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尼罗罗非鱼无乳链球菌病的病理学研究

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摘要: 为了解尼罗罗非鱼感染无乳链球菌后各组织的病理变化,运用革兰氏染色和电镜负染技术对一株从自然发病的尼罗罗非鱼上分离的无乳链球菌进行形态观察,采用组织切片和超薄切片电镜技术对患病尼罗罗非鱼的肝脏、脾脏、肾脏、脑、心肌、骨骼肌、肠、鳃等 8 种组织进行病理学研究,探讨该病的致病机理。结果显示,革兰氏染色呈阳性,负染后透射电镜观察多数细菌呈链状排列;组织病理学变化主要是各内脏器官的广泛充血、水肿、变性和炎性细胞浸润,严重的细胞坏死;超微病理显示,大量球菌侵染脾脏等内脏组织,破坏细胞结构和各种细胞器;细胞界限模糊,细胞核畸形,线粒体肿大,嵴断裂,溶解;粗面内质网肿大、核糖体脱落;细胞质空泡化严重;心肌和骨骼肌纤维断裂、紊乱、肌节长短不一;肠微绒毛排列不整齐、长短不一;眼中有纤维性沉积。研究表明,无乳链球菌能造成尼罗罗非鱼全身性组织器官损害和炎症反应,尤其是肝脏、脾脏、肾脏和脑等重要器官功能障碍和衰竭,最后导致鱼体死亡。

关键词: 尼罗罗非鱼; 无乳链球菌; 病理学; 超微结构

中图分类号: S 941　　**文献标志码**:A

无乳链球菌(*Streptococcus agalactiae*)又名 B 族链球菌(group B streptococcus,GBS),革兰氏阳性球菌,是自然界中广泛存在的一种条件致病菌,根据其荚膜多糖抗原性的不同,可分为 10 种血清型(Ⅰa、Ⅰb、Ⅱ~Ⅸ型),它的宿主广泛,包括新生儿,猪、牛等陆生脊椎动物和鱼类等水生动物[1]。无乳链球菌是温水性鱼类的重要病原菌,受感染的鱼类包括海水和淡水鱼类,流行水温 26℃以上,通常认为是夏季季节性暴发疾病,目前已有 12 个国家报道了该病的暴发和流行,是全球罗非鱼养殖业危害最严重的病害[2-9]。我国 2008 年以前罗非鱼病害较少,且以海豚链球菌病为主,无乳链球菌病只有零星报告,2009 年在我国海南省首次大规模暴发,主要危害亲鱼及 100 g 以上的罗非鱼[2]。此后,无乳链球菌就成为危害我国罗非鱼产业最严重的病原菌,且发病呈现区域扩大至整个南方主产区、发病率和死亡率居高不下、易感规格蔓延至鱼苗和鱼种等新特征,给我国罗非鱼养殖业造成危害[2-3,8-9]。

尽管已经明确了无乳链球菌对我国罗非鱼产业的危害性,但迄今为止,人们对罗非鱼无乳链球菌的了解仅局限于疾病描述、病原鉴定[2-3],药敏试验[2-3],PCR 检测方法(双重 PCR,三重 PCR,巢式 PCR)[10-12],中草药防治[13]和相关疫苗的研制[14-16],而对疾病的发生与发展规律缺乏深入了解。国外虽有文献对无乳链球菌引起罗非鱼的组织病理变化做了广泛研究[5,17-22],但尚未见相关的超微病理学变化报道。本实验以一株无乳链球菌(16 rRNA 分子鉴定的 GenBank 登录号是:JQ990153)引起自然发病的尼罗罗非鱼(*Oreochromis niloticus*)为研究对象,较为系统地研究了该病原菌引起的组织病理学和超微病理学变化特征,旨在为罗非鱼无乳链球菌病的临床诊断,致病机理探索及预防治疗提供基础资料。

1 材料与方法

1.1 病鱼来源

患病尼罗罗非鱼鱼取自广西壮族自治区柳州

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市某养殖场,选择具有典型症状的濒死尼罗罗非鱼,体质量为(500 ± 75)g。

1.2 病原分离与观察

取症状典型的濒死病鱼,用 70% 的酒精棉球擦拭两遍病鱼的体表和腹部,用灭菌剪刀剪开腹腔,无菌条件下取少量的肝脏、脾脏、肾脏和脑等组织在血平板培养基上划线分离病原菌,28 ℃培养 24 h 后观察。

