

土肉桂萃取液誘導飢餓素表現舒緩D-核糖引發之HepG2細胞死亡

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摘要

糖尿病患者中，因需長期服用降糖藥物、施打胰島素或糖代謝功能不彰等問題，常常伴隨有脂肪肝及藥物傷害等肝損傷問題存在。其中，據研究肉桂葉萃取液有改善糖尿病患者脂肪代謝及胰島素阻抗情形；本研究以核糖（D-ribose）誘發細胞內高氧化壓力，作為模擬糖尿病之環境，探討土肉桂萃取液對肝癌細胞株 - HepG2細胞之保護作用。本實驗採用三種本土不同化學類型土肉桂萃取液進行研究，分別為：桂皮醛-桂皮乙酸酯型（Cinnamaldehyde-cinnamylacetate type）（G2）、混合型（Mixed type）（P3）和台北市農會待測品種（TFA），實驗結果顯示皆可明顯減少細胞凋亡，並與已知用於糖尿病治療之用藥 - 氨基胍（Aminoguanidine, AG）效用相似（AG, P3, G2及TFA）分別各有：18.2%，22.4%，24.7%等恢復成效。土肉桂萃取液可促進飢餓素表現，而且受影響的以第一型變異產物（variable）為主，但第三型表現不受土肉桂之影響。另外，於D-ribose及土肉桂萃取液添加下，膜結合醯基轉移[?]-4（MBOAT4）基因仍可表現，說明於HepG2細胞內，飢餓素仍有機會可受到醯化修飾。而Furin裂解蛋白[?]為修飾飢餓素使其成為成熟的飢餓素之關鍵，在HepG2細胞中亦可被RT-PCR偵測到。因此，HepG2細胞亦可能將飢餓素原裂解成具功能性之飢餓素。最後，本實驗將飢餓素表現載體轉染到HepG2細胞，顯示具有保護細胞不受核糖傷害之效果。故未來可進一步探討土肉桂萃取液實際用於糖尿病患者中，以期在幫助糖代謝之外，亦可避免肝損傷、肝纖維化及肝硬化等後續肝功能傷害等問題產生。

關鍵詞：土肉桂、飢餓素、核糖

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參考文獻

- 1.王振瀾與尹華文。1992。土肉桂精油萃取、組成、季節性變化與葉部精油組成之天然變異。土肉桂專論 - 林業叢刊（38）:47-61。台北，台灣。
- 2.行政院衛生署統計98年國人癌症十大統計數據：
http://www.doh.gov.tw/CHT2006/DM/DM2_2_p02.aspx?class_no=440&now_fod_list_no=11397&level_no=-1&doc_no=76512
- 3.林讚標。1992。土肉桂專論 - 林業叢刊（38）。台北，台灣。
- 4.胡家僑。2008。肉桂萃取液對細胞生長的影響及其在化妝品上的應用。大葉大學。分子生物科技學系。彰化。台灣。
- 5.張上鎮。2002。土肉桂葉精油的抗細菌活性與應用。林業研究專訊9（3）:23-28。
- 6.張上鎮。2003a。「傑出的生物化學家」- 台灣鄉土林木。科學發展（366）:6-11。
- 7.張上鎮。2003b。土肉桂葉子精油的生物活性與應用。台

