The Expression of Surfactant Protein A in Hypoplastic Lungs in Cases of Congenital Diaphragmatic Hernia

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Abstract

Background/Purpose: Babies born with congenital diaphragmatic hernia （CDH） are high-risk patients. The mortality of patients with CDH remains high, essentially due to severe pulmonary hypoplasia and pulmonary hypertension. In addition, a surfactant deficiency is considered to contribute to the pathophysiology of CDH. The aim of the present study is to compare the expression of surfactant protein A (SP-A) in hypoplastic lungs of fetal rats with CDH induced by Nitrofen with that in normal lungs. using an immunohistochemical method.

Methods: Pulmonary hypoplasia associated with CDH was induced in fetal Sprague Dawley （SD） rats by administering Nitrofen （100 mg/kg in 1 ml olive oil） to pregnant adult rats on day 9 of gestation. As a control, 1 ml of olive oil without Nitrofen was administered to other pregnant SD rats. Immunohistochemical examination of the fetal lungs was performed using anti-SP-A monoclonal antibody.

Results: Nitrofen-exposed fetuses showed significantly lower body weights and lung weights than control fetuses. The lungs of the fetuses with Nitrofen-induced CDH had severely collapsed alveoli as well as bleeding in the alveoli. The expression of surfactant in the alveoli in the fetuses with CDH was significantly different from that in the controls.

Conclusions: The hypoplastic lungs in rats with Nitrofen-induced CDH showed a reduced level of SP-A. This suggests that prophylactic administration of surfactant at birth might be beneficial in human cases of CDH.

Introduction

Neonates with congenital diaphragmatic hernia (CDH) exhibit a high mortality rate despite intensive medical and surgical management including extracorporeal membrane oxygenation and high-frequency oscillatory ventilation. Congenital diaphragmatic hernia is characterized by a diaphragmatic defect, pulmonary hypoplasia, and pulmonary hypertension. Pulmonary hypoplasia and persistent pulmonary hypertension are considered to be the principal causes of high mortality and morbidity in infants with CDH. The associated pulmonary hypoplasia is accompanied by an underlying biochemical deficiency that bears similarities to respiratory distress syndrome (RDS) in premature newborns. Alveolar type II cells produce and secrete a complex mixture of lipids and proteins called pulmonary surfactant, which functions to keep the alveoli from collapsing at the end of expiration. Surfactant protein A (SP-A) is a major protein component of surfactant.

Wigglesworth et al. have reported that by histological, morphological, and quantitative biochemical criteria, fetuses and newborns with CDH show many similarities to the premature, surfactant-deficient newborn with RDS[1]. Exogenous surfactant is frequently used as a supplement in RDS therapy, with high efficacy. Glick et al. described three high-risk newborns with prenatally diagnosed CDH who were successfully treated with exogenous surfactant therapy shortly after birth[1].

The embryotoxicity of Nitrofen in rats and mice is well known[2] and many investigators have used the Nitrofen-induced CDH model to study the pathogenicity of CDH[3-5].

The aim of this study is to compare the expression of

surfactant in the hypoplastic lungs of fetal rats with CDH to that in normal lungs using an immunohistochemical method. For this purpose, a rat model of pulmonary hypoplasia in association with Nitrofen-induced CDH was studied.

Materials and methods

Rat model

Adult Sprague-Dawley (SD) rats were bred in our laboratory after controlled overnight matings. Pregnancy was verified by positive smears and the day of verification was designated day 0. Water and food were supplied ad libitum. These rats were divided into two groups: a Nitrofen-exposed group and a control group. The control group received 1 ml of olive oil intragastrically on day 9 of gestation, while the Nitrofen-exposed group received 100 mg per kg bodyweight of Nitrofen (2, 4-dichlorophenyl-p-nitrophenyl ether; WAKO Chemical, Osaka, Japan) diluted in 1 ml of pure olive oil on the same day and by the same method.

Fetuses were delivered by cesarean section under general anesthesia and decapitated on day 21. After a physical examination and weighing, the fetuses were dissected for inspection of their diaphragmatic hernias. Lungs from the fetuses with Nitrofen-induced CDH and the control fetuses were studied by the immunohisto-chemical method. The fetal lungs were fixed in buffer formalin and then embedded in paraffin. They were sectioned to a thickness of 5 μm.

