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Apoptosis in experimentally infected chicks with *Salmonella gallinarum*

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Abstract

This experiment was performed to investigate apoptosis during undergoing patho-genesis of *Salmonella gallinarum*(SG)-infected chicks. 16 days old, 49 chicks was infected with SG (10^6-10^8 CFU/㎖) experimentally, they were autopsied to remove liver, spleen, intestine and lung at 1, 6, 12 hr, 1, 2, 4 and 7 day post infection(PI) respectively, for H-E and TUNEL staining. Grossly, white foci in the liver and enlarged spleen were seen on 4 day PI and coppery bronze liver, dark-red discolored intestine, green-yellowish discolored and enlarged spleen was observed on 7 day PI. Histopathologically, multi focal necrosis in the liver, follicle hyperplasia in the spleen and inflammatory cells infiltration in the intestine were shown from 2 day PI and more severely observed on 4 day and 7 day PI. In TUNEL analysis, apoptotic cells reached a maximum at 6 hr PI in the liver and intestine and at 12 hr PI in the spleen, and then decreased the levels of controls by 7 day PI.

Key words : Aopptosis, *Salmonella gallinarum*, Histopathology, TUNEL stain

Introduction

Salmonellae are gram negative bacilli and intracellular pathogens. After consumption of contaminated food or water, *Salmonellae* adhere to epithelial cells(M cell). Once the infected M cells of the ileac Peyer's patches are destroyed[1]. And then the bacteria gain access to the subepithelial lymph tissue and the lamina propria where they encounter macrophages, lymphocytes and neutrophils. Surviving this encounter is the key to a successful infection as many other facultative intracellular pathogens[2]. A novel set of *Salmonella* virulence gene located in an operon which was denoted *sip*(*Salmonella* invasion protein) containing five genes *sipE*, *sipB*, *sipC*, *sipD*, and *sipA*[3-5]. Invasive *Salmonella* induces the formation of membrane ruffles localized at the contact point between bacterium and host cell requiring *sip*B

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and is ultimately taken up in large vacuoles[6]. These vacuoles do not acquire lysosomal markers and may therefore represent a relatively safe intracellular site in which the bacteria can survive and multiply[7,8], consequently disseminating to the spleen and liver[9].

Salmonella gallinarum(SG) is causal organism of fowl typhoid(FT). Gross and microscopic lesions due to SG in chicks and poults include coppery bronze liver, enlarged spleen, dark brown bone marrow hepatitis, splenitis, typhlitis, omphalitis, myocarditis, ventriculitis, pneumonia, synovitis, peritonitis and ophthalmitis[10]. Transovarian infection resulting in infection of the egg and sub sequently the chick or poult is one of the most important modes of transmission[10]. FT can be detected serologically by use of a macroscopic tube agglutination test, rapid serum test, stained antigen whole blood test or microagglutination test[10]. Although FT is widely distributed throughout the world, the disease has been eradicated from commercial poultry in developed countries such as the United States of America, Canada and most countries of Western Europe[11]. FT steel has not been eradicated and is important disease in the poultry industry of Korea.

Apoptosis[12] is genetically programmed cell death[13,14] and a complex process that removes aging or injured cells from body[15] with features that distinguish it form necrosis. Apoptosis cells usually shrink and condense, display surface alterations and cleave DNA into large and often small oligonucleosomal-sized(200-bp) fragments that form a ladder pattern on agarose gels while organelles and the plasma membrane retain their integrity requiring ATP and the activation of specific proteases[16]. Necrotic death, on the other hand, comprises cell and organnelle swelling and ultimately followed by cell dissolution. As a result, necrosis is strongly proinflammatory *in vivo,* whereas apoptosis cells are rapidly phagocytosed and thus generate minimal inflammation[16]. It is conceivable that apoptotic pathways converge to one or very few common final executive steps. These comprise the tumor suppressor p53 or the regulatory role of Bcl-2 family members[17].

