



Contents lists available at ScienceDirect

Forensic Science International

journal homepage: www.elsevier.com/locate/forensiint



Immediate anaphylactic death following antibiotics injection: Splenic eosinophilia easily revealed by pagoda red stain

Nicoletta Trani ^{a,*}, Luca Reggiani Bonetti ^b, Giorgio Gualandri ^a, Giuseppe Barbolini ^b

^a Department of Diagnostic Services, Pathology and Legal Medicine, Section of Legal Medicine, University of Modena and Reggio Emilia, Modena, Italy

^b Department of Diagnostic Services, Pathology and Legal Medicine, Section of Pathology, University of Modena and Reggio Emilia, Modena, Italy

ARTICLE INFO

Article history:

Received 22 February 2008

Received in revised form 11 July 2008

Accepted 15 August 2008

Available online xxx

Keywords:

Anaphylactic death

Antibiotics

Splenic eosinophils

Splenic mast cells

Pagoda red

Immunohistochemistry

Shock organ

ABSTRACT

The comparison of 4 cases of immediate anaphylactic death following the intramuscular injection of antibiotics (different types of penicillin or cephalosporins) with 4 cases of immediate non-anaphylactic death (induced by different causes) recognized splenic eosinophilia, as the main feature for the differential diagnosis, in agreement with isolated previous studies.

The use of a stain (pagoda red) little known and seldom employed in Pathology and in Forensic Medicine, showed the concomitant massive presence (immunohistochemically confirmed) of mast cells and degranulated mast cells, the latter mainly located in splenic sinuses.

The whole of our findings led us to consider the spleen as the possible shock organ in man. Waiting for further judgment on our hypothesis, we seized the opportunity to remark the employment of the pagoda red stain when the contemporary demonstration of eosinophils, mast cells and degranulated mast cells is required.

© 2008 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

The concept of anaphylaxis derives from the observations concerned with the effects of actinotoxins on the blood pressure of dogs [1]. Anaphylactic shock is a well known example of the immediate type of hypersensitivity reaction inducing a diffuse organ hypoperfusion. It may be defined [2] as the failure of the peripheral circulation induced by an antigen(allergen)–antibody reaction in already sensitized subjects for a foreign substance.

If the hypoperfusion is complicated by increased capillary permeability, a rapidly fatal irreversible circulatory damage (anaphylactic death) takes place. In the exhaustive and detailed clinico-pathological study of Delage and Irely [2], based on 43 validated cases of drug-induced fatal anaphylactic shock, the predominant role of penicillin, subsequently confirmed [3–5], is reported. In these as well as in other cases preformed immunoglobulin E (IgE) antibodies to various allergens, present in

contrast media [5,6], sera [7], insect venom [8–10], and food [5,6,8,9] are thought to initiate anaphylactic reaction in patients previously (and often unknowingly) sensitized towards that allergen. A register including all fatal anaphylactic reactions in the UK is operative since 1992.

High affinity (IgE) receptors [4] are located in mast cells and basophils, whereas low affinity receptors are present in other cytotypes (eosinophils, macrophages, platelets). Systemic acute anaphylaxis results from degranulation of activated mast cells [11] with release, mainly, of histamine, tryptase and chymase, all important markers of mast cell activation. Unlike histamine, characterized by a very short half-life in the circulation, tryptase and chymase are stable post-mortem [12,13] and their sera measurements have been used in post-mortem diagnosis of acute anaphylaxis [8–10,12–14]. Other mediators like cytokines, chemokines, eosinophil chemotactic factor, leukotrienes and so on may be involved [4,14]. Tryptase is a serine protease stored mainly in mast cell granules and not found in circulating basophils, eosinophils, platelets or any other cell. There are mainly two varieties of tryptase: an active free form (β) and an inactive tetramer (α) [15,16]. The former is a protein released from mast cell granules during anaphylactic reactions, the latter is a similar protein secreted by resting mast cells and raised in mastocytosis [6,15]. Chymase is a mast cell-derived serine protease, characterized as an angiotensin

* Corresponding author at: Dipartimento integrato di Servizi diagnostici e di Laboratorio e di Medicina legale, Università degli Studi di Modena e Reggio Emilia. Azienda ospedaliero-universitaria Policlinico di Modena, Via del pozzo 71, 41100 Modena, Italy. Tel.: +39 059 4223150; fax: +39 059 4224450.

