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Article

Effects of the methanolic extract of Moutan cortex radicis on immune systems in colostrum

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Abstract: More than ten percentage of piglets die before weaning, and the transfer of immunoglobulins across the placenta is prevented unlike human. Because the new-born piglets are immunologically naive, they depend upon the sow for immune protection. Many cytokines in colostrum are correlated with concentrations in sow serum. Based on the phenomenon that the quality of colostrum influences on the growth of piglets, we tried to add a plant extract into livestock feed to change immune system of swine.Moutan cortex radicis is the bark of the root of Paeoniasuffruticosaused as oriental medicine. The aim of this research was to examine that the methanol extract of Moutan cortex radicis changes immune system of swine.

Keywords: colostrum; swine; Moutan cortex radices; maternal antibody; immunity

1. Introduction

Due to the pig industry's pressures to avoid use of antibiotics for livestock feed, improvement of passive immunity in the neonate piglet is raised on our head. More than 10% of piglets born alive die before weaning [1]. Unlike human, the transfer of immunoglobulins across the placenta is prevented because of the epitheliochorial nature of the placenta in swine [2]. Therefore, passive transfer of immunity via colostrum is important. The immune system of colostrum and milk plays important roles in supplying maternal antibodies to piglets and protecting the glands from the infection. Swine which cannot transfer maternal antibodies to their offspring during the period of maternity provide maternal antibodies through colostrum and milk [2]. The new-born piglet is therefore reliant on immunoglobulin G (IgG) absorbed from colostrum for humoral immune protection until its own immune system has sufficiently matured to respond (to produce antibodies against) to foreign antigens. Thus, the concentrations of IgG in the plasma of piglets shortly after birth are positively correlated with survival [3]. As transfer of intact macromolecules across the gastro-intestinal tract is only possible for a short time after birth, this period is particularly important. Most components of the immune system of the piglet are present at birth but are functionally undeveloped and several weeks of life are necessary before the immune system becomes fully developed [4]. Since the new-born piglet is immunologically naive and the time lag before its immune system develops fully covers at least the period from birth until weaning, the piglet is dependent on the sow for immune protection during that period [5].

There was no significant relationship between colostrum IgG intake and concentrations of IgG in piglet plasma at 24 h of age However, since in normally suckling piglets, the concentration of IgG in colostrum that any piglet first encounters will be determined by its position in the birth order and the length of farrowing, IgG acquisition by piglets late in the birth order may be prejudiced by low colostrum IgG concentrations [4]. Dead piglets had lower serum IgG concentrations than comparable surviving piglets [6]. Even though the placenta of sow prohibits transplacental passage of immunoglobulins, many cytokines such as interleukin (IL) -6, interferon-γ, IL-12 (Th1), IL-10, IL-4 (Th2), and tumor growth factor (TGF) -β1 (Th3) in colostrum are correlated with concentrations in sow serum except for tumor necrosis factor (TNF) -α and TGF-β1. [7]. Based on the phenomenon that the quality of colostrum effects on the growth of piglets, research to improve immunomodulation of neonatal immunity is required. As concentrations of IgG in maternal colostrum are very variable, an improved understanding of factors influencing colostrum IgG concentrations is desirable. The transmission of colostrum IgG to the piglet can be manipulated nutritionally, as demonstrated by the effects of

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maternal dietary fat soluble vitamin concentrations [8]. Conjugated linoleic acid had a positive effect on immunologic variables in lactating sows and piglets, and increased colostrum IgG in sows [9]. Sows supplemented with alkylglycerols and polyunsaturated fatty acids showed increment of the concentrations of IgG [10]. Feed ingredients altered colostrum composition [11]. Therefore, we tried to add a plant extract into livestock feed to change immune system of swine.

Moutan cortex radicis (MCR) is the bark of the root of Paeonia suffruticosa, which has been used as traditional Chinese medicine. Its biological effects were sedative, anti-inflammatory, radical scavenging effects, inhibitory effects on anaphylactic reaction, and antimicrobial activities [12]. Therefore, the aim of this research was to examine that the methanol extract of Moutan cortex radicis changes immune system of swine.

2. Materials and Methods

2.1. plant material and extraction

The dried root bark of Moutan cortex radicis was purchased from Kyungdong oriental pharmaceutical market in Seoul, Korea. Six kilograms of the dried sample were ground and dissolved in 18L methanol (three times for three days) at room temperature in a dark room. After filtering, the methanol extract were concentrated under reduced pressure with a rotary evaporator (Eyela, Tokyo, Japan) and the extract of 1,344.21 g was collected.