Salmonella infection induces apoptosis by stimulation of cells with cytokines(eg IFN-r in combination with TNF-*a*)[18,19] or by ligation of Fas receptor on the cell surface[20] and increases production of inducible nitric oxid synthase(iNOS) and nitric oxid(NO) generated at a high quantity by activated macrophages[21]. In general, it is appreciated that a massive production of NO initiates cell injury. NO-evoked death follows morphological and biochemical features that charaterize apoptosis[22,23]. In addition, invasive *Salmonella* induces macrophage apoptosis *in vitro*[24~27] and *in vivo*[28]. *Salmonella*-mediated macrophage apotosis requires sipB essentially[29,30] to activate capases which are the effector molecules of the apoptotic program.[31,32] *Salmonella* shares the ability of inducing macrophage apotosis with *Yersinia*[33,34] and *Shigella*[35] suggesting that this may represent a hallmark of, and perhaps a selective advantage for, the establishment of enterobacteria infection[36].

Apoptosis is inhibited or increased depending on kinds of diseases. The infectious diseases associated with inhibition of apoptosis are enterovirus infection[37], *papillomavirus* in woman uterine[38] and the infectious diseases associated with increased apoptosis are AIDS[39], *Salmonella typhimurium*[36], *Salmonella typhi* in human keratinocytes[40], *Escherichia coli*[41], *Shigella*

flexneri[42], *Helicobactor pylori* in human gastric epithelial cells[43], *Cytomegalovirus* in human retina[44], *Corinebacterim diphtherae*, *Bordetella pertussis* and *Listeria monocytogenes*[45].

Though a lot of studies have been reported about pathogenesis with *Salmonellae*, there are few studies of apoptosis with SG in chicks. In this study, we investigated the effects of SG-infection on apoptosis in various organ at early phase during undergoing pathogenesis in chicks.

Materials and Methods

Animals and Bacteria

This study used 16 days old chicks and *Salmonella galliarum*(SG) provided by Deasung Microbiological Lab Co LTD (Anyang, Korea). SG was cultured with brain heart infusion for 24h and diluted with saline into 10^6-10^8CFU/*ml*. 1*ml* brain heart infusion(10^6-10^8CFU/*ml* SG) was inoculated into 49 chicks and sterilized brain heart infusion was administrated to 21 controls orally. 1, 6, 12 hr, 1, 2, 4 and 7 day after infection, seven infected and three controls chicks were autopsied respectively and liver, intestine, lung and spleen were removed.

Histopathological analysis

Organs are fixed with 10% neutral buffered formailin, embede in paraffin, and serial sectioned to 5㎛ thickness. The sections were stained with H-E for light microscopic examination.

TUNEL staining

The sections were held with the ApopTag plus Peroxide Kit(Intergen, NY, USA) in a coplin jar. Paraffin sections were deparaffinized and pretreated with proteinase K(20 ㎍/㎖, Sigma Chemicals, NY, USA). After washing in phosphate buffered saline(PBS), sections were quenched endogenous peroxidase in 3.0% hydrogen peroxide and applied working strength TdT enzyme (incubation at 37℃ for 1 hr). The reaction was stopped by putting PBS. The sections were then incubated in a humidified chamber with Anti-Digoxigenin conjugate for 30 min at room temperature. After 4-times washing in PBS, the sections were applied with enough peroxidase substrate to develop color. And counterstained with hematoxylin, according to standard procedure.

Statistical analysis

TUNEL-positive cells counted at three fields of the sections made from every specimen each time were average out and expressed as number of TUNEL-positive cells per 1,000 cells. As no significant difference was among control groups during experiment, the number of TUNEL-positive cells in controls were combined. To compare the difference of treatment and control chicks, the results were analyzed with one-way analysis of variance and presented the level of significance as $p<0.01$.

