Relationship between virulence of *Streptococcus iniae* strains, induction of oxidative activity and cytotoxicity in rainbow trout (*Oncorhynchus mykiss*) spleen macrophages

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Abstract

In the present study, the effect of the high and low virulence *Streptococcus iniae* strains on oxidative activity and cytotoxic effects of trout spleen macrophages were studied. Spleen macrophages were collected from trout. Luminol was used as chemiluminigenic probe. Phorbolmyristate acetate (PMA) also was used as positive control activating agent of respiratory burst and significantly stimulated production of reactive oxygen species (ROS) by normal macrophages. Sensitivity of *S. iniae* strains to H2O2 and cytotoxicity of bacterial strains for trout macrophages was also determined using the antibacterial effect of H2O2 and the release of lactate dehydrogenase from infected macrophages, respectively. Spleen macrophages produced ROS upon stimulation with both bacterial strains. Approximately twofold as much ROS production was induced by the high virulence strain compared to the low virulence strain. The low virulence strain was killed approximately 6 times more by H_2O_2 compared to the high virulence strain. In spleen macrophages, the highly virulent strain caused approximately twice as much cytotoxic effects compared to the low virulent strain. Results indicate that, high virulence in *S. iniae* appears to be correlated with higher macrophage cytotoxicity and resistance to ROS. Moreover, significant differences between the strains in their capacity to stimulate the macrophages were observed ($P < 0.05$).

Keywords: rainbow trout, Streptococcus iniae, reactive oxygen species, chemiluminescence, macrophage.

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Introduction

Streptococcus iniae is a Gram-positive, sphere-shaped bacterium which most often occurs in long chains in broth culture (Austin & Austin 1999; Lau, Woo, Luk, Fung, Hui, Fong , Chow, Wong & Yuen 2006; Agnew & Barnes 2007). This bacterium is the causal agent of Streptococcusis in many fish species. Since its isolation from an Amazon freshwater dolphin, *S. iniae* has emerged as a leading fish pathogen in aquaculture operations worldwide. *S. iniae* has been reported to cause disease in more than two dozen species of fish from both freshwater and saltwater environments. This pathogen has also been associated with disease outbreaks in different commercial fish species such as rainbow trout, tilapia, channel catfish, gilthead sea bream or sea

bass (Baya, Lupiani, Hetrick, Roberson, Lukacovic, May & Poukish 1990; Zlotkin, Hershko & Eldar 1998; Soltani, Jamshidi & Sharifpour 2005; Agnew *et al*. 2007). In outbreaks of Streptococcusis, mortalities of 30 to 50% are reported (Kaige, Miyazaki & Kubota 1984; Zlotkin *et al*. 1998; Agnew *et al*. 2007;). In addition to its importance in aquaculture, *S.iniae* is capable of causing disease in humans who have recently handled infected fish from aquaculture farms (Weinstein, Litt, Kertesz, Wyper, Rose, Coulter, McGeer, Facklam, Ostach, Willey, Borczyk & Low 1997).

This bacterium was initially isolated from subcutaneous abscesses on Amazon freshwater dolphins (Pier & Madin 1976), and the pathology of the disease caused by this pathogen had been described in rainbow trout by Eldar and Ghittino (1999).

In spite of the importance of *S. iniae* as a fish pathogen and the significant increasing of the disease, the data relating to its pathogenesis to provide sufficient scientific information for an efficient control strategy, and the first step of the interaction between this pathogen and fish, are lacking. So far there is limited information available about the interaction of this bacterium and immune defence mechanisms of the fish.

