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枸杞子水提取物对 Bel-7402 人肝癌细胞增殖的影响 *

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摘要 目的:探讨枸杞子水提取物对 Bel-7402 人肝癌细胞增殖的影响。**方法:**用 RPMI-1640 完全培养液温育 Bel-7402 人肝癌细胞,分为空白对照组和药物组,空白对照组给予培养液处理,药物组分为低剂量组(100 μg/mL)和高剂量组(200 μg/mL),实验过程加入相应剂量的枸杞子水提取物。于倒置显微镜下观察三组细胞形态学变化,采用流式细胞仪观察细胞的凋亡状况及活性氧簇(ROS)含量,采用 MTT 法检测细胞增殖的抑制作用,采用反转录-聚合酶链式反应(RT-PCR)检测 Bel-7402 细胞中 bax mRNA 和 bcl-2 mRNA 的表达。**结果:**在倒置显微镜下观察,枸杞子水提取物使 Bel-7402 细胞生长受到抑制,表现为细胞贴壁减少、脱落增加,脱落的细胞浮悬于培养液中,呈圆形,体积缩小。枸杞子水提取物可促进 Bel-7402 细胞凋亡,药物组 Bel-7402 细胞的凋亡率比空白对照组明显增加($P<0.01$), G_0/G_1 期细胞比率减少($P<0.05$), G_2/M_2 期细胞比率增加($P<0.05$),且 ROS 含量增加($P<0.05$)。枸杞子水提取物对 Bel-7402 细胞的生长有明显抑制作用,且抑制率随着培养时间的延长而增加,且高剂量组在 96 小时抑制率最高。药物组 Bel-7402 细胞中 bax 基因灰度比值显著高于空白对照组($P<0.01$),bcl-2 基因灰度比值显著低于空白对照组($P<0.01$)。**结论:**枸杞子水提取物可抑制 Bel-7402 人肝癌细胞的增殖、阻滞细胞周期、诱导细胞凋亡,增加 ROS 含量,促进 bax 基因表达,抑制 bcl-2 基因表达,该实验结果可为枸杞子抗肝癌作用提供实验依据。

关键词:枸杞子水提取物;Bel-7402 人肝癌细胞;增殖;影响

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Effect of Lycium barbarum Water Extract on Proliferation of Bel-7402 Human Liver Cancer Cell*

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ABSTRACT Objective: To explore the effect of Lycium barbarum water extract on proliferation of Bel-7402 human liver cancer cell. **Methods:** Incubated the Bel-7402 human liver cancer cells with RPMI-1640 culture completely, and they were divided into blank control group and drug group. The blank control group was treated with culture medium. The drug group were divided into low dose group (100 g/mL) and high dose group (200 g/mL), and the Lycium barbarum water extract at corresponding dose was added in the experiment. Observed the cells' morphological changes under inverted microscope, tested the apoptosis of cell and ROS level by flow cytometry instrument, detected cell proliferation inhibition by MTT, the levels of bax mRNA and bcl-2 mRNA expressions were detected by reverse transcription polymerase chain reaction(RT-PCR). **Results:** Cells growth were inhibited by adding Lycium barbarum water extract through inverted microscope, showed the adherent cells were reduced, abscission was increased, which floated in culture, round and smaller. Lycium barbarum water extract could promote the Bel-7402 cells apoptosis. The apoptotic rate of Bel-7402 cells in the drug group was significantly higher than that in the blank control group ($P<0.01$), G_0/G_1 phase cells ratio was decreased($P<0.05$), G_2/M_2 phase cell ratio was increased($P<0.05$), and level of ROS was increased($P<0.05$). Lycium barbarum water extract had obvious inhibitory effect on growth of Bel-7402 cells, and the inhibition rate was increased with the extension of incubation time, and the inhibition rate was the highest in the high dose group at 96 hours. The levels of bax gene gray ratio in drug group was significantly higher than that of the blank

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control group ($P<0.01$), while bcl-2 gene gray ratio was lower than that of blank control group ($P<0.01$). **Conclusion:** The Lycium barbarum water extract can inhibit the proliferation, block cell cycle and induce apoptosis of Bel-7402 liver cancer cell. It can increase ROS level, promote bax gene expression and inhibit bcl-2 gene expression. The results can provide experimental evidence for the anti liver cancer effect of Lycium barbarum.