Results

Clinical signs and gross lesions

First death was 2 day post infection(PI) without clear lesions. Total 5 infected chicks were died during the study, which were role outed from the results. From 5 day PI, chicks showed clinical signs such as drop in food consumption, depression and thin and greenish yellow feces. Grossly, the liver showed swelling, friability, multifocal white foci and

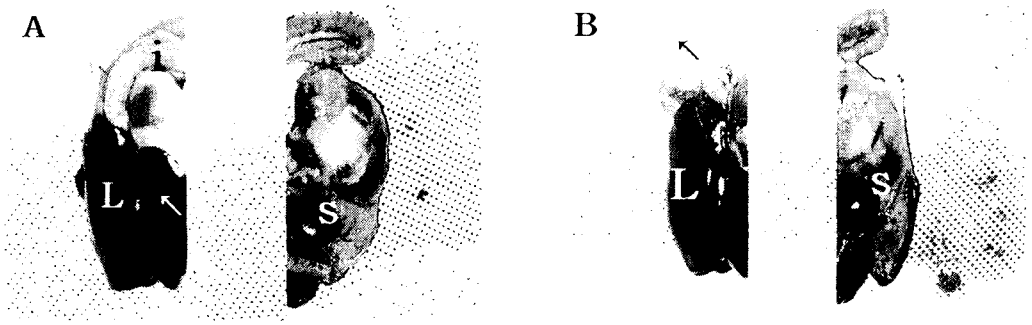

Fig 1. Gross lesions of chicks infected with *Salmonella gallinarum*.
A. 4 day PI, showing white foci (↑) of the liver (L), enlarged spleen (s) and congested intestine(i).
B. 7 day PI., showing coppery bronze liver (L), dark-red discolored intestine (i) and green-yellowish spleen (s).

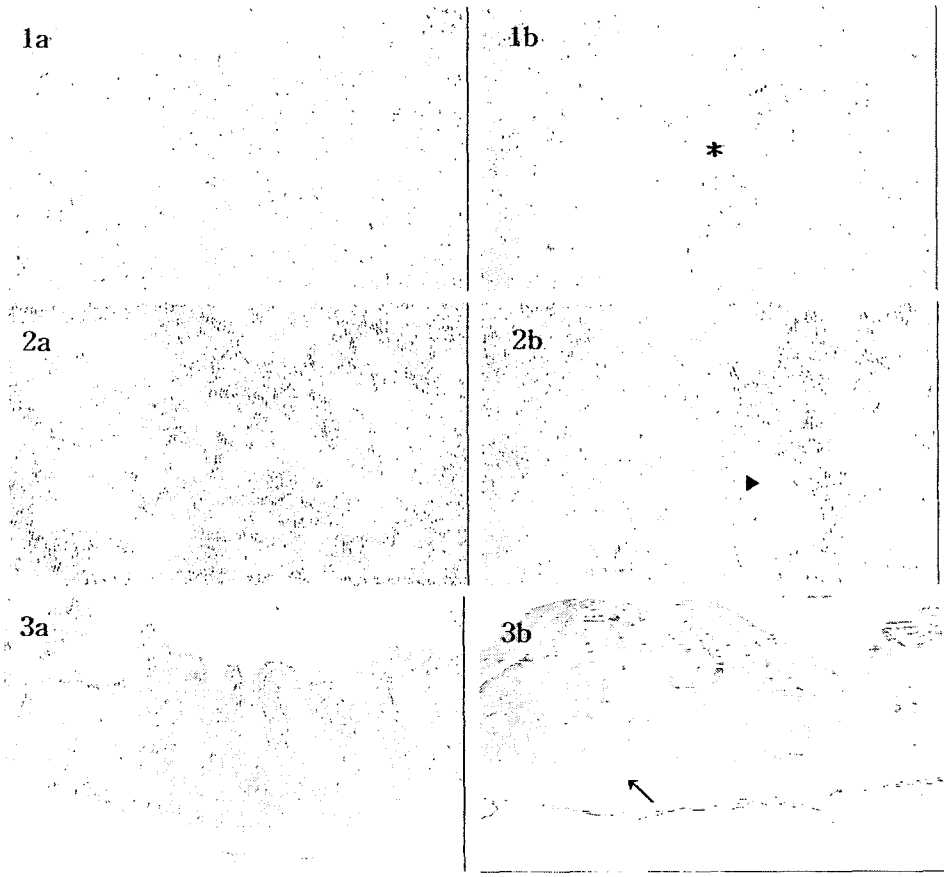

Fig 2-1. Liver : a, 6 hr PI, showing no specific changes(× 200). b, 2 day PI, showing mild necrosis (*) of liver parenchyma. H-E, × 100.
Fig 2-2. Spleen : a, 12 h PI., showing no specific changes. b, 2 day PI., showing mild follicle hyperplasia(▶). H-E, × 100.
Fig 2-3. Intestine : a, 6 hr PI, showing no specific changes. b, 7 day PI, showing mild inflammatory cells infiltration(↑) in lamina propria and crypt of vili. H-E, × 100.

