

# The functional assessment of kefir on the postponement of bone loss in ovariectomized mice

賴冠達、陳小玲

E-mail: 345502@mail.dyu.edu.tw

## ABSTRACT

Postmenopausal women would be more prone to osteoporosis, because the ovary was atrophied and unable secret enough estrogen and isoflavones, which can promote the growth of osteoblasts and inhibit the activity of osteoclast. In previous studies, casein phosphopeptides (CPPs) were found that exhibit the ability to bind calcium and to enhance calcium absorption into the body. CPPs may be the functional materials to postpone the bone loss. Kefir is a kind of fermented milk with various bioactivities reported in many previous research papers. However, there is few osteoporosis-related papers published. Therefore, this study was conducted to verify the bioactivity of osteoporosis prevention in kefir. Experiment were divided into two part (1) the assessment of kefir on the postponement bone loss in the old aged ovariectomied mice. Eight-week-old B6 ovariectomized mice was used to be the animal model to simulate menopause women. There were five treatment, including ovariectomized groups (Water / OVX), sham groups (Water / Sham), calcium and carbonate supplement groups (Ca / OVX), kefir supplement groups (Kefir / OVX) and kefir combined with calcium carbonate supplement groups (Kefir + Ca / OVX). Mice were sacrificed after 16 months treatment. Micro-CT was used to analyze trabecular bone in the knee growth plates. Result showed that three-dimensional structure, proportion of bone volume/tissue volume (BV / TV), trabecular bone mineral density (BMD), trabecular thickness degree (Tb.Th), trabecular number (Tb.N), trabecular separation (Tb. Sp) and cortical bone density (BMD), did not had significant difference between various treatments. (2) the assessment of kefir on the postponement bone loss in the ovariectomied mice. Four-month old sexual matured and ovariectomied mice were used. There were five treatment as described above. Mice were sacrificed after 2 months treatment. The results of Micro-CT showed that the Kefir / OVX group was significantly different compared with the other group in three-dimensional structure of trabecular bone in knee growth plate. In the proportion of bone tissue and bone volume/tissue volume (BV / TV), Kefir / OVX was about 75% higher than Water / OVX ,group. In trabecular bone mineral density (BMD), Kefir / OVX group was 27% higher than Water / OVX group. In trabecular bone thickness (Tb.Th) and trabecular bone number (Tb.N), Kefir / OVX group was 24% and 55% higher than Water / OVX group. In trabecular bone separation (Tb.Sp), Water / OVX group was 75% higher than Kefir / OVX group. In addition, the result of SEM showed that the Kefir / OVX group was higher then the other groups. Kefir / OVX group has the highest amount of trabecular bone in five groups. According to the results described above, kefir exhibited the ability of postponement bone loss.

Keywords : ovariectomized mice、kefir、computerized tomography、scanning electron microscopy

## Table of Contents

|                                                             |       |                                       |     |
|-------------------------------------------------------------|-------|---------------------------------------|-----|
| 封面內頁 簽名頁 中文摘要.....                                          | iii   | 英文摘要.....                             | vii |
| 要.....                                                      | v     | 誌謝.....                               | vii |
| 目錄.....                                                     | viii  | 圖目錄.....                              | ix  |
| 表目錄.....                                                    | xviii | 1. 前言.....                            | 2   |
| 1. 文獻回顧.....                                                | 2     | 2.1 骨質疏鬆症介.....                       | 2   |
| 2.2 骨骼的組成結構、生合成以及代謝.....                                    | 5     | 2.2.1 破骨細胞 (Osteoclast).....          | 6   |
| 2.2.2 骨細胞 (Osteocytes).....                                 | 9     | 2.2.3 骨吸收 (Bone resorption).....      | 10  |
| 2.2.4 骨生成 (Bone remodeling).....                            | 10    | 2.3 動物模式.....                         | 12  |
| 2.4 分析儀器 - 掃描式電子顯微鏡 (Scanning Electric Tomography:SEM)..... | 13    | 2.4.1 分析儀器 - 電腦斷層掃描.....              | 13  |
| 3. 材料與方法.....                                               | 16    | 3.1 實驗架構規畫.....                       | 16  |
| 3.2 材料方法 (一).....                                           | 16    | 3.2.1 (一) 克弗爾在摘除卵巢老齡鼠的骨質流失延緩功效評估..... | 16  |
| 3.2.1.1 小鼠來源.....                                           | 16    | 3.2.1.2 動物試驗分組.....                   | 17  |
| 3.2.1.2.1 偽手術組.....                                         | 17    | 3.2.1.2.2 切除卵巢組.....                  | 18  |
| 3.2.1.2.3 營養補充組.....                                        | 18    | 3.2.1.2.4 克弗爾來源及劑量.....               | 18  |
| 3.2.1.3 鼠骨試片樣本處理及分析.....                                    | 19    | 3.2.1.3.1 次氯酸鈉處理.....                 | 19  |
| 3.2.1.3.2 脫水處理.....                                         | 20    | 3.2.1.4 儀器分.....                      | 20  |