Key words: Water extract of Lycium barbarum; Bel-7402 human liver cancer cell; Proliferation; Effect

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前言

肝癌即肝脏恶性肿瘤,可分为原发性和继发性两大类,其在我国是高发性、危害极大的肿瘤类型^[1]。手术是治疗肝癌的首选方法,但对于不能切除的肝癌,则一般采用药物和放射治疗^[2]。近年来,中药常配合放射治疗用于肝癌的治疗,其可以提高机体抗病力,减少放射治疗造成的不良反应等^[3]。枸杞作为药食两用的中药,其主要活性成分为枸杞多糖(Lycium barbarum polysaccharide, LBP),LBP是由阿拉伯糖、半乳糖、甘露糖、鼠李糖、木糖等6种单糖组成的传统中药材枸杞的主要活性成分之一^[4,5]。随着近年来人们对LBP的研究,发现其具有抗肿瘤、调节机体免疫、延缓机体衰老等功能^[6,7],动物实验提示其具有保护肝细胞、神经元细胞及降血脂血糖作用^[8,9]。本实验采用枸杞子水提取物干预Bel-7402人肝癌细胞,观察枸杞子水提取物对Bel-7402人肝癌细胞的作用。

1 材料与仪器

1.1 试剂与试剂盒

枸杞子(为茄科植物宁夏枸杞的干燥成熟果实)购自中国北京同仁堂饮片有限责任公司,RPMI1640细胞培养基购自GIBCOBRL公司,小牛血清购自杭州四季青生物工程有限公司,四甲基偶氮唑盐MTT购自Serva公司,二甲基亚砜DMSO、DAFC抗体、RNA提取试剂Trizol、RT-PCR试剂盒购自Sigma公司,引物核苷酸片段由上海生工生物技术有限公司合成。

1.2 实验设备

酶标仪(Sunrise型,TECAN公司),CO₂恒温培养箱(HH-CP型,上海博迅公司),Olympus倒置显微镜(XDP-1,Leica公司)。

1.3 细胞来源及培养 人肝癌细胞株Bel-7402购自美国Sciencell公司,用含有青霉素、链霉素和10%小牛血清各100 U/mL的RPMI-1640培养液中培养,并在37℃、5% CO₂培养箱中培养。

1.4 方法

1.4.1 枸杞水提取物的准备 称取干燥枸杞子500 g,以3000 mL水加热,回流收集,加热至沸腾后保持2小时,加2000 mL水再提取1次,把2次的提取液混合及滤过,于旋转蒸发器浓缩,得到初膏100 g,80℃烘干,研磨,获枸杞提取物25 g,再以蒸馏水配制成浓度100 mg/mL的药液(即2 g生药/mL)待用。

1.4.2 实验分组和处理 实验包括3个组,其中空白对照组在实验时加入培养液,药物组分为低剂量组(100 μg/mL)和高剂量组(200 μg/mL),实验过程加入相应剂量的枸杞子水提取物。

1.4.3 倒置显微镜观察 Bel-7402 的形态 取上述单层贴壁生长的Bel-7402细胞,以0.25%胰蛋白酶消化,均匀吹打后加入含10%小牛血清的RPMI-1640培养液以悬浮细胞,调整细胞数量至 5×10^4 /mL后取3 mL接种于6孔板,每组设5个复孔,培养24小时后,待细胞贴壁生长状态好时(每孔中细胞覆盖孔底部约90%)吸取培养液,按照上述分组分别加入相应药液100 μL,空白对照组加入新鲜培养液100 μL,于CO₂培养箱内继续培养48小时后,于倒置显微镜下观察Bel-7402细胞的形态学变化并拍照。

1.4.4 流式细胞仪检测枸杞子水提取物对 Bel-7402 细胞周期和细胞凋亡的影响 取各组细胞样品1000 r/min离心10分钟,收集并用PBS洗涤2次,调整制成 10^6 个/mL悬液,以75%、-20℃冷乙醇固定24小时。测定前离心去除乙醇,PBS洗涤2次,加以不含Dnase的RNA酶、EB染液,于室温条件下反应30分钟后上机检测细胞周期,采用CELLQUEST软件分析细胞凋亡率。