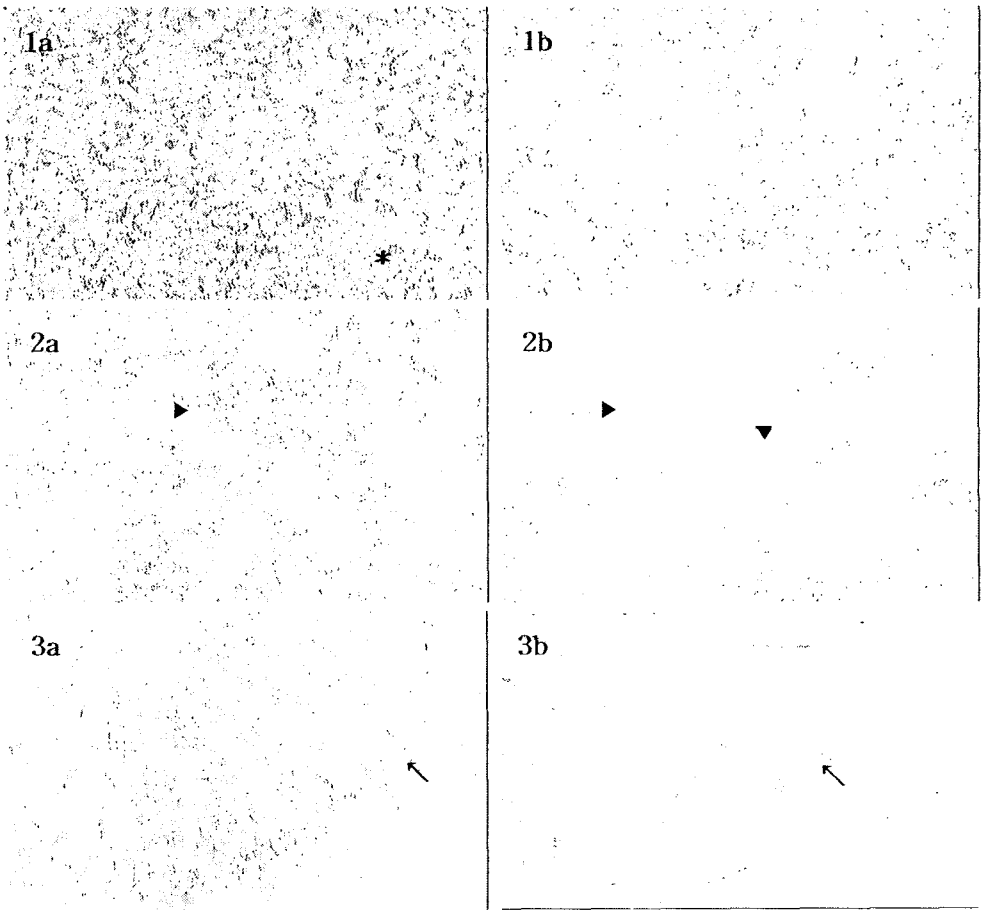

Fig 3-1. Liver : a, 4 day PI, showing multi focal necrosis (*) and congestion. b, 7 day PI, showing severe necrosis (*) of liver parenchyma. H-E, × 200.

Fig 3-2. Spleen : a, 4 day PI, showing marked follicle hyperplasia (▼). b, 7 day PI, showing severe follicle hyperplasia (▼). H-E, × 200.

Fig 3-3. Intestine : a, 4 day PI, showing inflammatory cells infiltration (↑) in lamina propria and crypt of vili. b, 7 day PI, showing large cysts (↑) in the mucosa. H-E, × 100.