Immunohistochemical study

The immunohistochemical examination of the lungs was performed using anti-SP-A monoclonal antibody, which was purified from rats supplied by Dr. T. Akino and Dr. Y. Kuroki, from the Department of Biochemistry, Sapporo University School of Medicine, Sapporo, Japan[6].

After deparaffinization and rehydration, all sections were treated with 0.3% hydrogen peroxide in methanol for 30 min to inhibit endogenous peroxidase, and were subsequently incubated at room temperature and treated with the reagents listed below in order. The sections were washed with phosphate-buffered saline (PBS) after each treatment.

    1) 1.0% normal bovine serum albumin (BSA) for 30 min.

    2) rat anti-mouse SP-A diluted 1/500 in 1% BSA for 1 h.

    3) biotinylated rabbit anti-rat diluted 1/200 in PBS for 30 min.

    4) avidin-biotin complex diluted 1/100 in PBS for 30 min.

    5) 3,3'-diaminobenzidine tetrachloride containing 0.05% H_2O_2 for 10 min.

The sections were then washed, stained with hematoxylin as a control stain, dehydrated, cleared in xylene, and mounted.

Controls were set up with PBS containing 1.0% normal BSA instead of the primary antisera.

After the sections were stained, they were examined under a light microscope.

The data are reported as the mean ± standard deviation. Differences were tested by Student's t test. P values less than 0.05 were considered to be statistically significant.

This experiment was conducted in accordance with the Guide for Care and Use of Laboratory Animals of Kagoshima University.

Results

In the control group, three pregnant rats were given 1 ml of pure olive oil on day 9. Thirty-three fetuses were delivered by cesarean section on Day 21. None of the fetuses had CDH.

In the Nitrofen-exposed group, six pregnant rats were given 100 mg/kg of Nitrofen in 1 ml of olive oil on day 9. Fifty-eight fetuses were delivered and 32 of these fetuses had CDH (65.5%). Eighteen fetuses (31.0%) had right-sided diaphragmatic hernias, 12 had left-sided ones, and two had bilateral ones.

The mean body weight of fetuses born from Nitrofen-exposed rats (4.2±0.3 g) differed significantly from that of the control fetuses (5.6±0.4 g) ($P<0.05$). The mean total lung weight of the Nitrofen-exposed fetuses (89.1± 20.1 mg) also differed significantly from that of the control fetuses (148.3±16.4 mg) ($P<0.05$). The total lung weight/body weight ratio (mg/g) was 21.2 in the Nitrofen-exposed fetuses and 26.5 in the control fetuses ($P<0.05$). Thus, Nitrofen-exposed fetuses showed significantly lower body weights, total lung weights, and total lung weight/body weight ratios than the control fetuses.

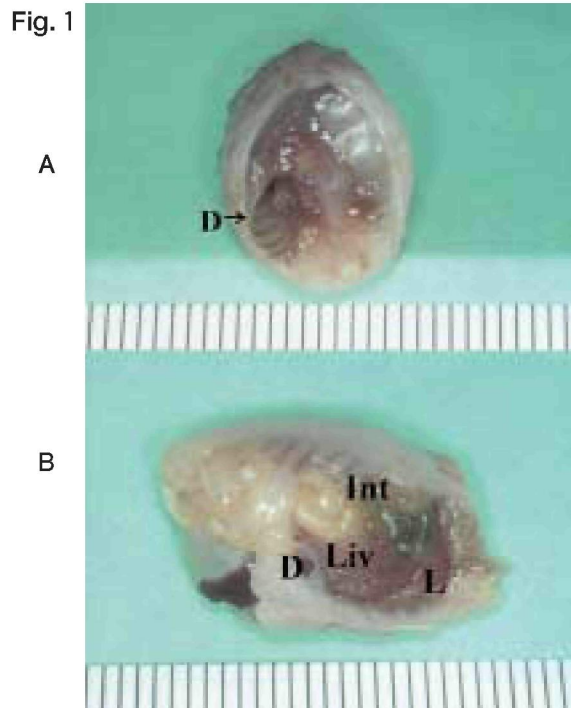Figures 1 parts A) and B) show a fetal rat with a

Fig. 1

Fig. 1. A) A bottom view of diaphragm from a transversely-sectioned fetal rat with CDH. There is a right-sided postero-lateral muscular defect allowing intra-abodominal organs to fill the left hemithorax. D; defect of diaphragm. B) A left sided view of another rat with several ribs removed. The small intestine, and liver can be seen in left hemithorax. Int; small intestin, Liv.; liver, L; lung, D; diaphragm.

