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黄芪多糖和当归多糖对维氏气单胞菌诱导鲫细胞凋亡的影响

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摘要: 为研究黄芪多糖(APS)和当归多糖(ASP)对维氏气单胞菌诱导鲫细胞凋亡的影响, 实验设阴性对照组、阳性对照组, 黄芪多糖组和当归多糖组, 通过对鲫用维氏气单胞菌攻毒处理, 用流式细胞仪测定血细胞的凋亡比例和细胞周期变化, 并用荧光显微镜和透射电镜观察凋亡细胞的形态。结果显示, 维氏气单胞菌攻毒后阳性对照组鲫血细胞的细胞凋亡率均极显著高于多糖组和阴性对照组; 多糖组能显著降低鲫的细胞凋亡率且黄芪多糖组效果更显著; 攻毒后鲫肝细胞和肾脏淋巴细胞出现染色质凝集、细胞核固缩边集和细胞空泡化及凋亡小体; 同阴性对照组相比; 维氏气单胞菌攻毒可以引起鲫血细胞周期中S/G2+M期细胞比例极显著下降, sub-G1极显著升高, 抑制细胞分裂诱发凋亡; 多糖组则G0/G1期细胞极显著降低, S/G2+M期细胞极显著升高, sub-G1极显著降低, 促进细胞分裂抑制凋亡。黄芪多糖和当归多糖添加量在1%时能抑制维氏气单胞菌攻毒引起的细胞凋亡。

关键词: 鲫; 黄芪多糖; 当归多糖; 维氏气单胞菌; 细胞凋亡
中图分类号: S 941.42 **文献标志码:** A

细胞凋亡(apoptosis, APO)是细胞生命活动中的重要组成部分, 由英国阿伯丁大学Kerr等[1-2]于1972年最早提出, 指细胞在接受某种信号或受到某些因素刺激后, 为了能够维持机体内环境的稳定而出现的一种细胞主动的自杀性死亡现象。细胞凋亡是目前发现的程序性细胞死亡(programmed cell death, PCD)中研究最多的一种重要类型。中药多糖为中草药提取物的主要活性成分, 因其具有毒副作用较小, 无耐药性等特性而引起国内外学者的关注[3]。中药多糖能够促进水生生物的生长、增强饵料利用率[4]、提升先天性免疫反应[5-6]、增加抗菌活性[7-8]等。维氏气单胞菌(*Aeromonas veronii*)是近年来发现的气单胞菌属(*Aeromonas* sp.)的一个新种, 是一种人鱼共患的细菌性疾病病原菌, 可引起鱼类和其他水产动物感染疾病而大量死亡[9]。该菌能够引起小鼠巨噬细胞的细胞毒性和细胞凋亡[10-11], 研究报道由于氧化应激线粒体去极化, 维氏气单胞菌能够诱导人上皮细胞发生凋亡, 引起典型的线粒体途径细胞凋亡[12]。此外该细菌毒素可以引起非洲绿猴(*Chlorocobus sabaeus*)肾脏细胞的胞浆空泡形成, 线粒体病变及凋亡信号通路的激活导致细胞凋亡[13]。目前, 水产养殖中对细胞凋亡的研究较少, 主要有凋亡对鱼性别分化的影响[14], 传染性胰脏坏死病毒感染引起大鳞大麻哈鱼(*Oncorhynchus tshawytscha*)胚胎细胞的凋亡和坏死[15], 以及脂多糖诱导引起鲫(*Carassius auratus*)头肾淋巴细胞线粒体途径的凋亡作用等[16], 维氏气单胞菌诱导鱼类细胞凋亡尚未见报道。本实验采用流式细胞仪对攻毒后鲫血细胞凋亡比例和细胞周期变化进行检测并结合电子显微镜和荧光显微镜对细胞凋亡形态进行观察分析, 研

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究黄芪多糖(*Astragalus* polysacharin, APS)与当归多糖(*Angelica sinensis* polysaccharide, ASP)对鲫由维氏气单胞菌攻毒引起细胞凋亡是否具有抑制作用，从而为黄芪多糖和当归多糖在水产养殖病害预防方面的研究提供理论依据。

1 材料与方法

1.1 实验用鱼和中药多糖

实验鲫购自重庆荣昌罗梅水产养殖场，实验中选用540尾体质量(70±3.5) g 、体质健康、个体均匀的实验鱼于暂养缸中暂养2周，暂养期间确保良好的生长环境，投喂鲫基础饲料。实验所用中药多糖干粉均购自西安草根生物工程有限公司，有效成分75%，其余成分为赋形剂。

1.2 实验设计及分组

实验分别设置阴性对照组(negative control group)、阳性对照组(positive control group)，黄芪多糖组(APS group)和当归多糖组(ASP group)4个组，每组设3个重复，试验鱼暂养结束后每个重复组随机放入30尾鲫。实验期间多糖组实验鱼分别投喂在基础饲料中添加了1.0%黄芪多糖和1.0%当归多糖的饲料，阳性对照组和阴性对照组投喂鲫基础饲料，日投饵量为2.0%。实验周期为19 d，多糖组饲喂中药多糖2周后，第15天用维氏气单胞菌96 h LC_{50}攻毒。凋亡率检测及荧光观察采样时间分别为攻毒后24、48、72和96 h，细胞周期及细胞凋亡电镜观察采样时间为攻毒后24 h，随机从每组取3尾试验鱼采样。

1.3 主要试剂及仪器

AnnexinV-FITC/PI凋亡检测试剂盒(南京建成生物工程研究所有限公司)，细胞凋亡及周期检测试剂盒(北京华迈科生物技术有限责任公司)，流式细胞仪(美国BECKMAN COULTER-FC500)，奥林巴斯荧光显微镜(日本OLYMPUS BX51)及CCD扫描成像系统拍照，超薄切片机(美国RMC Power Tome-XL)，生物透射电子显微镜(日本JEOL-1400 plus)。

1.4 维氏气单胞菌及半致死浓度

实验菌为西南大学鱼类繁育与健康养殖研究中心分离并保存的维氏气单胞菌(登录号：KC663719)。实验时将菌种在固体培养基进行单菌落划线培养，28 °C条件下培养24 h，然后4 °C冷藏备用。采用麦氏比浊法和改良寇氏法测出维氏气单胞菌的96 h LC_{50}=1.6×10^7 CFU/mL。