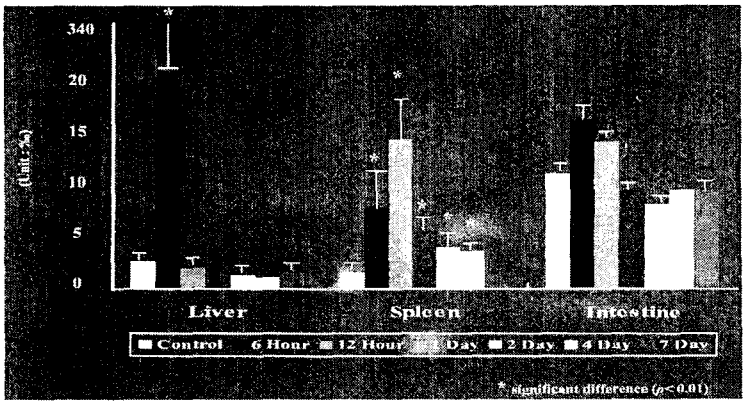

Fig 4. Summary of apoptotic cells in organs

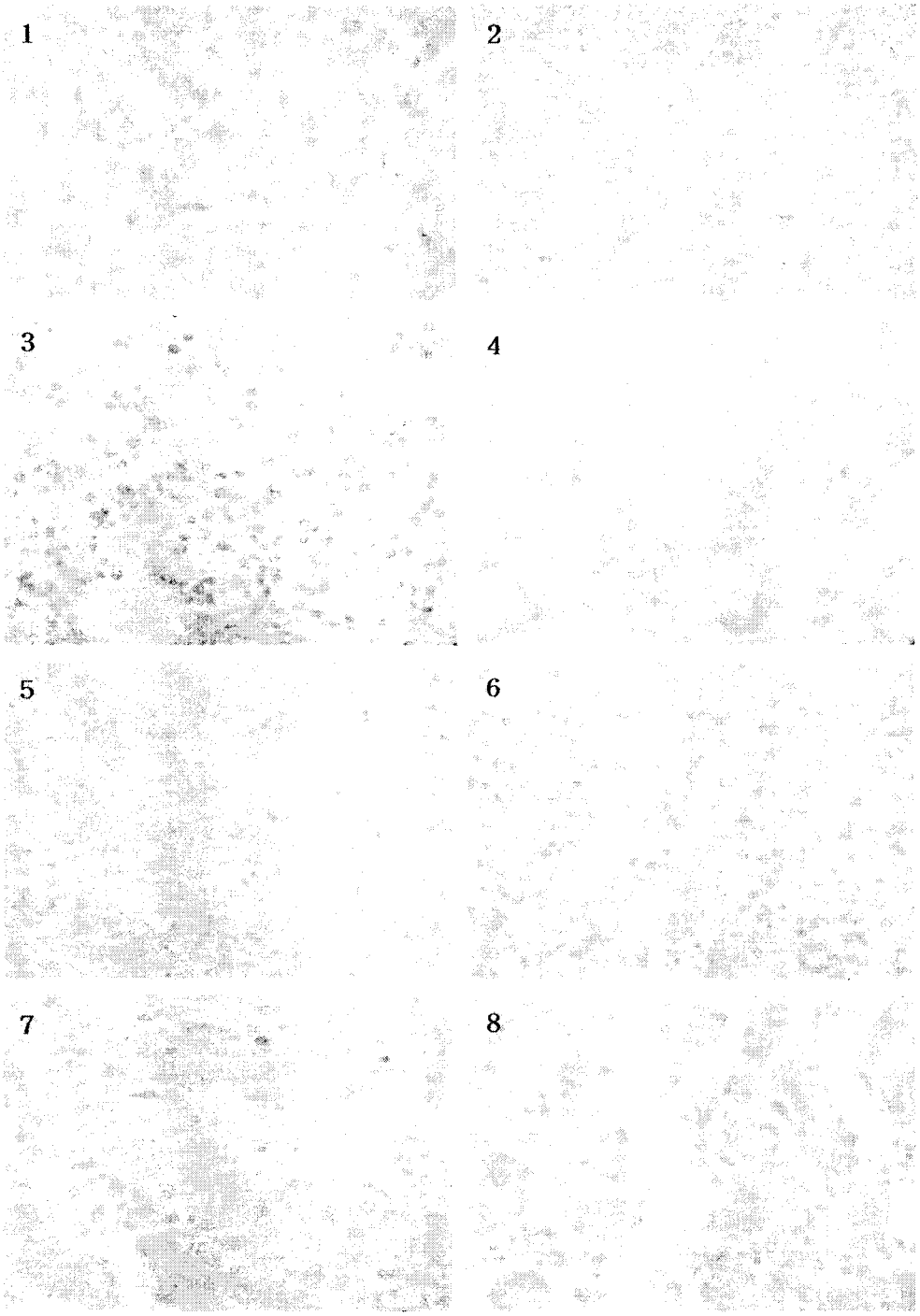

Fig 5. Liver, showing increase of apoptotic cells on 6 hr PI and decrease with time-course(1 : contro 2 : 1 hr, 3 : 6 hr, 4 : 12 hr, 5 : 1 day, 6 : 2 day, 7: 4 day, 8: 7 day). TUNEL stain, × 400.