1.4.5 酶标仪检测枸杞子水提取物对 Bel-7402 细胞内活性氧水平的影响 各组细胞样品按 1×10^5 密度接种在96孔板中,每孔100 μL,每组设3个复孔,于培养箱中培养,待细胞贴壁后,用无血清培养基培养12小时,弃培养基,空白对照组加入150 ppm1640培养液,处理组则加入上述1640培养液和不同剂量的枸杞子提取物,培养48小时后舍弃培养基,再加入用无血清培养基按1:1000稀释的DCFH-DA(终浓度为10 μmol/L),直至液体体积盖住细胞表面,于培养箱中培养20分钟,以无血清培养基洗涤细胞3次以充分洗去DCFH-DA,最后加入终浓度为100 μmol/L H₂O₂,培养2小时后置于多功能酶标仪上检测活性氧簇(reactive oxygen species, ROS)含量,激发波长488 nm,发射波长525 nm。

1.4.6 MTT 法检测枸杞子水提取物对 Bel-7402 细胞的影响 取单层贴壁的Bel-7402细胞,以0.25%胰蛋白酶消化,均匀吹打后加入含10%小牛血清的RPMI-1640培养液以悬浮细胞,调整细胞数量至 2.5×10^5 /mL后取100 μL接种于96孔板,每组设5个复孔,在CO₂培养箱里培养24小时后,舍弃原培养液,按照上述分组分别加入相应溶液,CO₂培养箱内继续培养48小时、72小时、96小时后,再向每孔中加入PBS配制的浓度为5 mg/mL的MTT溶液10 μL,于37℃培养4小时,吸去培养液。加入DMSO100 μL,于37℃用荡器震荡10 min,经酶标仪570nm处测定OD值,计算细胞增殖抑制率,计算公式为抑制率(%)=(OD值对照孔-OD值观察孔)/OD值对照孔 $\times 100\%$ 。

1.4.7 枸杞子水提取物对 Bel-7402 细胞 bax mRNA 和 bcl-2 mRNA 表达的影响 取贴壁生长的细胞,经上述方法处理并

调整细胞数量后加入相应药液,空白对照组以等体积生理盐水代替药液,作用 48 小时后,采用反转录-聚合酶链式反应(RT-PCR)测定 bax 表达。 β -actin 引物上游:5'-ATGTGGCAC-CACACCTTCTA-3';下游:5'-CGTCATACTCCTGCTTGCTG-3',扩增片段为 838bp;bax 引物上游:5'-GTTTCATCCAGGATC-GAGC-3',下游:5'-GGAAGTCCAATGTCCAGC-3',扩增片段为 345bp;bcl-2 引物上游:5'-GACTTCGCCGAGATGTCCAG-3';下游:5'-CAGGTGCCCGGTTTCAGGTACT-3',扩增片段为 225bp。设置反应条件为 95℃ 预变性 2 分钟,94℃ 45s,55℃ 退火 1 分钟,72℃ 延伸 1 分钟,共 35 循环,72℃ 复性 5 分钟,产物经 1% 琼脂糖凝胶电泳,溴化乙锭染色,采用凝胶成像系统分析得出 bax mRNA 和 bcl-2 mRNA 的相对表达量,再以 bax/ β -actin

和 bcl-2/ β -actin 灰度比作为相对定量。

1.4.8 统计学处理 采用 SPSS19.0 软件进行数据分析。各测量指标的结果用均数 $\pm$ 标准差($\bar{x}\pm s$)表示,组间比较采用 One Way ANOV 分析,两两比较采用 LSD-t 检验, $P<0.05$ 为差异具有统计学意义。

2 结果

2.1 倒置显微镜观察 Bel-7402 细胞的形态变化

在倒置显微镜下观察,可见空白对照组 Bel-7402 细胞生长正常,细胞呈梭形,贴壁生长,核质比例正常,核膜完整;药物组的细胞生长受到抑制,表现为细胞贴壁减少、脱落增加,脱落的细胞悬浮于培养液中,呈圆形,体积缩小,见图 1。