2.2. marker substance in Moutan cortex radicis

Several chemical components contained in Moutan cortex radicis have been reported [13-15]. Of them, paeonol and paeoniflorin can be candidates for marker substances. The former is contained in the methanolic extract of Moutan cortex radicis more than the latter, so that it was decided to be a marker substance. Paeonol and paeoniflorin were purchased from Sigma-Aldrich (99%, St. Louis, MO) and used without further purification. They were analyzed using Agilent 1260 Infinity high performance liquid chromatography (HPLC) system (Santa Clara, CA) with the reversed phase column (Luna C18, 250 x 4.6 mm, 5μm diameter, Phenomenex, Torrance, CA) at 30°C. The flow rate and injection volume were 1.0 ml/min and 10 μl, respectively.

2.3. preparation of livestock feed

The extract of MCR was dissolved in 50% aqueous ethanol and homogenized at 10,000 rpm. It was immobilized using wheat powder, silica, and ethanol and coated with zeolites. The livestock feed was prepared by mixing with corn powder (58.78%), soybean peel (26.52%), soybean oil (2.5%), limestone (1.05%), CaHPO4 (1.20%), NaCl (0.1%), lysine (0.25%), Grobig-SW (Bayer Healthcare, Monheim, Germany, 0.2%), biotin (0.1%), beet pulp (6.6%), wheat bran (2.2%), and microorganisms (0.5%), and the final concentration of the extract of MCR was adjusted to 0.5%. The materials used here except materials whose sources were given in parentheses were supplied by a local company in Korea.

2.4. animals experiments

Animal experiments were carried out in Swine science division, National Institute of Animal Science, Rural Development Administration, Korea, and all animals were prepared in the same laboratory. Ingredient and chemical composition of the experimental diets are listed in Table 1. A total of 11 crossed Landrace × Yorkshire sows (10 sows randomly allocated per each dietary group, second parity sows) were fed corn-soybean meal diets with or without supplementation of 0.5% MCR extract, from 14 d before their expected farrowing date until 0 d postpartum.

Table 1. Ingredient and chemical composition of the experimental diets.

Ingredients, %	Gestating	Lactating
Corn	70.38	58.78
Soybean meal	19.85	26.52
Wheat bran	2.20	2.20
Soybean oil	2.50	2.50
Beat pulp		6.60
Limestone	0.80	1.05
Calcium phosphate	1.40	1.20
Salt	0.30	0.10
Lysine	0.28	0.25
Hairagase[a]	0.20	0.50
Mix-vitamins + minerals[b]	0.69	0.30
Total	100.00	100.00
Calculated value		
Crude protein	14.50	17.50
Calcium	0.85	0.75
Phosphorus	0.60	0.60
Total lysine	0.92	1.18
Total methionine+cysteine	0.49	0.55
Digestible energy, kcal/kg	3,400	3,450

[a]Supplied per kg feed : Diastase 10g, Dry yeast 20g, Ponciruc trifoliate rafin 5.5g, Gentiana 2.0g, Scopolia extract 0.5g, Calcium lactate 2.0g, Calcium carbonate 30.0g

[b]Supplied per kg feed : Vit A 5,000,000IU, Vit D3 1,000,000IU, Vit E 1,000mg, Vit B1 150mg, Vit B2 300mg, Vit B12 1,500mg, Niacin amide 1,500mg, DL-calcium pantothenate 1,000mg, Folic acid 200mg, Vit H 10mg, Choline chloride 2,000mg, Mn 3,800mg, Zn 1,500mg, Fe 4000mg, Cu 500mg, I 250mg, Co 100mg, Mg 200mg.

2.5. *reproductive performance and growth rate*

The reproductive performance, including the total number of piglets born per litter, the number of suckled piglets per litter, the number of weaned piglets per litter, and piglet mortality rate of sows were investigated from 0 day postpartum to 25 days postpartum. The piglets of both experimental groups were weighed at 0 and 25 days postpartum. The average daily weight gain of the piglets was calculated, and the average piglet weight per litter was used for statistical analysis.

