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草鱼肝细胞外泌体的分离鉴定及其对肝细胞miR-122/33和免疫相关基因表达的影响

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摘要：外泌体是具有磷脂双分子膜结构的纳米级囊泡，能够参与机体多种生理过程。实验探讨了草鱼肝细胞外泌体的分离鉴定方法，并初步研究外泌体对草鱼肝细胞中miRNAs及免疫相关基因表达的影响。实验以草鱼肝细胞L8824为材料，通过超速离心获得外泌体，利用电子显微镜观察外泌体形态，采用纳米颗粒示踪分析(nanoparticle tracking analysis，NTA)技术检测外泌体粒径和数量，同时利用Western blot分析其标志蛋白CD63的表达，最后用正常肝细胞和油酸诱导的脂肪肝细胞源外泌体孵育草鱼肝细胞，通过Real-time qPCR技术检测两种不同来源的外泌体对草鱼肝细胞中miR-122/33及免疫相关基因(TNF-α，NF-κB，IL-1β，IL-6和IL-10)转录水平的影响。结果显示，草鱼肝细胞外泌体为30~150 nm的不均匀囊泡，呈圆形或椭圆形，有完整的膜结构；外泌体标志蛋白CD63呈阳性表达；NTA技术检测显示外泌体囊泡占所有囊泡的50%以上；脂肪肝细胞源外泌体显著提高了肝细胞中miR-122及炎症因子TNF-α、IL-1β和IL-6的mRNA转录水平。研究表明，通过超速离心法可成功分离草鱼肝细胞外泌体，且脂肪肝细胞源外泌体在草鱼肝细胞免疫调节中可能发挥重要作用。

关键词：草鱼；肝细胞；外泌体；分离；鉴定；免疫调节

中图分类号：Q 785; S 917.4　　**文献标志码：**A

外泌体(exosome)是细胞内溶酶体颗粒向内凹陷形成的多泡体、在刺激下多泡体外膜与细胞膜融合释放到细胞外隙或体液中的囊泡，其直径一般在30~150 nm[1]。外泌体能够被大多数细胞所分泌，且广泛存在于各种体液环境，如尿液、血清、唾液和乳液等[2]，其通过转运内部装载的蛋白质、脂质、mRNAs和miRNAs等来影响或改变受体细胞的行为，已被证明是一种细胞间通讯的新模式。

目前养殖鱼类普遍存在肝脏脂质代谢紊乱而导致的脂肪过度蓄积及炎症等问题[3-5]。研究发现外泌体及miRNAs参与了陆生动物脂质代谢及免疫调节过程[6-8]，但外泌体在水生动物上的分离鉴定及相关功能的研究较少，目前仅见Lovy等[9]在圆腹雅罗鱼(*Leuciscus idus*)脾脏朗格汉斯样细胞中发现了外泌体；Zhang 等[10]在中国大鲵(*Andrias davidianus*)胃部端粒细胞(TCs)附近发现了外泌体；Chen[11]以中华鳖(*Pelodiscus sinensis*)附睾为模型，探讨纳米级外泌体在精子储存和成熟过程中的作用；大西洋鲑(*Salmo salar*)白细

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胞来源的外泌体中含有主要组织相容性复合体II类(MHCII)[12]；在半滑舌鳎(*Cynoglossus semilaevis*)和虹鳟(*Oncorhynchus mykiss*)的血液中分离鉴定了外泌体[13-14]；在斑马鱼(*Danio rerio*)中发现外泌体可以作为载体携带抗癌药物通过血脑屏障治疗脑癌[15]。截至目前，脂肪肝细胞源外泌体及miRNAs在草鱼肝脏脂代谢紊乱及诱导的炎症中的作用尚未被探究。

1 材料与方法

1.1 实验材料与仪器

MEM培养基(Gibco)；胰蛋白酶(Gibco)；胎牛血清(Hyclone)；油酸(Sigma)；兔源CD63单克隆抗体(Cell Signaling Technology)；细胞裂解液、蛋白抽提试剂(Solarbio)和ECL 化学发光试剂盒(Thermo Fisher)等。

倒置相差显微镜(Nikon TS100)；超速离心机(美国Beckman Culter，OPTIMAXPN 100)；CO_2细胞培养箱(Thermo Scientific Forma)；透射电子显微镜(日立HT7700)；扫描电子显微镜(日立JSM-7800F)；纳米颗粒示踪仪(NanoSight NS300，Malvern)；荧光定量PCR仪(罗氏LightCycler96)等。