It has been postulated previously that macrophages are extremely important in specific as well as non-specific immune responses against invading microorganisms. Spleen has also shown to be a rich source of macrophages in fish (Secombes & Fletcher 1992; Ellis 1999). Respiratory burst generated by macrophages has been recognized as one of the major contributors to the killing of pathogens. This phenomenon is accompanied by the production and release of highly reactive oxygen metabolites. Reversion of the unstable oxygen metabolites to their ground state is responsible for the emission of photons, which has been termed chemiluminescence (Rossi, Bianca & de Togni 1985). Additionally, it has been found that the spleen tissue from naturally infected fish with *S. iniae* revealed the presence of phagosomes and residual bodies indicative of extensive lysosomal activity within the phagocytes (Bromage & Owens 2002; Kaige *et al*. 1984). However, the appearance of numerous encapsulated coccus bacteria and extensive degeneration of the spleen tissue suggested that the response to the pathogen was not effective (Eldar, Frelier, Assenta, Varner, Lawhon & Bercovier 1995; Agnew *et al*. 2007; Soltani, Nikbakht, Mousavi & Ahmadzadeh 2008).

In the present study, the effect of a high and a low virulence *S. iniae* strains on oxidative activity and cytotoxic effects of trout spleen macrophages were investigated. This study aims at deciphering in greater depth of the association between bacterial virulence and interactions of *S. iniae* with trout macrophages.

Materials and Methods

Fish

Twelve rainbow trout (mean weight 750 g) were obtained from a commercial fish farm, and kept in a flow through system (1000L tank) containing aerated well water (14-16°C) for two weeks prior to experimentation. Fish were fed daily with a commercial diet. All fishes were clinically healthy and found to be free of external parasite infestations. The presence of *S. iniae* was assessed by streaking swabs from the spleen and kidney onto tryptic soy agar (TSA) supplemented with 5% de-fibrinated sheep blood. Plates were incubated at 27-30°C for 24–48 h (Soltani *et al*. 2008). *S. Iniae* was not isolated from randomly selected fish.

Macrophages

Spleen macrophages were collected according to the procedures of Nematollahi, Pasmans, Haesebrouck & Decostere (2005). Briefly, fish were euthanized using an overdose of a solution of benzocaine (ethilaminobenzoate) in ethanol (1g/10 mL). The spleen (3±1 g) was aseptically removed and pushed through a 150 µm nylon mesh (Solana NV, Schoten) with Dulbecco's Modified Eagle Medium (DMEM, Gibco) containing 5% Foetal Calf Serum (Jitegro, aaDierea), 1% non-essential amino-acids (Gibco), 1% Glutamine (Gibco), 100 IU mL^{-1} penicillin (Gibco) 100 mg mL^{-1} streptomycin (Gibco), 100 mg mL^{-1} kanamycine sulphate (Gibco) and 10 IU mL^{-1} heparin. Then, the cell suspension was

layered onto a discontinuous 34% / 51% Percoll gradient (Pharmacia Biotech). After centrifugation (400 × g, 25 min, 4°C), the band of the cells lying at the 34-51% interface of Percoll suspension was collected and washed once with sterile Hanks' balanced salt solution (HBSS; Gibco) without Ca/Mg. For identification of cells, air-dried smears of the different samples were stained with Heamacolor® stain (Merck) and observed under the light microscope at magnification x 1000. The presence of non-specific esterase activity was determined using the α-naphtyl butyrate esterase staining kit (Sigma Diagnostic). The cells were counted using a Burker counting chamber and their viability was determined by exclusion of trypan blue. The cells were then adjusted to 10^7 macrophages mL^{-1} and 100 µL volumes were cultivated in 96-well plates. To assess whether spleen macrophages adhere well to the 96-well plate, the cells seeded in plate with flat bottom (Iwaki Microplate) and incubated for 24 h at 17°C and 5% CO_2. After gently rinsing two times with PBS, the cells were harvested by adding 20 µL trypsin solution per well, based on 88 mL trypsin diluent (NaCl 8 g, KCl 0.2 g, KH2PO4 0.12 g, NaH2PO4 0.91 g, phenolred 0.5% and aqua dest 1000 mL), 10 mL trypsin stock 2.5% (Gibo) and 2 mL versenate (2 g EDTA in 100 mL trypsin diluent). After 10 min incubation (37°C, 5% CO2), the cells were re-suspended in 100 µl DMEM and counted using a Burker counting chamber.