2.6. measurement of milk composition, immunoglobulin G, and cytokines

The milk composition in colostrum was analyzed using a LactoScope automatic analyzer (Delta Inst., Drachten, Netherlands). IgG was measured with Porcine IgG kit (ECOS Inst., Miyagi, Japan). Insulin was measured with Porcine insulin Enzyme-linked immunosorbent assay kit (ELISA, Catalog no 10-1113-01, Mercodia Inst., Uppsala, Sweden). IL-1β and TNF-α was measured with Porcine IL-1β and TNF-α ELISA kit (R&D Systems Inst., Minneapolis, MN). The total phenolics were determined based on the Folin-Ciocalteu method [16]. A 250 µL amount of undiluted Folin-Ciocalteu-reagent was added to 50 µL of sample. After 1 min, 750 µL of 20% (w/v) aqueous Na2CO3 was added, and the volume was made up to 5.0 mL with distilled water. After 2 h of incubation at 25°C, the absorbance was measured at 760 nm and compared to a gallic acid calibration curve.

2.7. analysis of proteomes

Colostrum sample was centrifuged at 8,000 rpm and resuspended with buffer (0.1M Tris-HCl, pH 7.5, 14 mM Mercapto-ethanol, 5 mM EDTA, 10% Glycerol), and it was sonicated for 2 min at 4˚C. The supernatant was centrifuged for 90 min at 39,000 x g and 4˚C using Micro-Ultra centrifuge (Hitachi, Tokyo, Japan). Protein analysis was carried out by the Bradford method, using bovine serum albumin as a standard. The concentration of proteins present in colostrum sample was 10 ug/ul. Fifty µg protein was separated using sodium dodesylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). It was washed again with washing buffer (50 mM ammonium bicarbonate : acetonitrile = 1 : 1 v/v) until decoloring Coomassie blue used for SDS-PAGE. The washed slice was dehydrated with acetonitrile, which was dried using Speed-vac (Maxi Dry Lyo, Heto-Holten, Denmark). The dried gel was incubated in trypsin solution (30 ul of Promega trypsin buffer, 570 ul of 50 mM ammonium bicarbonate) at 37˚C for 16 hr. The tryptic peptides were desalted and dried using Speed-vac. It was dissolved in 0.1% formic acid, which was used as a peptide solution for liquid chromatography (LC) – mass spectrometry (MS) – MS analysis. The above process was iterated three times, so that three peptide solutions were collected. The peptide solutions were analyzed using a linear trap quadrupole mass spectrometer (LTQ-XL, Thermo Fisher, Waltham, MA) coupled online with a Nano LC system (Eksigent, Dublin, CA), which was loaded on a microcapillary column (Home-made C18, 75 µm x 120 mm, 5 µm particles). The flow rate was 0.3 µl/min. The mobile phase was a gradient of increased organic solvent (buffer A : 0.1% formic acid in D.W, buffer B : 0.1% formic acid in acetonitrile). The molecular weight was ranged between 300 and 2,000 m/z. The MS data was analyzed using SEQUEST database search algorithm and the library search was carried out using Xcalivar 1.3 software and TurboSEQUEST program.

3. Results and Discussion

3.1. marker substance in Moutan cortex radicis

Paeonol used as a marker substance was dissolved in methanol (HPLC grade, Burdick and Jackson, Indianapolis, IN) and its concentrations was adjusted to 2.18 mg/ml. Its chromatogram was detected at 273 nm. The mobile phase was 40% aqueous acetonitrile. The retention time was 13.1 min. The methanol extract of Moutan cortex radicis was prepared in methanol and its concentration was 20 mg/ml. The concentration of paeonol contained in the methanol extract of MCR were 9.47% [Fig. 1]. The livestock feed containing 0.5% extract of MCR was analyzed. Since 0.48 mg/ml paeonol was detected using HPLC analysis, the livestock feed was considered to contain approximately 0.5% MCR extract.