E-mail address: nicoletta.trani@alice.it (N. Trani).

II-generating enzyme [13] and used to determine mast cells and thus to assess the timing of wounds after deaths [17,18]. It is quite stable in serum and a significant positive correlation between serum chymase and tryptase levels was found in post-mortem diagnosis of anaphylaxis [13]. Heterogeneity of human mast cells is known [19,20] and recently different subsets of mast cells (MC) are distinguished by immunohistochemistry [21], as follows:

- MC-TCs (formerly connective tissue mast cells) mainly composed of histamine, heparin, tryptase, chymase, cathepsin G and carboxypeptidase, preformed and stored in granules.
- MC-T (formerly mucosal mast cells) lacking or containing only small amounts of chymase, carboxypeptidase and cathepsin.
- MC-C lacking tryptase and not further characterized.

The quantity of advanced laboratory data is in contrast with the scarcity of significant macroscopic findings, since post-mortem findings reported in many anaphylactic deaths are rather indefinite and not pathognomonic [5,6]. In fact immediate anaphylactic death is basically due to a diffuse hypoperfusion with increased capillary permeability, mainly following the release of both tryptase and chymase from activated and degranulated mast cells. The aim of this study was to verify the possibility to demonstrate the presence of activated and degranulated mast cells, even intermingled with eosinophils, by using the pagoda red stain [22,23] and to add, in histological routine practice, this simple stain to other means used to validate the diagnosis of anaphylactic death. We paid particular attention to this demonstration at spleen level, where eosinophilia, occasionally reported [2,28,29], is unlike other organs (respiratory tract, lymphnodes, joints, etc.), in our experience an absolutely unusual finding.

2. Materials and methods

The study was carried out on 3 groups, each of 4 cases, distinguished in the following way:

- Immediate anaphylactic death (IAD);
- Immediate non-anaphylactic death (INAD, negative controls);
- Inflammatory nasal polyps, with eosinophilia (stain controls).

2.1. Immediate anaphylactic death (IAD)

Besides circumstantial data we followed mainly the criteria listed by Di Maio and Di Maio [5] and by Pumphrey and Robert [6]. Year, sex, age, drug and time between injection and death are listed in Table 1.

2.2. Immediate non-anaphylactic death (INAD)

Year, sex, age and type of death are reported in Table 2. Absence of asthma and splenomegaly was ascertained in the selected patients.

2.3. Inflammatory sino-nasal polyps

The four cases selected for the number of eosinophils and mast cells were used as stain- and immunohistochemistry-controls for both cytotypes.

Table 1
Essential data of IAD-series.

File/year	Case, sex	Age	Drug ^a	Time between injection and death
61/02	1, M	53	Antibiotic: Unasyn [®] , penicillins and beta-lactamase inhibitors, ampicillin and sulbactam	Immediately after the injection (few minutes)
58/05	2, M	64	Antibiotic: Rocephin [®] , cephalosporins III generation and anesthetic: ceftriaxone sodium, lidocaine	Immediately after the injection (few minutes)
189/05	3, M	65	Antibiotic: Tazobac [®] , penicillins and beta-lactamase inhibitors, piperacillin sodium, tazobactam sodium	After 5 min
29/06	4, F	58	Antibiotic: Fidato [®] , cephalosporins III generation and anesthetic: ceftriaxone sodium, lidocaine	Immediately after the injection (few minutes)

^a All protected penicillins cross-reacting with the cephalosporins.

Table 2
Essential data of INAD-series.

