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FATAL AMNIOTIC FLUID EMBOLISM: AN OVERVIEW OF POSTMORTEM BIOMARKERS

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Abstract: Amniotic fluid embolism (AFE) is a rare clinical condition with a high maternal fatality rate. The definitive diagnosis in fatal cases is based on various postmortem biomarkers: histochemical (amniotic fluid components in the maternal pulmonary vessels visualized by routine and special stains), immunohistochemical (cytokeratin from fetal squamous cells visualized by AE1/AE3 antibody cocktail, tryptase release from mast cell degranulation assessed by AA1 monoclonal anti-tryptase antibody, complement C3a by anti-C3a, mucin-type glycoprotein sialyl Tn visualized by monoclonal antibody TKH-2), and serum biomarkers, especially the specific fetal antigen sialyl Tn (determined by immunoradiometric competitive inhibition assay) and the mast cell tryptase (quantified by fluorescence enzyme immunoassay with capsulated cellulose polymer solid-phase). Herein we present a comprehensive overview of postmortem biomarkers for AFE diagnosis and their usefulness.

Keywords: amniotic fluid embolism, pregnancy, postmortem, biomarkers, immunohistochemistry, serum specimens.

INTRODUCTION

Amniotic fluid embolism (AFE) is an infrequent, unpredictable and often fatal condition unique to obstetrics. Uniform diagnostic criteria for research reporting of AFE are clinical: onset during labor or within 30 min of delivery of the placenta, no fever during labor, and sudden onset of cardiorespiratory arrest, or both hypotension and respiratory compromise (dyspnea, cyanosis, or peripheral capillary oxygen saturation less than 90%), documentation of overt disseminated intravascular coagulation (DIC) following the appearance of these initial signs or symptoms (coagulopathy must be detected prior to loss of sufficient blood to itself account for dilutional or shock-related consumptive coagulopathy) [1].

AFE is recognized initially by its clinical course only, including severe genital bleeding and DIC with uterine atony soon after delivery and cardiopulmonary collapse during the peripartum period [2]. A recent analysis of the largest United States-based current international AFE registry reported a maternal and neonatal mortality reduction consecutive to perinatal health care improvement. Regarding risk factors, AFE is associated with maternal age greater than 35, in vitro fertilization and abnormal placentation. AFE is not significantly associated with induction of labor, but occurs more frequently during labor than cesarean section or during delivery. Moreover, an association with atopy is also possible [3, 4].

AFE continues to represent a daunting clinical challenge that needs an interdisciplinary approach involving obstetricians, intensive care physicians, laboratory immunologists and forensic pathologists [5-8]. Differential diagnoses include anaphylaxis, other causes of acute pulmonary embolism, cardiogenic, septic or obstetric hemorrhagic shock, DIC due to other causes [1, 4, 8]. Currently, only autopsy can confirm with a high degree of certitude the AFE diagnosis [9, 10]. The aim of this review is to provide comprehensive information on postmortem biomarkers for AFE diagnosis and to highlight their usefulness.

HISTOCHEMICAL AND IMMUNOHISTOCHEMICAL BIOMARKERS

The gold standard for the diagnosis of AFE is

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the histopathologic examination of lung tissue samples during forensic autopsy [11, 12]. At the postmortem examination, no remarkable macroscopic findings are found except for pulmonary edema and congestion, along with focal atelectasia and subpleural blood extravasation due to DIC [13-15].

The use of histochemical and immunohistochemical biomarkers (Table 1) are used for an accurate postmortem diagnosis. Bilateral pulmonary specimens are collected from each lobe together with a perihilar sample. All specimens are fixed in formalin and embedded in paraffin [14, 16]. The legal medicine diagnosis of AFE is based upon the microscopic recognition of fetal debris inside maternal pulmonary vessels. Such a foreign material consists of scales, lanugo fragments, mucin plugs, meconium droplets, and trophoblast elements. However, although morphologically remarkable, these components may be sporadic [11].

Histochemical biomarkers of amniotic fluid particulate components presented in the maternal pulmonary vessels in AFE are represented by fetal epithelial squamous debris and lanugo hair, vernix caseosa cells and intravascular mucin. These components of amniotic fluid emboli have been identified as epithelial squames shed from the fetal skin, lanugo hairs (increased numerically with the length of gestation period), vernix caseosa as fatty material, mucin derived from the fetal intestine and associated with the passage of meconium, and bile pigments derived from the meconium. From the formalin-fixed paraffin-embedded tissues, 5 μm thick sections are prepared and routinely stained with hematoxylin-eosin, elastic van Gieson, Prussian blue, periodic acid-Schiff (PAS) and Luxol fast blue/PAS. Squamous cells embedded in microthrombi and decidual cells in the pulmonary artery, along with infiltration of the vessel wall by granulocytes may be visualized with haematoxylin-eosin stain, while meconium in the lung capillaries with PAS [12, 17].