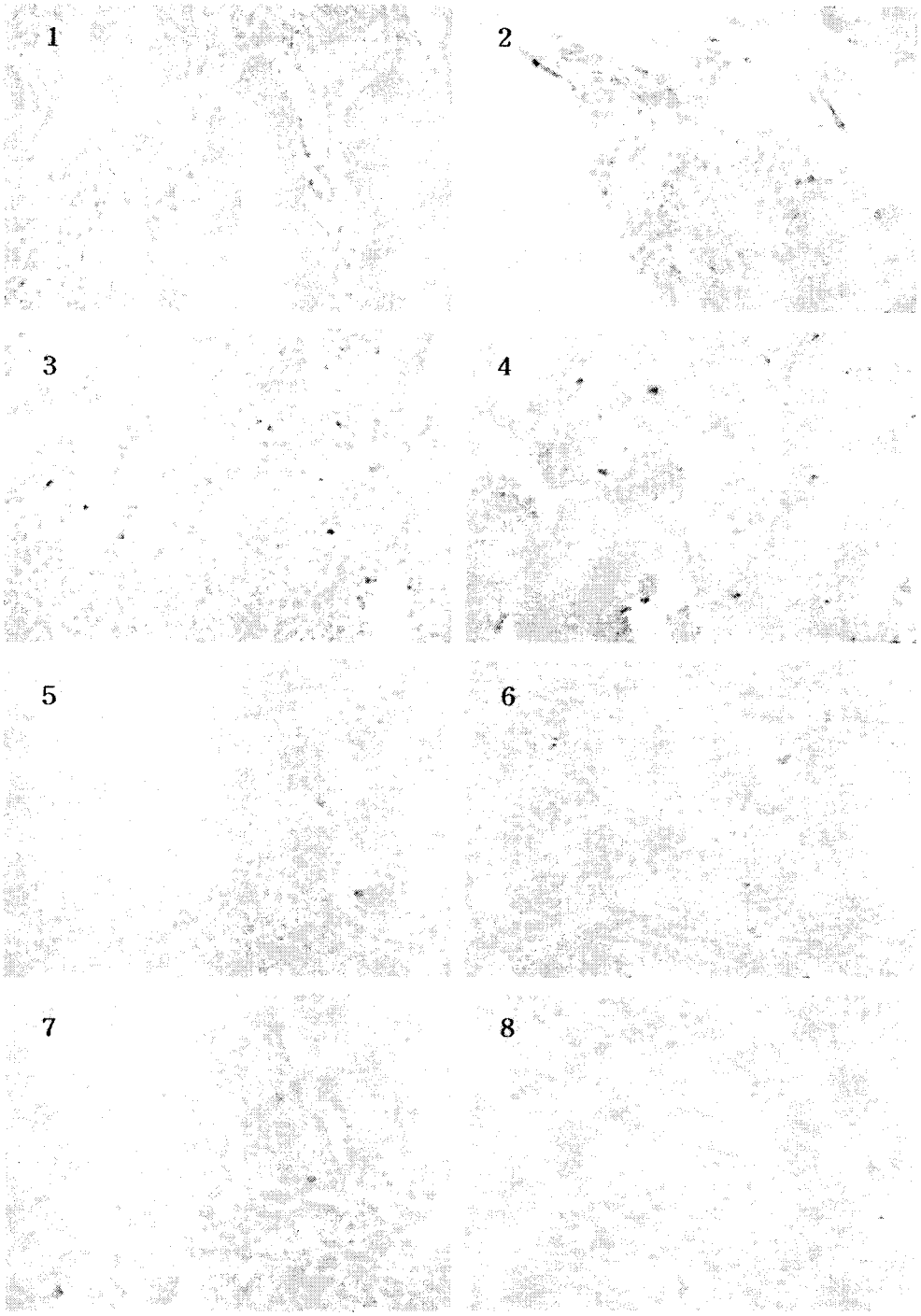

Fig 6. Spleen, showing increase of apoptotic cells(↑) on 6 hr and 12 hr PI, and decrease with time-course(1 : control , 2 : 1 hr, 3 : 6 hr, 4 : 12 hr, 5 : 1 day, 6 : 2 day, 7 : 4 day, 8 : 7 day). TUNEL stain, × 400.

Fig 7. Intestine, showing increase of apoptotic cells (↑) on 6 hr PI and decrease with time-course(1 : control, 2 : 1 hr, 3 : 6 hr, 4 : 12 hr, 5 : 1 day, 6 : 2 day, 7 : 4 day, 8 : 7 day). TUNEL stain, x400

dark red color on 4 day PI and coppery bronze liver was seen on 7 day PI. The spleen was enlarged and dark red discolored on 4 day PI and got green-yellowish color and mild multifocal white foci on 7 day PI. The small intestine contained viscous slimy, bile-stained materials(Fig 1). No specific lesion was found in the other organs.

Histopathological observation(Fig 2, Fig 3)

Microscopic changes were characterized in the liver by multi focal necrosis, congestion and infiltration of inflammatory cells. Mild multi-focal necrosis began on 12 hr PI, got more severe on 4 day PI and necrotic area was enlarged on 7 day PI. In the intestine, the portion of crypt and lamina propria showed infiltration of inflammatory cells and mild necrosis of vili from 2 day PI. More severe changes was seen on 4 day PI and especially large cysts were detected in the mucosa on 7 day PI. In the spleen, moderate to severe congestion, follicle hyperplasia, mild necrosis and inflammatory cells infiltration were seen from 2 day PI and sever follicle hyperplasia and inflammatory cells infiltration were observed severely by 7 day PI. On the other hand, there were no pathologic changes in the lung.

