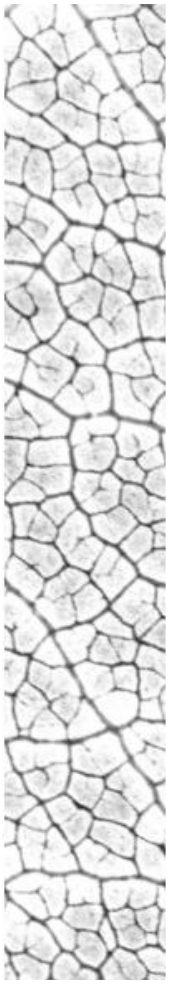

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Effect of cigarette and water-pipe smoking on osteocalcin and RANKL serum levels among Iraqi university students

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Abstract
Osteoporosis is most common skeleton illness characterized by low bone mass and increased bone fragility that putting patients at risk of fracture and smoking considered the most important environmental risk factor in the development of osteoporosis. The present study aimed to assess osteocalcin and receptor activator of the nuclear factor-kappa B ligand (RANKL) levels in two smokers groups of university students. The first include 61 cigarette-smokers and the second include 64 cigarette/water-pipe smokers, as well as 70 non-smokers (control group). A significant reduction was seen in osteocalcin serum level especially in the second group as compared to non-smokers, while RANKL serum level was significantly elevated. The osteocalcin/RANKL ratio was also significantly decreased in cigarette-smokers. These effects were exaggerated in cigarette/water-pipe smokers. In conclusion, the two mediators; osteocalcin and RANKL, may be good predictors for the development of osteoporosis especially in young adults.

Keywords: smoking, osteocalcin, RANKL, osteoporosis

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INTRODUCTION

In recent years, water-pipe tobacco-smoking (Narghileh) has gained popularity among Iraqi population especially adolescents and young adults. Actually, it has become a daily habit and a social activity. Therefore, increased risks for cancer and cardiovascular diseases, as well as other morbidities, are expected (Milara, & Cortijo, 2012., Bassiony, et al. 2015). Osteoporosis and bone fracture are among the morbidities that have been associated with tobacco-smoking (Christos, et al. 2015. Alsaadawi 2016. Uçar, et al. 2019). It has been revealed that tobacco-smoking influences the imbalance of bone turnover; rendering bone vulnerable to osteoporosis and fracture due to a lower consequent bone mass (Law & Hackshaw 1997. Ward & Klesges 2001). The influence of tobacco-smoking on bone development and bone mass can be indirect and direct. The indirect influence is related to alterations in the general biological functions, such as physiological and immunological status, which may influence the bone modeling and remodeling (Yoon, et al. 2012. Eissenberg & Shihadeh 2009). However, bone modeling and remodeling are also subjected to the effects of regulatory factors involved in the metabolism of bones; such as osteocalcin and RANKL (receptor activator of nuclear factor kappa-B ligand), which are suggested to be a target for tobacco-smoking. Therefore, both features of bone (osteogenesis and angiogenesis) are directly influenced by the two mediators (Al-Bashaireh et al. 2018).

Osteocalcin is the most abundant non-collagenous bone protein produced by the bone-forming cells osteoblasts. It is generally presented as a marker of bone formation through involvement in the process of mineralization (Holvik et al. 2014). Serum levels of intact osteocalcin are considered as biomarkers of bone formation. Further, levels of circulating osteocalcin are suggested to reflect a histological evidence of bone formation rate (Seibel 2005). Osteocalcin synthesis is dependent on the actions of the vitamin D metabolite $1,25(OH)_2D_3$, which impacts osteocalcin gene and promotes the transcriptional activation (Price & Baukol 1980; Pande, et al , (2015). However, expression and serum level of osteocalcin can be influenced by drugs and chemical compounds. For instance, inhaled corticosteroids were associated with a decreased serum level of osteocalcin (Hanania et al. 1995. Barnes, 2010). Nicotine (a prominent component of tobacco-smoke) has also been demonstrated to have a similar effect, and accordingly, tobacco-smoking has been considered as a candidate risk factor for osteoporosis (Pouresmaeili, et al. 2018).