|                                            |                                                                   |
|--------------------------------------------|-------------------------------------------------------------------|
| 析.....                                     | 20 3.3.1.4.1 高解析度微型 X光電腦斷層掃描儀 (Micro-CT)                          |
| .....                                      | 20 3.2.1.4.2 掃描式電子顯微鏡(SEM)..... 21 3.2.2 (二) 克弗爾在摘除卵巢小鼠           |
| 相對女性中年更年期的骨質流失延緩功效評估.....                  | 22 3.2.2.1 小鼠來                                                    |
| 源.....                                     | 22 3.2.2.2 動物試驗分組..... 23 3.2.2.3 偽手術                             |
| 組.....                                     | 23 3.2.2.4 切除卵巢組..... 23 3.2.2.5 營養補充                             |
| 組.....                                     | 24 3.2.3 克弗爾來源及劑量..... 24 3.2.4 分析方                               |
| 法.....                                     | 25 3.3 統計分析..... 25 4. 結果                                         |
| .....                                      | 26 4.1 克弗爾在摘除卵巢老齡鼠的骨質流失延緩功效評估.. 26 4.1.1                          |
| 小鼠體重變化.....                                | 26 4.1.2 股骨外觀圖..... 26 4.1.3                                      |
| 以Micro-CT分析膝蓋端生長板骨小樑的3D立體結構.....           | 26 4.1.4 以Micro-CT                                                |
| 分析膝蓋端生長板骨小樑與組織所佔之比例.....                   | 27 4.1.5 以Micro-CT分析膝蓋端生                                          |
| 長板骨小樑厚薄度.....                              | 27 4.1.6 以Micro-CT分析膝蓋端生長板骨小樑數量..... 27 4.1.7 以Micro-CT分析膝蓋端生長板骨小 |
| 樑分散程度. 28 4.1.8 以Micro-CT分析膝蓋緻密骨骨礦物密度..... | 28 4.2 克弗爾在摘除卵巢小鼠相對女性中年更年期的                                       |
| 骨質流失延緩功效評估.....                            | 29 4.2.1 小鼠體重變化..... 29                                           |
| 4.2.2 股骨外觀圖.....                           | 29 4.2.3 以Micro-CT分析膝蓋端生長板骨小樑的3D立體結構                              |
| .....                                      | 30 4.2.4 以Micro-CT分析膝蓋端生長板骨小樑與組織所佔之比                              |
| 例.....                                     | 30 4.2.5 以Micro-CT分析膝蓋端生長板骨小樑骨礦物密度                                |
| .....                                      | 30 4.2.6 以Micro-CT分析膝蓋端生長板骨小樑厚薄度..... 31 4.2.7                    |
| 以Micro-CT分析膝蓋端生長板骨小樑數量.....                | 31 4.2.8 以Micro-CT分析膝蓋端生長板骨小樑分散程度. 32 4.2.9                       |
| 以Micro-CT分析膝蓋緻密骨骨礦物密度.....                 | 32 4.2.10 以掃描式電子顯微鏡觀察膝蓋端生長板骨小樑的外觀切片                               |
| .....                                      | 33 4.2.11 以Micro-CT分析膝蓋端生長板骨小樑的3D縱切立體結構                           |
| .....                                      | 33 4.2.12 五種處理組小鼠臟器外觀觀察..... 33 5. 討論                             |
| .....                                      | 35 5.1 克弗爾在摘除卵巢老齡鼠的骨質流失延緩功效評估... 35 5.2 克                         |
| 弗爾在摘除卵巢小鼠相對女性中年更年期的骨質流失延緩功效評估.....         | 36 6. 結                                                           |