1.5 血细胞凋亡比例检测

试验鱼尾静脉取血，迅速加入含有1%肝素钠的抗凝管中混匀。取5 μL抗凝血加入含有1 mL PBS的1.5 mL离心管中于4 °C、3000 r/min离心5 min，弃上清液收集细胞，用PBS轻轻重悬细胞，取1×10^5~5×10^5的重悬细胞重复离心操作，弃上清液后加入500 μL结合液轻轻重悬细胞，加入5 μL Annexin V-FITC轻轻混匀，25 °C水浴避光孵育10 min，上机前加入5 μL碘化丙啶(PI)混匀，随即上机检测[17]。检测前清洗仪器通道并用荧光微球校正。设置空白管、Annexin V-FITC单染管、PI单染管、阳性双染管进行准确荧光补偿和阈值设定，建立凋亡检测方案(图1-a，b)，检测中全部低速进样。

1.6 细胞周期测定

取10 μL血细胞加入1 mL预冷的PBS中，4 °C、3000 r/min重复离心5 min 2次，重悬细胞，70%预冷酒精固定24 h后清洗细胞，加入PI染色工作液37 °C避光水浴30 min。流式细胞仪检测后，经CXP数据处理分析得出细胞周期各时相比例。流式检测主要步骤：建立FS-SS、AUX-FL3 Lin双参数图，阈值为30，在FS-SS图中设门“A”圈定细胞(图2“A”)；在AUX-FL3图中设门“B”并建立FL3直方图(图3“B”)。阴性对照组对新建Cell Cycle检测方案调试后即进行样品检测。

1.7 凋亡细胞的荧光观察

在暗室取“血细胞凋亡比例检测”中制好的细胞样品20 μL滴加在洁净的载玻片上，用无色指甲油加盖玻片封闭，荧光显微镜下蓝光激发，快速进行观察并拍照。依据细胞着色情况区分正常、凋亡和坏死细胞[18]。

1.8 鲫肝脏和头肾组织凋亡细胞形态观察

实验鱼麻醉后快速取肝脏和头肾组织，用经高压灭菌过的一次性双面剃须刀片迅速将组织切成1 mm^3大小的小块，放入4%戊二醛固定液中4 °C固定2 h，0.1 mol/L PBS漂洗3次，经1%锇酸4 °C固定2 h，后用0.1 mol/L PBS重新漂洗3次；再用1%醋酸铀块染和不同浓度丙酮梯度脱水后经包埋剂(环氧树脂)浸透包埋聚合修块，进行超薄切片、观察、拍照。

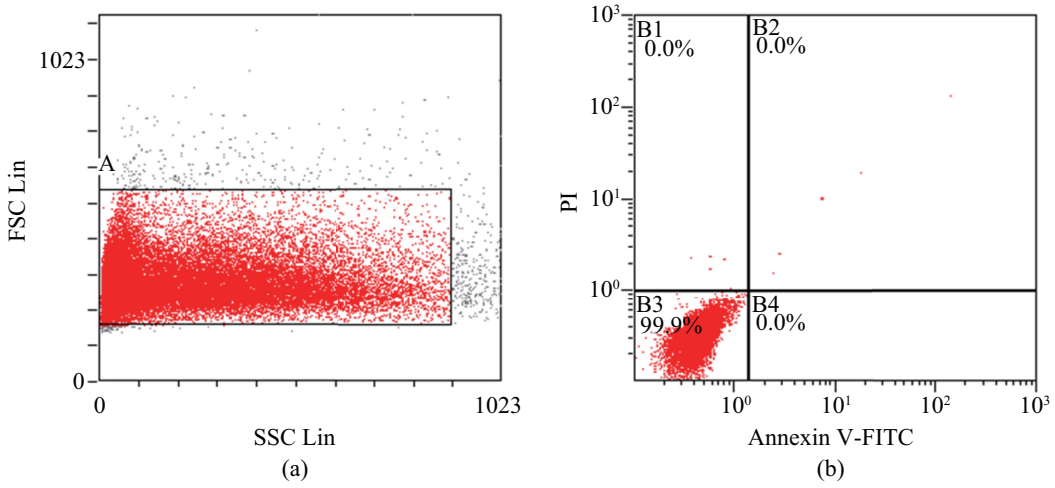

图 1　鲫全血Annexin V-FITC/PI凋亡检测方案图

(a)为鲫全血SSC-FSC散点图、(b)为FL1-FL3通道电压调节图；(b)中根据十字形门细胞分布被依次划分为B1、B2、B3、B4 4个区域；其中B1区域代表PI阳性和FITC阴性细胞群为坏死细胞；B2区域为PI和FITC的双阳性细胞群，为凋亡晚期细胞；B3区域代表PI和FITC的双阴性细胞群为正常细胞；B4区域代表PI阴性和FITC阳性细胞群，为早期凋亡细胞

Fig. 1　The whole blood apoptosis chart of *C. auratus* detected by Annexin V-FITC/PI

(a) is the SSC-FSC scatter plots of crucian carp, the (b) is the voltage regulation diagram of FL1-FL3; according to the cross gate in (b), cells were divided into B1, B2, B3 and B4 four regions, cells in the B1 region representing PI positive and FITC negative were necrosis cells; cells in the B2 region were PI and FITC double positive cells, which were called late stage apoptotic cells; cells in the B3 region representing PI and FITC double negative were normal cells; cells in the B4 region were PI negative and FITC positive cells, which were early stage apoptotic cells