1.2 细胞培养

草鱼(Ctenopharyngodon idella)肝细胞(L8824)购自中国典型培养物保藏中心(China Center for Type Culture Collection，CCTCC)，细胞为贴壁生长。用含10%胎牛血清(FBS V/V，Hyclone)、青链霉素(Solarbio)各10 万U/L的MEM培养基(Gibco)传代培养。脂肪肝细胞通过本课题组前期建立的方法即采用80 μg/mL油酸孵育肝细胞24 h诱导而成。细胞在28 °C，5% CO_2培养箱中培养，每天用倒置相差显微镜观察细胞生长状况，当细胞生长至70%~80%汇合时，收集草鱼肝细胞及草鱼脂肪肝细胞培养液以富集外泌体。

1.3 草鱼肝细胞外泌体的分离

从草鱼肝细胞(脂肪肝细胞)培养液中分离外泌体，简易操作流程如图1所示。首先，草鱼肝细胞培养液以800 × *g*离心10 min除去细胞，然后3 000 × *g*离心20 min 除去残留较大的微泡；将上清液转移到新的离心管，以8 000 × *g*离心30 min除去大的碎片；在12 000 × *g*离心30 min除去细胞碎片后，将上清液通过0.22 μm滤膜过滤；过滤后的上清液在4 °C 120 000 × *g*超速离心2 h；弃上清液，将沉淀溶解在PBS中，在4 °C 120 000 × *g*下离心1 h，用30 μL无菌1×PBS将外泌体沉淀重悬，储存在−80 °C冰箱用于后续实验。

1.4 透射电子显微镜检测外泌体形态和粒径

取10 μL外泌体样品滴加在密封膜上，将载样铜网覆盖于样品上，在室温下静置1 min，用滤纸吸去多余液体，10 μL 2%(*W*/*V*)磷钨酸溶液室温负染1 min后用PBS轻洗1次后室温干燥2 min，在透射电子显微镜观察并照相。

1.5 扫描电子显微镜检测外泌体形态及粒径

将分离获得的外泌体用2.5%戊二醛固定，涂在盖玻片上，用滤纸从边缘处吸去浮液。将载有样品的玻璃片放入干燥盘中用不同浓度的乙醇脱水，用叔丁醇干燥后放入真空冷冻干燥机中进行干燥，用离子溅射仪在样品表面喷镀一层金属膜，在扫描电子显微镜下对样品进行成像并观察外泌体形态及粒径大小。

1.6 Western blot检测外泌体CD63蛋白表达

在分离的外泌体中加入RIPA高效裂解液，通过BCA法对外泌体蛋白进行定量。用SDS-PAGE凝胶分离外泌体中的蛋白质，将蛋白质转移到PVDF膜上，用溶解在TBST缓冲液中的5%脱脂奶粉将膜封闭2 h。5%脱脂奶粉中加入兔CD63单克隆抗体(1∶400) 4 °C孵育过夜。TBST漂洗后加入辣根过氧化物酶(HRP)标记的羊抗兔二抗(1∶1 000)，室温孵育1 h，加入适量PBS，在水平摇床上摇动洗涤10 min，TBST漂洗3次，ECL显色。

1.7 NTA技术分析外泌体粒径和浓度

NTA检测的具体步骤为取灭菌水1 mL重悬外泌体用于Nanosight NS300分析仪进行粒径分布检测，NTA软件可以直接观察并自动跟踪每一个带有散射光颗粒的布朗运动轨迹，再结合颗粒的散射强度数据，在悬浮液中能实时直接成像和可视化直径范围为10~2 000 nm的特定外泌体和微泡，同时NTA技术提供高分辨率粒度分布图和浓度信息。