| 論.....                                     | 39 參考文獻.....                                                      |
| 70 圖目錄 圖1 骨骼微結構.....                       | 41 圖2 成破骨細胞生長示意圖                                                  |
| .....                                      | 42 圖3 五種處理組之試驗動物處理時程..... 43 圖4 五種處理組小鼠經16                        |
| 個月餵食後之 Micro-CT 掃描影像圖.....                 | 44 圖5 以Micro-CT分析軟體CTan                                           |
| 分析膝蓋端生長板骨小樑之圈選範圍.....                      | 45 圖6 五種處理組之試驗動物處理時程                                              |
| .....                                      | 46 圖7 五種處理組小鼠餵養16個月後之體重變化..... 47 圖8 五種處理組小鼠餵養16個月後               |
| 之股骨頭外觀圖.....                               | 48 圖9 五種處理組小鼠餵養16個月後之膝蓋端生長板                                       |
| 骨小樑Micro-CT合成圖.....                        | 49 圖10 五種處理組小鼠餵養16個月後利用Micro-CT分析膝                                |
| 蓋端骨小樑與組織所佔之比例比較.....                       | 50 圖11 五種處理組小鼠餵養16個月後利用Micro-CT分析膝蓋端                              |
| 生長板骨小樑之厚薄度比較.....                          | 51 圖12 五種處理組小鼠餵養16個月後利用Micro-CT分析膝蓋端生長                            |
| 板之骨小樑數量比較.....                             | 52 圖13 五種處理組小鼠餵養16個月後利用Micro-CT分析膝蓋端生長板之                          |
| 骨小樑分離度比較.....                              | 53 圖14 五種處理組小鼠餵養16個月後利用Micro-CT分析膝蓋緻密骨之骨礦物                        |
| 密度比較.....                                  | 54 圖15 小鼠與人類年齡相對圖..... 55 圖16 五種處理組                               |
| 小鼠餵養2個月後之體重變化.....                         | 56 圖17 五種處理組小鼠餵養2個月後之股骨頭外觀圖..... 57 圖18 五種處理                      |
| 組小鼠餵養2個月後之膝蓋端生長板骨小樑合圖.....                 | 58 圖19 五種處理組小                                                     |
| 鼠餵養2個月後之膝蓋端生長板骨小樑側面合成圖.....                | 59 圖20 五種處理組小鼠餵                                                   |
| 養2個月後利用Micro-CT分析膝蓋端生長板之骨小樑與組織所佔之比例比較..... | 60 圖21 五種處理組小鼠餵養2個                                                |
| 月後利用Micro-CT分析膝蓋端生長骨小樑之骨礦物密度比較.....        | 61 圖22 五種處理組小鼠餵養2個月後利                                             |
| 用Micro-CT分析膝蓋端生長板骨小樑之厚薄度比較.....            | 62 圖23 五種處理組小鼠餵養2個月後利                                             |
| 用Micro-CT分析膝蓋端生長板骨小樑之數量比較.....             | 63 圖24 五種處理組小鼠餵養2個月後利                                             |
| 用Micro-CT分析膝蓋端生長板之骨小樑分離度比較.....            | 64 圖25 五種處理組小鼠餵養2個月後利                                             |
| 用micro-CT分析膝蓋緻密骨骨礦物之密度比較.....              | 65 圖26 五種處理組小鼠餵養2個月後利用掃描                                          |
| 式電子顯微鏡分析膝蓋端生長板之骨小樑比較.....                  | 66 圖27 五種處理組小鼠餵養2個月後之膝蓋端生長                                        |
| 板骨小樑縱切面合成圖.....                            | 67 圖28 五種處理組小鼠餵養2個月後臟器外觀圖                                         |
| .....                                      | 68 表目錄 表1 世界衛生組織 (WHO) 骨質疏鬆症分級方式..... 69                          |