灣林業28 (6) :31-35. 8.張上鎮, 鄭森松, 王升陽. 2009. 土肉桂飄香 – 葉子的神奇功效及應用. 台灣林業35 (1) : 116-122. 9.許文馨. 2010. 土肉桂葉精油在STZ-糖尿病大鼠之降血糖作用. 中山醫學大學. 營養學研究所. 台中. 台灣. 10.Ariyasu H, Takaya K, Hosoda H, Iwakura H, Ebihara K, Mori K, Ogawa Y, Hosoda K, Akamizu T, Kojima M et al: Delayed short-term secretory regulation of ghrelin in obese animals: evidenced by a specific RIA for the active form of ghrelin. *Endocrinology* 2002, 143 (9) : 3341-3350. 11.Ariyasu H, Takaya K, Iwakura H, Hosoda H, Akamizu T, Arai Y, Kangawa K, Nakao K: Transgenic mice overexpressing des-acyl ghrelin show small phenotype. *Endocrinology* 2005, 146 (1) :355-364. 12.Baldanzi G, Filigheddu N, Cutrupi S, Catapano F, Bonisconi S, Fubini A, Malan D, Baj G, Granata R, Broglio F: Ghrelin and des-acyl ghrelin inhibit cell death in cardiomyocytes and endothelial cells through ERK1/2 and PI 3-kinase/AKT. *J Cell Biol* 2002, 159 (6) :1029-1037. 13.Bataller R, Brenner DA: Liver fibrosis. *J Clin Invest* 2005, 115 (2) :209-218. 14.Baynes JW: Role of oxidative stress in development of complications in diabetes. *Diabetes* 1991, 40 (4) :405-412. 15.Bierman EL, Baker EM, Plough IC, Hall WH: Metabolism of d-ribose in diabetes mellitus. *Diabetes* 1959, 8:455-458. 16.Brenner DA, Waterboer T, Choi SK, Lindquist JN, Stefanovic B, Burchardt E, Yamauchi M, Gillan A, Rippe RA: New aspects of hepatic fibrosis. *J Hepatol* 2000, 32 (1 Suppl) :32-38. 17.Brownlee M: The pathobiology of diabetic complications: a unifying mechanism. *Diabetes* 2005, 54 (6) :1615-1625. 18.Camina JP, Campos JF, Caminos JE, Dieguez C, Casanueva FF: Obestatin-mediated proliferation of human retinal pigment epithelial cells: regulatory mechanisms. *J Cell Physiol* 2007, 211 (1) :1-9. 19.Carlini VP, Schioth HB, Debarioglio SR: Obestatin improves memory performance and causes anxiolytic effects in rats. *Biochemical and biophysical research communications* 2007, 352 (4) :907-912. 20.Chang ST, Chen PF, Chang SC: Antibacterial activity of leaf essential oils and their constituents from *Cinnamomum osmophloeum*. *J Ethnopharmacol* 2001, 77 (1) :123-127. 21.Chou JY, Mansfield BC: Gene therapy for type I glycogen storage diseases. *Curr Gene Ther* 2007, 7 (2) :79-88. 22.Chung H, Kim E, Lee DH, Seo S, Ju S, Lee D, Kim H, Park S: Ghrelin inhibits apoptosis in hypothalamic neuronal cells during oxygen-glucose deprivation. *Endocrinology* 2007, 148 (1) :148-159. 23.D'Amico G, Pagliaro L, Bosch J: The treatment of portal hypertension: a meta-analytic review. *Hepatology* 1995, 22 (1) :332-354. 24.Du XL, Edelstein D, Rossetti L, Fantus IG, Goldberg H, Ziyadeh F, Wu J, Brownlee M: Hyperglycemia-induced mitochondrial superoxide overproduction activates the hexosamine pathway and induces plasminogen activator inhibitor-1 expression by increasing Sp1 glycosylation. *Proc Natl Acad Sci U S A* 2000, 97 (22) :12222-12226. 25.Fuller CJ, Chandalia M, Garg A, Grundy SM, Jialal I: RRR-alpha-tocopheryl acetate supplementation at pharmacologic doses decreases low-density- lipoprotein oxidative susceptibility but not protein glycation in patients with diabetes mellitus. *Am J Clin Nutr* 1996, 63 (5) :753-759. 26.Ghosh K: Thrombohaemorrhagic balance in diabetes mellitus. *J Indian Med Assoc* 2002, 100 (7) :428, 430, 432-423. 27.Granata R, Settanni F, Biancone L, Trovato L, Nano R, Bertuzzi F, Destefanis S, Annunziata M, Martinetti M, Catapano F: Acylated and unacylated ghrelin promote proliferation and inhibit apoptosis of pancreatic beta-cells and human islets: involvement of 3',5'-cyclic adenosine monophosphate/protein kinase A, extracellular signal-regulated kinase 1/2, and phosphatidylinositol 3-Kinase/Akt signaling. *Endocrinology* 2007, 148 (2) :512-529. 