Histological examination of lung samples with hematoxylin-eosin stain reveals the presence of fetal squamous cells, lanugo hairs and vernix caseosa in the pulmonary artery vasculature, along with acute emphysema, focal atelectasis, and pulmonary edema. With special stains such as Alcian blue and Alcian green-phloxine tartrazine (Attwood modified), squamous cells and mucin are evident. Alcian blue stain is used to confirm the presence of acidic mucosubstances and acetic mucins from amniotic fluid in the pulmonary vessels. Pinacyanole chloride stain may be used to identify vernix caseosa cells. Nuclei of fetal squamous cells and lanugo are also clearly visible by confocal laser scanning microscopy. In addition, aggregates of fetal squames are present in small- and medium-sized pulmonary vessels, with the occasional commingled presence of platelet thrombi adjacent to fetal squames. Microthrombi in the pulmonary septal capillaries and small cerebral and renal vessels are observed due to DIC, along with subpleural and subepicardial hemorrhages. Microscopic examination of the uterus samples is usually unremarkable [11, 13-15, 18, 19].

The evidence of fetal squamous cells in the maternal pulmonary artery circulation is considered suggestive but not pathognomonic for AFE. In patients with many critical conditions, including complicated preeclampsia and septic shock, distal port aspirates from pulmonary artery catheters reveal that trophoblasts and squamous cells are often transported in these pulmonary vessels [8, 20, 21]. More than thirty years ago, initial AFE reports were based on detection of squamous cells and sporadically other debris of fetal or placental origin in the maternal pulmonary circulation at autopsy. However, subsequent data revealed that these findings are not always specific to AFE. In addition, such reports included critically ill and fatal cases demanding autopsy, eliminating less severe cases. Therefore, evaluations based only on histologic biomarkers may be biased [1, 22, 23].

Regarding fetal cell identification in maternal circulation, only in about half of the AFE cases fetal products are detected. Nowadays, available evidence does not clearly support the use of these fetal biomarkers to firmly confirm or exclude AFE diagnosis [5, 24].

Uterine AFE is considered when fetal debris and amniotic fluid components are found in the uterus in pathological conditions such as severe uterine hemorrhage after placental removal, in the absence of other obstetric hemorrhagic complications [25].

Criteria for the diagnosis of AFE in the UK and Australia consist in clinical diagnosis of AFE (acute hypotension or cardiac arrest, acute hypoxia,

Table 1. Postmortem biomarkers for amniotic fluid embolism

Biomarker type	Example
Histochemical	Amniotic fluid components in the maternal pulmonary vessels
Immunohistochemical	Cytokeratin, tryptase, complement C3a, sialyl Tn
Serum biomarker	Sialyl Tn, mast cell tryptase, complement C3 and C4

or coagulopathy in the absence of any other potential explanation for signs and symptoms observed), or pathologic/postmortem diagnosis with the presence of fetal squames or hair/debris in the pulmonary circulation, but proposed uniform diagnostic criteria for research reporting of AFE do not include such histochemical biomarkers [1,26].

Immunohistochemical biomarkers for the postmortem diagnosis of AFE are linked to the pulmonary intravascular presence of fetal squamous cells and pathophysiology mechanisms related to mast cell degranulation and complement activation. Cytokeratin, tryptase release and complement C3a responses in autopsy specimens reveal strong staining patterns for cytokeratin and degranulated tryptase, and weak expression of complement C3a in AFE cases. Few fetal debris can also be noticed in the lung capillaries of women without AFE during peripartum period [15, 27].

An immunohistochemical examination is focused on keratin scales because they are considered a hallmark of AFE. Cytokeratin stain on lung samples reveals intense intravascular positivity of fetal squamous cells in AFE. AE1/AE3 cytokeratin (CK) immunostain reacts with CKs 1-8, 10, 14-16 and 19 [15, 28]. In single immunohistochemistry with anti-pancytokeratin clones AE1/AE3, after antigen retrieval with Tris-EDTA buffer, antigen-antibody complexes are highlighted with the brown chromogen 3,3-diamninobenzidine tetrahydrochloride. Scales recognition may be difficult with this approach because other keratin containing cellular elements, mainly bronchial/bronchiolar epithelial cells and pneumocytes, normally found almost everywhere in the lung tissue, are creating a so-called "background noise".