File/year	Case, sex	Age	Causes of death	Time elapsing to the death
118/03	1, M	36	Airplane fall	Few minutes
170/03	2, M	16	Road accident	Few minutes
144/04	3, M	36	Industrial accident	Few minutes
51/05	4, M	34	Suicide by train	Few minutes
88/05	5, M	29	Industrial accident	Few minutes
138/05	6, M	56	Suicide by train	Few minutes
30/07	7, F	21	Road accident	Few minutes
48/07	8, M	24	Suicide by train	Few minutes
51/07	9, M	45	Road accident	Few minutes
61/07	10, M	85	Industrial accident	Few minutes
85/07	11, M	21	Road accident	Few minutes
89/07	12, M	64	Industrial accident	Few minutes
130/07	13, M	19	Industrial accident	Few minutes
140/07	14, M	19	Industrial accident	Few minutes
156/07	15, M	51	Road accident	Few minutes
160/07	16, M	39	Industrial accident	Few minutes
197/07	17, M	51	Industrial accident	Few minutes
199/07	18, M	61	Suicide by fall	Few minutes
207/07	19, M	23	Road accident	Few minutes

2.4. Stains and histochemistry

Besides haematoxylin–eosin, we performed the pagoda red (Dylon International LTD, England) stain, as follows:

Method:

- (1) Bring paraffin sections to water.
- (2) Stain in 1% aqueous pagoda red, 1 h at 55 °C.
- (3) Wash in water.
- (4) Counterstain nuclei with haematoxylin, 3 min.
- (5) Wash in tap water.
- (6) Dehydrate in alcohols, clear in xylene and mount in a suitable synthetic resin.

2.5. Immunohistochemistry for human eosinophil proteins

Monosan clone BMK-13 (Sanbio, Uden, The Netherlands), which specifically binds to the 10 kD eosinophil major basic protein (EMBP) of both resting and activated eosinophils was used. This monoclonal murine IgG1 antibody is considered a pan-eosinophil marker.

2.6. Immunohistochemistry for mast cells

The following two monoclonal antibodies were used:

- DIA PATH (Lab Vision, Ca, USA) mast cell chymase Ab-1 (clone CC1);
- DIA PATH (Lab Vision, Ca, USA) mast cell tryptase Ab-2 (clone AA1);
- A third polyclonal antibody was also used:
- DAKO-Japan CD 117 (clone 104,D2), minor (and not specific) marker for mast cells of epithelial and hematopoietic tissues.

3. Results

3.1. Pathologic findings

The main features of both IAD- and INAD-series are reported in the following Tables 3 and 4, from which splenic eosinophilia

Table 3

Pathologic findings of IAD-series.

Pathologic findings	Number of cases (4)
Cerebral edema	4
Pulmonary congestion	4
Pulmonary edema	2
Mucous plugging of bronchi	0
Laryngeal edema	1
Slight focal myocardial sclerosis	2
Spleen weight (less than 250 g)	4
Splenic (clusters) eosinophils	4
Lung (scattered) eosinophils	1
Liver (scattered) eosinophils	1