28.Granata R, Settanni F, Gallo D, Trovato L, Biancone L, Cantaluppi V, Nano R, Annunziata M, Campiglia P, Arnoletti E: Obestatin promotes survival of pancreatic beta-cells and human islets and induces expression of genes involved in the regulation of beta-cell mass and function. *Diabetes* 2008, 57 (4) :967-979. 29.Gutierrez JA, Solenberg PJ, Perkins DR, Willency JA, Knierman MD, Jin Z, Witcher DR, Luo S, Onyia JE, Hale JE: Ghrelin octanoylation mediated by an orphan lipid transferase. *Proc Natl Acad Sci U S A* 2008, 105 (17) :6320-6325. 30.Horie Y, Yamagishi Y, Kato S, Kajihara M, Kimura H, Ishii H: Low-dose ethanol attenuates gut ischemia/reperfusion-induced liver injury in rats via nitric oxide production. *J Gastroenterol Hepatol* 2003, 18 (2) :211-217. 31.Iseri SO, Sener G, Saglam B, Ercan F, Gedik N, Yegen BC: Ghrelin alleviates biliary obstruction-induced chronic hepatic injury in rats. *Regul Pept* 2008, 146 (1-3) :73-79. 32.Kajimoto Y, Matsuoka T, Kaneto H, Watada H, Fujitani Y, Kishimoto M, Sakamoto K, Matsuhisa M, Kawamori R, Yamasaki Y: Induction of glycation suppresses glucokinase gene expression in HIT-T15 cells. *Diabetologia* 1999, 42 (12) :1417-1424. 33.Kamegai J, Tamura H, Shimizu T, Ishii S, Tatsuguchi A, Sugihara H, Oikawa S, Kineman RD: The role of pituitary ghrelin in growth hormone (GH) secretion: GH-releasing hormone-dependent regulation of pituitary ghrelin gene expression and peptide content. *Endocrinology* 2004, 145 (8) :3731-3738. 34.Kapica M, Zabielska M, Puzio I, Jankowska A, Kato I, Kuwahara A, Zabielski R: Obestatin stimulates the secretion of pancreatic juice enzymes through a vagal pathway in anaesthetized rats - preliminary results. *J Physiol Pharmacol* 2007, 58 Suppl 3:123-130. 35.Khan A, Safdar M, Ali Khan MM, Khatkhat KN, Anderson RA: Cinnamon improves glucose and lipids of people with type 2 diabetes. *Diabetes Care* 2003, 26 (12) :3215-3218. 36.Kiefer MC, Tucker JE, Joh R, Landsberg KE, Saltman D, Barr PJ: Identification of a second human subtilisin-like protease gene in the *fes/fps* region of chromosome 15. *DNA Cell Biol* 1991, 10 (10) :757-769. 37.Kim SH, Hyun SH, Choung SY: Anti-diabetic effect of cinnamon extract on blood glucose in db/db mice. *J Ethnopharmacol* 2006, 104 (1-2) :119-123. 38.Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K: Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* 1999, 402 (6762) :656-660. 39.Kui L, Weiwei Z, Ling L, Daikun H, Guoming Z, Linuo Z, Renming H: Ghrelin inhibits apoptosis induced by high glucose and sodium palmitate in adult rat cardiomyocytes through the PI3K-Akt signaling pathway. *Regul Pept* 2009, 155 (1-3) :62-69. 40.Lebrec D: Pharmacological treatment of portal hypertension: hemodynamic effects and prevention of bleeding. *Pharmacol Ther* 1994, 61 (1-2) :65-107. 41.Liang TJ, Heller T: Pathogenesis of hepatitis C-associated hepatocellular carcinoma. *Gastroenterology* 2004, 127 (5 Suppl 1) :S62-71. 42.McGlynn KA, Hunter K, LeVoyer T, Roush J, Wise P, Michielli RA, Shen FM, Evans AA, London WT, Buetow KH: Susceptibility to aflatoxin B1-related primary hepatocellular carcinoma in mice and humans. *Cancer Res* 2003, 63 (15) :4594-4601. 43.Meszarosova M, Sirotkin AV, Grossmann R, Darlak K, Valenzuela F: The effect of obestatin on porcine ovarian granulosa cells. *Animal reproduction science* 2008, 108 (1-2) :196-207. 44.Moller P, Wallin H, Knudsen LE: Oxidative stress associated with exercise, psychological stress and life-style factors. *Chem*