1.8 外泌体对草鱼肝细胞miR-122/33及相关免疫基因表达的影响

草鱼肝细胞在培养瓶中培养，待细胞长满后传代至六孔培养板，当细胞生长至培养板的

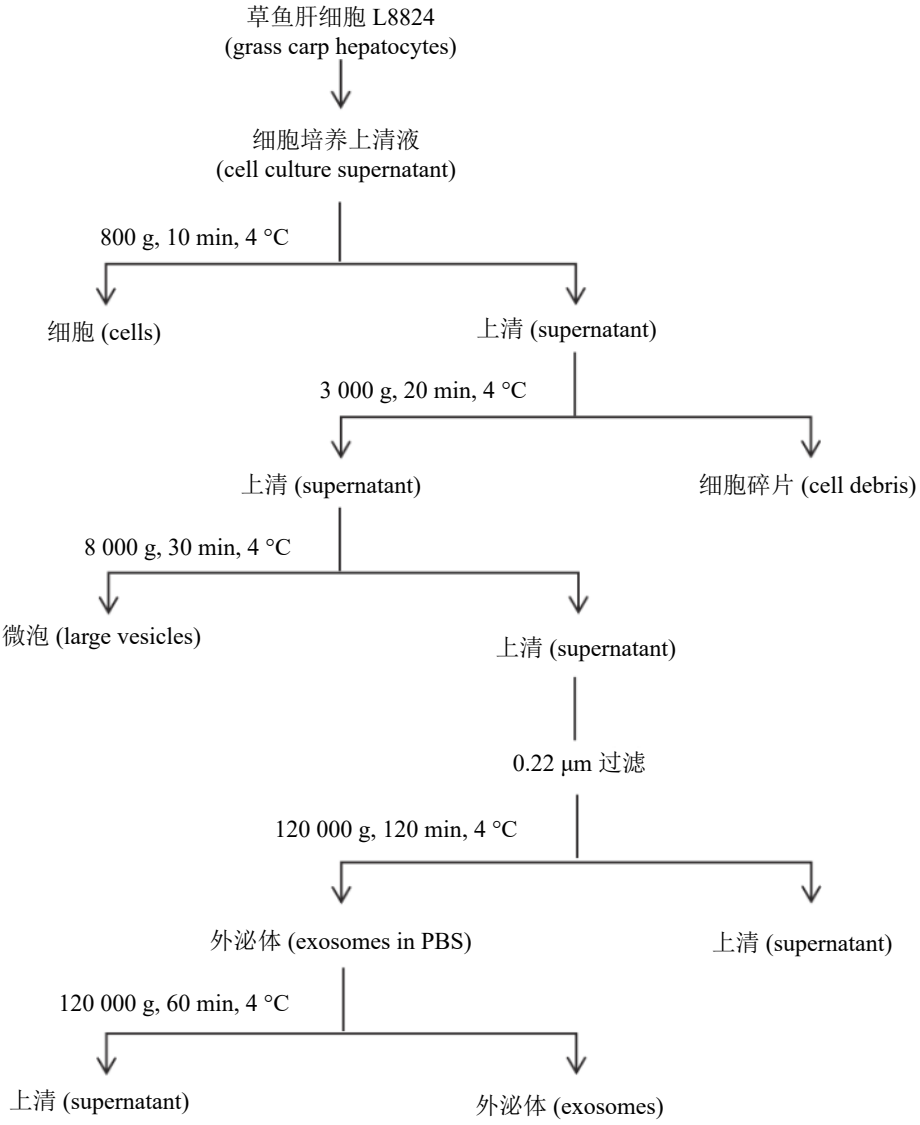

图 1　草鱼肝细胞外泌体分离的流程图

Fig. 1　Flowchart for exosomes isolation from the grass carp hepatocytes

70%~80%密度时，将收集的草鱼肝细胞和脂肪肝细胞源外泌体按终浓度为200 μg/mL分别加入到六孔培养板和细胞共孵育24 h后，收集肝细胞，按照RNAiso Plus说明书提取细胞中的总RNA。

采用qRT-PCR技术，检测肝细胞中miR-122/33及TNF-α, NF-κB, IL-1β, IL-6和 IL-10的相对表达量。内参18S及基因引物见表1。利用PrimeScript RT试剂盒(perfect real time)反转录获得cDNA第一链。参照TB Green Premix Ex Taq (Tli RNase Plus)说明书，进行qRT-PCR。PCR反应体系为20 μL：TB Green Premix Ex Taq (2X) 10 μL，PCR 上游引物(10 μmol/L) 0.6 μL，PCR 下游引物(10 μmol/L) 0.6 μL，cDNA模板2 μL，加ddH_2O至总体积为20 μL。PCR程序采用两步法：95 °C 30 s；95 °C 5 s、60 °C 20 s，共35个循环。反应结束后进行溶解曲线分析，以验证产物特异性。每个样品6个平行，反应结束后进行数值分析。

