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Disseminated Intravascular Coagulation in Experimental Fowl Cholera of Chickens

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Introduction

Fowl cholera is an infectious disease which affects practically not only domestic fowls but also wild birds. It usually appears as a septicemic disease associated with high morbidity and mortality. In chickens, the disease is sometimes acute and affected birds may be found dead with no premonitory signs of infection. It occurs sporadically or enzootically in most countries of the world.[5]

Pasteurella multocida(*P. multocida*) the causative agent, is a Gram-negative, non-motile, non-spore-forming and produces in *vivo* endotoxin. Endotoxins are integral components of the Gram-negative bacterial cell wall, and large macromolecular complexes are chemically lipopolysaccharides. Even small amounts of these substances exert profound and varied biological effects on body system.[8] The infections with Gram-negative bacteria are often accompanied by shock, and it is believed that endotoxin is the shock producing factor. Shock resulting from Gram-negative bacteria is common in mammals[1,14] and is often attributed to the action of endotoxins.[15]

In addition to shock, Gram-negative sepsis is often accompanied by disseminated intravascular coagulation(DIC).[2,12] The term DIC is defined as the pathological activation of the coagulation mechanism which leads to generalized intravascular clotting involving particularly the arterioles and capillaries. In veterinary medicine, DIC was first reported by Welti,[16] in rabbits subjected to experimental burns, it was not until 1961, that Schulz et al.[13] described the occurrence of intravascular thrombosis as a result of intravascular hemoconcentration in porcine edema disease.[12]

Although there are many studies for demonstration of intravascular arteriolar, capillary or venular fibrin deposits in multiple organs of mammals, study of DIC in chicken diseases is scant[3,6] and the cause of death in acute fowl cholera is still not known well.[9] It is needed to study of the role *P. multocida* endotoxins in disease process of fowl cholera. The purpose of this study was to survey the development or distribution of DIC in multiple organs and the role of *P. multocida* endotoxin in relation to the cause of death in fowl cholera.

Materials and Methods

1. Chickens : For this experiment, 108 Leghorn were reared in an indoor isolation facility. Consecutive infection trials for 80 chickens were undertaken as the birds reached to 10 through 32 weeks of age. Infection trials were done in an isolated room. Chickens were housed in standard wire poultry cages that were cleaned and disinfected between trials. Chickens that received different dosage or inoculation route of bacteria were kept in separated cages. Twenty eight control chickens were housed together in a separated healthy chicken room to avoid accidental contact with bacteria.

2. Bacteria : *P. multocida* strain was 2623V that obtained from Houghton Poultry Research Station in London. It was cultured aerobically in blood

agar at 37°C for 18 hours and passed once through mice, 3 times in chickens and then used for this experiment. The suspension contained approximately 2-3 × 10^9 organisms per ml as determined parallel plate counts.

3. Experiment design and bacterial challenge: Chickens were challenged by various inoculation route of the appropriate dosage of culture. It was divided into seven inoculation route: intravenously into wing vein with 1ml, 0.5ml and 0.25ml, intraperitonealy with 1ml, intramuscularly into thigh muscle with 1ml, subcutaneously into dorsal part with 1ml, into ear with intranasally and per oral with 2ml, respectively. Control birds were inoculated with saline by the same method. Blood samples were taken at 12, 24 and 48 hours after the bacterial inoculation to the chickens. Blood smears were prepared, stained with 3% methylene blue solution and evaluated microscopically for evidence of bacteremia.

All the chickens were necropsied as soon as possible after death. A record was kept for all mortality, time of death, and lesion distribution. One week (168 hours) after initiation of the experiment, all surviving chickens and control chickens were killed and necropsied.