Bacterial strains

Two *S. iniae* strains were used: Strain 1(high virulent) and Strain 2 (low virulent). Strain 1 was isolated from trout spleen and kidney in a Streptococcusis outbreak in Fars province with 50% mortality, Iran (Akhlaghi & Keshavarzi 2002). Strain 2 was recovered in Shahrekord, Iran from the internal organs of rainbow trout with 10% mortality. In order to preserve virulent properties, both strains were stored virtually immediately following *in vivo* isolation. After intraperitoneal inoculation, Strain 1 proved to be of highly virulent whereas Strain 2 was found to be of low virulent (Akhlaghi & Keshavarzi 2002). The isolate was grown in tryptic soy broth for 24 h at 27-30°C, and then the culture was frozen at -70 °C in 0.2 mL aliquots with 15% glycerol (v/v). After thawing, the bacteria were grown for 24-48 h in 4 mL of tryptic soy broth at 27-30°C. Subsequently, the cultured broth was centrifuged (3000 g, 10 min, 15°C) and the resulting pellet and supernatant were separated. The pellet was re-suspended in DMEM medium without phenolred. The number of colony forming units (CFU) was determined by plating 10-fold serial dilutions on TSA supplemented with 5% de-fibrinated sheep blood plate (Soltani *et al*. 2008).

Oxidative activity of spleen macrophages

The generation of O_2- by rainbow trout macrophages (ROS production) was determined by chemiluminescence (CL) assay (Rossi *et al*. 1985). The assay was performed in a microplateluminometer (Thermo Labsystems), using sterile 96-well plates with clear bottom (Greiner bio-one GmbH) at 27°C. Luminol (Sigma Biosciences) was used as chemiluminigenic probe. Luminol was dissolved in dimethyl sulfoxide (Sigma) to give a concentration of 104 mM. Prior to use, the luminol stock was thawed and diluted in HBSS to final concentration of 200 µM. After 24 h incubation at 16°C and 5% CO_2 allowing adherence of the cells, the cell culture medium was replaced by 100 µl HBSS and 25 µl of diluted luminol solution in HBSS. Then, the plates were placed in luminometer and the CL background value was recorded for 10 min. Data were collected at 2 min interval times. After stabilisation of the background value, 50 µl of either of the bacterial strains to be tested, were added per well (first stimulation). The addition of 50 µl of phorbolmyristate acetate (PMA) (Sigma), final concentration 70 µg mL^{-1}, to non-infected macrophages was used as positive control. Negative control samples were also included in each CL assay. The plates then were placed in luminometer and the CL value was recorded for 2 h. In order to assess the macrophages viability after the first stimulation, PMA (25 µl) with concentration 160 µg mL^{-1} were added to both control and infected wells and the CL responses measured for 1 h (second stimulation). Each CL assay was repeated four times in triplicate, depending on the macro-

phage yield. The ROS productions are presented as area under the curve (AUC).

Sensitivity of *S. Iniae* strain to H_2O_2

In order to determine the role of ROS in bacterial killing, the antibacterial effect of H_2O_2 was evaluated. Briefly, 5 mL of tryptic soy broth containing 40 mM H_2O_2 were mixed with 5 mL of either of the bacterial suspensions containing 108 CFU mL^{-1} and incubated at 27°C. The number of CFU was enumerated at t = 30 and t = 60 min after incubation time by plating ten-fold serial dilutions on TSA plates in triplicate (Barnes, Bowden, Horne & Ellis 1999; Nematollahi *et al*. 2005).