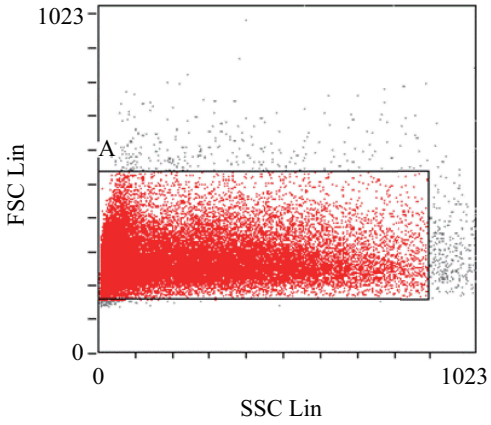

图 2　鲫全血SSC-FSC散点图

Fig. 2　SSC-FSC scatter plots of *C. auratus*

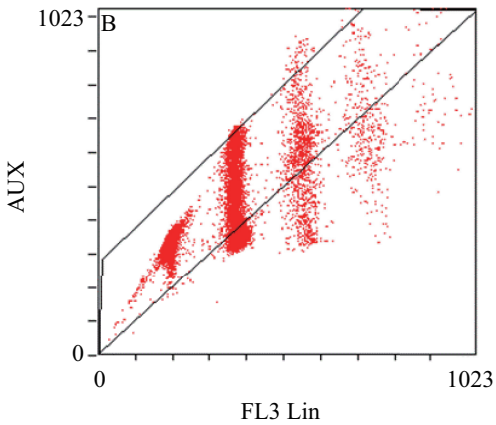

图 3　FL3-AUX“门(B)”示意图

Fig. 3　The schematic diagram of settine up “GATE” in AUX-FL3 (GATE B)

1.9　数据处理

实验结果数据采用平均值±标准差(mean ± SD)表示，用SPSS 19.0对数据分别进行One-Way ANOVA单因素方差分析和Duncan多重比较。差异显著性水平为$P<0.05$，极显著水平为$P<0.01$。

2　结果

2.1　鲫血细胞凋亡比例

鲫血细胞凋亡流式检测显示(图版Ⅰ)，攻毒后阳性对照组血细胞凋亡率在相同检测时间点均极显著高于多糖组和阴性对照组($P<0.01$)，且阳性对照组在攻毒后24 h凋亡率为最高，达到19.80%；黄芪多糖组和当归多糖组在攻毒后24 h血细胞凋亡率无显著差异($P>0.05$)，从48 h到96 h相同检测时间段，黄芪多糖组凋亡率均极显著低于当归多糖组($P<0.01$)；阳性对照组和多糖组凋亡率均极显著高于阴性对照组($P<0.01$)(图4)。结果显示黄芪多糖和当归多糖具有抑制攻毒后

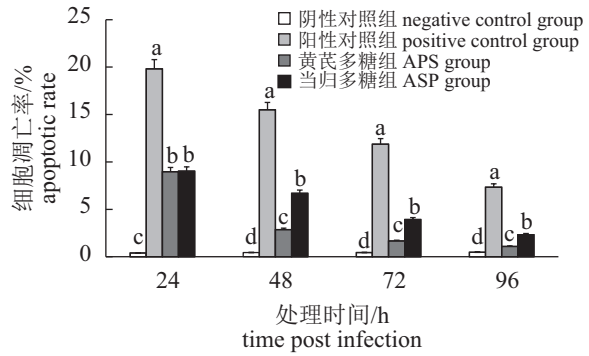

图 4 各组鲫血细胞凋亡率

图中同一检测时间标有不同小写字母表示实验结果差异极显著(P<0.01)

Fig. 4 Apoptosis rate in *C. auratus* blood cells

Data with different lowercase means significant difference from other groups at the same time (P<0.01)

鲫血细胞凋亡的功效，且黄芪多糖组凋亡率极显著低于当归多糖组(P<0.01)，表明黄芪多糖的凋亡抑制效果更强。

2.2 攻毒后鲫血细胞的细胞周期变化

攻毒后24 h鲫血细胞的细胞周期变化结果显示，同阴性对照组(1.9%)相比，阳性对照组sub-G1期细胞比例(20.4%)极显著增高(P<0.01)，S/G2+M期细胞明显减少。黄芪多糖组G1期细胞(46.23%)和当归多糖组G1期细胞(57.10%)极显著低于阴性对照组(P<0.01)，S/G2+M期细胞明显增多。同阳性对照组相比，多糖组sub-G1期细胞则极显著降低(P<0.01)，G1期细胞极显著降低(P<0.01)，S/G2+M期细胞极显著升高(P<0.01)。结果显示，黄芪多糖和当归多糖能促进被攻毒后的鲫血细胞由G0/G1期向S/G2+M转换(图版Ⅱ，表1)。

表 1 攻毒后24 h各组鲫血细胞的细胞周期变化

Tab. 1 The cell cycle changes of the experimental fish blood cells infected with *A. veronii* after 24 h %

组别 group	Go/G1	S	G2+M	sub-G1
阴性对照组 negative control group	81.17±3.15^{a}	5.37±1.11^{c}	11.62±2.28^{b}	1.84±0.07^{d}
阳性对照组 positive control group	69.67±3.60^{b}	4.67±0.68^{c}	5.27±1.19^{c}	20.40±0.56^{a}
黄芪多糖组 APS group	46.23±1.78^{d}	26.10±1.04^{a}	17.16±0.91^{a}	10.50±0.70^{c}
当归多糖组 ASP group	57.10±1.35^{c}	12.77±0.32^{b}	16.13±2.21^{a}	14.00±0.62^{b}

注：表中同一列标有不同小写字母表示实验结果差异极显著(P<0.01)

Notes: data with different lowercase means significant difference from other groups at the same column (P<0.01)

2.3 鲫血细胞的凋亡形态荧光观察

早期凋亡细胞因细胞膜磷脂酰丝氨酸外翻可被Annexin V-FITC标记，经蓝光激发，细胞膜呈现绿色，因细胞膜结构尚完整所以细胞核未能被PI染色，故不能被激发出特异荧光(图5中“1”)；晚期凋亡细胞伴随细胞膜磷脂酰丝氨酸外翻，细胞通透性增大，细胞膜被Annexin V-FITC标记的同时，细胞核会被PI染色，蓝光激发后细胞膜呈现绿色荧光，细胞核呈现红色(图5中“2”)；坏死细胞及死细胞因膜结构破坏，仅细胞核会被PI染色，蓝光激发后呈现红色(图5中“3”)；正常细胞不能被标记染色，激发后为无色。

阴性对照组血细胞在24~96 h均只有极少量的凋亡细胞(图版Ⅲ)。阳性对照组攻毒后24 h凋亡细胞数量最多，黄芪多糖组和当归多糖组在攻毒后24 h均被激发出大量具有特异性激发荧光的凋亡细胞。攻毒后48~96 h，阳性对照组、黄