4. Analytical methods : Samples of lung, liver, kidney, heart, spleen, brain, pancreas, thymus, thyroid gland, small and large intestine were fixed in 10% neutral formalin. Paraffin sections 4-6μm in thickness were prepared and stained with hematoxylin and eosin (H & E) for microscopic examination. For tinctorial confirmation of fibrinous thrombi and bacteria, special stains were used as required including phosphotungstic acid hematoxylin (PTAH) stain and Gram's stain.[7]

The histopathologic criterion of DIC was the presence of fibrinous thrombi primarily in small blood vessels. The frequency of thrombi in each organ was determined microscopically by counting the maximum numbers of fibrinous thrombi in each section and graded as follows: one or two as +, three or five as ++, six to ten as +++, more than ten as ++++.

Tissues of major organs such as heart, liver, spleen and kidney were cultured on blood agar incubated aerobically for 18 hours at 37°C. Identification of *P. multocida* was made on the basis of in *vitro* biochemical reactions.

Results and Discussion

1. Disseminated intravascular coagulation : There were 40 cases of microscopically diagnosed as having DIC among the 80 infected chickens. No significant micro thrombi were observed in the major organs of the 4 chickens among the 44 chickens which were died within 72 hrs. after the inoculation. The chickens which were died at 96 through 168 hrs. after the dosing also showed no significant fibrinous thrombi in the major organs.

The frequency and extent of organ involvement by fibrinous thrombi are shown in Tab. 1 and 2. In the 40 cases, thrombi were observed most frequently in the lung followed by the liver, kidney, heart, spleen, brain, pancreas, thymus and thyroid gland.

Fibrinous thrombi were found in the lungs of 36 out of 40 cases (90 percentage). In addition to being the most frequently involved organ, the lung showed the largest number of fibrinous thrombi, and 30 cases ranged from ++ to +++ +. In addition to fibrinous thrombi occluding arterioles, capillaries and venules, thrombi were found in medium-sized arteries and veins(Fig. 1).

Fibrinous thrombi were found in the liver of 28 cases out of 40 cases. The number of fibrinous thrombi was relatively small in this organ, and all 28 cases ranged from+to +++. They were often found in the blood vessels and sinusoids.

Fibrinous thrombi were presented in the kidney in 24 of the 40 cases. Thrombi were frequently found in the afferent arterioles of glomeruli, large arteries and small veins.

The heart was affected in 8 DIC cases out of 40 cases, fibrinous thrombi were revealed in small blood vessels(Fig. 2).

In eight of the 40 cases fibrinous thrombi were also found in the spleen. Fibrinous thrombious were noted in arterioles and venules. Wide spread involvement

Table 1. Frequency of Organ Involvement by Fibrinous Thrombi

Organs	No. of cases*	Frequency(%)
Lung	36	90.0
Liver	28	70.0
Kidney	24	60.0
Heart	8	20.0
Spleen	8	20.0
Brain	6	15.0
Pancreas	4	10.0
Thymus	4	10.0
Thyroid gland	2	5.0

* The total number of cases with fibrinous thrombi was 40.

Table 2. Distribution of Fibrinous Thrombi in Organs

Organs	Grade of fibrin thrombi*				
	−	+	++	+++	++++
Lung	4	6	12	8	10
Liver	12	16	8	4	0
Kidney	16	10	10	2	2
Heart	32	6	0	2	0
Spleen	32	4	2	0	2
Brain	34	6	0	0	0
Pancreas	36	2	2	0	0
Thymus	36	0	2	2	0
Thyroid gland	38	0	0	2	0

* Number of fibrinous thrombi graded as follows: none as −, 1 or 2 per section as +, 3 to 5 as ++, 6 to 10 as +++, more than 10 as ++++.

Table 4. Multiple Organ Involvement with Fibrinous Thrombi

No. of organs	No. of cases
5	6
4	4
3	20
2	4
1	6

of sinuses was sometimes prominent in the spleen. In this cases, the fibrin appeared to be fluffy or loosely arranged, so that the PTAH staining was very useful for detection of thrombi(Fig. 3).