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Table 1. Serum level of osteocalcin and RANKL and Osteocalcin/RANKL ratio among cigarette-smokers, cigarette/water-pipe smokers and non-smokers

Parameter	Median (range)			p-value		
	CS (N = 61)	CWS (N = 64)	NS (N = 70)	CS vs. CWS	CS vs. NS	CWS vs. NS
Osteocalcin (ng/ml)	1.2 (0.5-2.7)	0.3 (0.1-1.5)	2.9 (0.7-4.6)	< 0.001	< 0.001	< 0.001
RANKL (pg/ml)	86.3 (53.5-126.0)	109.1 (42.6-236.0)	55.4 (37.8-212.7)	< 0.001	0.115	< 0.001
Osteocalcin/RANKL ratio (%)	1.6 (0.5-2.8)	0.4 (0.1-2.3)	5.7 (0.6-9.4)	< 0.001	< 0.001	< 0.001

CS: Cigarette-smokers; CWS: Cigarette/water-pipe smokers; NS: Non-smokers; p: Mann-Whitney U test probabilit

RANKL, also known as osteoclast differentiation factor, is a member of the tumor necrosis factor (TNF) cytokine family that functions as a key factor for differentiation and activation of osteoclasts (Garcia-Lopez, Villanueva, Meikle, 2013). It is expressed by osteoblasts, bone stromal cells, fibroblasts, osteocytes, macrophages and activated T cells (Ozc aka, et al. 2010). The activation of osteoclasts to initiate bone destruction is signaled by RANKL, RANK (receptor activator of nuclear factor kappa-B) and osteoprotegerin (OPG). RANKL attaches to RANK, a receptor on the cell surface of osteoclasts and osteoclast precursors, and such attachment stimulates the proliferation and differentiation of cells to form the osteoclast phenotype (Laceyet al. 1998. Teitelbaum, & Ross, 2003. Behfarnia, Saied-Moallemi, Javanmard, & Naseri, 2016). Serum This ligand can be found as a cell membrane-bound variant (mRANKL) or a primary soluble secreted form (sRANKL) that has been described in diseases of chronic inflammation and progressive bone loss; such as rheumatoid arthritis, periodontitis and gingival crevicular fluid (Sarlati, Sattari, Gazar, Rafsenjani, 2010). As in osteocalcin, serum level of RANKL can also be influenced by tobacco-smoking, but the evidence has not been conclusive (Kargin, et al. 2016. Al-Bashaireh, & Alqudah, 2020. Jordeet al. 2019).

This study hypothesized that tobacco-smoking may influence serum level of osteocalcin and RANKL. Therefore, serum level of both markers was determined in two groups of smokers (cigarette-smokers and cigarette/water-pipe smokers).

MATERIAL AND METHODS

Populations studied

A case-control study was conducted at the College of Education for Pure Science (Ibn Al-Haitham), University of Baghdad during October – December, 2019. A total sample of 195 university male students was enrolled in the study, and their age mean was 21.5 year (range: 18 – 24). The selection criteria of participants were based on their health and cigarette-smoking. They were healthy and did not complain of any inflammatory or chronic disease. According to cigarette-smoking status, they were distributed into three groups; 70 non-smokers (control group), 61 cigarette-smokers and 64 cigarette/water-pipe smokers. The participant was considered as cigarette-smoker when he smoked at least 20 cigarettes per day during the last 12 months. For cigarette/water-pipe smokers, in addition for being cigarette-smokers, they smoked water-pipe tobacco at least two times per week during the last 12 months.

Methods

From each participating subject, 3 ml of venous blood was collected and left for 15 minutes to clot at room temperature. The clotted blood was centrifuged (3000 rpm for 15 minutes), and serum was collected. The separated sera were frozen at -20°C until laboratory assessments. Osteocalcin and RANKL levels were determined in sera using enzyme linked immunosorbet assay (ELISA) kits (Koma Biotech, Korea) and instructions of manufacturer were followed.