Cytotoxicity of *S. iniae* strains for rainbow trout macrophages

In order to measure cytotoxic effects of a low and a high virulent strain of *S. iniae* to spleen macrophages, the release of lactate dehydrogenase (LDH, Roche) from infected macrophages was assessed according to the method of Korzenieweski and Callewaert (1983) with some modifications. Inoculation of the macrophages was performed as described previously. Un-inoculated wells were used as negative control. Positive control samples consisted of macrophages in 175 µl medium, lysed with 25 µl of 2% Triton X-100 for 15 min. Following centrifugation (250 × g, 10 min, 4°C) the LDH level of the supernatant was determined according to the manufacturer's instructions. The absorbency at 492 nm was recorded Spectrophotometrically. The percentage cytotoxicity was calculated adopting the following formula:

Cytotoxicity =Absorbency – Average absorbency negative control ×100 / Average absorbency positive control – Average absorbency negative control

Statistical analysis

The results of each experiment were compared using the one way ANOVA test (computer program Sigma Stat, Analytical Software). Results were expressed as mean ± standard error of experiments. A value of $P < 0.05$ was considered statistically significant.

Results

Collection of macrophages

Approximately 10^7 macrophages were harvested from each rainbow trout spleen. The average proportion of esterase positive cells (macrophages) was more than 95% of the cells in suspension. The viability also exceeded 98%. No significant differences in adhesive capacity were noted between the spleen macrophages before and after rinsing.

Oxidative activity of spleen macrophages

Results of ROS production were given in Figure 1. Spleen macrophages produced ROS upon stimulation with a high and a low virulence strain of *S. iniae* as measured using CL. PMA was used as a positive control activating agent of the respiratory burst and this significantly stimulated ROS production by normal macrophages (Fig. 1). However, the level of ROS during the first stimulation (after 2 h) following infection with strain 1 was higher than strain 2. Similarly, significant differences were found in area under the curve (AUC) values between *S. iniae* strains and PMA ($P < 0.05$). A twofold higher ROS production was induced by the high virulence strain compared to the low virulence strain ($P < 0.05$). All negative controls included in the first stimulation of each CL assay gave low AUC values. Likewise, overall ROS production was demonstrated a significant difference between high and low virulence strains ($P < 0.05$). Regarding the viability of macrophages after each CL assay, the macrophages could still be stimulated by PMA, 1 h later (second stimulation). The addition of PMA to the infected macrophages did not increase ROS production above the level of control non-infected macrophages and the level of ROS was significantly lower than control ($P < 0.05$) (Fig. 2).

Sensitivity of *S. iniae* strain to H_2O_2

Results of bacterial survival of two *S. iniae* strains after exposure to H^2O^2 were summarized in Figure 2. As shown, both strains were viable after 30 and 60 min, respectively. The low virulence strain was killed approximately 6 times more than the high

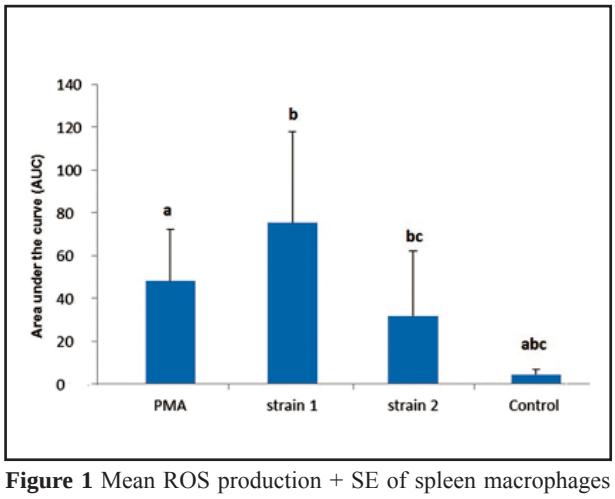

Figure 1 Mean ROS production + SE of spleen macrophages from rainbow trout after exposure to PMA, a high (Strain 1) or of a low (Strain 2) virulence strain of *S. iniae*. Data represents the mean of 5 independent experiments (+SE). The same letter (a–c) refers to a statistically significant difference ($P < 0.05$).