The occurrence of fibrinous thrombi was less frequent in other organs. In the brain, six cases of thrombi were identified in blood vessels of neural parenchyma and leptomeninges(Fig. 4).

The thrombi obsorved in the pancreas were 4 chickens, in the thymus were 4 chickens and in the thyroid gland were 2 chickens of the 40 chickens, respectively.

Since the frequency of thrombi in an organ does not necessarily parallel the frequency of organ involvement by thrombi, a calculation was made to obtain a "density index" of thrombi in the major organs(Table 3). For example, the sum of grades derived from Table 2 on the frequency of thrombi in the livers of all 28 cases is 44 + and division of this figure by the number of cases(28) yields 1.57, which was called the density index of the liver. In the Table 3, the lung also shows the highest density index because this organ was contained also the greatest number of fibrinous thrombi, thus making the

Table 3. Density Index of Major Organs Involved with Fibrinous Thrombi

Organs	Sum of grades from Table 2 (No. of+)	Frequency of organ involvement (No. of cases)	Density index: sum of grades / No. of cases
Lung	94	36	2.61
Liver	44	28	1.57
Kidney	44	24	1.83
Heart	12	8	1.50
Spleen	16	8	2.00

density index 2,61, followed by spleen, kidney, liver and heart in the descending order.

Multiple organ involvement is shown in Table 4. Among the 40 DIC cases, 20 cases of thrombi were involved in the three major organs with fibrinous thrombi.

2. Bacteriology : *P. multocida* were cultured in the lung, liver, heart, spleen and kidney tissues of the 45 dead chickens. Blood agar platess treaked with tissues from these chickens developed heavy growth of bacteria biochemically indentical with those used as inoculum.

3. Blood smear for bacteremia : Blood samples taken from chickens 12 hours post inoculation had large numbers of circulating blood cells containing bipolar stainning bacteria. Samples drawn at 24 hours postinoculation from chickens that proceeded to die had a massive bacteremia with very few degenerating phagocytics evident. Chickens that survived from bacterial challenge had no evidence of bacteremia in either at 24 or 48 hours post inoculatio nblood smear.

In the present study, the lungs, the most frequently affected organ, were particulary susceptible to blood-borne toxins by virtue of their anatomical position, and they were one of the most frequently damaged organs in septic shock. In human medicine, the association between DIC and various clinical conditions, including severe infections and malignancies is well documented.[2] The most common was Gram-negative bacteria and fungi. Septicemia due to Gram-negative bacilli was thought to cause endotoxemia, which may induce the DIC. In addition, shock following severe infections may be important for triggering DIC.[4] In all forms of shock(endotoxin, hemorrhagic, burn, traumatic, cardiogenic, anaphylactic etc.), there are microthrombi or hyaline globular material in practically every organ including the kidney, hypophysis, brain, heart, intestine, liver and especially the lung. The frequency of microthrombi in human material was highest in endotoxin-shock(78%) and lowest in hemorrhagic shock (20%). The lung is the most frequently involved organ in shock containing thrombi in 80% of the cases.[11] It is unclear if DIC occurs in chickens because of differences in avian internal clotting mechanisms and the unknown function of the avian nucleated thrombocyte, and if the same pathogenetic mechanism of chicken's DIC to mammal's DIC, remains an enigma.

In this investigation, among the 80 cases *P. multocida* inoculated chickens, 36 chickens which were mostly inoculated by oral, intranasal and into ear died approximately 3 days after inoculation, and they revealed no microthrombi pathologically. The usual explanations for these cases included: in *vivo* or post mortem lysis of fibrinous thrombi, insufficient time for producing DIC and misdiagnosis.

Rhodes[10] who inoculated *P. multocida* into the nasal cleft of the chickens, described that the most pronounced and significant lesions obserbed in the infected chickens were acute generalized passive hyperemia. Since there was no apparent impairment of pulmonary circulation, the hyperemia must have been produced by cardiac insufficiency, loss of vascular tone or a combination of these conditions. In any event, the increased amount of blood in the peripheral vessels was necessarily accompanied by reduced effective cardiac output and is indicative of the syndrome of shock.