Statistical analysis

Serum level of osteocalcin and RANKL were tested for normality using Kolmogorov-Smirnov and Shapiro-Wilk test. The test revealed that the data did not follow normal distribution; therefore, they were given as median and range. Significant differences between medians were assessed Mann-Whitney test.

The data were also distributed according to their relation to median (> and ≤ median). Based on such distribution, the odds ratio (OR) together with its 95% confidence interval (CI) were estimated for each biomarker. A *p*-value ≤ 0.05 was considered significant. The statistical package SPSS version 16.0 was used to carry out these analyses.

RESULTS

Populations studied

A case-control study was conducted at the College of Education for Pure Science (Ibn Al-Haitham), University of Baghdad during October – December, 2019. A total sample of 195 university male students was enrolled in the study, and their age mean was 21.5 year (range: 18 – 24). The selection criteria of participants were based on their health and cigarette-smoking. They were healthy and did not complain of any inflammatory or chronic disease. According to cigarette-smoking status, they were distributed into three groups; 70 non-smokers (control group), 61 cigarette-smokers and 64 cigarette/water-pipe smokers. The participant was considered as cigarette-smoker when he smoked at least 20 cigarettes per day during the last 12 months. For cigarette/water-pipe smokers, in addition for being

Table 2. Serum level of osteocalcin distributed according to median (> median and ≤ median) among cigarette-smokers, cigarette/water-pipe smokers and non-smokers

Group	Osteocalcin (ng/ml)				OR	95% CI	p-value
	(> median		≤ median				
	N	%	N	%			
Non-smokers (N = 70)	56	80.0	14	20.0	Reference		
Cigarette-smokers (N = 61)	24	39.3	37	60.7	0.16	0.07 - 0.35	< 0.001
Cigarette/water-pipe smokers (N = 64)	12	18.7	52	81.3	0.06	0.02 - 0.14	< 0.001

OR: Odds ratio (versus non-smokers); CI: Confidence interval; p: Two-tailed Fisher exact probability.

Table 3. Serum level of RANKL distributed according to median (> median and ≤ median) among cigarette-smokers, cigarette/water-pipe smokers and non-smokers

Group	RANKL (pg/ml)				OR	95% CI	p-value
	(> median		≤ median				
	N	%	N	%			
Non-smokers (N = 70)	21	30.0	49	70.0	Reference		
Cigarette-smokers (N = 61)	30	49.2	31	50.8	2.26	1.11 - 4.60	0.031
Cigarette/water-pipe smokers (N = 64)	45	70.3	19	29.7	5.53	2.65 - 11.53	< 0.001

OR: Odds ratio (versus non-smokers); CI: Confidence interval; p: Two-tailed Fisher exact probability.

Table 4. Osteocalcin/RANKL ratio distributed according to median (> median and ≤ median) among cigarette-smokers, cigarette/water-pipe smokers and non-smokers

Group	Osteocalcin/RANKL ratio (%)				OR	95% CI	p-value
	(> median		≤ median				
	N	%	N	%			
Non-smokers (N = 70)	56	80.0	14	20.0	Reference		
Cigarette-smokers (N = 61)	31	50.8	30	49.2	0.26	0.12 - 0.56	0.001
Cigarette/water-pipe smokers (N = 64)	6	9.4	58	90.6	0.03	0.01 - 0.07	< 0.001

OR: Odds ratio (versus non-smokers); CI: Confidence interval; p: Two-tailed Fisher exact probability.

cigarette-smokers, they smoked water-pipe tobacco at least two times per week during the last 12 months.

Methods

From each participating subject, 3 ml of venous blood was collected and left for 15 minutes to clot at room temperature. The clotted blood was centrifuged (3000 rpm for 15 minutes), and serum was collected. The separated sera were frozen at -20°C until laboratory assessments. Osteocalcin and RANKL levels were determined in sera using enzyme linked immunosorbet assay (ELISA) kits (Koma Biotech, Korea) and instructions of manufacturer were followed.