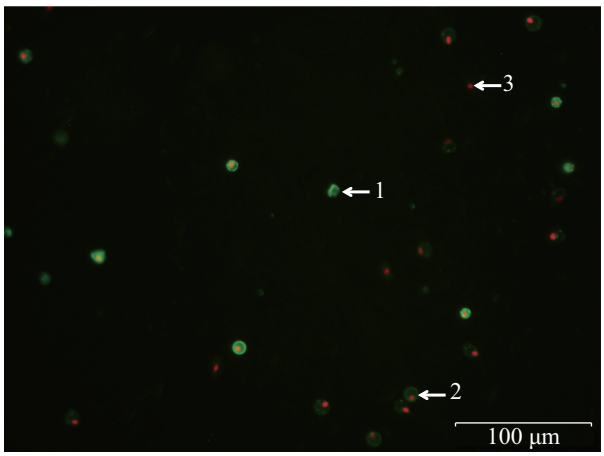

图 5 鲫血细胞凋亡Annexin V-FITC/PI双染荧光图(×200)

1. 早期凋亡细胞；2. 晚期凋亡细胞；3. 坏死细胞

Fig. 5 The fluorescent image of *C. auratus* apoptosis blood cells stained by Annexin V-FITC and PI

1. early stage apoptotic cells; 2. late stage apoptotic cells; 3. necrotic cells

芪多糖组和当归多糖组细胞的凋亡比例均出现降低，且同一检测时间凋亡比例从高到低依次为阳性对照组>当归多糖组>黄芪多糖组>阴性对照组，荧光观察结果同流式凋亡检测结果一致。

2.4　鲫凋亡肝细胞和头肾细胞超微结构观察

阴性对照组肝细胞的细胞器结构完整，细胞核规则(图版Ⅳ-1)；攻毒24 h后阳性对照组细胞核皱缩、染色质凝集，核周围出现明显凋亡小体(图版Ⅳ-2)；黄芪多糖组出现明显的细胞核染色质边集和凋亡小体(图版Ⅳ-3)；当归多糖组同样出现显著的染色质凝集及凋亡小体(图版Ⅳ-4)。

阴性对照组头肾组织的淋巴细胞细胞核结构完整、规则(图版Ⅴ-1)；阳性对照组淋巴细胞细胞核分解，出现与凋亡具有密切关系的空泡化现象和典型的凋亡小体(图版Ⅴ-2)；黄芪多糖组出现细胞核固缩、染色质凝集和细胞边缘空泡化现象(图版Ⅴ-3)；当归多糖组同样出现细胞核固缩，且细胞核边缘出现显著空泡化现象(图版Ⅴ-4)。

3　讨论

3.1　维氏气单胞菌对细胞凋亡的诱导

Zychlinsky等[19]于1992年首次报道福氏志贺菌(*Shigella flexneri*)入侵人结肠黏膜能够引起感染细胞发生凋亡，将细胞凋亡研究拓展至细菌感染领域。维氏气单胞菌是一种革兰氏阴性菌，主要通过非LPS(内毒素)毒力因子(气溶素、肠毒素、粘附因子等)诱导机体免疫细胞和器官实质细胞出现细胞凋亡[20-22]。细胞凋亡伴随着诸多特异性的形态变化过程，凋亡早期细胞的染色质出现凝集、细胞核固缩并出现细胞核边缘化典型特征，凋亡后期细胞核分解，会形成凋亡小体并被吞噬细胞吞噬消化[23]。本实验中发现维氏气单胞菌攻毒后，鲫血细胞凋亡率极显著升高，sub-G1峰(凋亡峰)细胞比例极显著高于阴性对照组，说明维氏气单胞菌攻毒可以诱导鲫发生细胞凋亡现象。鲫部分肝细胞和头肾淋巴细胞在维氏气单胞菌攻毒后出现明显的核固缩、染色质凝集现象，并有凋亡小体出现，该结果与Martins等[13]研究报道维氏气单胞菌气溶素诱导非洲绿猴肾脏细胞细胞核凝集及出现显著凋亡小体结果相一致。Krzymińska等[12]发现维氏气单胞菌感染能够引起人肠上皮细胞因氧化损伤而诱导发生细胞凋亡，如核固缩、染色质凝集、凋亡小体生成等，分析认为通过诱导组织细胞凋亡，造成组织损伤，是该菌提升其致病能力的一种方式[24]。此外研究报道维氏气单胞菌作为病原体侵入宿主后，能够诱导靶细胞凋亡，进而自我保护与扩散造成宿主出现实质器官病理改变，从而形成了诱导靶细胞凋亡并杀死免疫细胞的作用机制[11]。

3.2　中药多糖对细胞凋亡和细胞周期的影响

细胞凋亡参与了免疫系统中免疫细胞的发育、免疫调节、免疫效应等诸多生理病理过程，是机体免疫系统发挥功能和维持动态平衡必不可少的条件[25]，研究发现，在多种疾病影响下机体会出现细胞凋亡这种效应机制[26]。中药多糖为中药主要有效成分之一，在非特异性和特异性免疫调节方面均具有重要作用。中药多糖能提高机体免疫监控系统，包括红细胞、天然杀伤细胞、巨噬细胞、杀伤性T细胞、T细胞、淋巴因子激活的杀伤细胞、白细胞介素和其他细胞因子的活性[27]。据报道黄芪多糖、当归多糖在体内外都有激活补体的作用，并可诱导产生细胞因子，如黄芪、当归多糖均能诱导白细胞介素2(IL-2)和肿瘤坏死因子(TNF)的产生，IL-2和TNF可引起靶细胞凋亡[28-29]。本研究中发现，黄芪多糖和当归多糖对鲫由维氏气单胞菌诱导的细胞凋亡具有一定抑制作用。实验结果同Hsu等[30]发现灵芝多糖能够对人中性粒细胞自发凋亡和Fas诱导的细胞凋亡抑制结果相一致。张杰等[31]研究发现SFPS(羊栖菜多糖)能通过调控BAX和*caspase*-3基因表达抑制高脂诱导的胰岛β细胞凋亡。此外朱贝贝等[32]研究发现当归红芪多糖能够通过抑制辐射诱导的心肌细胞线粒体凋亡通路的激活，降低凋亡从而发挥心肌细胞保护作用。Weyts[33]等发现细胞凋亡是保存在鱼体中的一种重要的免疫调节机制，鱼体可以有效地通过调控细胞凋亡来维持免疫平衡。黄芪多糖和当归多糖对鲫由维氏气单胞菌诱导的细胞凋亡产生抑制作用，可能由于两种中药多糖能够通过增强鲫机体的免疫抵抗力从而调节其凋亡代谢过程使得凋亡率降低，从而保持鲫免疫平衡趋向稳定。