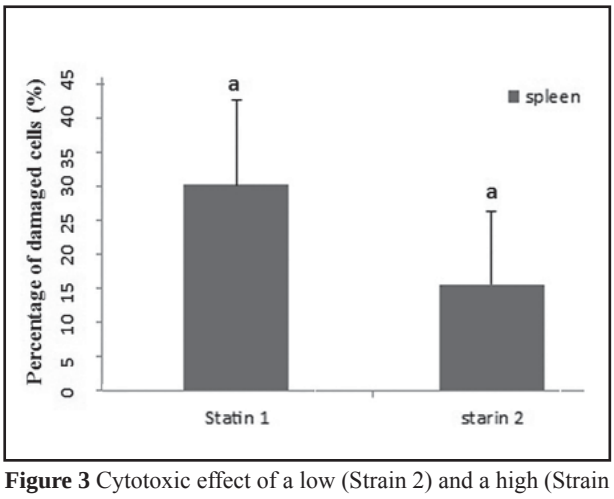

Figure 3 Cytotoxic effect of a low (Strain 2) and a high (Strain 1) virulence strain of *S. iniae* on spleen macrophages from rainbow trout. The data are presented as mean % of damaged cells + SE of 4 independent experiments. The same letter refers to a statistically significant difference ($P < 0.05$).

virulence strain ($P < 0.05$) over the 30 and 60 min periods.

Cytotoxicity of *S. iniae* strains for rainbow trout macrophages

Both *S. iniae* strains were cytotoxic for spleen macrophages (Fig. 3). In spleen macrophages, the highly virulent strain caused twice as much cytotoxic effects compared to the low virulent strain ($P < 0.05$).

Discussion

Fish rely on both specific and non-specific immune

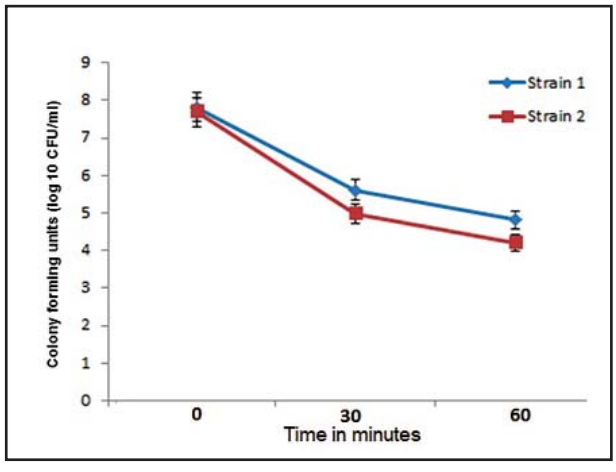

Figure 2 Survival of a low (Strain 2) and a high (Strain 1) virulence strain of *S. iniae* in the presence of H2O2. The data are presented as the average log (10) of the number of CFU between 0, 30 and 60 min after exposure ± SE.

mechanisms to protect themselves against various pathogens. Macrophages are the most important cells involved defense mechanisms in fish. Several ROS are produced by fish macrophages during the oxidative activity such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), singlet oxygen ($1O_2$) (Ellis 1999; Secombes & Fletcher 1992). ROS establish an important component of the innate immune response against invading pathogens by fish macrophages. Therefore, fish pathogens must overcome the toxic effects of ROS to establish infections. Bacteria have evolved systems to protect themselves from these toxic radicals. One of these protective pathways involves the production of detoxifying enzymes such as superoxide dismutases (SODs) and catalases. Productions of SOD and catalase enzymes have been reported to contribute to the virulence of a great number of fish pathogens (Austin & Austin 1999; Barnes *et al*. 1999; Ellis 1999; Barnes, Young, Horne & Ellis 2003; Nematollahi *et al*. 2005).