Summary

Chickens from 10 to 32 weeks of age were inoculated with *P. multocida* via seven routs(intravenous, intramuscular, intraperitoneal, subcutaneous, into ear, intranasal, per oral). The development or distribution of disseminated intravascular coagulation (DIC) in multiple organs and the role of *P. multocida* endotoxins in disease process of fowl cholera were studied. The histological diagnosis of DIC was made by demonstration of fibrinous in arterioles, capillaries, venules and medium-sized blood vessels.

The presence of fibrinous thrombi in blood vessels of multiple organs was observed in chickens which died within approximately 3 days post inoculation. Fibrinous thrombi were observed most frequently in the lung(90% of all cases with

DIC) followed by liver (70%), kidney (60%), heart(20%), spleen, brain, pancreas, thymus and thyroid gland. The density of fibrinous thrombi (i.e. the number of thrombi per section) was greatest in the lung, followed by spleen, kidney, liver and heart.

It is thought that the widespread hemorrhage of acute fowl cholera is also caused by *P. multocida* endotoxin which initiates DIC in variety of organs. The cause of death for the chickens after infection with acute fowl cholera is probably due to an endotoxin (septic) shock accompanied with DIC in multiple organs.

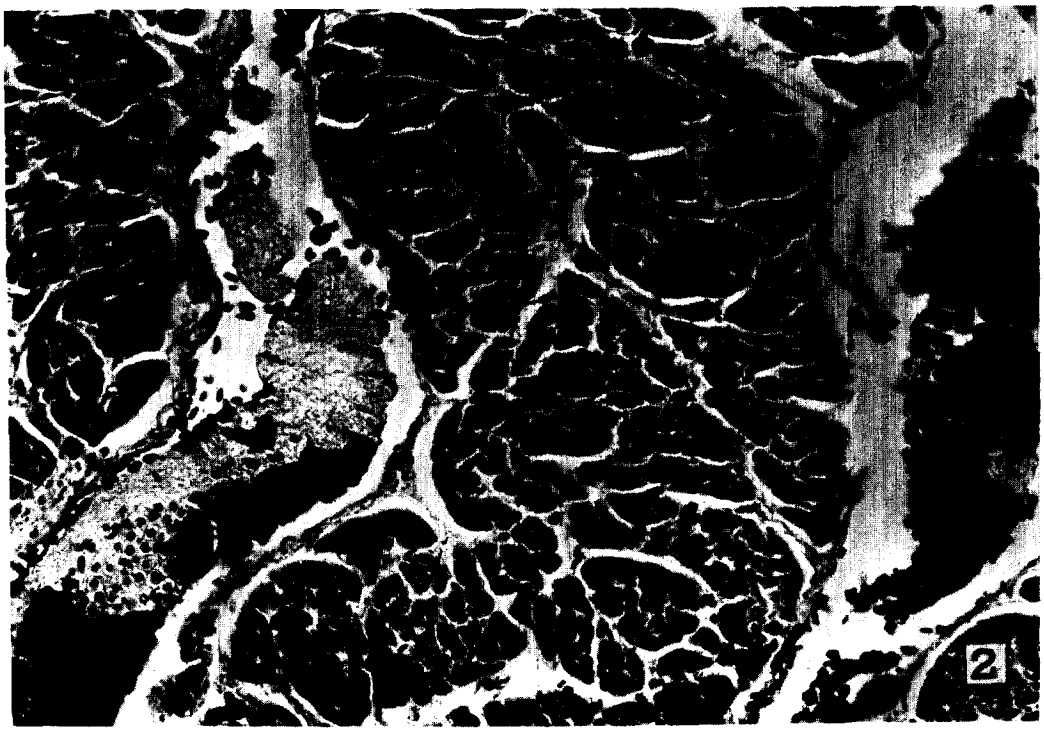
Legends for Figures

Fig. 1. A fibrinous thrombus in a lung vessel, 13 weeks old chicken died at 18 hours post inoculation phosphotungstic acid hematoxylin. Phosphotungstic acid themaoxylin (PTAH) stain, × 40.