miRNA由All-in-One™ miRNA First-Strand cDNA Synthesis Kit反转录获得miRNA第一链cDNA。根据All-in-One™ miRNA qPCR Kit说明书进行qRT-PCR，反应体系如下：All-in-OneTM qPCR Mix (2×) 10 μL; All-in-OneTM miRNA qPCR primer (2 μmol/L) 2 μL; Universal Adaptor PCR primer (2 μmol/L) 2 μL; First-strand cDNA 6 μL，反应体积共20 μL。采用两步法：95 °C 10 min，95 °C 10 s，60 °C 35 s，共35个循环。由扩增曲线得出各样品qRT-PCR扩增的Ct值，根据$2^{-\Delta\Delta C}$算法计算出样品中miRNA相对表达丰度。

表 1 实时定量引物

Tab. 1 Primers used for quantitative real-time PCR

引物名称 primer name	序列(5′→3′) forward (5′→3′)	序列(5′→3′) reverse (5′→3′)	GenBank登录号 GenBank accession no.
miR-122	GGAGTGTGACAATGGTGTTTGAA		NR_030058.1
miR-33	TTCCCTTTGTCATCCTATGCCTA		KU670831.1
TNF-α	GATTGGAGAGTGAACCAGGAC	CCTGGCTGTAGACGAAGTAAAT	HQ696609
NF-κB	GAACATTTAACCCACGCAAGAG	GGCCTTTCCTGCCATCTAATA	KY613789.1
IL-1β	ATGCCAACCTCCTGTTCTTC	CACTTCCACCTGCTCCATATC	JN705663.2
IL-6	CGTATGAAGGTGTCAGGATCAG	CCACGTCAGGACACTGTAAAT	KC535507.1
IL-10	CGCTTCTACTTGGAGACCATTC	CCATATCCCGCTTGAGATCTTG	HQ388294.1
18S	TGGAATGAGCGTATCCTAAACC	TCTCCCGAGATCCAACTACAA	EU047719.1

1.9 统计分析

实验数据通过SPSS 20.0软件进行单因素方差分析，Duncan氏多重比较法进行组间差异性检测，结果以平均数±标准差(mean±SD)表示，若$P<0.05$，表示有显著性差异。

2 结果

2.1 电子显微镜观察草鱼肝细胞外泌体形态和粒径

透射和扫描电镜结果显示，草鱼肝细胞外泌体呈圆形或椭圆形，直径在30~150 nm，有完整的膜结构，内含低密度物质(图2)。透射电子显微图像显示外泌体具有典型的“杯托”状和双层膜结构(黑色箭头所示)(图2-a，b)，符合外泌体的形态特征。扫描电子显微图像可以观察到分离得到的外泌体大小均一，数量较多，纯度较好(图2-c，d)，可以用于后续实验。

2.2 草鱼肝细胞外泌体标志蛋白CD63的鉴定

CD63为外泌体标志蛋白。Western blot结果显示，CD63呈阳性表达(图3)，表明超速离心所获得的沉淀主要由外泌体组成。

2.3 NTA技术检测草鱼肝细胞外泌体粒径和浓度

通过NTA技术分析外泌体的大小分布和浓度，在所有获得的纳米颗粒中，直径30~200 nm的纳米颗粒数量超过50% (图4)。外泌体粒径主峰位于100 nm附近。外泌体的平均浓度为4.67e+008个拷贝/mL，据此提取的纳米颗粒可以确认是外泌体。

2.4 不同来源外泌体对草鱼肝细胞miR-122/33表达的影响

为进一步检测不同来源外泌体在肝细胞免疫调节中的作用，本实验采用qRT-PCR检测草鱼肝细胞源外泌体(exos)和草鱼脂肪肝细胞源外泌体(OA-exos)孵育肝细胞24 h 对miR-122和miR-33的表达影响。结果显示，OA-exos组肝细胞中miR-122表达量显著高于exos对照组的($P<0.05$)，而miR-33的表达差异不显著($P>0.05$)(图5)。说明miR-122可能在草鱼脂肪肝细胞的外泌体中被富集，进而通过脂肪肝细胞源外泌体进入到孵育的肝细胞中，使肝细胞中miR-122的表达量升高，而miR-33则没有发生这种变化。