## REFERENCES

1.楊榮森 (1997) 骨質疏鬆症-病因、診斷、治療。合記圖書出版社，台北市。 2.黃永彥 (1997) 骨質疏鬆症-基礎與臨床。合記出版社，台

北市。3.陳力俊 (1990) 材料電子顯微鏡學。251-285。4.林良平 (1991) 科儀新知。12 (5):17。5.沈清良 (1991) 科儀新知。12 (5):10。6.陳力俊 (1990) 材料電子顯微鏡學，行政院國科會精密儀器發展中心，1-20、49-50。7.賴永沛 (1996) 長壽地區的發酵乳Kefir。食品資訊，124:38-41。8.郭卿雲(1996) 療效乳製品「克弗爾」。科學農業，44: 57-61 9.細野明義 (1990) 牛乳發酵???機能性?向上。New Food Industry. 32: 51-64。10.林慶文。1993。乳製品之特性與機能性，140-159、358-367 華香園出版社，台北。11.Aitken, A., Collinge, D.B., Heusden, B.P., Isobe, T., Roseboom, P.H., Rosenfeld, G., and Soll, J. (1992) Proteins: a highly conserved, widespread family of eukaryotic proteins. Trends. Biochem. Sci. 17: 498-501. 12.Ash, P., Loutit, K.M., and Townsen, M.S. (1980) Osteoclasts derived from haematopoietic stem cells. Nature 283: 669-670. 13.Aubin, J.E., and Liu, F. (1996) The osteoblast lineage. In: Bilezikian J.P., Raisz L.G., Rodan G.A., eds. Principles of bone biology 13: 655-663. 14.Blair, H., Teitelbaum, C., Ghiselli, S.L., and Gluck, S. (1989) Osteoclastic bone resorption by a polarized vacuolar proton pump. Science 245: 855-857. 15.Bouhallab S., and Bougle, D. (2004) Biopeptides of milk: caseinophosphopeptides and mineral bioavailability. Reprod. Nutr. Dev. Sep-Oct;44(5): 493-8. 16.Bouxsein, M.L., Myers, K.S., Shultz, K.L., Donahue, L.R., Rosen, C.J., and Beamer, W.G. (2005) Ovariectomy-induced bone loss varies among inbred strains of mice. J. Bone. Miner. Res. 20(7): 1085-92. calcium absorption in rats. J. Nutr. Sci. Vit. 41: 95-104. 17.Cano, A., Dapia, S., Noguera, I., Pineda, B., Hermenegildo, C., Caeiro, JR., and Garcia-Perez, MA. (2008) Comparative effects of 17 $\beta$ -estradiol, raloxifene and genistein on bone 3D microarchitecture and volumetric bone mineral density in the ovariectomized mice. Osteoporos Int. Jun;19(6):793-800. 18.Chen, H., Shoumura, S., and Emura, S. (2004) Ultrastructural changes in bones of the senescence-accelerated mouse (SAMP6): a murine model for senile osteoporosis. Histol. Histopathol. 19(3): 677-85. 19.Cheong, S.H., and Chang, K.J. (2009) The preventive effect of fermented milk supplement containing tomato (lycopersicon esculentum) and taurine on bone loss in ovariectomized rats. Adv Exp Med Biol.;643:333-40. 20.Chonan, O., and M. Watanuki. (1995). Effect of galatooligosaccharides on 21. Ducy, P., Schinke, T., and Karsenty, G. (2000) The osteoclast: A sophisticated fibroblast under central surveillance. Science 289: 1501-1504. 22.Emerton, K.B., Hu, B., Woo, A.A., Sinofsky, A., Hernandez, C., Majeska, R.J., Jepsen, K.J., and Schaffler, M.B. (2010) Osteocyte apoptosis and control of bone resorption following ovariectomy in mice. Bone 46(3): 577-83. 23.Flurkey, K., Curren, J.M., and Harrison, D.E. (2007) " The Mouse in Aging Research. " In The Mouse in Biomedical Research, 2nd edition. American College Laboratory Animal Medicine. Publishe: 637 – 672. 24.Fujioka, M., Uehara, M., Wu, J., Adlercreutz, H., Suzuki, K., Kanazawa, K., Takeda, K., Yamada, K., and Ishimi, Y. (2004) Equol, a metabolite of daidzein, inhibits bone loss in ovariectomized mice. J. Nutr. 134(10): 2623-7. 25.Fujioka, M., Uehara, M., Wu, J., Adlercreutz, H., Suzuki, K., Kanazawa, K., Takeda, K., Yamada, K., and Ishimi, Y. (2004) Equol, a metabolite of daidzein, inhibits bone loss in ovariectomized mice. J. Nutr: 2623-7 26.Gennard, D.E. (2007) Forensic Entomology. John Wiley and Sons Ltd., England: 64-66. 27.Guide for the Care and Use of Laboratory Animals, revised. NIH Publication Number 85-23. National Institutes of Health, Bethesda, MD, 1996. 28.Hinton, H.E. (1945) A Monograph of the Beetles Associated with Stored Products, Volume I. British Museum (Natural History), England: 261-268. 