In the present study, trout spleen macrophages produced ROS upon stimulation with both *S. iniae* strains. A twofold higher ROS production was induced by the high virulence (strain 1) compared to the low virulence one (strain 2). However, when the macrophages were infected with the low virulence strain, the ROS production was suppressed compared to stimulate with high virulence strain

and non-infected control macrophages. This lack of induction of ROS was observed both at first and second stimulation. This finding suggests that ROS production was inhibited by the low virulence strain or alternatively that this strain did not activate the production of ROS. The latter seems unlikely as the infected macrophages were unresponsive to PMA, suggesting inhibition or exhaustion of ROS.

Our finding is in agreement with Buchanan, Colvin, Vicknair, Patel, Timmer & Nizet (2008) who reported that the a virulent strain of *S. iniae* was susceptible to reactive oxygen species and rapidly killed within macrophages. The avirulent strain was found to be markedly more susceptible to killing by hydrogen peroxide and singlet oxygen than other virulent strains.This group also reported that the loss of virulence in strain of *S. iniae* is not associated with decreased hemolytic activity, cellular adherence, or intracellular invasion (Buchanan *et al*. 2008). Several studies have reported the high affinity of high-virulent strains of *S. iniae* to macrophage reach organs such as kidney, spleen and liver. However, this strong affinity is not resulted to higher killing by macrophages (Akhlaghi & Mahjor 2004; Soltani *et al*. 2005). The results of the present study revealed a lower sensitivity of the high virulence strain to killing by H_2O_2 compared to the low virulence strain. It seems that in spite of stronger induction of ROS by spleen macrophages against high virulence strain, this strain was killed to a lesser extent (Soltani *et al*. 2005).

Several virulence factors have been identified in *S. iniae* species. The first one which present in all isolates is capsule however, in serotype II, this part is providing more coverage of surface antigens, thereby conferring additional anti-phagocytic properties. Capsule has also been shown to play an important role in resistance to phagocytic clearance by fish macrophages (Barnes *et al*. 2003; Buchanan, Stannard, Lauth, Ostland, Powell, Westerman & Nizet 2005). Protection against the macrophage bactericidal mechanisms enables the bacteria to reside inside these cells, protected against both humoral response and host complement. Therefore, resistance to bacterial killing by the fish macrophages may constitute an important virulence factor of *S. iniae* confirming the results of Buchanan *et al*. (2008). Based on our results, the spleen macrophages produced more ROS and killed more high virulent bacteria than the low virulent one. Interestingly, *S. iniae* bacteria proved to be cytotoxic for rainbow trout macrophages, resulting in fairly high losses of viable macrophages at 2 h post inoculation. This finding suggests that spleen macrophages are more tolerant for harbouring the low virulent *S. iniae* bacteria and hence may constitute a safer site for this bacterium to reside in. This hypothesis is supported by earlier studies, in which the spleen proved to be one of the target organs of the *S. iniae* (Akhlaghi & Mahjor 2004; Soltani *et al*. 2005). However, it is important to study the interaction of macrophages with live bacteria as many pathogens can subvert ROS and survive in macrophages such as Renibacteriumsalmoninarum (Campos-Pérez, Ellis & Secombes 1997), Aeromonassalmonicida (Barnes *et al*. 1999), Vibrio anguillarumserogroup O1 and O2a (Boesen, Larsen, Larsen & Ellis 2001), Flavobacterium psychrophilum (Nematollahi *et al*. 2005). Likewise, there is very few information available on the interaction of salmonid macrophages with *S. iniae* and the role of ROS in bactericidal activity of macrophages. Buchanan *et al*. (2008) demonstrated that *S. iniae* bacteria were able to induce ROS in phagocytes. In this process, they found that the metabolites and/or bacterial components of *S. iniae* were able to stimulate phagocytes and they did observe significant differences between the strains in their capacity to stimulate the phagocytes. Indeed, adherence of the pathogen to the phagocyte membrane or vice versa can be sufficient to cause the release oxidative radicals. It was speculated that the differences between *S. iniae* strains may be present in the stages following initial contact of the bacterium phagocyte interaction reflecting an inequality in virulence (Buchanan *et al*. 2008).