2.5 不同来源外泌体对草鱼肝细胞免疫相关基因表达的影响

进一步检测了草鱼肝细胞源外泌体(exos)和草鱼脂肪肝细胞源外泌体(OA-exos)对草鱼肝细胞免疫相关基因表达的影响。与exos组相比，OA-exos处理草鱼肝细胞后其促炎因子TNF-α，IL-1β和IL-6 mRNA表达水平显著升高($P<0.05$)，而NF-κB和 IL-10则无显著性差异($P>0.05$)(图6)。

3 讨论

外泌体是一类形态大小均一、直径为30~100 nm、密度为1.10~1.18 g/mL的脂质双层膜囊泡样小体。其作为载体通过运输蛋白质、脂质、mRNA和miRNAs等来影响或改变受体细胞的行为[16]，参与机体多种生理和病理功能，如免疫调节、脂肪代谢、血管生成和肿瘤发生等[6-8,17-18]。目前外泌体的研究虽取得了很大进展，但总体

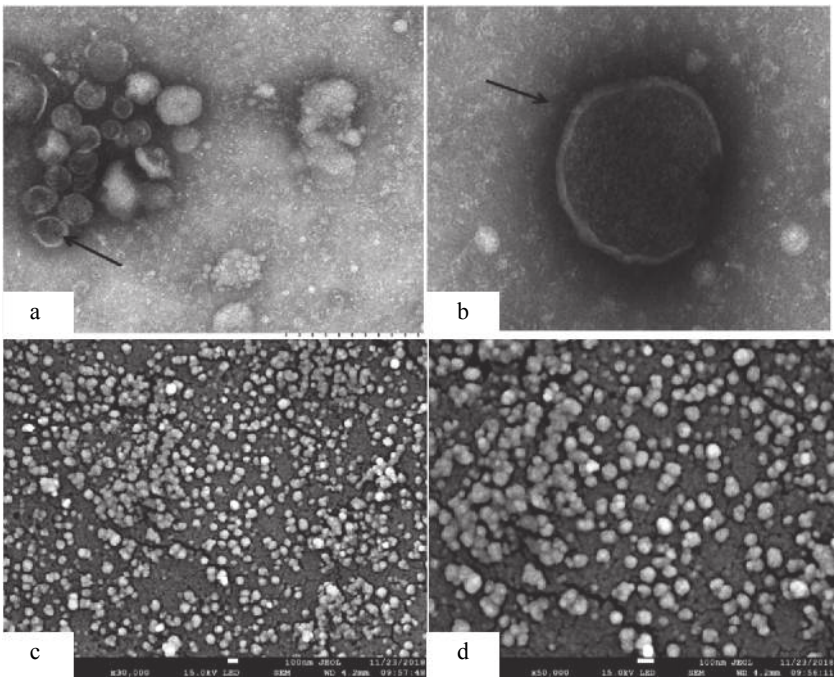

图 2　电子显微镜观察草鱼L8824细胞中外泌体颗粒的形态(黑色箭头)

(a)，(b) 草鱼肝细胞外泌体的透射电子显微镜图像(200 nm)；(c) 草鱼肝细胞外泌体的扫描电子显微镜图像(×30 000)；(d) 草鱼肝细胞外泌体的扫描电子显微镜图像(×50 000)

Fig. 2　Electron micrograph of an exosome from the grass carp hepatocyte (black arrow)

(a), (b) Transmission electron microscopy image of exosome from the grass carp hepatocyte (The scale bar represents 200 nm). (c) Scanning electron microscopy image of exosome from the grass carp hepatocyte (×30 000). (d) Scanning electron microscopy image of exosome from the grass carp hepatocyte (×50 000)