29.Jane, B.J., and Gary, S.S. (1996) Osteoblast biology. Osteoporosis. San Diego, California, U.S.A: Academic Press: 23-59. 30.Kanis, J.A., Johnell, O., Oden, A., Jonsson, B., De Laet, C., and Dawson, A. (2000) Risk of hip fracture according to the World Health Organization criteria for osteopenia and osteoporosis. Bone 27 (5) 585-590. 31.Katsuyoshi, T., Nobuyuki, S., and Higashi, K. (1998) Osteoclast differentiation factor mediates an essential signal for bone resorption induced by 1 $\alpha$ ,25(OH) $_2$ D $_3$ , prostaglandin E $_2$ , or parathyroid hormone in the microenvironment of bone. Basin. Bur. Act. Camp. 246: 337-341. 32.Kim, D.W., Yoo, K.Y., Lee, Y.B., Lee, K.H., Sohn, H.S., Lee, S.J., Cho, K.H., Shin, Y.K., Hwang, I.K., Won, M.H., and Kim, D.W. (2009) Soy isoflavones mitigate long-term femoral and lumbar vertebral bone loss in middle-aged ovariectomized mice. J. Med. Food 12(3): 536-41. 33.Kroger, M., (1993). Kefir. Cult. Dairy Prod. J. 28(2): 26-29. 34.Li, C.Y., Schaffler, M.B., Wolde-Semait, H.T., Hernandez, C.J., and Jepsen, K.J. (2005) Genetic background influences cortical bone response to ovariectomy. J. Bone Miner. Res. 20(12): 2150-8. 35.Lynch, M., Stein, P. and Stein, L. (1994) Apoptosis during in vitro bone formation. J. Bone Miner. Res. 9: S352. 36.Marks, S.C. Jr., and Walker, D.G. (1981) The hematogenous origin of osteoclasts: experimental evidence from osteoporotic (microphthalmic) mice treated with spleen cells from beige mouse donors. Am. J. Anat. 161(1): 1-10. 37.McCabe, L.R., Last, T.J., Lynch, M., Lian, J., Stein, J., and Stein, G. (1994) Expression of cell growth and bone phenotypic genes during the cell cycle of normal diploid osteoblasts and osteosarcoma cells. J. Cell. Biochem. 56: 274-282. 38.McNamara, K.B., Brown, R.L., Elgar, M.A., and Jones, T.M. (2008) Paternity costs from polyandry compensated by increased fecundity in the hide beetle. Behavioral Ecology 19: 433-440. 39.Milhaud, G. (1987) Deficiency of calcitonin in age related osteoporosis. Biomedicine 29: 272-281. 40.Omi, N., and Ezawa, I. (1995) The effect of ovariectomy on bone metabolism in rats. Bone 17(4 Suppl): 163S-168S. 41.Otis, E.M., and Brent, R. (1954) Equivalent ages in mouse and human embryos. Anat. Rec. 120: 35 – 63. 42.Owen, T.A., Aronow, M., Shalhoub, V., Barone, L.M., Wilming, L., Tassinari, M.S., Kennedy, M.B., Pockwinse, S., Lian J.B., and Stein, G.S. (1990) Progressive development of the rat osteoblast phenotype in vitro: reciprocal relationships in expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extracellular matrix. J. Cell. Physiol. 143: 420-430. 43.Pilvi, T.K., Korpela, R., Huttunen, M., Vapaatalo, H., Mervaala, E.M. (2007) High-calcium diet with whey protein attenuates body-weight gain in high-fat-fed C57Bl/6J mice. Br. J. Nutr. 98(5): 900-7. 44.Reszka, A.A., Halasy, N.J.M., Masarachia, P.J. and Rodan, G.A. (1999) Bisphosphonates act directly on the osteoclast to induce caspase cleavage of mst1 kinase during apoptosis. A link between inhibition of the mevalonate pathway and regulation of an apoptosis-promoting kinase. J. Biol. Chem. 274: 34967-34973. 45.Riggs, L., and Melton, J. (1982) Evidence for two distinct syndromes of involutional osteoporosis. American Journal of Medicine 73: 899-901. 46.Riggs. B.L. (1991) Overview of osteoporosis. West J. Med. 154: 63-75. 47.Roodman, G.D. (1996) Advances in bone biology: The Osteoclast. Endocr. Rev. 17: 308-331. 48.Roodman, G.D. (1999) Cell biology of the osteoclast. Exp. Hematol. 27: 1229-1241. 49.Rubin, M.A., Rubin, J., and Jasiuk, I. (2004) SEM and TEM study of the hierarchical structure of C57BL/6J and C3H/HeJ mice trabecular bone. Bone 35(1): 11-20. 50.Tang,