细胞周期同细胞凋亡的关系密切，由于很多能够诱导细胞凋亡的因素对细胞周期也具有特异性，因此造成细胞周期调控的异常可能是

引发细胞凋亡的重要原因之一，通常能够通过细胞周期的改变得以体现，此变化一般发生在细胞凋亡出现之前[34]。细胞周期改变的原因既可能是周期调控受损，也可能是细胞自我保护的方式，DNA轻微损伤能够造成细胞周期出现停滞，DNA损伤严重则会使得细胞出现凋亡[35]。细胞周期在正常状态下会受到周期调节因子严格地调控，促使不同细胞周期事件有序协调，细胞周期中存在两个非常关键的调控点：一个是位于G1/S期的过渡点，此调控点决定细胞能否进入细胞周期，所以被认为是细胞周期调控的关键[36]；另一个调控点位于G2/M期之间，此点决定着细胞能否完成有丝分裂或是停滞一段时间[37]。本实验结果发现，攻毒后24 h阳性对照组细胞周期图中G0/G1、S/G2+M期细胞比例均出现极显著降低，sub-G1比例极显著增高，说明维氏气单胞菌攻毒后鲫血细胞进入细胞周期的调控点作用被抑制。牙龈卟啉单胞菌(*Porphyromonas gingivalis*)对成骨细胞[38]及铜绿假单胞菌(*P. aeruginosa*)诱导小鼠肠上皮细胞[39]，中间普氏菌(*Prevotella intermedia*)内毒素对人牙周膜细胞[40]的细胞周期和凋亡实验结果均与本研究结果相一致。此外攻毒后24 h黄芪多糖和当归多糖组同阳性对照组相比，sub-G1、G0/G1极显著降低，S/G2+M期细胞比例均出现极显著增高，分析认为可能是此两种中药多糖可有效促使细胞周期通过G0/G1期、进入S期和G2/M期，增强血细胞的增殖能力，延缓和抑制了细胞凋亡的发生。

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Effect of Astragalus polysaccharide and *Angelica sinensis* polysaccharide on apoptosis of *Carassius auratus* induced by *Aeromonas veronii*

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Abstract: The present study aimed to evaluate the effect of *Astragalus* polysaccharide (APS) and *Angelica sinensis* polysaccharide (ASP) on the cell apoptosis of *Carassius auratus* induced by *Aeromonas veronii*. Negative control group, positive control group, APS group and APS group (four groups) were set up in this experiment. Experimental fish was infected with *A. veronii*. The changes of apoptosis rate and cell cycle in blood cells were determined by flow cytometer. The morphology of apoptosis cells were observed by fluorescence microscopy and transmission electron microscopy (TEM). The results showed that the blood cell apoptosis rate of the positive control group was significantly higher than those of two polysaccharide groups and negative control group after being infected with *A. veronii*. Polysaccharide groups could significantly reduce the apoptosis rate and APS group had more significant effect than ASP group. Karyopyknosis appeared in the liver cells and the head kidney lymphocytes of crucian carp. Besides,chromatic agglutination adjacent to the nuclear membrane and apoptotic bodies appeared after the infection. Compared with the negative control group, the *A. veronii* infection could significantly decrease the percentage of S/G2+M phase cells and increase sub-G1 phase cells in crucian carp blood cell cycle and the infection had an effect on inhibiting cell division and inducing apoptosis. Compared with the negative control group, the proportion of G0/G1 and sub-G1 cells in the polysaccharide group was significantly reduced, and the proportion of S/G2+M phase cells was significantly increased, which indicated that polysaccharide groups can promote cell division and inhibition of apoptosis. Based on the above results, we found that APS and ASP could effectively inhibit crucian carp apoptosis induced by *A. veronii* when the addition was 1%.

Key words: *Carassius auratus*; *Astragalus* polysacharide; *Angelica sinensis* polysaccharide; *Aeromonas veronii*; apoptosis