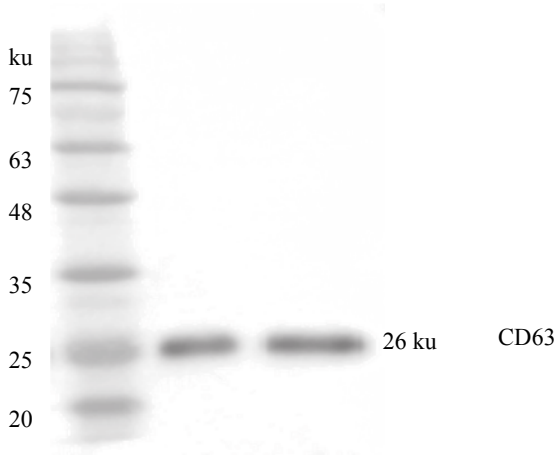

图 3　蛋白质印迹技术检测
草鱼肝细胞外泌体CD63蛋白的表达

Fig. 3　Immunoblot analysis of samples of exosomes isolated from the grass carp hepatocyte using ultracentrifugation for exosomes markers CD63

还处于研究的初始阶段。因此可靠、标准化的外泌体分离与鉴定方法是本研究的基础和前提。Shao等[19]综述了很多分离和鉴定外泌体的方法，如富集外泌体常用方法包括：超速离心法、梯度超速离心法、共沉淀法、尺寸排阻色谱和场流分级法；以及新的富集方法(微流体法、无接触分选法、免疫亲和富集法)等。Oksvold等[20]介绍了磁珠的免疫捕获技术，即通过抗体结合磁珠实现特异性捕获外泌体的方法。近年来，市场上出现了各种外泌体提取试剂盒，然而有时试剂盒提取的外泌体中的杂质蛋白质污染比较严重且价格昂贵[21]。密度梯度离心是将样本和蔗糖梯度材料一起超速离心，使样品中不同组分沉降到各自的等密度区域，从而实现外泌体的分离[22]，通过该方法获得的外泌体的纯度很高，但该过程麻烦且耗时。超速离心是目前分离外泌体最常用、最广泛的方法[23-24]。它通过不断提高转速，依次去除样品中的细胞、细胞碎片和大分子蛋白质等物质，最后分离纯化获得大量的外泌体，该方法获得的外泌体量多且纯度可靠。

本实验采用超速离心法分离外泌体，收集草鱼肝细胞上清液100 mL，使分离的外泌体量能满足后续所用。首先要确定沉淀的纳米颗粒是外泌体，采用电子显微镜分析外泌体大小和形态被认为是鉴定外泌体的金标准[25-26]。本实验观

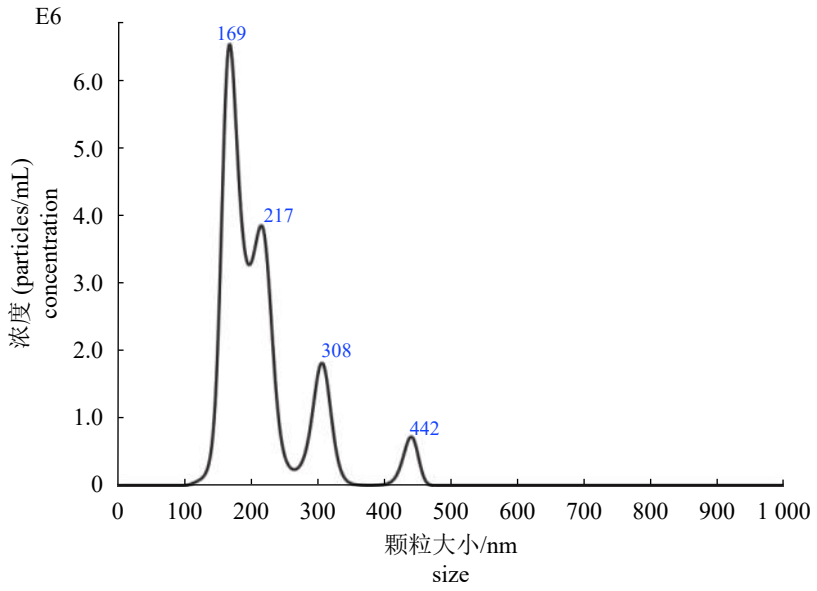

图 4 NTA技术分析草鱼肝细胞中外泌体的颗粒大小分布和浓度

Fig. 4 Analysis of size distributions and concentration of exosomes in grass carp hepatocytes by nanoparticle tracking analysis