A.C., Nakazawa, M., Romeo, R.D., Reeb, B.C., Sisti, H., and McEwen, B.S. (2005) Effects of long-term estrogen replacement on social investigation and social memory in ovariectomized C57BL/6 mice. *Horm Behav.* Mar;47(3):350-7.

51. Teitelbaum, S.L. (2000) Bone resorption by osteoclasts. *Science* 289: 1504-1508.

52. Teti, A., Grano, M., Colucci, S., Cantatore, F.P., Loperfido, M.C., and Zallone, A. Z. (1991) Osteoblast-osteoclast relationships in bone resorption : osteoblasts enhance osteoclast activity in a serum-free coculture system. *Biochem. Biophys. Res. Commun.* 179: 634-640.

53. Teti, A., Grano, M., Colucci, S., Cantatore, F.P., Loperfido, M.C., and Zallone, A. Z. (2002) Bone Cell Culture: Osteoblasts, Osteoclasts and Osteocytes ECTS PhD Training course 54.

Tortora, G.J. (1999) Principles of Human Anatomy. Benjamin/Cummings Science Publishing, 8th edition, USA, 547-562.

55. Tsuchita, H., Suzuki, T., and Kuwata, T. (2001) The effect of casein phosphopeptides on calcium absorption from calcium-fortified milk in growing rats. *Br. J. Nutr.* 85(1): 5-10.

56. Veer, V., Negi, B.K., and Rao, K.M. (1996) Dermestid beetles and some other insect pests associated with stored silkworm cocoons in India, including a world list of dermestid species found attacking this commodity. *Journal of Stored Products Research* 32: 69-89.

57. Wronski, T.J., Walsh, C.C., and Ignaszewski, L.A. (1986) Histologic evidence for osteopenia and increased bone turnover in ovariectomized rats. *Bone*: 7(2): 119-23.