将分离的病原菌进行革兰氏染色,尼康 80i 显微镜下观察拍照;将过夜培养的病原菌用 0.85% 的无菌 NaCl 溶液制成 10^8 cfu/mL 的菌悬液,将菌悬液滴到 250 目有碳膜的铜网上面,静置 3 min,用滤纸条从液滴边缘吸去多余液体,待稍干燥后加入 1 滴饱和醋酸双氧铀染液,染色 3 s,用滤纸条从边缘将多余液体吸去,自然干燥后置于日立 H-7650 透射电子显微镜观察拍照。

1.3 组织病理观察

取病鱼肝脏、脾脏、肾脏、脑、心肌、骨骼肌、肠、鳃等组织,用 Bouin 液固定;将组织从固定液里取出,用 70% 酒精洗涤至无色;组织依次放入 70%、80%、90% 酒精中各 1 h,然后放入 95%、100%(2 次)酒精里各 30 min 进行梯度脱水;脱水后用 100% 酒精∶二甲苯(1∶1)透明 30 min,二甲苯透明 30 min(2 次);石蜡包埋;KD2258 型切片机连续切片,厚度为 4 ~ 7 μm;经展片和烘片后,将组织切片分别放入二甲苯及各梯度浓度的酒精中进行脱蜡和水化,其中二甲苯 20 min(2 次),二甲苯∶100% 酒精(1∶1),100% 酒精(2 次),95% 酒精,80% 酒精,70% 酒精,50% 酒精,蒸馏水各 5 min;苏木精 - 伊红(H. E)染色后将切片放入梯度酒精里脱水;然后依次放入二甲苯 + 100% 酒精(1∶1)20 min、二甲苯 10 min(2 次)进行透明;中性树胶封片后用尼康 80i 显微镜观察并拍照保存。

1.4 超微细胞病理观察

将病鱼的肝脏、脾脏、肾脏、脑、心脏、骨骼肌,肠和眼等组织分别切成约 1 mm^3 小块迅速用 2.5% 戊二醛固定。然后以 0.1 mol/L 磷酸缓冲液冲洗,再用 1% 锇酸固定 2 h,磷酸缓冲液冲洗后置于梯度乙醇和丙酮中脱水,Epon812 环氧树脂包埋,LKB28800 超薄切片机切片,厚度约 70 nm。切片经醋酸铀和柠檬酸铅双重染色,日立 H-7650 透射电子显微镜观察并拍照,拍照时电压 60 kV。

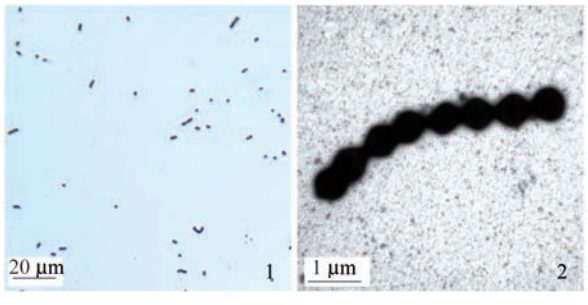
2 结果

2.1 临床症状

自然感染的病鱼表现出典型的链球菌病症状:体色发黑,突眼(单突或双突),角膜混浊,泳姿失衡,在水面间歇性打转,在池边离群慢游,昏睡消瘦,食欲不振。鳃盖下缘,胸鳍基部腹部体表具瘀点状或斑块出血。解剖见肝脏、脾脏和肾充血,脾脏尤其肿大,胆囊肿大,胆汁稀薄,腹腔充满淡黄色液体。

2.2 病原分离与形态观察

培养 24 h 后血平板出现形态一致,边缘光滑,圆润细小的白色菌落,具有 β 溶血活性。革兰氏染色后在光学显微镜下显示为阳性球菌(图版Ⅰ-1),呈单菌落或链状排列。负染后透射电镜下显示为球形或卵圆形,直径约为 0.6 ~ 1.0 μm,多数呈链状排列(图版Ⅰ-2)。



图版Ⅰ 无乳链球菌革兰氏染色及负染电镜照片

1. 细菌革兰氏染色呈阳性,球形或卵圆形,单独或呈链状排列; 2. 细菌负染电镜照片

Plate Ⅰ Gram stain of *S. agalactiae* and the bacterial morphology by negative stain under electron microscopy