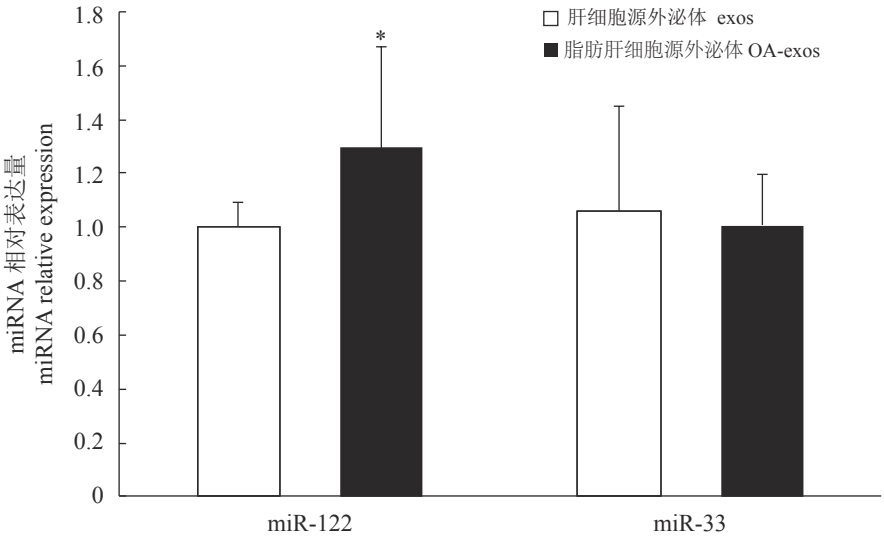

图 5 外泌体对草鱼肝细胞中miR-122和miR-33 mRNA 的表达影响

图中数据表示为平均值±标准差(n=6)，标*表示差异有显著性(P<0.05)

Fig. 5 Effects of exosomes on the expression of miR-122 and miR-33 mRNA in grass carp hepatocytes

Error bars indicate the mean and standard deviation (n=6). *indicates statistical difference (P<0.05)

察到来自草鱼肝细胞的外泌体是圆形的、大小约为30~200 nm (图2-a，b)，这与先前在哺乳动物中描述的形状类似[27-29]。但最近的一项研究表明，来自不同卵巢上皮癌细胞的外泌体大小不一[30]，在使用冷冻电子显微镜(cryo-EM)研究外泌体时，也观察到了具有非球形形状且没有任何明显支架结构的外泌体[19,31-34]。外泌体富含四跨膜蛋白(例如，CD9，CD63和CD81)，这是一种具有四个跨膜结构域的蛋白质超家族[35-36]，被认为是外泌体重要的生物标志物。本实验采用Western blot技术结果显示CD63呈阳性表达。NTA技术是一种光学粒子示踪方法，用于确定粒子的浓度和粒度分布[37-39]。本实验通过NTA技术发现所分离的囊泡主峰位于100 nm左右(图4)，可以确认分

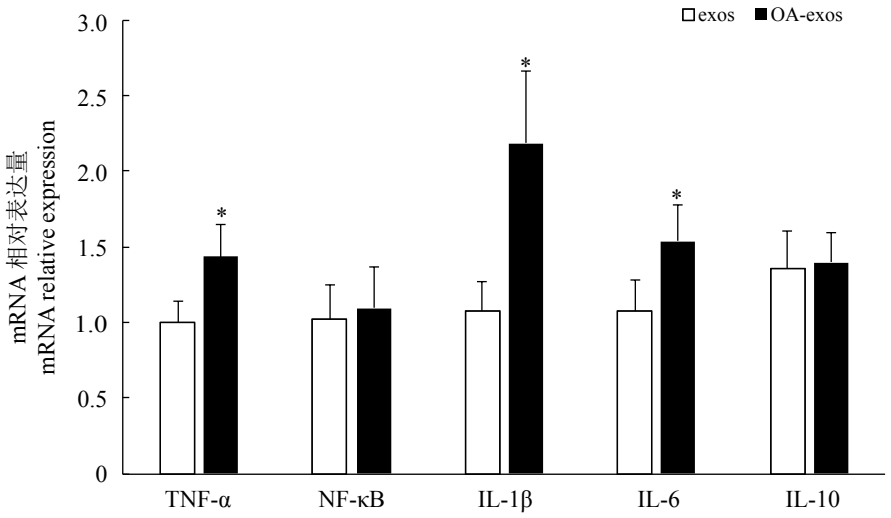

图 6　外泌体对草鱼肝细胞中免疫相关基因mRNA表达的影响

图中数据表示为平均值±标准差(n=6)，标*表示差异有显著性(P<0.05)