Table 4

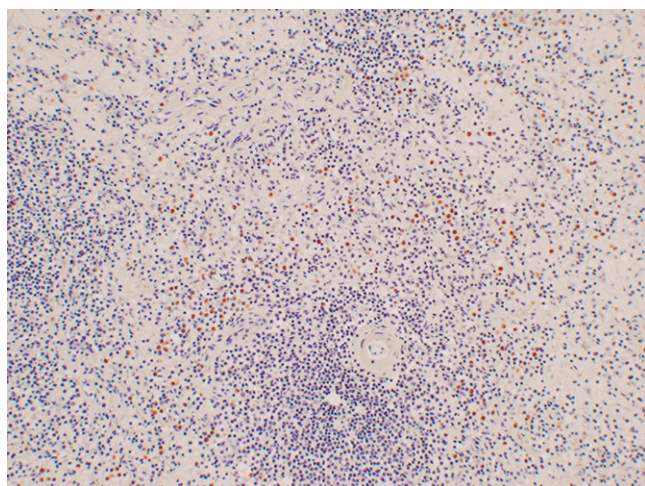
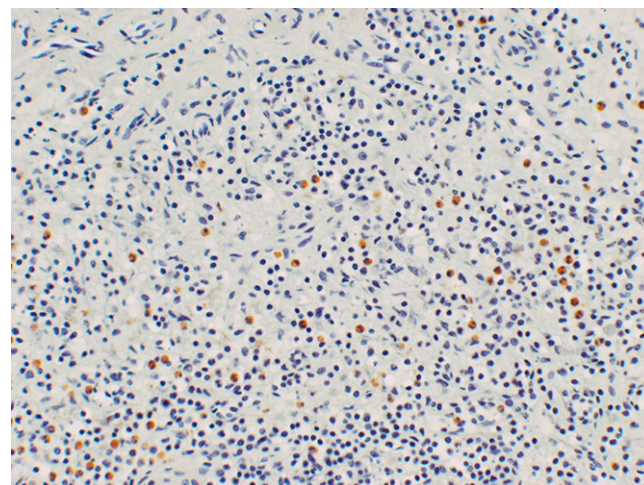
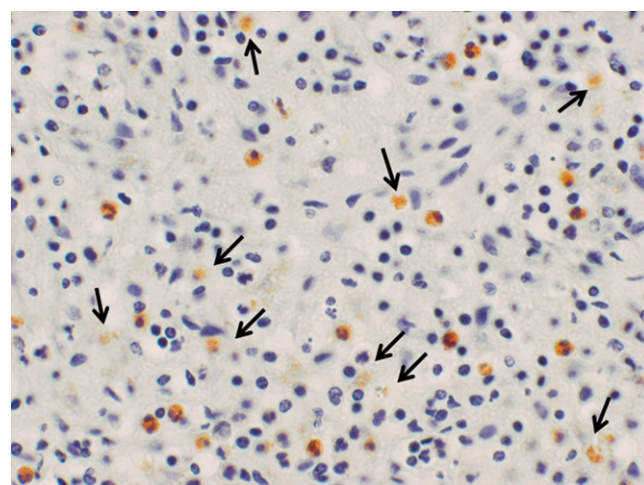
Pathologic findings of INAD-series.

Pathologic findings	Number of cases (4)
Cerebral edema	4
Pulmonary congestion	4
Pulmonary edema	2
Mucous plugging of bronchi	0
Laryngeal edema	0
Slight focal myocardial sclerosis	1
Spleen weight (less than 250 g)	4
Splenic (clusters) eosinophils	0
Splenic (scattered) eosinophils	1
Lung (scattered) eosinophils	0
Liver (scattered) eosinophils	0

appears as a useful support in the differential diagnosis of our two series: marked and diffuse in the IAD-series, totally absent or just in the form of scattered eosinophils in INAD-series.

3.2. Histochemical findings

The Pagoda red stain evidenced in all cases of IAD-series a diffuse, perifollicular, cordonal and endosinusal splenic eosinophilia and also the close presence of intermingled mast cells, even degranulated mainly at endosinusal level (Figs. 1–3). In INAD-series (case 6) scattered eosinophil and not degranulated mast cells were found only (Fig. 4). Clusters of eosinophils and not degranulated mast cells were easily recognized in the cases of inflammatory sino-nasal polyp (Fig. 5).

**Fig. 1.****Fig. 2.****Fig. 3.**

3.3. Immunohistochemical findings

The specific employed antibodies revealed either eosinophils or mast-cells without any cross-reaction between the cytotypes (Table 5). CD 117 always displayed a negative reaction.

4. Discussion

As pointed out in Literature [2–6], anaphylactic death is not easy to prove since pathological findings (laryngeal edema, pulmonary acute emphysema, mucous plugging of bronchi, etc.) may be inconstant or liable to subjective evaluation. Therefore tryptase concentrations in femoral blood (not influenced by prone position at death or resuscitation efforts) [12] and serum chymase and tryptase levels [13] have been suggested in post-mortem cases to validate the diagnosis of anaphylactic deaths.