1. gram stain positive, sphere-shaped bacteria were alone or arranged in chains; 2. the bacterial morphology by negative stain under electron microscopy

2.3　组织病理变化

肝脏　　肝脏充血,细胞索紊乱,肝细胞肿胀,严重空泡变性,胞浆中充满大小不等的空泡,部分肝细胞胞浆及胞核淡染、溶解、消失,只剩下细胞轮廓(图版Ⅱ-1)。溶解、消失的细胞相互融合,形成模糊、无结构的溶解坏死灶。强嗜酸性纤维素样物质渗出,偶见少量含铁血黄素沉积。

脾脏　　中央动脉周围大量红细胞浸润和含铁血黄素大量沉积。白髓体积缩小,几乎完全消失,以致淋巴细胞减少,仅在中央动脉周围有少量残余(图版Ⅱ-2)。红髓广泛性淤血,脾窦内充满大量红细胞,巨噬细胞呈弥漫性浸润。脾实质细胞水肿,胞核固缩,胞质透明空泡,血浆蛋白渗出呈淀粉样变性(图版Ⅱ-3)。

肾脏　　肾小囊扩张,肾小球毛细血管严重扩张、充血。肾小管上皮细胞肿胀,核清晰,胞浆中充满红染的细小颗粒。部分肾小管上皮细胞坏死,溶解、消失,管内有大量管型出现(图版Ⅱ-4)。

脑　　脑膜疏松水肿,小血管和毛细血管扩张淤血,脑基质水肿,疏松,呈现出多孔的海绵状,并伴有淋巴细胞浸润(图版Ⅱ-5)。

其他器官　　心肌纤维间隙增大,断裂,红细胞浸润(图版Ⅱ-6);骨骼肌纤维断裂,弯曲不齐,肌纤维间隙增大(图版Ⅱ-7)。肠上皮细胞坏死脱落,散落于肠腔,伴随淋巴细胞浸润(图版Ⅱ-8),肌层也失去原有结构,肌纤维散乱,呈玻璃样变。鳃小片充血、水肿、坏死,呼吸上皮细胞增生、融合,毛细血管肿胀、充血,鳃丝末端膨大弯曲(图版Ⅱ-9)。

2.4　超微病理变化

透射电子显微镜观察,在脑、肝脏、脾脏,眼和肠中观察到大量球状细菌,肾脏中未发现球菌,所有组织都未观察到病毒颗粒。

肝脏　　肝细胞界限模糊,细胞核膜溶解、核固缩、核沟出现,细胞质空泡化严重,染色质边集,线粒体肿大,嵴断裂、凋亡,内质网肿大,粗面内质网核糖体脱落,呈板层状排列(图版Ⅲ-1)。

脾脏　　大部分巨噬细胞中病原菌没有被消化,仍保持完整形态,可导致核固缩,甚至使巨噬细胞凋亡。严重病例可见大量细菌聚集,完全破坏细胞结构和各种细胞器,细胞膜和核膜均被溶解(图版Ⅲ-2)。被巨噬细胞吞噬的少部分细菌正在被消化溶解,在细胞质中可见有一些细菌残体,仅存少量完整细菌(图版Ⅲ-3)。

肾脏　　核溶解,核固缩,出现核沟,细胞界限模糊,线粒体增生,肿大,嵴减少、断裂、凋亡(图版Ⅲ-4)。

脑　　巨噬细胞细胞质中大量溶酶体增生,核溶解,细胞膜上有伪足,线粒体肿大,嵴断裂、凋亡,内质网肿大、断裂,粗面内质网核糖体脱落(图版Ⅲ-5)。

其他器官　　心肌纤维断裂、紊乱,线粒体肿大,嵴断裂,凋亡,细胞质空泡化(图版Ⅲ-6)。骨骼肌肌节长短不一,肌原纤维灶性溶解、断裂,Z线排列紊乱,呈锯齿状或阶梯状(图版Ⅲ-7)。肠细胞核固缩,微绒毛排列不整齐、长短不一(图版Ⅲ-8),杯状细胞周围有大量溶酶体增生。眼中有纤维性沉积(图版Ⅲ-9)。