Fig. 2

Fig. 2. A microscopic view of a typical example of a diaphragmatic hernia in a fetal rat. A part of the liver and lung can be seen in the same thoracic cavity. Liv.; liver, L; lung, D; diaphragm, H; heart, C; costae.
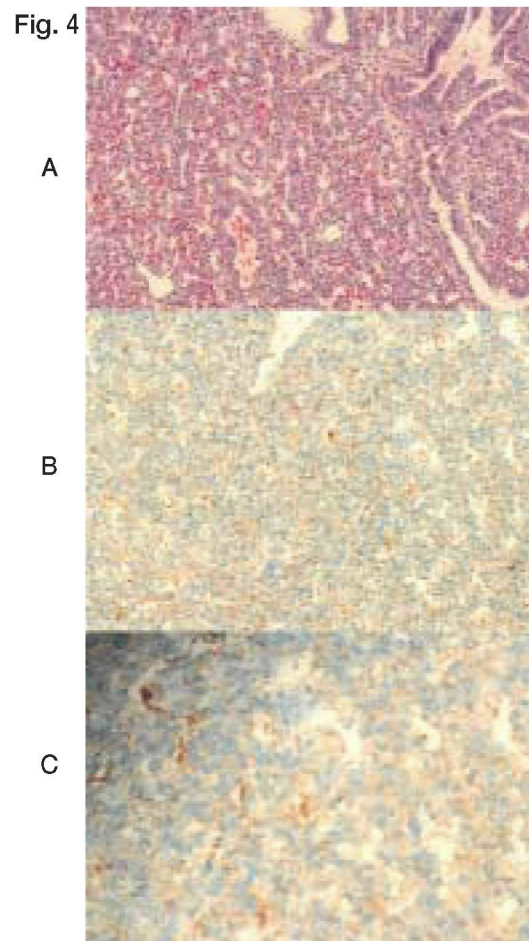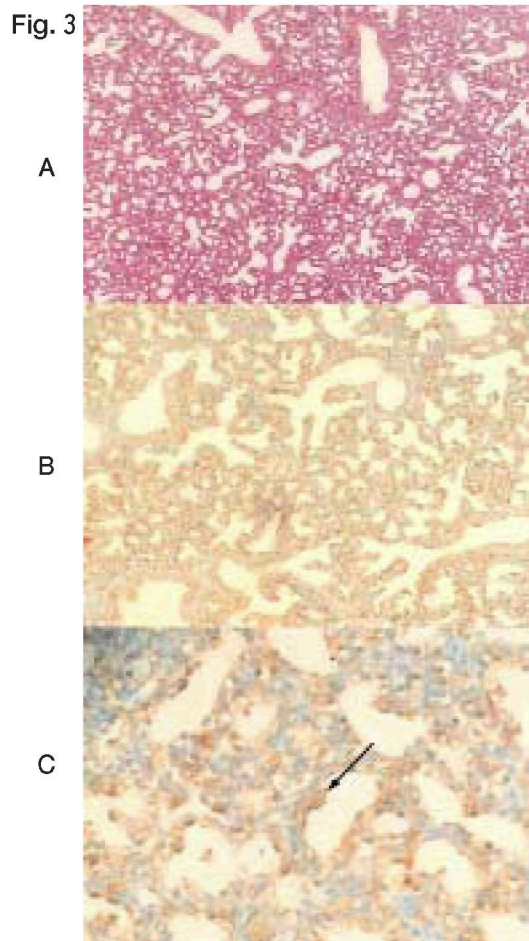
Fig. 3

Fig. 4

Fig. 3. Microscopic findings in control lung. Hematoxin & Eosin staining (A) and immunohistochemical staining with SP-A antibody (B and C). An arrow indicates an alveolar type II pneumocyte (C). Original magnification: 100× for A, 200× for B and 400× for C

Fig. 4. Microscopic findings in hypoplastic lung with Nitrofen-induced CDH. Hematoxin & Eosin staining (A) and immunohistochemical staining with SP-A antibody (B and C). Original magnification: 100× for A, 200× for B and 400× for C.

congenital diaphragmatic hernia. Figure 1A is a bottom view of the diaphragm from a transversely-sectioned fetal rat with CDH. There is a right-sided postero-lateral muscular defect that allows intra-abdominal organs to fill the left hemithorax. Figure1B is a view of another rat with several ribs removed. The small intestine and liver can be seen in the left hemithorax. Overall, the lesion is strikingly similar to a human one.