In this study, however, splenic eosinophilia was the more significant feature for the differential diagnosis between immediate anaphylactic death and immediate non-anaphylactic death. Nevertheless it must be specified that it was a particular model of eosinophilia characterized by a number of eosinophils intermingled with mast cells and degranulated mast cells. However the mixing of the cytotypes, contemporaneously on the same slide,

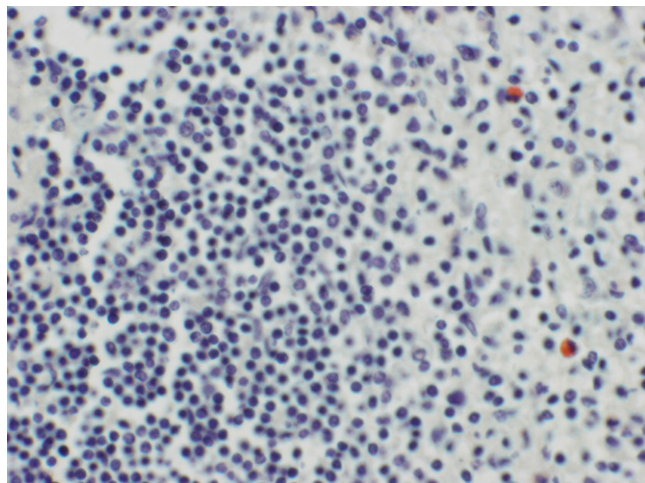


Fig. 4.

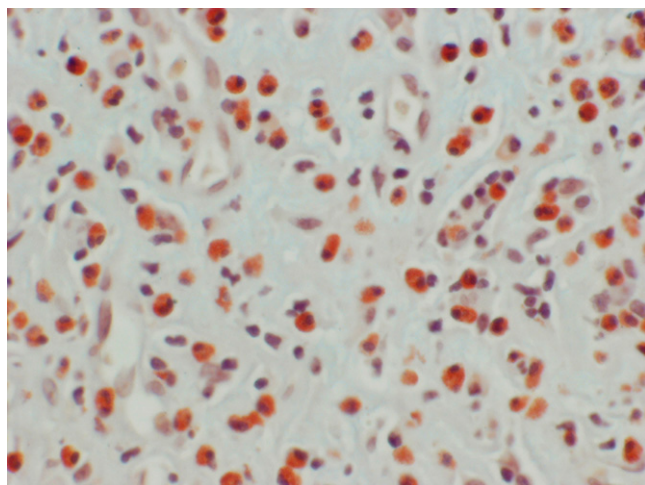


Fig. 5.

was easily displayed by pagoda red stain only, since degranulated mast cells and their outside granules appear brilliant red over a pale blue background.

Pagoda red is a dye originally employed in the industrial field to tint clothes and able in our experience to selectively trace eosinophils and mast cells. Moreover the dye has been successfully employed for the histological demonstration of several substances with fibrillar periodical structure, like amyloids [22,23], cellulose, siloxanes, polysiloxanes and polyethylene polymers [24,25].

In cytopathology the stain has been able to selectively trace eosinophils and mast cells in case of nasal [26] or ocular [27] allergic processes possibly related to the presence of airborne, non-human elements.

It is therefore clear that capability and specificity of pagoda red to demonstrate so different findings are related to cytohistological

and morphostructural diagnostic criteria and not to an histochemical specificity. Performed immunohistochemistry has been used to confirm specificity and sensibility of the pagoda red stain for mast cells and eosinophils.

Our findings, according to previous studies related to splenic eosinophilia [2,28,29] led us to hypothesize that the spleen in man could be that part of the vascular system where the trigger is initially pressed by the responsible allergen to initiate IAD. As reported from the outstanding work of Delage and Irey [2] shock organs and shock tissues are different in the different laboratory animals in the following manner:

- In the cat: the lungs. Death occurs from acute pulmonary emphysema [30].
- In the dog: the liver. Death derives from circulatory collapse of portal or splanchnic circulation [2].
- In the guinea pig: the bronchial smooth musculature. Death occurs from asphyxia secondary to severe broncospasm [2].
- In the mouse: the capillary bed. Death results from insufficient oxygenation of the tissues due to severe hypovolemia following capillary bed dilatation [31].
- In the rabbit: the pulmonary arteriolar tree. Death is related to the obstruction of pulmonary circulation [2].
- In the rat: the intestinal mass. Death follows circulation failure in the splanchnic area [32].