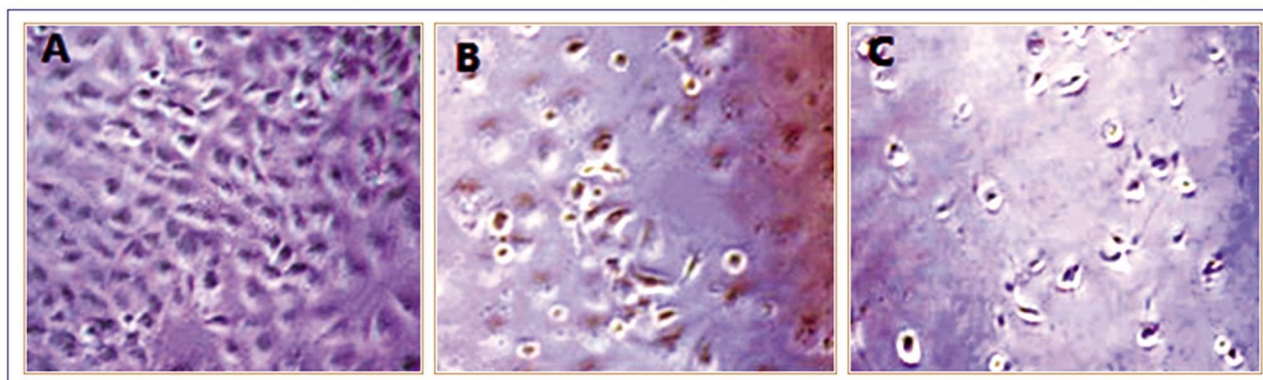


图 1 枸杞子水提取物对 Bel-7402 细胞的形态学影响($\times 200$)

Fig.1 Effects of water extract of Lycium barbarum on Bel-7402 cells morphology($\times 200$)

A:Blank control group; B:Low dose group; C:High dose group

2.2 流式细胞仪检测枸杞子水提取物对 Bel-7402 细胞凋亡的影响

从结果发现,随着枸杞子水提取物浓度提高,Bel-7402 细

胞的凋亡率明显高于空白对照组,枸杞子水提取物可以使 G_0/G_1 期细胞比率减少、 G_2/M_2 期细胞比率增加,见表 1、图 2。

表 1 枸杞子水提取物对 bel-7402 癌细胞细胞周期和凋亡率的影响($\bar{x}\pm s$)

Table 1 Effect of Lycium barbarum extract on cell cycle and apoptosis rate of BEL-7402 cancer cells($\bar{x}\pm s$)

Groups	cell cycle(%)			Apoptosis rate(%)
	G_0/G_1	S	G_2/M_2	
Blank control group	69.81 $\pm$ 6.08	25.96 $\pm$ 3.6	18.13 $\pm$ 4.37	3.34 $\pm$ 0.18
Low dose group	49.21 $\pm$ 3.07 [@]	22.13 $\pm$ 5.14	29.72 $\pm$ 4.68 [@]	11.08 $\pm$ 0.18 [#]
High dose group	45.44 $\pm$ 2.26 [#]	21.03 $\pm$ 5.21	31.58 $\pm$ 4.66 [#]	25.16 $\pm$ 2.01 [*]
F	2.935	0.917	3.564	7.946
P	<0.05	>0.05	<0.05	<0.01

Note: compared with blank control group, [@] $P<0.05$, [#] $P<0.01$, ^{*} $P<0.001$.

2.3 枸杞子水提取物对 Bel-7402 细胞内活性氧含量的影响

已知 H_2O_2 会损伤细胞,使用不同浓度的枸杞子水提取物处理 Bel-7402 细胞后,低剂量组、高剂量组 ROS 含量分别为 (31.72 $\pm$ 1.52)、(77.36 $\pm$ 1.05),明显高于空白对照组的 (12.476 $\pm$ 3.641),差异具有统计学意义($P<0.05$),提示枸杞子水提取物可增加胞内 ROS 含量,并且随着药物浓度递增。

2.4 MTT 法检测枸杞子水提取物对 Bel-7402 细胞的作用

实验通过比较枸杞子水提取物对体外培养 Bel-7402 细胞

的作用,其对 Bel-7402 细胞的抑制率随着培养时间的延长而提高,高剂量组在 96 小时抑制率最高,见表 2。

2.5 枸杞子水提取物对 Bel-7402 细胞 bax mRNA、bcl-2 mRNA 表达的影响

经相应药液作用 48 小时后,药物组的 bax 基因灰度比值均高于空白对照组,bcl-2 基因灰度比值则低于空白对照组,差异具有统计学意义($P<0.05$),见表 3。