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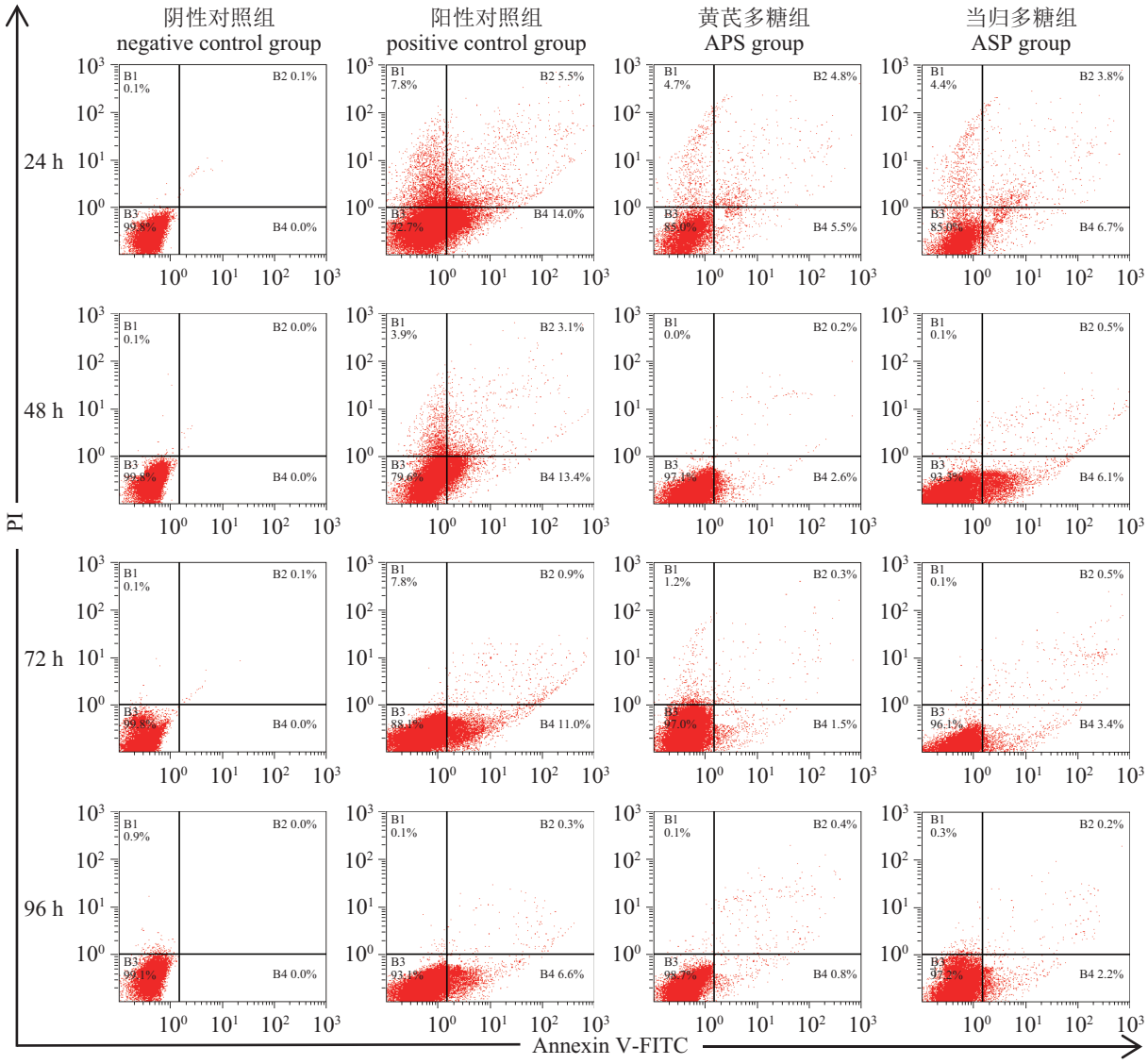
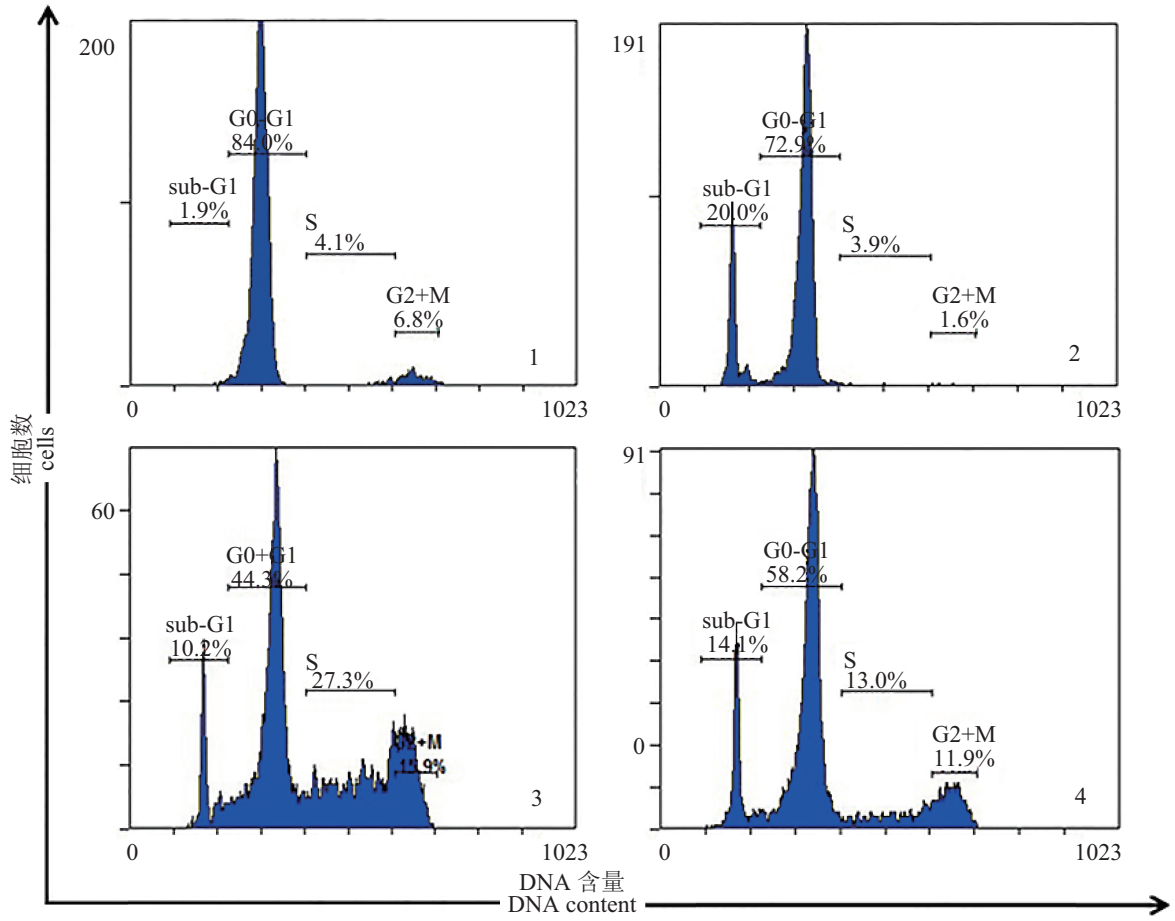