The hypothesis to consider the spleen as a shock organ in humans is supported by the following considerations.

- It is well known that the spleen is supplied with a double circulation: open and closed. During the closed circulation the blood flow (arteriolar in origin) is periodically stopped in the spleen sinuses to allow the margining of aged erythrocytes and their subsequent destruction in the Billroth's cord. This stop also immobilizes mast cells and basophils (usually few in circulating blood) and facilitates contact between their high affinity (IgE) receptors and allergens, represented in our IAD-series by injected antibiotics.
- The following degranulation of activated mast cells leads to the release of tryptase, chymase and ECF (eosinophils chemotactic factor) with possible subsequent appearance of eosinophilia in other sites (for example, liver and lung) as reported in Literature [2,7] and found in our cases 4 and 6.

Obviously, further research to confirm our hypothesis is required. Anyway, independently from any confirmation, we would like to stress that the use of a simple stain like pagoda red may be very useful in the simultaneous demonstration of eosinophils and mast cells in tissues.

References

- C. Richet, Des effets anaphylactiques de l'actinotoxine sur la pression artérielle, C.r. Soc. Biol. 54 (1902) 837–838.
- C. Delage, N.S. Irey, Anaphylactic deaths: a clinicopathologic study of 43 cases, J. Forensic Sci. 17 (1972) 525–540.
- S.M. Menchel, P.D. Gorevic, N.F. Adkinson, An immunologic approach to the evaluation of deaths due to penicillin anaphylaxis, in: Presented at the 39th Annual Meeting of the American Academy of forensic sciences, San Diego, CA, February, 1987.
- W. Weedn, Anaphylactic death (Letters to the Editor), J. Forensic Sci. 33 (5) (1988) 1108–1110.
- V.J. Di Maio, D. Di Maio, Forensic Pathology, second ed., CRC Press, Boca Raton, 2001.
- R.S.H. Pumphrey, S.D. Roberts, Postmortem findings after fatal anaphylactic reactions, J. Clin. Pathol. 53 (2000) 273–276.
- B.M. Vance, G. Strassmann, Sudden death following injection of foreign protein, Arch. Pathol. 34 (1942) 849–865.

Table 5

Panel of identification of eosinophils and mast cells: a comparative evaluation.

Cytotypes	EMBP	Chymase	Tryptase	CD117	Pagoda red
Eosinophils	++	—	—	—	+++
Mast cell	—	+++	+++	—	+++

(—) negative reaction; (++) moderate reaction; (+++) strong reaction.