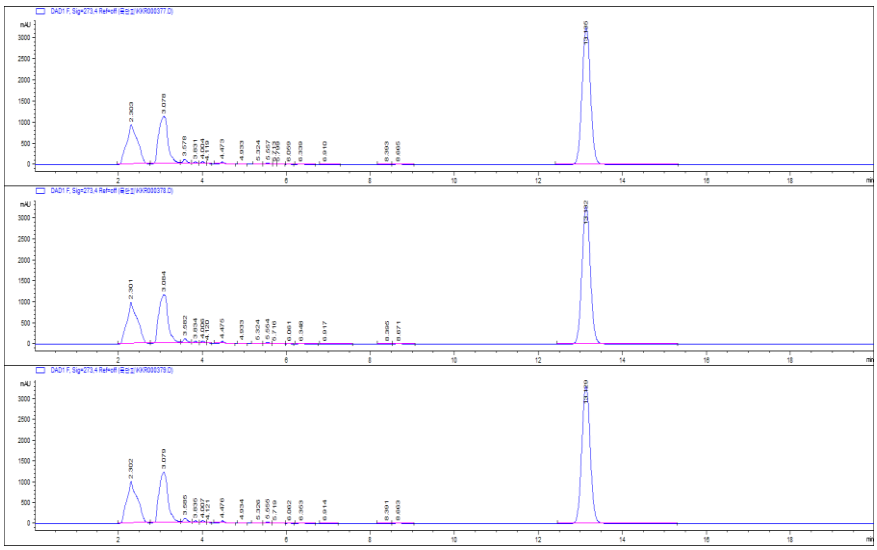

Fig. 1. The chromatograms of HPLC to analyze the concentration of paeonol contained in the methanol extract of MCR. (The peak at 13.4 min of the retention time indicates paeonol.)

3.2. animals experiments and measurement of milk composition

Additives were added to the feed by replacing the same amount of Dried Distillers Grains with Solubles. Over the gestation periods, individual feed allowances were intended to provide at farrowing a 20-mm back fat thickness and a body weight adjusted on parity. During pregnancy the experimental animals were housed in environmental controlled, slatted-floored individual gestation crates in an enclosed facility with temperatures averaging 21 ± 4°C. At 7 d before farrowing, the sows were moved to individual farrowing accommodations where they were housed under an automatic environmental control system. The sows received 2.4 kg of feed daily from the beginning of the experiment until 1 d before farrowing. Upon farrowing, they were initially fed 1.2 kg of diet and 0.32 kg of feed was then cumulatively added each subsequent day until 7 d postpartum. After 7 d postpartum, the sows were basically fed 3.4 kg/day of diet and were supplied 0.2 kg/day of diet per piglet head in addition. The sows were allowed access to this diet at 08:00 and 15:00, and were allowed ad libitum access to water from a nipple during the experiment. All piglets were supplied with feed formulated to meet National Research Council recommendations for all nutrients from the 10th day after birth and were allowed ad libitum access to water from a nipple. The animal care was in accordance with the Guide for the Care and Use of Laboratory Animals (National Institute of Animal Science, Animal Care Committee of Korea). The colostrum was obtained by squeezing facility within 3 h postpartum and stored at -24°C for the determination of milk composition, IgG, and cytokines. The total phenolics determined as gallic acid equivalents and the contents of immunochemical in colostrum are listed in Table 2.

Table 2. The The contents of immunochemicals in colostrum.

	Control	MCR	SEM*
IgG (mg/mL)	46.55	50.31	1.25
Insulin (μg/L)	1.73	1.93	0.13
IL-1β (pg/mL)	47.09	49.36	14.74
TNF-α (pg/mL)	8.91	29.34	8.97
Total phenolics (μg/mL)	1786.13	2700.00	199.01

* standard error of the mean

3.3. analysis of proteomes

The 153 proteins were identified from the LC/MS/MS analysis. Of them, 21 proteins identified from colostrum of sow provided with livestock feed containing the MCR extract were three times as up-regulated as that without the MCR extract as listed in Table 3. Especially, five immunoglobulins were observed. Immunoglobulin M (IgM) is a basic antibody that is produced by B cells. IgM is by far the physically largest antibody in the human circulatory system. It is the first antibody to appear in response to initial exposure to antigen. Immunoglobulin D (IgD) is an antibody isotype that makes up about 1% of proteins in the plasma membranes of immature B-lymphocytes where it is usually coexpressed with another cell surface antibody called IgM. Immunoglobulin mu is the class of heavy chains found in IgM. They have a molecular weight of approximately 72 kDa and they contain about 57 amino acid residues arranged in five domains and have more oligosaccharide branches and a higher carbohydrate content than the heavy chains of IgG. Polymeric immunoglobulin receptor is a protein that in humans is encoded by the polymeric immunoglobulin receptor gene. It is a Fragment, crystallizable (Fc) receptor which facilitates the secretion of immunoglobulin A and IgM. The poly-Ig receptor is expressed on several glandular epithelia including those of liver and breast. It mediates transcellular transport of polymeric immunoglobulin molecules. It is a member of the immunoglobulin superfamily. The receptor has 5 units with homology to the variable units of immunoglobulins and a transmembrane region, which also has some homology to certain immunoglobulin variable regions. Lactotransferrin is a multifunctional protein of the transferrin family, which is a globular glycoprotein with a molecular mass of about 80 kDa that is widely represented in various secretory fluids, such as milk, saliva, tears, and nasal secretions. It is one of the components of the immune system of the body, and it has antimicrobial activity (bacteriocide, fungicide) and is part of the innate defense, mainly at mucoses [17]. Butyrophilin genes constitute a subgroup of at least 10 genes in the Ig superfamily identified in human, mouse, cow, goat and other species [18]. Hemopexin is known as beta-1B-glycoprotein. Its function is scavenging the heme released or lost by the turnover of heme proteins such as hemoglobin and thus protects the body from the