Fig. 2. Fibrinous thrombi and erythrocytes with bacteria in the cardiac vessels, 24 weeks old chicken died at 36 hours post inoculation. PTAH stain, × 250.

Fig. 3. A fibrinous thrombus in the spleen, 19 weeks old chicken died at 30 hours post inoculation. PTAH stain, × 400.

Fig. 4. Fibrinous thrombi, in the vein, of leptomeninges, 20 weeks old chicken died at 35 hours post inoculation. Hematoxylin & Eosin stain, × 250.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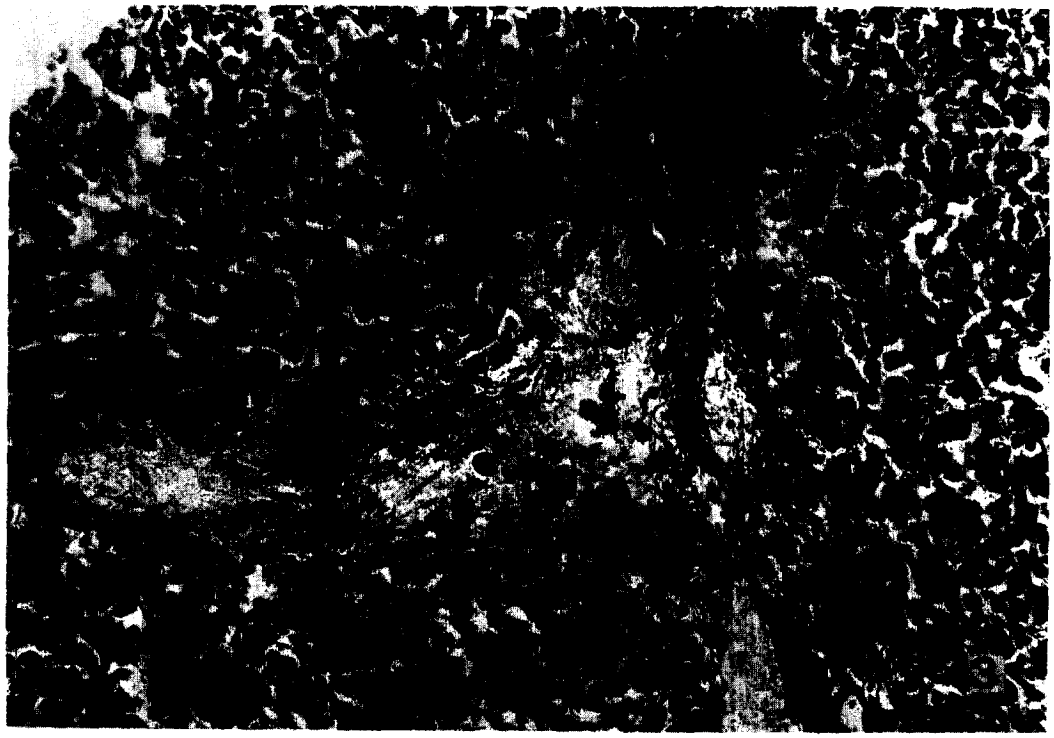