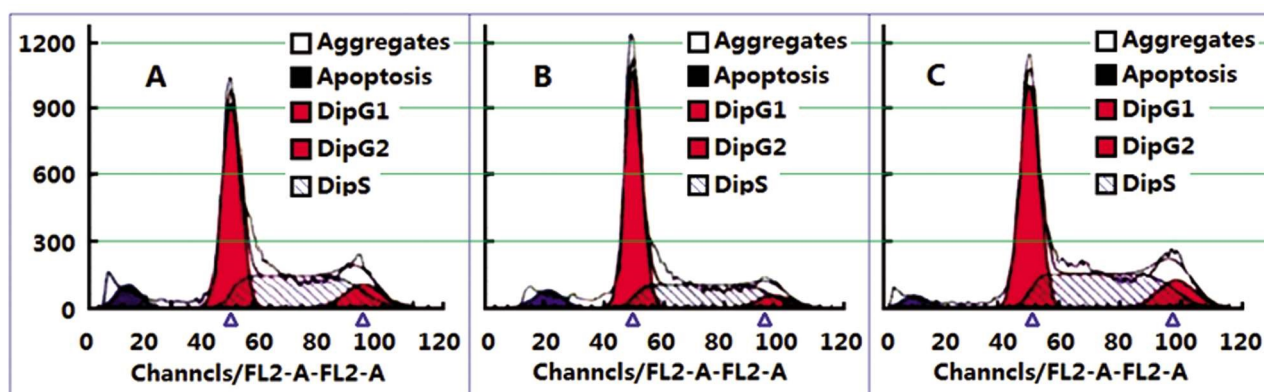


图 2 枸杞子水提取物对 bel-7402 癌细胞细胞周期和凋亡率的影响

Fig.2 Effect of Lycium barbarum extract on cell cycle and apoptosis rate of BEL-7402 cancer cells

A: Blank control group; B: Low dose group; C: High dose group

表 2 枸杞子水提取物对 Bel-7402 细胞的抑制作用

Table 2 Inhibitory effect of Lycium barbarum extract on Bel-7402 cells

Groups	n	Inhibition rate(%)			F	P
		48 h	72 h	96 h		
Blank control group	5	0.00± 0.09	0.00± 0.05	0.00± 0.02	1.147	>0.05
Low dose group	5	24.61± 0.99	35.81± 1.23	65.11± 1.34	5.121	<0.05
High dose group	5	52.74± 2.14	74.11± 1.18	83.61± 1.02	3.254	<0.05
F		5.126	6.623	11.327		
P		<0.05	<0.01	<0.001		

表 3 枸杞子水提取物对 Bel-7402 细胞 baxmRNA 和 bcl-2mRNA 表达的影响

Table 3 Effects of Lycium barbarum extract on expression of baxmRNA and bcl-2mRNA in Bel-7402 cells

Groups	bax/β-actin	bcl-2/β-actin
Blank control group	0.37± 0.03	0.79± 0.02
Low dose group	0.48± 0.04	0.48± 0.07
High dose group	0.75± 0.06	0.22± 0.05
F	18.741	22.157
P	<0.01	<0.01

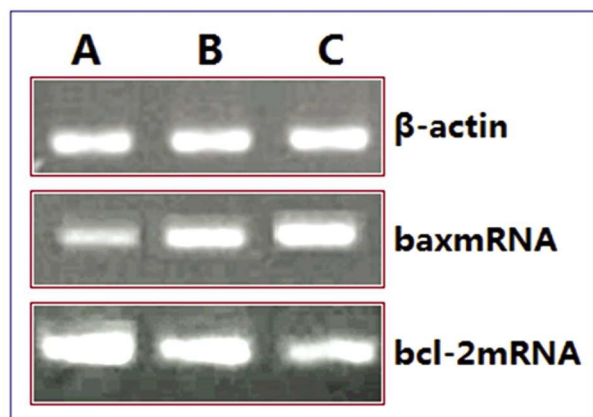


图 3 Bel-7402 细胞 bax mRNA、bcl-2 mRNA 的表达

Fig.3 Expression of Bax mRNA and Bcl-2 mRNA in Bel-7402 cells

(M: 100bp Marker A:Blank control group; B: Low dose group; C: High dose group)