3　讨论

临床症状的描述和之前关于罗非鱼链球菌病报道基本一致[2-7],表现出典型的链球菌病症状,如体色发黑,眼球突出,混浊发白,游姿平衡失调,体表具点状或斑块出血,内脏器官广泛出血等。病变组织中分离获得病原菌革兰氏染色呈阳性;进一步的负染电镜观察显示,菌体为球形或卵圆形,多数呈链状排列;结果证实分离的病原菌为链球菌,但因为海豚链球菌与无乳链球菌分类地位接近,病原形态相似,且都容易感染罗非鱼[23],需进一步进行 16S rRNA 分子鉴定。通过与 GenBank 数据库中的菌株进行 Blast 比较发现,此次感染罗非鱼的病原菌为无乳链球菌。通过透射电镜观察,在脑、眼、肝脏、脾脏和肠等组织中,尤其是脾脏组织中发现,有大量球菌,形态与光学显微镜下结果相似,但未发现任何病毒粒子,在细胞水平证明该菌为患病罗非鱼的致病菌。

组织病理学观察发现,患病罗非鱼主要症状是全身组织器官广泛性充血、出血,肝脏、脾脏、肾脏、脑、心、骨骼肌、肠和鳃均发生不同程度病变,细胞肿胀、变性、局灶性坏死和炎性细胞浸润,造成全身多器官功能障碍,属于全身性感染。肝细胞肿胀,变性,广泛性坏死,引起鱼体内的水、钠等的代谢紊乱,引起腹部肿大,腹水等临床症状[24]。与已有研究[21-23]不同的是,脾脏除了巨噬细胞弥漫性浸润外,还发现髓窦内含铁血黄素大量沉积,当组织内出血时,从血管中逸出的红细胞被巨噬

细胞吞入并由其溶酶体降解,使来自红细胞血红蛋白的 Fe^{3+} 与蛋白质结合成含铁血黄素,说明红细胞大量被破坏,溶血严重,造成脾脏造血功能较低,导致贫血以及免疫功能的下降,使鱼体更容易感染病原菌。肾小球毛细血管扩张引起血管球肿胀,血流受阻,致使有效循环血量降低,使肾小球血流量进一步减少,从而导致肾小球的滤过效率降低,体内渗透压及水盐失衡[7,22]。除了肾小管上皮细胞细胞水肿、坏死等变化外,肾小管内蛋白管型比较突出,其原因可能是肾小体受损后通透性失调,血浆蛋白从滤过屏障过量进入肾小管[23]。淋巴细胞浸润使脑膜增厚,导致脑膜脑炎,由于脑的损伤,导致病鱼出现泳姿失衡和在水中打转等神经性症状[19-22]。无乳链球菌还引起心肌、骨骼肌、肠和鳃等其他器官组织出现不同程度的病变。心肌纤维断裂,心肌收缩力减弱,引起心脏功能衰竭[25];骨骼肌纤维坏死,断裂,影响肌肉的活动,造成游泳障碍;肠上皮细胞坏死,造成消化吸收障碍,使病鱼的摄食能力降低,严重的甚至不食;由于鳃小片上皮和基部细胞增生,鳃小片肿大弯曲,并粘连成棒状,血管输送氧的能力降低,从而使病鱼的呼吸作用和鳃的渗透调节作用受到影响,造成病鱼呼吸障碍,加速了病鱼的死亡[23,26]。