图版 Ⅰ 各组鲫血细胞凋亡流式检测图

Plate Ⅰ The blood cell apoptosis flow chart of *C. auratus*

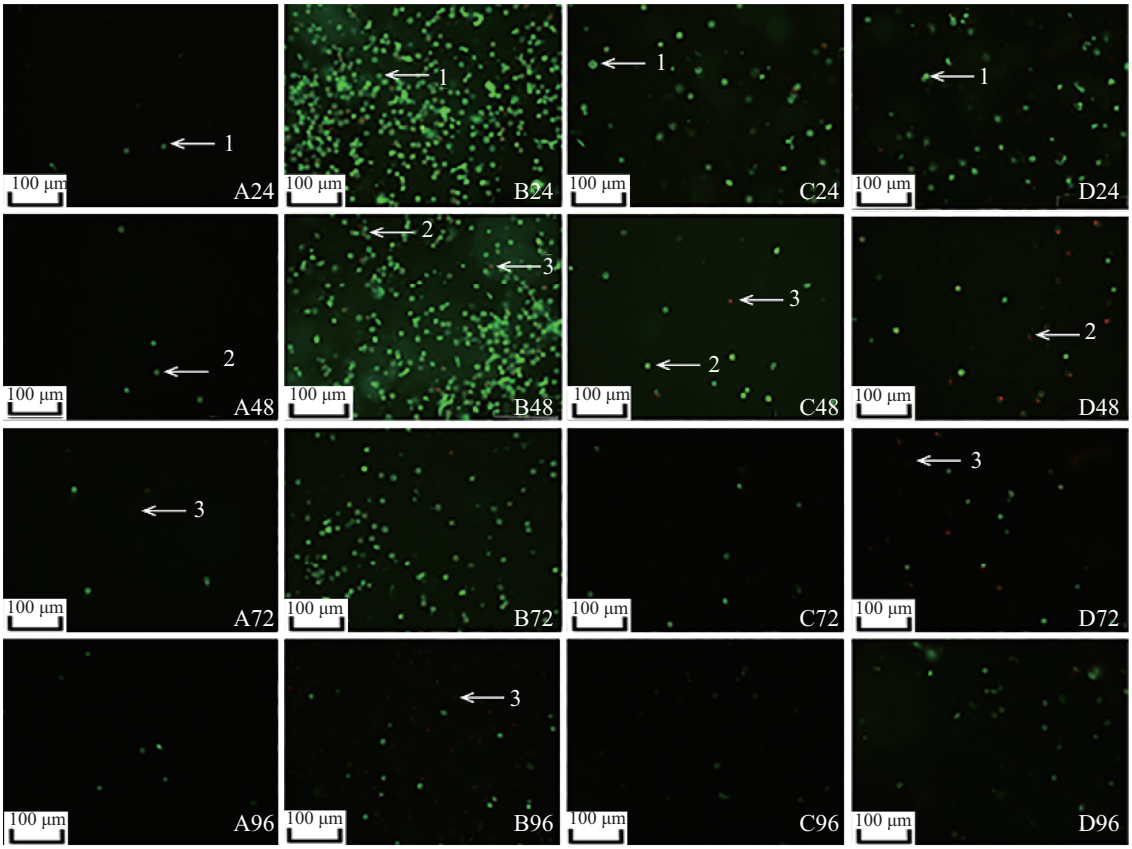


图版 Ⅱ　攻毒后24 h各组鲫血细胞周期检测流式图

1、2、3、4分别为阴性对照组、阳性对照组、黄芪多糖组、当归多糖组实验鱼攻毒后24 h鲫血细胞周期流式检测图

Plate Ⅱ　The cell cycle flow chart of *C. auratus* blood infected with *A. veronii* after 24 h

1, 2, 3, 4 represent the *C. auratus* blood cell cycle flow chart detected after 24 h of negative control group, positive control group, APS group and ASP group, respectively

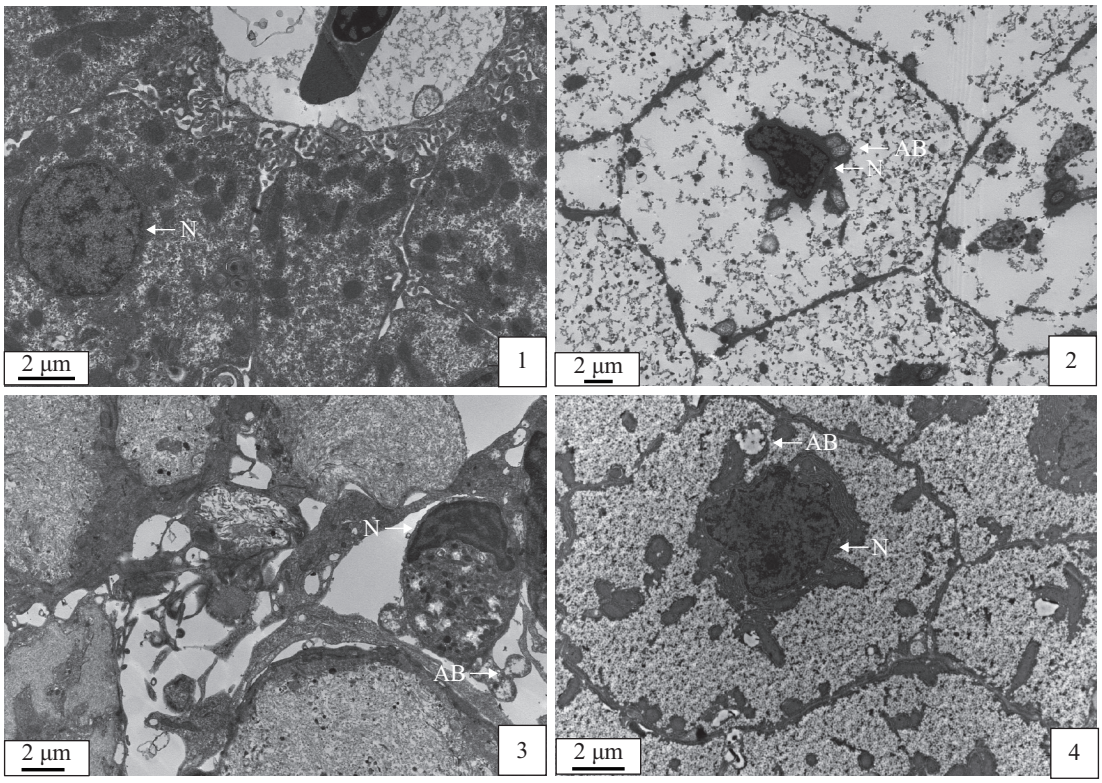


图版 Ⅲ　鲫血细胞凋亡不同检测时间Annexin V-FITC/PI双染荧光图(×200)

A、B、C、D分别为阴性对照组、阳性对照组、黄芪多糖组和当归多糖组。24、48、72、96分别代表攻毒后的小时数。图中箭头所示1. 凋亡早期细胞；2. 凋亡晚期细胞；3. 坏死细胞

Plate Ⅲ　The fluorescent images of *C. auratus* apoptosis blood cells stained by Annexin V-FITC/PI at different detection time (×200)

A, B, C and D respectively represent the negative control group, positive control group, APS group and ASP group. 24, 48, 72 and 96 respectively represent the hours after the attack. The arrowhead in figure means: 1. early stage apoptotic cells, 2. apoptotic cells at the middle and late stages, 3. necrotic cells