TUNEL analysis

The results of TUNEL analysis were summarized in Fig 4. 1 hr PI, apoptotic cells were detected in the liver(2.6 ‰), spleen(1.6 ‰) and intestine(11 ‰). In the liver, apoptotic cells showed dramatical changes reaching a maximum at 6 hr PI(340 ‰), sharp decreasing at 12 hr PI(2 ‰) (Fig 5). In the spleen, marked increasing of apoptotic cells was seen at 6 hr PI and peaked at 12 hr PI and then decreased gradually returning to a level of the control by 7 day PI(Fig 6). In the intestine, apoptotic cells did not show significant difference as compared with the control(Fig 7). In contrast, the lung was devoid of apoptotic cells(Data not shown).

Discussion

The first death was 2 day PI without clear lesions and the mortality was not high by 7 day PI. The mortality could be influenced by dietary such as Fe and protein source[46,47]. In chicks infected with SG, iron from iron dextran or ferric ammonium citrate (DFe and CFe, respectively), in dose of 20 or 50 ㎎/㎏ given intramuscularly at the time of infection rose the survival rate sharply[46]. In addition, the influence of protein source on the survival of chicks inoculated with SG was significantly more chicks survived among those given beef powder as the protein supplement in a starch-based diet than among those fish flour replaced beef powder[47].