- [8] J.W. Yunginger, D.R. Nelson, D.L. Squillace, et al., Laboratory investigation of deaths due to anaphylaxis, *J. Forensic Sci.* 36 (1991) 857–865.
- [9] R.S.H. Pumphrey, Lessons for management of anaphylaxis from a study of fatal reactions, *Clin. Exp. Allergy* 30 (2000) 1141–1150.
- [10] K.J. Riches, D. Gills, R.A. James, An autopsy approach to bee sting-related deaths, *Pathology* 34 (2002) 257–262.
- [11] P.W. Ewan, ABC of allergies. Anaphylaxis, *BMJ* 316 (1998) 1442–1445.
- [12] E. Edston, O. Eriksson, M. Van Hage, Mast cell tryptase in postmortem serum-reference values and confounders, *Int. J. Legal Med.* 121 (2007) 275–280.
- [13] H. Nishio, S. Takai, M. Miyazaki, et al., Usefulness of serum mast cell-specific chymase levels for postmortem diagnosis of anaphylaxis, *Int. J. Legal Med.* 119 (2005) 331–334.
- [14] L.B. Schwartz, Mediators of human mast cells and human mast cell subsets, *Ann. Allergy* 58 (1987) 226–235.
- [15] L.B. Schwartz, K. Sakai, T.R. Bradford, et al., The alpha form of human tryptase is the predominant type present in blood at base line in normal subjects and is elevated in those with systemic mastocytosis, *J. Clin. Invest.* 96 (1995) 2702–2710.
- [16] M.Q. Ansari, J.L. Zamora, M.F. Lipscomb, Postmortem diagnosis of acute anaphylaxis by serum tryptase analysis: a case report, *Am. J. Clin. Pathol.* 99 (1993) 101–103.
- [17] H. Urata, A. Kinoshita, K.S. Misono, F.M. Bumpus, A. Husain, et al., Identification of a highly specific chymase as the major angiotensin II-forming enzyme in human heart, *J. Biol. Chem.* 265 (1990) 22348–22357.
- [18] A. Bonelli, S. Bacci, G.A. Norelli, Affinity cytochemistry analysis of mast cells in skin lesions: a possible tool to assess the timing of lesions after death, *Int. J. Legal Med.* 117 (2003) 331–334.
- [19] N. Weidner, K.F. Austen, Heterogeneity of mast cells at multiple body site. Fluorescent determination of avidin binding and immunofluorescent determination of chymase, tryptase and carboxypeptidase content, *Pathol. Res. Pract.* 189 (1993) 156–162.
- [20] A.M. Irani, L.B. Schwartz, Human mast cell heterogeneity, *Allergy Proc.* 15 (1994) 303–308.
- [21] N. Perskvist, E. Edston, Differential accumulation of pulmonary and cardiac mast-cell-subsets and eosinophils between fatal anaphylaxis and asthma death. A postmortem comparative study, *Forensic Sci. Int.* 169 (2007) 43–49.
- [22] M. Yanaginara, A.H. Mehregan, D.R. Mehregan, Staining of amyloid with cotton dyes, *Arch. Derm.* 120 (1984) 1184–1185.
- [23] S. Battaglia, G. Barbolini, A.R. Botticelli, et al., Apoptotic amyloid: a study on prostatic amiloidosis with particular reference to corpora amylacea, *Appl. Pathol.* 3 (1985) 103–114.
- [24] F. Ferrara, R. Tranò, S. Fundarò, et al., Fibrosi costrittiva periprotetica mammaria: contributo anatomo-clinico ed immunoistochimico, *Riv. Ital. Chir. Plastica* 28 (1996) 101–105.
- [25] L. Piccagliani, R. Ferrone, E. Costa, et al., Evaluation of biocompatible materials during reparation of abdominal wall defects: results in the long term, three and six months, *Chirurgia* 19 (2006) 183–187.
- [26] F. Rivasi, G. Bergamini, Nasal cytology in allergenic processes and other syndromes caused by hyper reactivity, *Diagn. Cytopathol.* 4 (1988) 99–105.
- [27] F. Rivasi, G.M. Cavallini, L. Longanesi, Cytology of allergenic conjunctivitis. Presence of airborne, non human elements, *Acta Cytol.* 36 (1992) 492–498.
- [28] L.P. James jr., K.F. Austen, Fatal systemic anaphylaxis in man, *N. Engl. J. Med.* 270 (1964) 597–603.
- [29] J. Voigt, Eosinophils in the spleen in cases of lethal anaphylactic shock, *Med. Sci. Law.* 6 (3) (1966) 162–163.
- [30] H.B. McCusker, I.D. Aitken, Anaphylaxis in the cat, *J. Pathol. Bacteriol.* 91 (1) (1966) 282–285.
- [31] J. Munoz, R.K. Bergman, Mechanism of anaphylactic death in the mouse, *Nature* 205 (1965) 199–200.
- [32] J. Lecompte, La masse intestinale commune organe du choc au cours de l'anaphylaxie active du rat, *Arch. Int. Physiol.* 75 (1967) 325–326.