Statistical analysis

Serum level of osteocalcin and RANKL were tested for normality using Kolmogorov-Smirnov and Shapiro-Wilk test. The test revealed that the data did not follow normal distribution; therefore, they were given as median and range. Significant differences between medians were assessed Mann-Whitney test. The data were also distributed according to their relation to median (> and ≤ median). Based on such distribution, the odds ratio (OR) together with its 95% confidence interval (CI) were estimated for each biomarker. A p-value ≤ 0.05 was considered significant. The statistical package SPSS version 16.0 was used to carry out these analyses.

DISCUSSION

This study demonstrated that cigarette-smoking impacted serum level of osteocalcin and RANKL with an opposite effect in young adults. It decreased the level of osteocalcin, while RANKL level was elevated in cigarette-smokers. The effect in both cases was exaggerated when cigarette-smoking was accompanied with water-pipe tobacco smoking. The ratio of osteocalcin / RANK was similarly influenced. These findings suggest that cigarette-smoking interfered with synthesis of both biomarkers, and their levels were consequently negatively and positively regulated, respectively.

With respect to osteocalcin, previous studies reported conflicting results regarding cigarette-smoking effects on osteocalcin. Several authors reported that cigarette-smoking had no effect on osteocalcin in humans in general or young adult men and levels of osteocalcin showed no variation between smokers and non-smokers (Nafarzadeh, et al. 2015. Laroche, et al. 1994. Xue, et al. 2014. Nafazadeh, et al. 2015). However, a further study demonstrated that saliva osteocalcin level was significantly decreased in cigarette-smokers. The authors also suggested that suppression effect of smoking on osteocalcin synthesis may be correlated with periodontal health (Gurlek, Lappin, & Buduneli, 2009). In a more recent study, influence of chronic cigarette-smoking on osteo-immunoinflammatory markers (including osteocalcin) was evaluated. Although no significant difference was observed, a decreased level of osteocalcin was reported in peri-implant crevicular fluid of smokers was observed (Negri, et al. 2016). In hemodialysis patients, cigarette-smoking was also associated with a significant decreased level of osteocalcin (Fusaro, et al. 2018). In the case of RANKL, effects of tobacco-smoking also showed conflicting results (Behfarnia, Saied-Moallemi,

Javanmard, Naseri, 2016. Aldahlawi, 2019. Jorde, et al. 2019. Buduneli, Buduneli, & Kütükçüler, 2009). However, gene-expression results were more consistent. Evaluating gene expression of bone resorption-related markers in nicotine-treated periodontal ligament cells demonstrated an elevation in RANKL expression. According, it was suggested that nicotine acted as a stimulus for bone resorption. Further studies also shared similar findings (Ribeiro, et al. 2020. Wu, et al. 2013. Chen, et al. 2014).). Further, it is generally agreed that RANKL alterations in smokers is due to the direct cellular effects of smoke on bone cells. Accordingly, cigarette-smoking is suggested as a risk factor for osteoporosis and osteoporotic fractures (Taes, et al. 2010).

In spite of the inconsistencies, cigarette-smoke seems to be effective in modulating serum level of osteocalcin and RANKL, probably due to the toxic effects of nicotine, which has been demonstrated to cause severe changes in different aspects of bone metabolism, especially at increased doses (Lappin, et al. 2007. WONG, Christie, & Wark, 2007).). The present study results favor such generalization, because more devastating effects were noted in cigarette /water-pipe smokers. Water-pipe smoking sessions typically last 20-80 minutes, during which the smoker may take 50 - 200 puffs; therefore water-pipe smokers may inhale as much smoke during one session as cigarette smokers would inhale (Eissenberg, & Shihadeh, 2009. Djordjevic, Stellman, & Zang, 2000. Shihadeh, Azar, Antonios, & Haddad. 2004).In addition, water-pipe smokers are also exposed to other toxic substances such as heat-sourced carbon.

CONCLUSION

They study concluded that cigarette-smoking had a negative effects on osteocalcin, while positive effects on RANKL were suggested. Such effects were much more exaggerated with water-pipe tobacco smoking.