Figure 2 shows a typical example of a diaphragmatic hernia in a fetal rat. Portions of the liver and lung can be seen in the same thoracic cavity.

Microscopic examination of the lungs from the control group showed the structure of the alveoli was normal and the structure of the lungs displayed characteristics of the terminal sac phase (Fig. 3A). The pulmonary alveolar surface and the alveolar type II pneumocytes were stained by anti-SP-A monoclonal antibody (Fig. 3 B, C).

Microscopic examination of the lungs from fetuses with CDH showed the affected lungs had severely collapsed alveoli as well as bleeding in the alveoli and thick muscularized walls (Fig. 4). The structure of the lungs displayed characteristics of the pseudoglandular phase. The alveolar type I and II pneumocytes were not detected and the expression of surfactant in the alveoli was not prominent (Fig. 4 B, C).

Discussion

Despite major advances in neonatal resuscitation and intensive care, newborns with CDH still have high morbidity and mortality rates. Lung hypoplasia and persistent pulmonary hypertension are considered to be the principal causes of high mortality and morbidity in infants with CDH.

The pathogenesis of CDH with pulmonary hypoplasia is poorly understood. So far, most researchers have speculated that a diaphragmatic defect in the viviparous term allows the intrathoracic cavity to be occupied by the intra-abdominal organs, causing obstruction of normal pulmonary growth and resulting in lung hypoplasia. Some animal models with surgically induced CDH were produced in accordance with this hypothesis. In 1967, de Lorimier et al. induced CDH in fetal lambs[7]. At term, the lungs of these lambs were hypoplastic, showing a 23% to 75% reduction in lung weight and air capacity compared with normal lungs. Subsequently, a number of other researchers created experimental animal models to study the effects of CDH. Our study also used rabbits as surgical experimental animals, although with some limitations. What all these experiments have in common is the need for surgical intervention in a relatively late stage of lung development, because any earlier, the fetus is too small to be operated on successfully.

Since 1971, the embryotoxicity of Nitrofen in rats and mice has been well known[8]. In 1984, Iritani reported the successful generation of neonatal CDH in mice after oral administration of Nitrofen for prolonged periods during gestation[9]. Nitrofen is an herbicide with potent teratogenic activity in mice and rats. After exposure to Nitrofen, several malformations were observed in mice and rats, affecting the heart, kidneys, diaphragm, and lungs. Manson reported that Nitrofen exerts a teratogenic effect via alterations in the thyroid hormone status[12].

Kluth et al. reported that most hernias occurred after 100 mg/kg of Nitrofen on days 9 and 11[14]. Left-sided hernias were observed only after exposure to Nitrofen on day 9. We gave 100 mg/kg of Nitrofen to pregnant rats on day 9, which resulted in left-sided hernias, right-sided hernias, and bilateral hernias.

Respiratory distress syndrome is caused by a surfactant deficiency, due to a decrease in surfactant synthesis or release by alveolar type II pneumocytes. A surfactant deficiency has been considered to contribute to the pathophysiology of CDH. Moya reported that there are decreased surfactant components in amniotic fluid in many pregnancies complicated by CDH, which may reflect fetal lung immaturity or hypoplasia[11]. Exogenous surfactant is frequently used as a supplement in RDS therapy, with high efficacy. Glick et al. described three high-risk newborns with prenatally diagnosed CDH who were successfully treated with exogenous surfactant therapy shortly after birth[2]. Lotze et al reported tracheal aspirate surfactant protein-A concentrations were initially low in infants with CDH on extracorporeal membrane oxygenation[15]. The results of our study support these findings by the Nitrofen-induced CDH rat model.