超微病理学观察发现,病鱼主要器官的细胞均有不同程度的损伤,尤其是线粒体、内质网和细胞核的损伤特别明显,和感染海豚链球菌的罗非鱼的细胞病理学变化基本一致[23],但不同的是,除了脾脏之外,在脑、肝脏、眼和肠都发现大量的球菌。相对于无乳链球菌,Chen 等[20]认为,罗非鱼先天免疫系统能更好地抑制住海豚链球菌,无乳链球菌可能有另外的机制来逃避宿主免疫系统的清除,从而在罗非鱼组织中成功繁殖。线粒体是细胞内重要的能量代谢场所,线粒体肿胀,嵴断裂和溶解消失,线粒体受损影响能量供应,钠泵的作用受到影响,造成细胞内 Na^+ 潴留,从而使细胞外的水分过多进入细胞,导致细胞的肿胀和变性;能量供应不足还引起心、肝、脾脏和脑等重要器官的功能衰竭,并随着病程的发展,细胞损伤进入不可恢复阶段,发生广泛性坏死,造成多器官功能障碍[24]。内质网是细胞内蛋白质和脂质合成基地,粗面内质网肿胀,核糖体脱粒是蛋白质的合成受到干扰的形态学表现,蛋白质合成障碍引起肝脏和脑等重要器官的功能衰竭。细胞核的变化主要表现为变形、染色质固缩、溶解,与细胞凋亡很类似[23]。此外,心肌和骨骼肌的超微病理变化与组织病理变化一致,都表现为断裂、造成心肌收缩能力减弱和游泳障碍;而肠微绒毛排列不整齐、长短不一,造成消化吸收障碍;眼中纤维性沉积或与突眼和角膜混浊有关[21]。超微病理的研究进一步表明,无乳链球菌感染后病鱼的脾脏中的炎症反应以巨噬细胞为主,与组织病理观察结果一致[5,20,22]。在细胞超微水平上进一步的研究证明,无乳链球菌感染的链球菌病主要是以巨噬细胞吞噬来参与机体的炎症应答反应。被巨噬细胞吞噬的少部分细菌被消化溶解,大部分病原菌在巨噬细胞内仍保持完整形态,甚至使巨噬细胞凋亡。当细菌的增殖超过细胞的吞噬能力时,巨噬细胞能够携带无乳链球菌,以“特洛伊”木马的形式突破血脑屏障,进入血液系统和中央神经系统[27-28],病原菌因此能逃避鱼体免疫系统的清除作用,使链球菌更容易扩散至其他器官和组织,引起多器官功能障碍和细菌性败血症,最后导致鱼体死亡。

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Pathological changes in tilapia (*Oreochromis niloticus*) naturally infected by *Streptococcus agalactiae*

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Abstract: *Streptococcus agalactiae* was isolated and identified as the pathogen from diseased Nile tilapia (*Oreochromis niloticus*) populations in China. The pathogen was gram stained through a standard protocol, negative stained and then photoed under electron microscopy. Tissue samples from infected fish were collected and examined for pathological changes under optical and transmission electron microscopes to elucidate the mechanism of infection of *Streptococcus agalactiae* in tilapia. The result showed that the pathogen was gram positive. And sphere-shaped bacteria were alone or arranged in chains. The most marked pathology observed was severe congestion in the liver, spleen and kidney. Edema, degeneration and acute multifocal necrosis were commonly observed in many organs. There was also evidence of an infiltration of numerous inflammatory cells. Ultrastructural pathology examination indicated that *S. agalactiae* were widely distributed in many internal organs, especially in the spleen. Moreover, a series of pathological changes were found in the target tissue cells. The boundaries between the cellular compartments were less defined, resulting in the loss of a normal cytoplasmic compartmentalization. There were numerous cases in which cells containing aberrant nuclei appeared singly or in clusters. The cytoplasm often contained degenerated mitochondria, which swelled with disintegration and lyses of the cristaes. Degranulation of the rough endoplasmic reticulum as well as dilatation of the reticulum cisternae was also prevalent. Cells containing such features were frequently accompanied by vacuolar degeneration in cytoplasm. Cardiac and dorsal muscle fibers were fractured and disordered. Sarcomere length of dorsal fiber bundles differed. Microvillus arranged disorderly and in different length. Fibrin precipitated in necrotic foci of the eye. These observations indicated that *S. agalactiae* can cause simultaneous development of multiple-organ lesions with an acute systemic inflammation in the host, especially in the liver, spleen, kidney and brain, which leads to the death of the fish.

Key words: *Oreochromis niloticus*; *Streptococcus agalactiae*; pathology; ultrastructure