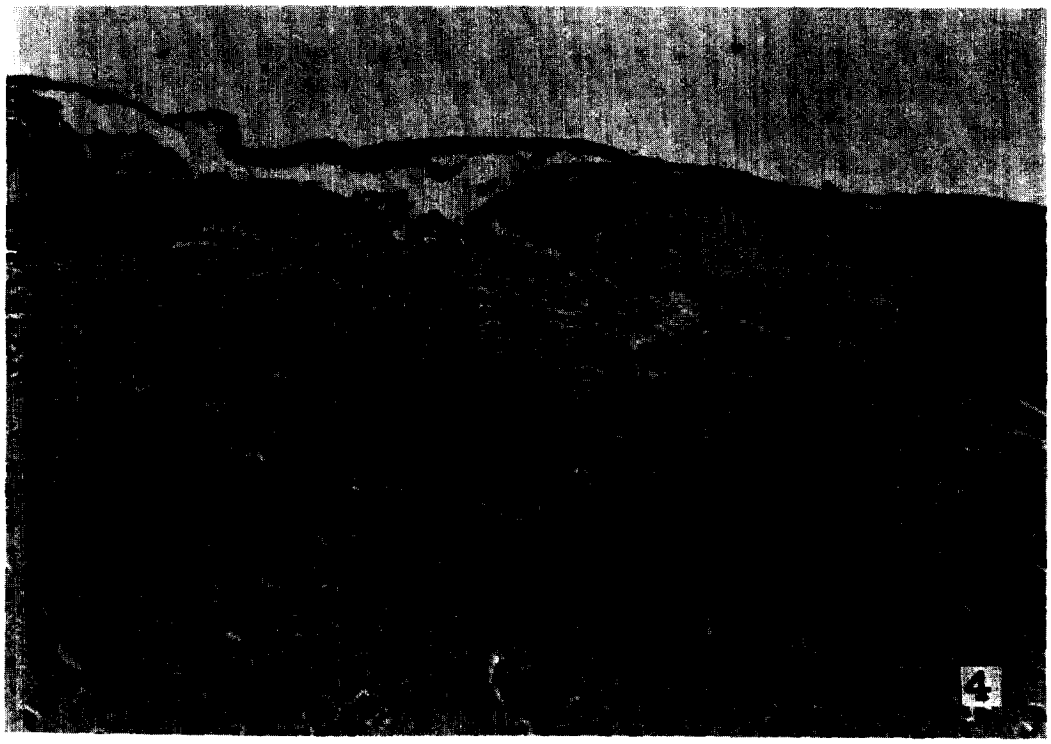
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닭의 家禽 콜레라 感染時의 播種性 血管內 凝固症

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抄 錄

닭의 急性 家禽 콜레라의 斃死原因과 機轉을 究明하고자 生後 10~32週齡 닭에 *P. multocida*菌을 七個 經路(靜脈, 筋肉, 皮下, 鼻腔, 口腔, 腹腔 및 귀)를 통해 注入해서 家禽 콜레라를 發病시키고 播種性 血管內 凝固의 發顯與否와 그 分布 및 本 疾病 進行 過程中 *P. multocida*의 endotoxin 役割에 대하여 硏究하였다.

播種性 血管內 凝固의 病理組織學的인 診斷은 小動脈, 小靜脈, 毛細血管 그리고 다소 큰 血管內에 纖維素性 血栓의 證明으로 이루어졌다.

各種 臟器內 播種性 血管內 凝固는 主로 3日 以內에 斃死된 닭에서 쉽게 觀察할 수 있었고, 臟器中 肺는 血栓의 發顯頻度가 가장 높았으며(90%) 그 다음으로 肝(70%), 腎臟(60%), 心臟(20%), 脾臟, 腦, 膵臟, 胸腺 및 甲狀腺의 順이었다. 纖維素性 血栓의 密度(組織切片當 血栓의 數) 역시 肺가 가장 높고 脾臟, 腎臟, 肝 및 心臟의 順이었다.

急性 家禽 콜레라 感染時 汎發性 出血은 播種性 血管內 凝固를 일으키는 *P. multocida*菌의 endotoxin에 基因된 것으로 思料되며 닭의 急性 家禽 콜레라의 斃死原因은 單純한 出血性 敗血症이 아니라 全身的으로 發生되는 播種性 血管內 凝固를 수반하는 endotoxin(septic) shock死임이 밝혀졌다.