Fig. 6　Effects of exosomes on the expression of immune-related gene mRNA in grass carp hepatocytes

Error bars indicate the mean and standard deviation (n=6). *indicate statistical difference (P<0.05)

离的纳米颗粒沉淀大部分为外泌体。

目前关于水生动物外泌体的研究仍然很少，仅见Iliev等[12]采用蛋白质组学方法，发现大西洋鲑鱼头肾白细胞培养液上清中分离的外泌体含有主要组织相容性复合体Ⅱ类(MHCⅡ)；在虹鳟的血液及肝细胞中分离鉴定了外泌体，并发现虹鳟血浆外泌体中富含乙酰胆碱酯酶(Acetylcholinesterase，AChE)，而AChE主要在免疫反应中发挥着重要作用[40]。这些研究提示外泌体在鱼的免疫反应中可能发挥重要作用[14]。Bala等[41]研究发现外泌体中富集的miR-122和脂肪肝炎密切相关，在酒精性脂肪肝病人血清中miR-122显著增多。小鼠酒精性肝炎中miR-122由外泌体介导从肝细胞进入到单核细胞中参与免疫调节[6]。众所周知，miR-122是第一个发现在肝细胞中特异高丰度表达的miRNA，且在脂肪代谢中发挥重要作用[6,42-43]。miR-122转染脂多糖(LPS)处理的斑马鱼肝脏细胞，随着时间的延长其TLR1、TLR3、TLR4a和TNFα的表达量均显著降低[44]。上述研究均显示miR-122或外泌体中的miR-122在调节炎症和脂代谢中起到了关键作用。本研究也发现，脂肪肝源外泌体上调了草鱼肝细胞中miR-122及促炎因子TNFα，IL-1β和IL-6的表达，证明了外泌体尤其外泌体中的关键miRNA可能在免疫调节中发挥重要作用。

研究结果证明，本实验获得的纳米颗粒是外泌体，并初步证明了脂肪肝源外泌体有可能通过调节miR-122及相关免疫基因的表达而在脂肪肝细胞免疫调节中发挥重要作用。本研究将为水生动物特别是鱼类的外泌体分离和鉴定提供一种有效的方法，也为研究外泌体在免疫调节中的作用提供了思路。

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Isolation and identification of hepatocellular exosomes and their effects on the expression of miR-122/33 and immune-related genes in grass carp(*Ctenopharyngodon idella*)

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Abstract: Exosomes are nano-scale vesicles with a phospholipid bimolecular membrane, which can participate in many physiological processes. The aim of this study was to investigate the isolation and identification methods of extracellular exosomes from grass carp hepatocyte and to conduct preliminary study on the effects of exosomes secreted from fatty hepatocyte on the expression of miRNAs and immune-related genes in grass carp hepatocytes. In this study, The exosomes of grass carp hepatocytes L8824 were extracted by ultracentrifugation. The morphology of exosomes was observed by Electron Microscopy, the parcicle size and quantity of exosomes were determined by Nano-particle Tracking Analysis. Meanwhile, we analyzed the expression of the specific protein CD63 using Western blot. Then, we used exosomes,which wwew secreted from normal hepatocytes and fatty hepatocyte induced by oleic acid to incubate grass carp hepatocytes, and the effects of two different exosomes on the transcription levels of miR-122/33 and immune-related genes, which are TNF-α, NF-κB, IL-1β, IL-6 and IL-10, were detected by real-time qPCR. Our results indicated that the exosomes secretion of grass carp hepatocytes culture medium were uneven vesicles of 30-150 nm, round or oval, and had a complete membrane structure. The expression of exosomes marker protein CD63 was positive, and the NTA results showed that the exosome vesicles accounted for more than 50 % of all the vesicles. Additionally, the exosomes of fatty liver cells significantly increased the expression of miR-122 and the immunogenic genes (TNF-α, IL-1β and IL-6) in hepatocytes (P<0.05). These results suggest that the exosomes of grass carp hepatocytes can be isolated successfully by ultracentrifugation. Moreover, exosomes may play a key role in the regulation of immunity of grass carp hepatocytes.

Key words: *Ctenopharyngodon idella*; hepatocytes; exosomes; isolation; identification; immunoregulation

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