Pulmonary surfactant is a complex mixture of lipids and proteins, and plays a role in reducing the surface tension of the alveolar interface. Surfactant protein A (SP-A) is a major protein component of surfactant and a glycoprotein with a reduced denatured molecular mass of 26-38 kDa in the rat[6]. Our immunohistochemical study

showed a reduction of SP-A within the hypoplastic lungs of rats with Nitrofen-induced CDH.

Phospholipids are the major components of pulmonary surfactant, making up 80-90% of its weight. Pulmonary SP-A binds sphingomyelin and disaturated phosphatidylcholine (DSPC).

Cogo et al. were the first to measure surfactant DSPC kinetics in newborn infants with CDH. These patients exhibited significantly lower DSPC in their tracheal aspirate[16].

Utsuki et al. determined the level and distribution of lung surfactant using the monoclonal antibody to sphingomyelin and DSCP[17]. They revealed that surfactant phospholipid in Nitrofen-treated fetuses was mainly in the form of intracellular granules, probably causing the hypoplastic lungs and then CDH, in contrast to the uniform distribution of surfactant phospholipid on the pulmonary alveolar surface in control fetuses. These findings support the results of our study.

Because treating pulmonary hypoplasia and pulmonary hypertension in newborns with CDH is difficult, antenatal tracheal ligation and glucocorticoid administration have been used in an attempt to prevent pulmonary hypoplasia and pulmonary hypertension. Kitano et al. showed the induction of proliferative lung growth by fetal tracheal occlusion in the rat model of Nitrofen-induced CDH[18]. Mann et al. report that a combination of prenatal maternal glucocorticoids and postnatal NO inhalation significantly improved the survival rate of newborn rats with Nitrofen-induced CDH[20]. However, these procedures are not as successful when put into practice on humans.

Conclusion

Solving each clinical problem brought about by pulmonary hypoplasia and hypertension will lead to the improvement of the prognosis of infants with CDH. Our study showed that hypoplastic lungs in rats with Nitrofen-induced CDH showed a reduced level of SP-A. This suggests that prophylactic administration of surfactant at birth might be beneficial for human cases of CDH.

Acknowledgements

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先天性横隔膜ヘルニアの低形成肺における
Surfactant Protein A の発現について

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　背景／目的：先天性横隔膜ヘルニア（CDH）は，死亡率の高い疾患であるが，その原因は，高度の肺低形成と肺高血圧症に起因する。加えて surfactant の欠乏もその一因と考えられている。この研究は，Nitrofen（2,4-dichlorophenyl-p-nitrophenyl ether）誘導により作成された CDH のラットモデルにおいて，低形成肺における surfactant protein A（SP-A）の発現を，Nitrofen を投与していない対照群と免疫組織学的に比較したものである。

　実験方法：妊娠9日目のラットに Nitrofen 100mg/kg を olive oil に溶解し胃内に投与した群（Nitrofen群）と olive oil のみを投与した群（対照群）を作成した。妊娠を継続させ妊娠21日目に帝王切開により胎仔を回収し，CDH の有無を確認後，肺を採取した。Nitrofen 群から得られた CDH 胎仔と対照群から生まれた対照胎仔の2群の肺をSP-Aに対するモノクロナル抗体を用いて免疫組織学的に染色し，比較検討した。

　結　　果：CDH 胎仔は，対照胎仔と比較し，有意に体重量，肺重量，肺重量／体重量比が小さかった。CDH 胎仔の肺は，組織学的に，肺胞の虚脱や，肺胞内の出血がみられた。SP-A 抗体を用いた免疫組織染色では，対照胎仔では，肺胞内の上皮に SP-A が十分に発現しているのに比べ，CDH 胎仔では，その発現は乏しかった。

　結　　論：今回の実験結果は，Nitrofen で誘導されたラットの CDH モデルの低形成肺では，解剖学的低形成のみでなく surfactant の産生能も低下していることを示唆している。