REFERENCES

Al-Bashaireh A.M., Haddad L.G., Weaver M., Chengguo X., Kelly D.L. & Yoon S.(2018). The Effect of Tobacco Smoking on Bone Mass: An Overview of Pathophysiologic Mechanisms. Journal of Osteoporosis,2018:1-17.

Al-Bashaireh, A.M. & Alqudah, O.(2020). Comparison of Bone Turnover Markers between Young Adult Male Smokers and Nonsmokers. Cureus.; 27:e6782.

Aldahlawi, S.(2019). Evaluation of Serum RANKL and OPG Concentrations in Patients with Periodontitis in Saudi Arabia. Int.J. Pharm. Res. Allied Sci., 8:45-51.

Alsaadawi T. (2016).Smoking is Associated with Increased Risk of Osteoporosis in Diabetes Mellitus Patients. J Bone Rep Recommendations.2:4.

Barnes P.J.(2010). Inhaled Corticosteroids. Pharmaceuticals , 3:514-540.

Bassiony, M. A., Aqil, M., Khalili, M., Radosevich, J. A., & Elsabaa, H. M. (2015). Tobacco consumption and oral, pharyngeal and lung cancers. *The Open Cancer Journal*, *8*(1).

Behfarnia, P., Saied-Moallemi, Z., Javanmard, S.H. & Naseri, R. (2016). Serum, saliva, and GCF concentration of RANKL and osteoprotegerin in smokers versus nonsmokers with chronic periodontitis. Adv Biomed Res., 5:80.

Behfarnia, P., Saied-Moallemi, Z., Javanmard, S.H., Naseri, R. (2016).Serum, saliva, and GCF concentration of RANKL and osteoprotegerin in smokers versus nonsmokers with chronic periodontitis. Adv Biomed Res.;5:80.

Buduneli, N., Buduneli, E. & Kütükçüler, N. (2009).Interleukin-17, rankl, and osteoprotegerin levels in gingival crevicular fluid from smoking and non-smoking patients with chronic periodontitis during initial periodontal treatment. J Periodontol.;80:1274-80.

Chen, B., Wu, W., Sun, W., Zhang, Q., Yan, F. & Xia, Y.(2014). RANKL Expression in Periodontal Disease: Where Does RANKL Come from?. BioMed Research International.,2014:1-7.

Christos, K. F., Maria, P. E., Ioanna, P. V., Polina, P., Ioannis, K., Sofia, D., ... & Lamprini, K. (2015). Smoking is associated with osteoporosis development in Primary care population. *Am J Nurs*, *4*, 96-101.

Djordjevic, M.V., Stellman, S.D. & Zang, E. (2000).Doses of nicotine and lung carcinogens delivered to cigarette smokers. J Natl Cancer Inst., 92:106-111.

Eissenberg T. & Shihadeh A.(2009). Waterpipe Tobacco and Cigarette Smoking Direct Comparison of Toxicant Exposure. Am J Prev Med.,37: 518–523.

Eissenberg, T. & Shihadeh, A.(2009). Waterpipe Tobacco and Cigarette Smoking Direct Comparison of Toxicant Exposure. Am J Prev Med.,37: 518–523.

Fusaro, M., Gallieni, M., Aghi, A., Iervasi G., Rizzo, M.A., Stucchi, A., Noale, M., Tripepi, G., Nickolas, T., Veronese, N., Fabris, F., Giannini, S., Calo, L., Piccoli, A., Mereu, M.C., Cosmai, L., Ferraro, A., Magonara, F., Spinello, M.,

Sella, S., Plebani, M.(2018). Cigarette Smoking is Associated with Decreased Bone Gla-protein (BGP) Levels in Hemodialysis Patients. Curr Vasc Pharmacol.,;16:603-609.