Rainbow trout macrophages also generate H_2O_2 during the oxidative burst (Secombes, Chung & Jeffries 1988). This study revealed that the susceptibility of low virulence strain was not similar to that of high virulence strain. There also seems to be a

difference in induction of H_2O_2 by the two *S. iniae* strains. However, unlike the high virulence strain, the resistance of low virulence strain to H_2O_2 was not up-regulated when exposed to H_2O_2 during growth. This might be a necessary in vivo resistance mechanism to avoid macrophage killing that the low virulence bacterium does not possess and therefore it will have less chance of surviving in the fish than high virulence strain. Several studies demonstrated H_2O_2 production by trout macrophages infected with *V. anguillarum* and *A. Salmonicida* (Boesen *et al*. 2001; Barnes *et al*. 2003).

In conclusion, the present study documented that high virulence in *S. iniae* strains appears to be correlated with higher macrophage cytotoxicity and resistance to ROS and, therefore, with enhanced resistance to bacterial killing by rainbow trout spleen macrophages.Additional study is needed in regard to development of in vitro assays to assess virulence factors of *S. iniae*.

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مطالعه ارتباط بین حدت سویه‌های استرپتوکوکوس اینیایی (*Streptococcus iniae*) با القاء فعالیت اکسیداتیو و سمیت سلولی در ماکروفاژهای طحال قزل آلای رنگین کمان (*Oncorhynchus smykiss*)

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چکیده

در مطالعه حاضر تاثیر سویه‌های پر حدت و کم حدت استرپتوکوکوس اینیایی بر روی فعالیت‌های اکسیداتیو و اثرات سیتوتوکسیک ماکروفاژهای طحال ماهی قزل آلا بررسی گردید. بدین منظور نسبت به جمع آوری ماکروفاژهای طحال از قزل آلا اقدام واز لومینول به عنوان کاوشگر کمیلومینسانس استفاده شد. استات فوربول میریستات (PMA) نیز به عنوان کنترل مثبت فعال کننده عامل انفجار تنفسی استفاده شد و بطور قابل توجهی تولید گونه‌های اکسیژن فعال (ROS) توسط ماکروفاژها عادی را تحریک کرد. حساسیت سویه‌های استرپتوکوکوس اینیایی به پراکسید هیدروژن (H_2O_2) و اثر سمیت سویه‌های باکتریایی برای ماکروفاژهای ماهی نیز به ترتیب با استفاده از اثر ضد باکتریایی H_2O_2 و انتشار لاکتات دهیدروژناز از ماکروفاژهای آلوده مشخص شد. به منظور تعیین نقش ROS در کشتن باکتری، اثر ضد باکتری پراکسید هیدروژن برای هر دو سویه مورد ارزیابی قرار گرفت. مقدار تولید ROS ناشی از سویه پر حدت باکتری، تقریبا دو برابر سویه کم حدت آن بود. سویه کم حدت باکتری حدود ۶ برابر بیشتر توسط H_2O_2 نسبت به سویه پر حدت کشته شد. در ماکروفاژهای طحال، سویه پر حدت تقریبا دو برابر اثرات سیتوتوکسیتی در مقایسه با سویه کم حدت باکتری ایجاد کرد. نتایج نشان می‌دهد که میزان زیاد حدت در استرپتوکوکوس اینیایی با سمیت سلولی بیشتر در ماکروفاژ و نیز مقاومت در برابر ROS ارتباط داشته باشد. علاوه بر این، تفاوت معنی داری بین سویه‌های باکتری درظرفیت تحریکی ماکروفاژها مشاهده گردید.

واژه‌های کلیدی: قزل آلای رنگین کمان، استرپتوکوکوس اینیایی، گونه‌های اکسیژن فعال، کمیلومینسانس، ماکروفاژ.

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