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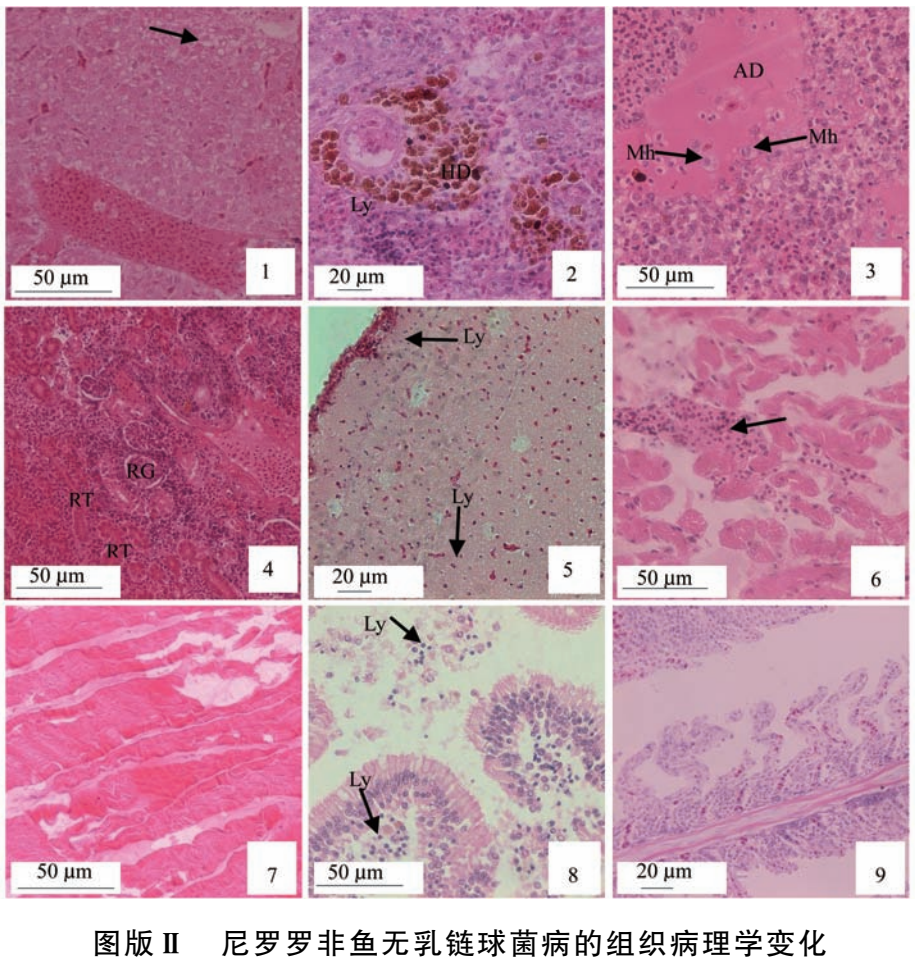


图版Ⅱ　尼罗罗非鱼无乳链球菌病的组织病理学变化

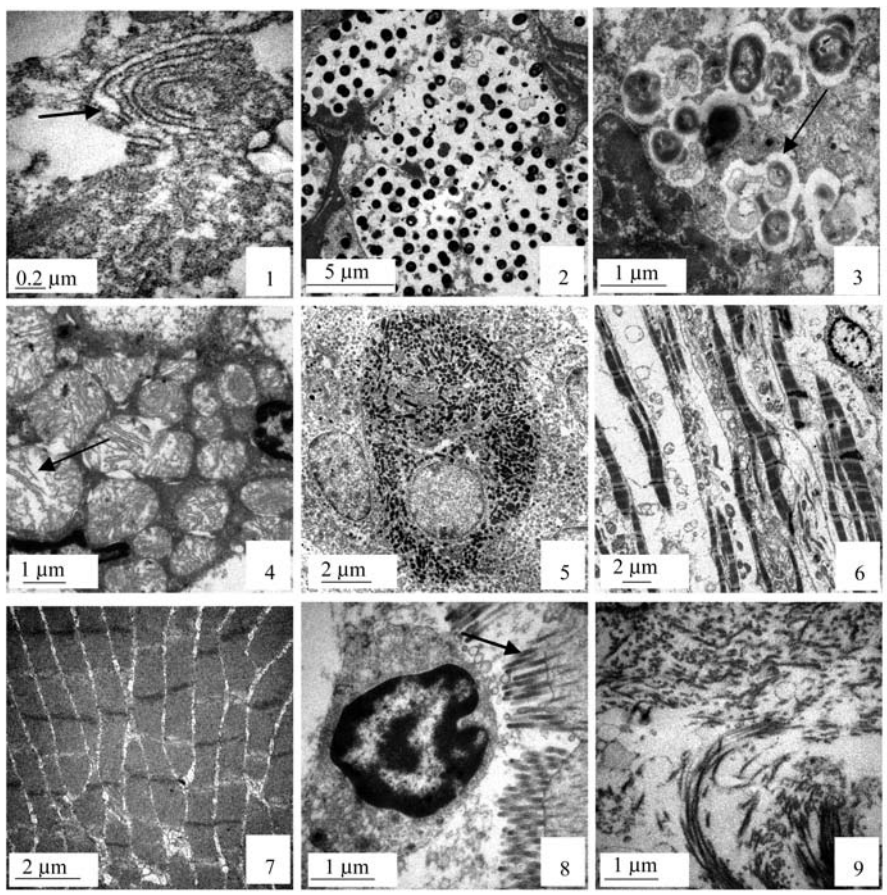
1. 肝脏充血,细胞严重空泡变性和坏死(↑),肝索排列紊乱; H. E. 2. 脾脏含铁血黄素大量沉积,中央动脉周围淋巴细胞浸润(↑); H. E. 3. 脾脏中巨噬细胞弥漫性浸润(↑),血浆蛋白渗出呈淀粉性样变; H. E. 4. 肾小管上皮细胞玻璃样变性与坏死; H. E. 5. 脑基质水肿,点状出血,疏松多孔呈海绵状,并伴有淋巴细胞浸润(↑); H. E. 6. 心肌间隙增大,断裂,红细胞浸润(↑); H. E. 7. 肌纤维断裂; H. E. 8. 肠上皮细胞坏死脱落,散落于肠腔,伴随淋巴细胞浸润(↑); H. E. 9. 鳃小片上皮细胞坏死,脱落,基底部细胞增生,鳃小片弯曲; H. E