Clear gross lesions were white foci in the liver, enlarged spleen, dark-red discoloration of the organs on 4 day PI and coppery bronze liver, dark-red discolored intestine and green-yellowish spleen were seen on 7day PI. These are the typical lesions of fowl typhoid[11]. In additionally, histopathological changes were shown from 2 day PI and more severely observed on 4 and 7 day PI in the liver, spleen and intestine. These time-course changes were correlated with previous report in which, 8-week-old broiler chicks SG-infected(1.5×10^9 CFU/㎖) were recorded 1.5×10^7, 1.3×10^2 and 1.2×10^2 CFU of SG from 1g of liver, spleen and blood 1 day PI. By 4 day corresponding data were 3.7×10^4, 4.8×10^3 and 1.1×10^3 respectively and 7 day PI 10^5 CFU were present in all three specimen. The liver and

spleen of dead bird were contaminated with more than 10^7 CFU per gram. The endotoxin was found an activity of 1.5, 12.0 and 15.0 endotoxin unit/㎖ 1 day, 4 day and 7 day PI, respectively[48].

The previous studies mentioned that tumor necrosis factor-α receptor(TNFRI) and Fas are both member of TNF receptor family that are expressed at high levels on hepatocytes[49,51]. These might be correlated with reason why our results showed the number of apoptosis of the liver were about a hundredfold higher than other tissues of SG-infected chicks. In case of hepatitis C virus(HCV)- and hepatitis B virus (HBV)-associated chronic liver diseases, liver-infiltrating lymphocytes that recognize the viral antigen on hepatocytes became activated and expressed cytolytic Fas ligand (FasL) molecules[52,53]. Meanwhile in the SG-infected liver, hepatocytes may exhibit enhanced Fas expression and become susceptible to FasL-mediated death. But this detection desires more studies as augmentation of the Fas system has also been observed in other liver diseases currently[54]. The commitment of cells to undergo apoptosis was at 12-18 hr after *Salmonella* infection in cultured human colon epithelial cell lines[55,56]. This observation is some different from our results that just a few increasing apoptosis from 1 hr PI and reached a maximum 6 hr PI in the intestine. Moreover the increasing of apoptosis was not significant compared with the control during experiment in the intestine. Other study investigated that *Salmonella*-induced activation of apoptosis was delayed for up to 6 hr after infection in epithelial cells in comparison with mouse monocyte-macrophage cell lines suggesting that epithelial cells activate antiapoptotic mechanisms[57]. The delayed onset of apoptosis might be explained, in part, by activation of the transcription factor NF-kB after bacterial entry[21]. Apoptosis showed a little different changes in the spleen form the liver and the intestine, though lesional and histological changes three of them were similar one another. In the spleen apoptosis was reached a maximum at 12 hr PI and the liver and intestine was at 6 hr PI. In addition apoptosis of liver showed sharp increasing and decreasing while slow changes of apoptosis was observed in the spleen. Despite of fowl typhoid occurs septicemic disease, no apoptotic positive cell was detected in the lung, and what is more specific lesional and hitopathological changes was not seen in the preserved study.

The previous study which reported the role of casp-1 which is a member of a family of cysteine proteases that induce apoptosis with mouse supports our opinion that apoptosis may be correlated with pathogenesis in SG-infected chicks. The study showed decrease in the number of apoptotic cells, intracellular bacteria, and the recruitment of polymorphonuclear lymphocytes of the Peyer's patches (PP) in mice lacking Casp-1(casp-1(-/)-mice). Furthermore, *Salmonella* did not disseminate systemically in the majority of casp-1 (-/)-mice. Thus it show that Casp-1 is essential for *Salmonella* to colonize the cecum and PP and subsequently cause systemic typhoid-like disease in mice[58].

In conclusion, we found SG-infection affected apoptosis at early phase of pathogenesis and can be considered to be useful data on pathogenetic mechanism of salmonellosis. Though our datum are lack to reveal the role of apoptosis in undergoing pathogenesis of SG-infected chicks, if further

studies are done, it seems that the researches could be valuable to control and diagnosis salmonellosis in chicks.

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