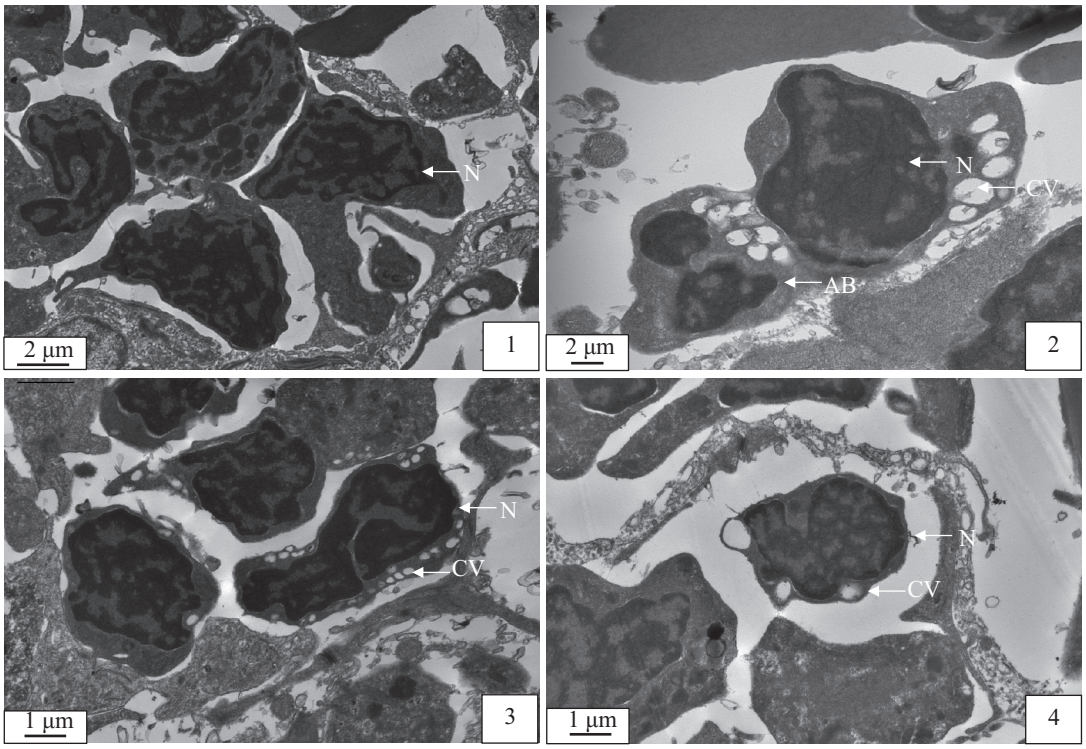


图版 Ⅳ　鲫凋亡肝细胞超微结构图

1~4. 阴性对照组、阳性对照组、黄芪多糖组和当归多糖组肝细胞超微结构。N表示鲫肝细胞的细胞核；AB表示凋亡小体。1. 阴性对照组鲫肝细胞的细胞核形态规则，染色质均匀分布(箭头N所示)；2. 阳性对照组鲫肝细胞染色质凝集，细胞核固缩(箭头N所示)，此外出现显著凋亡小体(箭头AB所示)；3. 黄芪多糖组鲫肝细胞染色质凝集，细胞核边集(箭头N所示)，细胞边缘出现凋亡小泡(箭头AB所示)；4. 当归多糖组鲫肝细胞细胞核固缩，染色质凝集(箭头N所示)及凋亡小体

Plate Ⅳ　The liver cell apoptosis ultrastructure of *C. auratus*

1–4 represented the crucian carp liver cells' ultrastructure of negative control group, positive control group, APS group and ASP group infected with *A. veronii* after 24 h respectively. N is the nucleus of the liver cells of *C. auratus*, and AB is the apoptotic body 1. in the negative control group, *C. auratus* liver cell nucleus presented regular shape and the chromatin distribution was very uniform (arrowhead N); 2. in the positive control group, the liver cell presented chromatin condensation, nuclear condensation (arrowhead N) and obvious apoptotic bodies (arrowhead AB); 3. in APS group, the liver cell presented chromatin condensation, nuclear marginalization (arrowhead N) and apoptotic vesicles appeared on the edge of the liver cell. (arrowhead AB); 4. in ASP group the liver cells appeared nuclear condensation, chromatin condensation (arrowhead N) and apoptotic bodies



图版 Ⅴ 鲫头肾组织淋巴细胞超微结构图

1~4. 阴性对照组、阳性对照组、黄芪多糖组和当归多糖组头肾组织淋巴细胞的凋亡超微结构。N表示鲫肾脏组织细胞的细胞核；CV表示细胞空泡化；AB表示凋亡小体。1. 阴性对照组鲫头肾淋巴细胞染色质分布均匀，细胞核结构正常(箭头N所示)；2. 阳性对照组鲫头肾淋巴细胞核染色质凝集(箭头N所示)，并出现显著的细胞空泡化现象(箭头CV所示)，此外有凋亡小体出现(箭头AB所示)；3. 黄芪多糖组鲫头肾淋巴细胞核染色质凝集(箭头N所示)，细胞边缘出现空泡化(箭头CV所示)；4. 当归多糖组鲫头肾淋巴细胞的细胞核固缩(箭头N所示)，细胞边缘出现显著空泡化(箭头CV所示)

Plate Ⅴ The ultrastructural changes of lymphocyte apoptosis in the head kidney of *C. auratus*

1–4 represent the crucian carp pronephros cells' ultrastructure of negative control group, positive control group, APS group and ASP group infected with *A. veronii* after 24 h respectively. N is the nucleus of the renal tissue cells of *C. auratus*, CV indicates vacuolization of cells and AB represents apoptotic bodies 1. in the negative control group, lymphocytes of head kidney presented uniform chromatin distribution and the nucleus appeared regular shape (arrowhead N); 2. in the positive control group, the lymphocyte nuclear presented chromatin condensation (arrowhead N) and there was a obvious cell cavitation phenomenon (arrowhead CV). Besides, there were there were some apoptotic bodies appear in the figure (arrowhead AB); 3. in APS group, the lymphocyte cell presented chromatin condensation(arrowhead N), and there was an obvious phenomenon of cavitation on the cell edge (arrowhead CV); 4: in ASP group, the lymphocyte cell presented nuclear condensation (arrowhead N) and there was an obvious phenomenon of cavitation on the cell edge (arrowhead CV)