AD:淀粉性样变; CV:中央静脉; HD:含铁血黄素 Ly:淋巴细胞; Mh:巨噬细胞; RG:肾小球; RT:肾小管

Plate Ⅱ　Histopathological changes of tilapia infected by *S. agalactiae*

1. The liver were congested and vacuolated with focal necrosis(↑), hepatic cord arranged irregularly; H. E. 2. hemosiderin deposition and lymphocytes infiltration, H. E. 3. the infiltration of macrophages and amyloidosis in the spleen(↑); H. E. 4. hyaline degeneration and necrosis appeared in renal tubular epithelia; H. E. 5. brain matrix edema, punctate hemorrhage and vacuoles with lymphocytes infiltration (↑); H. E. 6. cardiac muscle fibres twisted rupturing, arranged disorderly and numerous red blood cell infiltrates(↑); H. E. 7. skeletal muscle fibres fractured; H. E. 8. intestinal epithelial cells degeneration and necrosis, collapse, lymphocytes infiltrated in lamina propria (↑); H. E. 9. the epithelial cells of lamellae necrosis, collapse, the epithelial cells and base of lamellae cells hyperplasia; H. E

AD: amylodosis; CV: central vein; HD: hemosiderosis; Ly: lymphocyte; Mh: macrophage; RG: renal glomerular; RT: renal tubule



图版Ⅲ 尼罗罗非鱼无乳链球菌病的超微病理学变化

1. 肝细胞内质网水肿(↑),脱粒,呈板层状结构; 2. 脾巨噬细胞中大部分球菌仍保持完整形态,甚至使巨噬细胞凋亡; 3. 脾巨噬细胞中被吞噬的细菌仅留外壁,内部结构完全消失(↑); 4. 肾小管上皮细胞核畸形,线粒体增生,肿大,嵴断裂溶解(↑); 5. 脑巨噬细胞中初级溶酶体增生; 6. 心肌纤维断裂,坏死,线粒体减少; 7. 骨骼肌肌原纤维断裂,排列紊乱; 8. 肠微绒毛排列不整齐,长短不一(↑); 9. 眼中纤维性沉积

Plate Ⅲ Ultrastructural pathology changes of tilapia infected by *S. agalactiae*

1. degranulation and dilatation of rough endoplasmic reticulum in hepatocyte(↑), which were formed as confronting cisternae; 2. Most bacteria engulfed by macrophages showing no degenerative changes, even induceing apoptosis in macrophages; 3. bacteria phagocytised by macrophages, a residual structure evidently derived from the bacterial cell wall was identified(↑); 4. nuclear deformation, degenerated mitochondria proliferated and swelled with disintegration and lyses of the cristaes(↑); 5. primary lysosome proliferated in macrophages of brain; 6. cardiac muscle fibers fractured and disordered with mitochondria decreased and dissolved; 7. myofibril fractured and arranged disorderly; 8. microvillus arranged disorderly(↑); 9. fibrin precipitation in necrotic foci of eye