Garcia-Lopez S., Villanueva R., Meikle M.C.(2013). Alterations in the Synthesis of IL-1β, TNF-α, IL-6, and their downstream targets RANKL and OPG by mouse calvarial osteoblasts in vitro: Inhibition of bone resorption by cyclic mechanical strain. Front Endocrinol (Lausanne) ;4:160

Gurlek, O. Lappin, D.F. & Buduneli, N. (2009). Effects of smoking on salivary C-telopeptide pyridinoline cross-links of type I collagen and osteocalcin levels. Arch Oral Biol., 54: 1099–1104.

Hanania N.A., Chapman K.R., Sturtridge W.C., Szalai J.P. & Kesten S.(1995). Dose-related decrease in bone density among asthmatic patients treated with inhaled corticosteroids. J Allergy Clin Immunol.;96:571-9.

Holvik K., van Schoor N.M., Eekhoff E.M.W., Heijer M., Deeg D.J.H., Lips P. & de Jongh R.(2014). Plasma osteocalcin levels as a predictor of cardiovascular disease in older men and women: a population-based cohort study.European Journal of Endocrinology, 171:161-170.

Jorde, R., Stunes, A.K., Kubiak, J., Grimnes, G., Thorsby, P.M. & Syversen, U.(2019). Smoking and other determinants of bone turnover. PLoS One.;14: 1-16

Jorde, R., Stunes, A.K., Kubiak, J., Grimnes, G.,Thorsby, P.M. & Syversen, U. (2019). Smoking and other determinants of bone turnover. PLoS One. 14:e0225539.

Kargin, N.C., Marakoglu, K., Unlu, A., Kebapcilar, L.,& Korucu, E.N.(2016). Comparison of bone turnover markers between male smoker and non-smoker. Acta Medica Mediterranea, 32:317-323.

Lacey, D.L., Timms, E., Tan, H.L., Kelley, M.J, Dunstan, C.R., Burgess, T., Elliott, R.,Colombero, A., Elliott, g., Scully, S., Hsu, H., Sullivan, J., Hawkins, N., Davy, E., Capparelli, C., Eli, A., Qian, Y-X., Kaufman, S., Sarosi, I., Shalhoub, V., Senaldi, G., Guo, J., Delaney, J. & Boyle, W.J.(1998).Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell.,93:165-176.

Lappin, D., Sherrabeh, S., Jenkins,W. & Macpherson, L. (2007).Effect of smoking on serum RANKL and OPG in sex, age and clinically matched supportive-therapy periodontitis patients. Journal Of Clinical Periodontology, 34:271-277.

Laroche, M., Lasne, Y., Felez, A., Moulinier, L., Bon, E., Cantagrel, A., Léophonte, P., & Mazières, B.(1994). Osteocalcin and smoking. Rev. Rhum. Ed. Fr., 61:433-436.[Pubmed].

Law M.R. & Hackshaw A.K. (1997). A meta-analysis of cigarette smoking, bone mineral density and risk of hip fracture: recognition of a major effect. BMJ; 315:841–846

Milara, J., & Cortijo, J. (2012). Tobacco, inflammation, and respiratory tract cancer. *Current pharmaceutical design*, *18*(26), 3901-3938.

Nafarzadeh, S., Amooeianb, B., Jafari, S., Nikbind, M., Mostafazadehe, V. & Bijanif, A.(2015).The effect of smoking on salivary osteocalcin in patients with chronic periodontitis.J.Craniomax.Res.;2:133-137.

Nafazadeh, S., Amooeianb, B., Jafari, S., Nikbind, M., Mostafazadeh, V. & Bijanif, A.(2015).The effect of smoking on salivary osteocalcin in patients with chronic periodontitis.J. Craniomax.Res., 2:133-137.

Negri, B.M., Pimentel, S.P., Casati, M.Z., Cirano, F.R., Casarin, R.C. & Ribeiro, F.V.(2016). Impact of a chronic smoking habit on the osteo-immunoinflammatory mediators in the peri-implant fluid of clinically healthy dental implants. Arch Oral Biol.;70:55-61.