oxidative damage that free heme can cause. In addition, hemopexin releases its bound ligand for internalisation upon interacting with a specific receptor situated on the surface of liver cells. This function of hemopexin is to preserve the body's iron [19-21].

Table 3. Proteomes up-regulated by the extract of MCR in colostrum.

Protein description	Accession (NCBI ID)	Spectral Counts		% Ratio (MCR/Control)
		Control	MCR	
Immunoglobulin mu heavy chain constant region	gi|253878	38.3	115.6	300
IgM heavy chain constant region	gi|291202695	6.4	22.1	345
IgM VH2 chain	gi|1699400	3.2	9.8	306
IgD heavy chain constant region	gi|291202696	0	14.8	only MCR
Polymeric immunoglobulin receptor precursor	gi|47523406	3.2	43.1	1347
Lactotransferrin	gi|3915882	7.5	30.8	411
Lactotransferrin precursor	gi|47523782	23.4	98.4	421
Butyrophilin subfamily 1 member A1-like	gi|194039786	3.2	19.7	616
Hemopexin	gi|1708183	2.1	34.4	1638
Serotransferrin precursor	gi|347582654	38.3	179.6	469
Legumain-like	gi|350596128	0	3.7	only MCR
Fibrillin-2, partial	gi|350581063	0	3.7	only MCR
Titin-like, partial	gi|350593667	0	4.9	only MCR
Afamin	gi|335293652	0	6.2	only MCR
Fatty acid-binding protein, heart	gi|2811020	0	6.2	only MCR
Alpha-1B-glycoprotein-like	gi|311259609	0	20.9	only MCR
Inhibitor of carbonic anhydrase	gi|6016307	0	13.5	only MCR
Fibrinogen alpha chain-like, partial	gi|350587695	0	3.7	only MCR
Acyloxyacyl hydrolase isoform 2	gi|350595334	0	3.7	only MCR
Alpha-1-antichymotrypsin 2 precursor	gi|47523270	3.2	9.8	306
Serum albumin	gi|71152981	256.7	857.3	334

Serotransferrin is a glycoprotein that is thought to have been created as a result of an ancient duplication event that led to generation of homologous C and N-terminal domains, each of which binds one ion of ferric iron. The function of this protein is to transport iron from the intestine, reticuloendothelial system, and liver parenchymal cells to all proliferating cells in the body. Serotransferrin may also have a physiologic role as granulocyte/pollen-binding protein (GPBP) involved in the removal of certain organic matter and allergens from serum [22]. Legumain is a protein that in humans is encoded by the LGMN gene. This gene encodes a cysteine protease that has a strict specificity for hydrolysis of asparaginyl bonds. This enzyme may be involved in the processing of bacterial peptides and endogenous proteins for MHC class II presentation in the lysosomal/endosomal systems. Enzyme activation is triggered by acidic pH and appears to be autocatalytic [23-24]. Fibrillin is a glycoprotein, which is essential for the formation of elastic fibers found in connective tissue. It is secreted into the extracellular matrix by fibroblasts and becomes incorporated into the insoluble microfibrils, which appear to provide a scaffold for deposition of elastin [25]. Titin is a protein that in humans is encoded by the TTN gene and is a giant protein that functions as a molecular spring which is responsible for the passive elasticity of muscle. Titin is important in the contraction of striated muscle tissues. It has also been identified as a structural protein for chromosomes [26-29]. Afamin is a protein that in humans is encoded by the AFM gene which is a member of the albumin gene family, which comprises four genes that localize to chromosome 4 in a tandem arrangement. These four genes encode structurally related serum transport proteins that are known to be evolutionarily related. The protein encoded by this gene is regulated developmentally, expressed in the liver and secreted into the bloodstream [30-31]. The fatty-acid-binding proteins (FABPs) are a family of carrier proteins for fatty acids and other lipophilic substances such as eicosanoids and retinoids. These proteins are thought to facilitate the transfer of fatty acids between extra- and intracellular membranes. Some