第25回鹿児島栄養代謝研究会抄録

鹿児島栄養代謝研究会
（代表世話人：高松　英夫教授）

日時：平成15年6月23日（月曜日）　会場：鹿児島東急ホテル　桜島の間

特別講演

座長　鹿児島大学小児外科教授　高松　英夫

『**特殊栄養成分による生活習慣予防－殊に栄養療法に於ける微量栄養素，CoQ10の役割－**』

久留米大学小児外科講師　田中　芳明

一般演題Ⅰ　座長　鹿児島大学第一外科　大脇　哲洋

⑴　肥満度は同じでも合併症重症度と頻度は増悪している

鹿児島大学大学院小児発達機能病態学分野，鹿児島市医師会*

田中　覚，島子敦史，西　順一郎，河野幸春，野村裕一，吉永正夫，河野嘉文，清水信一郎*，有馬　桂*

最近小児のライフスタイルの変化は，肥満の程度は同じでも合併症の重症度や頻度を増悪させている可能性がある。1992年から2001年まで鹿児島市で行われている小児生活習慣病検診を受診した小学生を対象に前半期（1992－1996）と後半期（1997－2001）で比較したところ，肥満の程度が同じであっても総コレステロール値，LDL，コレステロール値，ALT値や，全体での異常値を示すこどもの割合は最近になるほど増悪しており，またこの関係は年齢，性も関与していた。

⑵　肝硬変患者において十分な食事量を摂取できない時の早朝空腹時のエネルギー代謝異常

鹿児島厚生連病院

今村也寸志，窪薗　修

一般演題Ⅱ　座長　鹿児島大学小児科　吉永　正夫

⑶　PEG より NST へ（第2報）－栄養科のとりくみ－

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浦底美由希，上之原美智子，岩下えりか，仮屋美幸，楠元千賀，立元紀子，藤岡俊昭*，有本之嗣*

当院では，平成8年より，経口摂取不良な患者様を対象にPEG（経皮内視鏡的胃瘻増設術）を施行している。平成15年5月までに計267例の症例を経験してきた（男性103例・助成164例　平均年齢83.2歳）。その間より良い栄養療法のために胃瘻委員会の設置やコーディネーターの導入，また PEG 術前検討会を導入してきたが，栄養士もチームの一員として関わってきた。特に PEG におけるチームでの検討は栄養について考える場と発展し，現在では自然発生的に NST（栄養療法チーム）の発足に至った。今回は，これらの経緯と現在行っている NST における栄養士の役割についてまとめたので報告する。

栄養療法チームの中での役割としては，病棟担当栄養士を配置し，発生する栄養に関する問題点の拾いあげや患者様の要望に答えている。また，使用している経腸栄養剤・栄養補助食品の一覧表の作成・広報・配布を行い院内外で活用している。さらに，月2回栄養療法委員会を開催し，栄養療法に関する学集会を定期的に行っている。

実際の患者様に対しての役割としては，PEG 術前検討会へ参加し，術前の身体計測を含む栄養評価を行い，必要エネルギー・水分量・経腸栄養剤についての情報提供を行っている。また，PEG 施工後の患者様に対して，入院期間中だけでなく，在宅・他施設においても連動的に身体計測や栄養評価を行っており，訪問による栄養指導も必要がある場合は随時実施している。その際全ての患者様に対して栄養カルテを作成し，情報の共有化を図っている。

現在このように NST に関わっているが，栄養管理はすべての治療の基本であり，私たち栄養士は資質の向上の必要性を痛切に感じ，日夜努力している。

⑷　400ml脱血に伴う血清タンパク喪失に対するバランス栄養食品の効果

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同種血輸血ならびに自己血輸血における急速脱血に伴う生体の変化に関しては，不明な点も多い。今回，われ

われは，400mL脱血直後のバランス栄養食品が血清タンパク喪失に与える影響を検討した。
＜材料と方法＞　対象：成人女性15名。方法：400mL脱血直後に，市販のバランス栄養食品（大塚製薬，カロリーメイトゼリー）2袋424gを摂食させ（以下，摂食群），末梢血の膠質浸透圧，血清タンパク量，および血液学的変化を経時的に検討し，対照群（摂食なし）と比較した。
＜結果とまとめ＞　1．脱血後1時間の膠質浸透圧は，脱血前に比較して両群ともに有意に（p<0.05）減少したが，摂食群においては，脱血後30分以後の減少が抑制された。2．血清タンパク量も，膠質浸透圧と同様の結果を示した。3．以上より，脱血直後に飲まれるタンパク質を含むバランス栄養食品は，脱血に伴う血清タンパク喪失を抑制する可能性があることが示唆された。