3 讨论

原发性肝癌 (primary hepatic carcinoma, PHC) 是世界癌症死亡第三大原因,在我国恶性肿瘤死亡率排第二位^[10],最常见的是肝细胞癌,因其进展迅速、易转移而导致患者预后极差。目前治疗 PHC 的主要手段是放化疗联合增敏剂、热疗、基因治疗、中医药、分子靶向治疗等^[11,12],而中药多糖在增强机体免疫力、增敏放化疗效果、抑制肝癌复发及转移等方面具有良好的疗效^[13]。近年来,作为我国上千年经久传承的中药枸杞子在临床治疗和预防保健方面具有广阔的开发前景,其主要药用成分 LBP 在抗肿瘤方面的研究越来越深入,LBP 可损伤 HepG2 细胞的结构和功能使肿瘤细胞增殖受抑制^[14],且 LBP 对人卵巢癌的抑制作用呈时间和剂量依赖性,可将癌细胞分裂周期阻滞在 G₀/G₁ 期,其作用随着 LBP 浓度升高而增加^[15];LBP 抗癌作用还与其调控机体外周血单核细胞、白介素 -12 (Interleukin-12,

IL-12)、肿瘤坏死因子- α (Tumor Necrosis Factor- α , TNF- α) 的表达有关,可激活 T 淋巴细胞和 B 淋巴细胞而调节人体免疫作用,加强抑制性 T 细胞(suppressor T cell, Ts),降低抗体排斥反应^[16-18]。

凋亡即细胞程序性死亡,其形态学表现为细胞核固缩、细胞体积缩小、变圆,细胞间连接消失而单个存在,细胞染色质 DNA 断裂并凝聚形成凋亡小体^[19-21]。细胞通过凋亡来保持机体组织动态平衡从而进行自我调控,由于肿瘤细胞生命力强、凋亡受限、存活期无限延长、繁殖过度并破坏机体正常细胞功能,甚至使正常细胞凋亡或坏死而发生肿瘤,故诱导肿瘤细胞发生凋亡便成为治疗肿瘤的靶点^[22-24]。本研究通过体外细胞实验,采用倒置显微镜观察枸杞子水提取物对人 Bel-7402 肝癌细胞形态影响,发现枸杞子水提取物药物组的细胞生长受到抑制,表现为细胞贴壁减少、脱落,部分悬浮于培养液中,细胞呈圆形,体积缩小,说明枸杞子水提取物可以抑制人 Bel-7402 肝癌细胞的生长,也提示凋亡机制可能参与枸杞水提取物抗肝癌作用;枸杞子水提取物对 Bel-7402 细胞的增殖周期亦有明显影响,可以使 G₂/M₂ 期细胞比率增加,提示枸杞子水提取物将人 Bel-7402 肝癌细胞阻滞于 G₂/M₂ 期,细胞损伤程度超过其修复范围,不易通过 G₂ 期发生凋亡,提示枸杞子水提取物可能通过诱导肿瘤细胞凋亡、抑制肿瘤细胞增殖发挥抗肿瘤的作用。

ROS 在生理水平时可参与防御、合成活性物质等有益活动,稍高水平时可抑制细胞增生并阻滞细胞周期,继续升高则会引起细胞凋亡甚至坏死^[25,26]。研究发现,自由基与肿瘤关系密切,肿瘤患者机体抗氧化水平降低,清除 ROS 的能力下降,而氧化应激水平高^[27],所以对 ROS 介导的凋亡更敏感。过量的 ROS 通过引起蛋白质、脂质、核酸等大分子物质发生氧化损伤,参与到肿瘤的发生和发展过程^[28,29],虽然高浓度 ROS 可诱导细胞凋亡发挥一定的治疗肿瘤的作用,但其浓度如何掌握以及如何使毒副作用最小,仍然需要更深入的研究。本实验结果显示,枸杞子水提取物可以有效提高 Bel-7402 细胞内 ROS 含量,这对今后筛选抗氧化活性植物提取物治疗肝癌提供了依据和参考;MTT 检测及流式细胞仪检测结果提示枸杞水提取物可明显抑制人 Bel-7402 肝癌细胞的生长,呈一定的时间依赖关系;bax 和 bcl-2 的水平高低在肿瘤细胞凋亡过程中起重要作用,bax 促进凋亡,bcl-2 抑制凋亡,二者对凋亡过程互相调节,控制着细胞的生存和死亡^[30],本实验 RT-PCR 结果显示枸杞子水提取物可以提高 Bel-7402 肝癌细胞中 bax 基因的灰度值、降低 bcl-2 基因的灰度值,提示其诱导凋亡的机制可能与激活 bax 基因、抑制 bcl-2 基因有关。

综上所述,枸杞子水提取物对 Bel-7402 人肝癌细胞的增殖有明显抑制作用,能促进细胞凋亡,可能与其增加 ROS 生成提高细胞抗氧化活性、上调 bax 基因表达、抑制 bcl-2 基因表达有关系。

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