family members are also believed to transport lipophilic molecules from outer cell membrane to certain intracellular receptors such as PPAR. Levels of fatty-acid-binding protein have been shown to decline with ageing in the mouse brain, possibly contributing to age-associated decline in synaptic activity [32-33]. Alpha-1-acid glycoprotein is an acute phase plasma alpha-globulin glycoprotein and is modulated by two polymorphic genes. Its only established function is to act as a carrier of basic and neutrally charged lipophilic compounds [34]. Fibrinogen alpha chain is a protein that in humans is encoded by the FGA gene. The protein encoded by this gene is the alpha component of fibrinogen, a blood-borne glycoprotein composed of three pairs of nonidentical polypeptide chains. Following vascular injury, fibrinogen is cleaved by thrombin to form fibrin, which is the most abundant component of blood clots. In addition, various cleavage products of fibrinogen and fibrin regulate cell adhesion and spreading, display vasoconstrictor and chemotactic activities, and are mitogens for several cell types. Mutations in this gene lead to several disorders, including dysfibrinogenemia, hypofibrinogenemia, afibrinogenemia, and renal amyloidosis. Alternative splicing results in two isoforms that vary in the carboxy-terminus [35]. Acyloxyacyl hydrolase removes from lipid A the secondary acyl chains that are needed for lipopolysaccharides. This reaction inactivates the lipopolysaccharide (endotoxin). Acyloxyacyl hydrolase is produced by monocyte-macrophages, neutrophils, dendritic cells, and renal cortical epithelial cells [36]. Alpha 1-antichymotrypsin is an alpha globulin glycoprotein that is a member of the serpin superfamily. It inhibits the activity of certain enzymes called proteases, such as cathepsin G that is found in neutrophils, and chymases found in mast cells, by cleaving them into a different shape or conformation. This activity protects some tissues, such as the lower respiratory tract, from damage caused by proteolytic enzymes. This protein is produced in the liver, and is an acute phase protein that is induced during inflammation. Deficiency of this protein has been associated with liver disease. Mutations have been identified in patients with Parkinson disease and chronic obstructive pulmonary disease. Alpha 1-antichymotrypsin is also associated with the pathogenesis of Alzheimer's disease as it enhances the formation of amyloid-fibrils in this disease [37]. Serum albumin, often referred to simply as albumin is a protein that in humans is encoded by the ALB gene. Serum albumin is the most abundant plasma protein in mammals. Albumin is essential for maintaining the osmotic pressure needed for proper distribution of body fluids between intravascular compartments and body tissues. It also acts as a plasma carrier by non-specifically binding several hydrophobic steroid hormones and as a transport protein for hemin and fatty acids. Too much serum albumin in the body can be harmful. Albumin is a soluble, monomeric protein which comprises about one-half of the blood serum protein. Albumin functions primarily as a carrier protein for steroids, fatty acids, and thyroid hormones and plays a role in stabilizing extracellular fluid volume. Albumin is a globular un-glycosylated serum protein of molecular weight 65,000. Albumin is synthesized in the liver as preproalbumin which has an N-terminal peptide that is removed before the nascent protein is released from the rough endoplasmic reticulum [38]. Carbonic anhydrases form a family of enzymes that catalyze the rapid interconversion of carbon dioxide and water to bicarbonate and protons (or vice versa), a reversible reaction that occurs rather slowly in the absence of a catalyst. The active site of most carbonic anhydrases contains a zinc ion; they are therefore classified as metalloenzymes. One of the functions of the enzyme in animals is to interconvert carbon dioxide and bicarbonate to maintain acid-base balance in blood and other tissues, and to help transport carbon dioxide out of tissues.

In conclusion, feeding livestock containing the methanol extract of Moutan cortex radicis resulted in an increase in the survival ability of offspring. Analysis of proteomes contained in colostrum of sows shows this result may be caused by the changes of proteins relating with immune system. Because the evidence for this relation is not clear based on the current experiments, however, further study is remained.

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