To better highlight fetal scales within maternal pulmonary vasculature in case of fatal AFE, a double immunohistochemistry stain utilizes endothelial biomarker CD31 in addition to the epithelial marker for CKs. CD31 is also known as platelet endothelial cell adhesion molecule (PECAM-1). After antigen retrieval, the anti-CD31 antibody (clone JC70) is then incubated for 28 min at 37°C, and antigen-antibody complexes are highlighted with the brown chromogen. This double immunohistochemical stain simultaneously visualize the cytokeratin AE1/AE3-positive scales in red, inside distal arteriolar and capillary vessels, and CD31-positive endothelial linings cells in brown [11, 29]. After washing out the 3,3-diamninobenzidine tetrahydrochloride chromogen excess, the anti-pancytokeratin AE1/AE3 antibodies are incubated at room temperature for 36 min and the antibody binding is highlighted by the red chromogen 4-chloro-2-methylbenzenediazonium (Fast Red). With such a double immunohistochemistry stain, "red" scales inside the "brown" ring of endothelial cells are rapidly and consistently identified. Single occluding intravascular scales are easily distinguished from pseudomicrothrombi due to desquamated endothelial cells and from fibrin microthrombi, the latter being extremely common in AFE complicated by DIC. Therefore, the anti-pancytokeratin and anti-CD31 double immunostaining of lung tissue sections are suggested to be used in order to support the postmortem diagnosis of AFE because the embolic obstructive microangiopathy of the lungs is a major AFE pathogenetic mechanism [11].

To facilitate identification of meconium- or amniotic fluid-derived mucin in postmortem lung specimens from patients suggesting AFE, immunohistochemical staining is performed with monoclonal antibody TKH-2 directed toward the core structure of O-linked mucin-type glycoprorein, sialyl Tn (NeuAc alpha 2–6GalNAc alpha 1-O-Ser/Thr) [21, 30]. Tissue samples are fixed in 10% neutral buffered formalin, embedded in paraffin, and cut into 5 μm serial sections for immunohistochemical, hematoxylin-eosin and Alcian blue staining. Formalin-fixed, paraffin-embedded lung tissue sections are stained using the streptavidin-biotin-immunoperoxidase method.

After incubation with fresh 3% hydrogen peroxide in methanol and washing with phosphate-buffered saline (PBS), monoclonal antibody TKH-2 is used as the primary antibody. TKH-2 is incubated 16 hours, at 4°C, and washed with PBS. Biotinylated anti-mouse IgG is added and washed with PBS. Streptavidin-peroxidase is then added, and after washing with PBS, slides are reacted with 3% 3-amino-9-ethylcarbazol substrate, rinsed with tap water, and then mounted.

In patients with AFE, notable positive TKH-2 immunostaining is observed within the pulmonary vasculature. Mucin emboli in the pulmonary artery produce two types of staining: dense staining parts, which are also positive for Alcian blue stain and correspond to a region of leukocytosis, mainly with neutrophils, and the faint staining parts, negative for a conventional Alcian blue stain with associated leukocytosis, regarded as the diluted amniotic mucin-type glycoprotein in the bloodstream. No mucin is usually recognized by routine hematoxylin-eosin staining. It is important to mention that a positive TKH-2 immunostaining in epithelial goblet cells is normal, and a negative TKH-2 staining may not exclude AFE in

the presence of clear amniotic fluid [30].

Regarding the role of mast cell degranulation in the maternal pulmonary vasculature as an AFE pathomechanism, the immunohistochemical data should be considered separately from the serological data, since the evidences are rather mixed in both cases. In fatal AFE, the available evidence suggests that mast cell degranulation does occur in the lungs dissimilar to other fatal pregnancy conditions [24]. It is speculated that a concomitant severe hypersensitivity reaction to amniotic fluid may be involved as a pathogenetic mechanism [31]. Mast cell degranulation releases tryptase, along with histamine and other vasoactive or procoagulant substances, similar to a systemic inflammatory response syndrome [32].

A numerical increase of pulmonary mast cells in patients who died of AFE is considered an additional immunohistochemical biomarker for the postmortem diagnosis, these cells being clearly involved in the systemic pathogenetic AFE mechanisms. Such a quantitative assessment needs a relatively large number of microscopic observations in different fields of the lungs using a video camera system and a computer program for quantitative morphometry. In each histological section, ten observations in different fields per slide equivalent to seventy observations for each single case are proposed. The elevated numbers of mast cells in fatal AFE are similar to those encountered in cases of death due to anaphylactic shock but higher than those found in cases of traumatic accidents [14].