Biol Interact 1996, 102 (1) :17-36. 45. Moreno M, Chaves JF, Sancho-Bru P, Ramalho F, Ramalho LN, Mansego ML, Ivorra C, Dominguez M, Conde L, Millan C: Ghrelin attenuates hepatocellular injury and liver fibrogenesis in rodents and influences fibrosis progression in humans. *Hepatology* 2010, 51 (3) :974-985. 46. Muller TD, Tschop MH, Jarick I, Ehrlich S, Scherag S, Herpertz-Dahlmann B, Zipfel S, Herzog W, de Zwaan M, Burghardt R: Genetic variation of the ghrelin activator gene ghrelin O-acyltransferase (GOAT) is associated with anorexia nervosa. *J Psychiatr Res* 2011, 45 (5) :706-711. 47. Nelson DL. and Cox MM.: *Lehninger principles of biochemistry*. Fourth Edition edition. W. H. Freeman 2005. 48. Niemela O, Parkkila S, Bradford B, Iimuro Y, Pasanen M, Thurman RG: Effect of Kupffer cell inactivation on ethanol-induced protein adducts in the liver. *Free Radic Biol Med* 2002, 33 (3) :350-355. 49. Nishikawa T, Edelstein D, Du XL, Yamagishi S, Matsumura T, Kaneda Y, Yorek MA, Beebe D, Oates PJ, Hammes HP: Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. *Nature* 2000, 404 (6779) :787-790. 50. Park JM, Kakimoto T, Kuroki T, Shiraishi R, Fujise T, Iwakiri R, Fujimoto K: Suppression of intestinal mucosal apoptosis by ghrelin in fasting rats. *Exp Biol Med (Maywood)* 2008, 233 (1) :48-56. 51. Pazos Y, Alvarez CJ, Camina JP, Casanueva FF: Stimulation of extracellular signal-regulated kinases and proliferation in the human gastric cancer cells KATO-III by obestatin. *Growth Factors* 2007, 25 (6) :373-381. 52. Rehermann B: Immune responses in hepatitis B virus infection. *Semin Liver Dis* 2003, 23 (1) :21-38. 53. Ren AJ, Guo ZF, Wang YK, Wang LG, Wang WZ, Lin L, Zheng X, Yuan WJ: Inhibitory effect of obestatin on glucose-induced insulin secretion in rats. *Biochemical and biophysical research communications* 2008, 369 (3) :969-972. 54. Roebroek AJ, Schalken JA, Leunissen JA, Onnekink C, Bloemers HP, Van de Ven WJ: Evolutionary conserved close linkage of the c-fes/fps proto-oncogene and genetic sequences encoding a receptor-like protein. *EMBO J* 1986, 5 (9) :2197-2202. 55. Romero A, Kirchner H, Heppner K, Pfluger PT, Tschop MH, Nogueiras R: GOAT: the master switch for the ghrelin system? *Eur J Endocrinol* 2010, 163 (1) :1-8. 56. Ryman BE: The glycogen storage diseases. *J Clin Pathol Suppl (R Coll Pathol)* 1974, 8:106-121. 57. Samson WK, White MM, Price C, Ferguson AV: Obestatin acts in brain to inhibit thirst. *American journal of physiology Regulatory, integrative and comparative physiology* 2007, 292 (1) :R637-643. 58. Schafer T, Scheuer C, Roemer K, Menger MD, Vollmar B: Inhibition of p53 protects liver tissue against endotoxin-induced apoptotic and necrotic cell death. *FASEB J* 2003, 17 (6) :660-667. 59. Schuppan D: Structure of the extracellular matrix in normal and fibrotic liver: collagens and glycoproteins. *Semin Liver Dis* 1990, 10 (1) :1-10. 60. Segal S, Foley J: The metabolism of D-ribose in man. *The Journal of clinical investigation* 1958, 37 (5) :719-735. 61. Stengel A, Goebel M, Wang L, Tache Y, Sachs G, Lambrecht NW: Differential distribution of ghrelin-O-acyltransferase (GOAT) immunoreactive cells in the mouse and rat gastric oxyntic mucosa. *Biochem Biophys Res Commun* 2010, 392 (1) :67-71. 62. Stickel F, Brinkhaus B, Kraemer N, Seitz HK, Hahn EG, Schuppan D: Antifibrotic properties of botanicals in chronic liver disease. *Hepatogastroenterology* 2002, 49 (46) :1102-1108. 63. Szentirmai E, Krueger JM: Obestatin alters sleep in rats. *Neuroscience letters* 2006, 404 (1-2) :222-226. 64. Takahashi T, Ida T, Sato T, Nakashima Y, Nakamura Y, Tsuji A, Kojima M: Production of n-octanoyl-modified ghrelin in cultured cells requires prohormone processing protease and ghrelin O-acyltransferase, as well as n-octanoic acid. *J Biochem* 2009, 146 (5) :675-682. 65. Thomson WH, Maclaurin JC, Prineas JW: Skeletal muscle glycogenesis: an investigation of two dissimilar cases. *J Neurol Neurosurg Psychiatry* 1963, 26:60-68. 66. Toshinai K, Mondal MS, Nakazato M, Date Y, Murakami N, Kojima M, Kangawa K, Matsukura S: Upregulation of Ghrelin expression in the stomach upon fasting, insulin-induced hypoglycemia, and leptin administration. *Biochem Biophys Res Commun* 2001, 281 (5) :1220-1225. 67. Vinik A, Flemmer M: Diabetes and macrovascular disease. *J Diabetes Complications* 2002, 16 (3) :235-245. 68. Wise RJ, Barr PJ, Wong PA, Kiefer MC, Brake AJ, Kaufman RJ: Expression of a human proprotein processing enzyme: correct cleavage of the von Willebrand factor precursor at a paired basic amino acid site. *Proc Natl Acad Sci USA* 1990, 87 (23) :9378-9382. 69. Zhang JV, Ren PG, Avsian-Kretschmer O, Luo CW, Rauch R, Klein C, Hsueh AJ: Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake. *Science* 2005, 310 (5750) :996-999. 70. Zhang Y, Ying B, Shi L, Fan H, Yang D, Xu D, Wei Y, Hu X, Zhang X, Wang T: Ghrelin inhibit cell apoptosis in pancreatic beta cell line HIT-T15 via mitogen-activated protein kinase/phosphoinositide 3-kinase pathways. *Toxicology* 2007, 237 (1-3) :194-202.