Ozc¸aka, O., Nalbantsoy, A. ,Kose, T. & Buduneli, N.(2010). Plasma osteoprotegerin levels are decreased in smoker chronic periodontitis patients. Australian Dental Journal, 55: 405-410.

Pande, A., Singh, S., Samad, J., Saurabh, K., & Haider, Z. A. (2015). Studies on Potential of Finger Millet (Eleusine Coracana Gaertn. L.) Amylases for Industrial Applications. The International Journal of Biotechnology, 4(4), 20-29.

Pouresmaeili F., Kamalidehghan B., Kamarehei M.& Goh Y.M. (2018). A comprehensive overview on osteoporosis and its risk factors. Ther Clin Risk Manag.;14: 2029–2049.

Price P.A.& Baukol S.A.(1980). 1,25-Dihydroxyvitamin D3 increases synthesis of vitamin K-dependent bone protein by osteosarcoma cells. J. Biol Chem;225: 11660-11663.

Ribeiro, L.N.S., Monteiro, P.M., Barretto, G.D., Luiz, K.G., Alves, S.Y.F. & Stuani, M.B.S.(2020). The Effect of Cigarette Smoking And Low-Level Laser Irradiation in RANK/RANKL/OPG Expression. Braz Dent J.;31:57-62.

Sarlati, F. Sattari, M., Gazar, A.G., Rafsenjani, A.N.(2010). Receptor Activator of Nuclear Factor Kappa B Ligand (RANKL) Levels in Peri-Implant Crevicular Fluid. Iran.J.Immunol.,7:226-233.

Seibel M.J.(2005). Biochemical Markers of Bone Turnover Part I: Biochemistry and Variability. Clin Biochem Rev.; 26: 97–122.

Shihadeh, A., Azar, S., Antonios, C. & Haddad. A. (2004).Towards a topographical model of narghile water-pipe café smoking: a pilot study in a high socioeconomic status neighborhood of Beirut, Lebanon. Pharmacol Biochem Behav.,79:75-82.

Taes, Y., Lapauw, B.,Vanbillemont, G., Bogaert, V., Bacquer, D.D., Goemaere, S., Zmierczak, H., & Kaufman, J.M.(2010). Early Smoking Is Associated With Peak Bone Mass and Prevalent Fractures in Young, Healthy Men. Journal of Bone and Mineral Research,25: 379-387.

Teitelbaum, S.L. & Ross, F.P.(2003) Genetic regulation of osteoclast development and function. Nat Rev Genet.,4:638-649.

Uçar, R. M., Marakoğlu, K., Gedik, G. K., Kızmaz, M., & Levendoğlu, F. (2019). The Comparison of Bone Mineral Density Between Male Smokers and Non-Smokers.

University of California - Irvine. “In first-of-its-kind study, researchers highlight hookah health hazards: Smokers exposed to toxic chemicals, ultrafine particles and carbon monoxide.” ScienceDaily. ScienceDaily, 12 August 2019. www.sciencedaily.com/releases/2019/08/190812144932.htm.

Ward K.D. & Klesges R.C.(2001). A Meta-Analysis of the Effects of Cigarette Smoking on Bone Mineral Density. Calcif Tissue Int.; 68: 259–270.

WONG, P.K.K., Christie, J.J. & Wark, J.D.(2007). The effects of smoking on bone health. Clinical Science, 113:233–241.

Wu, L., Duan, D., Liu, Y., Ge, X., Zhou, Z. & Wang, X.(2013).Nicotine favors osteoclastogenesis in human periodontal ligament cells co-cultured with CD4+ T cells by upregulating IL-1β.Int.J.Mol.Med.;31:938-942.

Xue, Peng-fei, Hou, Y.D., Zhang, Y.T., Pan, J.J., Sun, X & Sun, L.(2014). Smokeless tobacco extract affects biological properties of the pre-osteoblast cell line MC3T3-E1. Sichuan Da Xue Xue Bao Yi Xue Ban.,45:908-912.

Yoon V., Maalouf N.M, Sakhaee K.(2012).The effects of smoking on bone metabolism. Osteoporosis International 23:2081-2092