To estimate the mast cell population by immunohistochemical staining, anti-tryptase antibody is utilized as a mast cell-specific marker [16]. The mast cell tryptase antibody staining protocol for immunohistochemistry uses the avidin-biotin-complex method (ABC-method) on 5 μm paraffin sections. Enzyme pretreatment with proteinase K 0.01% at 37°C is necessary to facilitate antigen retrieval and to increase membrane permeability to antibodies. The primary mouse monoclonal anti-tryptase antibody, clone AA1, recognizes human mast cell tryptase, both alpha and beta isoforms, and is applied in a 1:100 ratio and incubated overnight at 4°C. The positive reaction is visualized by 3-diamino-9-ethylcarbazole, the sections are counterstained with Mayer's hematoxylin, mounted in low molecular aziridin homopolymer, and examined under a light microscope [14, 33, 34].

In all cases of death by AFE, the immunohistochemical study of mast cell tryptase is added to the measurements of serum tryptase levels. In these cases, an elevated number of pulmonary mast cells are identified by immunohistochemistry in the bronchial walls and capillary septa [14].

With another similar approach, a large increase in extracellular tryptase, consistent with mast cell degranulation, may be found in fatal AFE cases compared to other pregnant women who died from traumatic injuries. In such an immunohistochemical investigation with anti-tryptase, 4 μm paraffin sections may be used, mounted on slides covered with 3-amminopropyl-triethoxysilane. Pretreatment is necessary to facilitate antigen retrieval and to increase membrane permeability to antibodies. A refined avidin-biotin technique is utilized, in which a biotinylated secondary antibody reacts with peroxidise conjugated streptavidin molecules. Positive reactions are visualized by 3,3-diaminobenzidine peroxidation. The sections are counterstained with Mayer's hematoxylin. The samples are then examined using an optical microscope and a confocal laser scanning microscope. A halo of tryptase-positive material (golden reaction) around the mast cells reveals evidence of mast cell degranulation. Indication of degranulating mast cells with tryptase-positive material outside the cells may be seen in capillary septa (starry effect) and can be documented by confocal laser scanning microscope technique [15].

It is possible that complement activation in AFE may be an initial immune response and degranulation of mast cells, a secondary consequence [24]. In the same histology samples with evidence for tryptase release, immunohistochemical investigation may be performed utilizing anti-C3a in a similar manner. Boiling in EDTA buffer using microwaves is needed, and the primary antibody is applied in a ratio of 1:500 for C3a. A weak expression of complement C3a is expected in fatal AFE cases. Diminished pulmonary C3a is consistent with complement activation [15].

SERUM BIOMARKERS

Postmortem serum biomarkers studied in fatal AFE (Table 1) are represented by elevated levels of the specific fetal antigen sialyl Tn (STn) related to meconium- or amniotic fluid-derived mucin, and of mast cell tryptase revealing mast cell activation and degranulation [13].

The sialyl Tn antigen is expressed in fetal colonic mucosal cells, particularly in goblet cell mucin, and is a characteristic component in meconium and amniotic fluid [35]. Serum STn antigen levels may be determined by an immunoradiometric competitive inhibition assay that uses the anti-mucin monoclonal antibody

TKH-2 (a unique anti-STn generated by immunization of mice with ovine submaxillary mucins), in a one-step procedure. Beads coated with purified STn are incubated with the sample, and 125iodine-labeled TKH-2 antibody solution for 90 min. Radioactivity bound to beads is measured by a gamma counter [36].

Markedly elevated levels of serum sialyl Tn, even above 600 U/mL, may be detected in fatal AFE, compared with a control value, less than 20 U/mL; therefore, this biomarker may be a useful parameter for the postmortem diagnosis [13, 37]. More studies should resolve issues of sensitivity and specificity of sialyl Tn assays in AFE and other obstetric conditions [37].

Mast cell tryptase is considered to be a specific marker for systemic mast cell activation. This neutral protease has a longer serum half-life than other mediators such as histamine; therefore, its measurement is useful for postmortem assessment as well as antemortem diagnosis of anaphylaxis. Elevation of serum total tryptase is also reported in patients with AFE, revealing the pathophysiological role of mast cell activation and degranulation in these cases [13].

Serum total mast cell tryptase is not a routine part of the postmortem laboratory testing, and it is generally requested only when suspect anaphylaxis [38] and AFE, considered in the past an "anaphylactoid syndrome of pregnancy" [13].

Postmortem total mast cell tryptase levels are measured from femoral blood samples collected with a wide bore needle (18G) and syringe by gently aspirating from either or both femoral vessels or collected from a femoral vein cut down during evisceration. The blood specimens are placed into plain tubes, stored at 4°C, and analyzed within seven days by utilizing a commercially available fluorescence enzyme immunoassay with capsulated cellulose polymer solid-phase. This method determines the total tryptase levels, including all forms of alpha-tryptase and beta-tryptase. Its principle consists in the reaction of the tryptase antigen in the postmortem sample with anti-tryptase covalently coupled to a solid phase consisting of a cellulose derivative enclosed in a capsule. Beta-galactosidase-labeled antibodies against tryptase are added to form a complex incubated with 4-methylumbelliferyl-beta-D-galactoside, a developing agent. The fluorescence of the eluate is then measured. The higher the response value, the more tryptase is present in the specimen [39, 40].

Commonly, postmortem mast cell tryptase is a valuable test in supporting the diagnosis of anaphylactic death, and femoral total postmortem values of 58 μg/L and above provide a good sensitivity and specificity. Age, sex and resuscitation have no significant influences on autopsy femoral tryptase. A postmortem interval between death and autopsy of up to eight days (with appropriate storage and refrigeration) has no significant association with elevated postmortem tryptase [38].

The heart chambers contain higher concentrations of tryptase compared to venous blood. Resuscitation may be a potential factor causing elevation in postmortem tryptase levels in blood collected from cardiac cavity [41]. A cut-off level of 110 μg/L in aortic samples from fatal anaphylactic cases was reported, but femoral specimens are preferable [42].

In peripheral venous blood specimens obtained less than two hours postmortem elevated tryptase levels may be detected in fatal AFE [43].

High serum tryptase level in blood extracted from the cardiac cavity, above 60 μg/L, may be found in fatal AFE, compared with control cases. In contrast, in fatal cases of typical anaphylactic shock, remarkable elevations of serum tryptase levels may also be recorded, even above 600 μg/L [13,44].

An elevated beta-tryptase level may also be found in fatal AFE. The biotin-G5 immunoassay may be used to determine mature beta-tryptase stored in mast cell granules and released upon degranulation [6, 32].

Beta-tryptase determination in the pericardial fluid may be an alternative to postmortem femoral samples in anaphylaxis, especially in the early postmortem period, but vitreous humor and urine tryptase are not [45].

If high tryptase levels are found in patients with AFE, it is possible that specific IgE to fetal proteins may hypothetically be involved. For their detection polyacrylamide gel electrophoresis and Western blot techniques may be used, but, to our knowledge, such IgE sensitization assessment was not reported in postmortem blood samples [46].

Because complement activation is reported to be associated with AFE pathophysiology, serum C3 and C4 levels are quantitative determined by nephelometry [37]. For serum complement components determination, nephelometry detects the formation of immune aggregates in solution via light scatter with reference curves previously constructed by multipoint calibration [47, 48]. In fatal AFE, serum C3 and C4 levels may be lower than published postpartum control values [13]. This should be interpreted with caution due to limited reported data. Moreover, it seems that some degree of complement activation during normal labor, with declines of C3 and C4, is physiologic and peaks at

or shortly before birth and start to return to normal in the postpartum period [24, 37, 46].

Other potential serum biomarkers for the diagnosis of AFE, such as zinc coproporphyrin 1 [49], insulin-like growth factor binding protein 1 [50] or squamous cell carcinoma antigen from fetal epidermis [51], found in amniotic fluid in higher concentrations [6], are not studied yet in postmortem biological samples. The research for new candidate AFE biomarkers in this pregnancy-associated cytokine storm-like condition is ongoing [52].

In conclusion, AFE is recognized initially only by its clinical course and currently confirmed with a high degree of certitude only by autopsy. This devastating condition needs an interdisciplinary approach involving obstetricians, intensive care physicians, laboratory immunologists and pathologists. Histochemical and immunohistochemical postmortem evaluation is needed to assess amniotic fluid components in the maternal pulmonary vessels. Serum biomarkers, especially the specific fetal antigen sialyl Tn and the mast cell tryptase, are also supportive for the AFE diagnosis. Due to the difficult diagnosis challenges and complex medico-legal issues related to AFE, the creation of large-scale databases or registries with detailed clinical information along with the preservation of serum and tissue samples of AFE patients is important for scientific collaboration to improve medical care and research.

Conflict of interest

The authors declare that they have no conflict of interest.

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