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A semi-automated micro-method for the histological assessment of fat embolism

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Abstract A method of quantitatively determining the volume of fat emboli in a tissue using an image analysis system (I.B.A.S.) was developed. This procedure is an interactive, semi-automated tool allowing the quick and accurate gathering of large quantities of data from sections of different tissue samples stained by osmium tetroxide. The development of this procedure was aimed at producing a system which is reliable, reproducible and semi-automated thereby enabling epidemiological and serial studies to be made of a large number of histological sections from different tissues. The system was tested in a study of tissue sections from a series of fatalities from an aircraft crash in an attempt at correlating the incidence of fat emboli with the presence of multiple fractures and soft tissue injuries, the correlation to be made being between the quantitative presence of fat emboli and the extent and severity of injuries suffered.

Key words Fat embolism · Image-analysis, I.B.A.S. Osmium tetroxide

Zusammenfassung Mit Hilfe eines interaktiven Bildanalysen-Systems (I.B.A.S.) wurde eine Methode für die quantitative Bestimmung des Volumens von Fettemboli im Gewebe entwickelt. Es handelt sich um ein halbautomatisches Verfahren, das die rasche und genaue Auswertung zahlreicher Daten an Schnitten von mit Osmiumtetroxid behandelten Gewebsproben erlaubt. Das halbautomatische System arbeitet zuverlässig und liefert reproduzierbare Ergebnisse. Es ermöglicht epidemiologische Studien und Serienuntersuchungen an einer großen Anzahl histologischer Schnitte von verschiedenen Geweben. Die Testung des Systems erfolgte an Gewebsschnitten von Organen der Opfer eines Flugzeugabsturzes, wobei die Korrelation zwischen Ausmaß der Fettembolie und Schwere der erlittenen Verletzungen überprüft wurde.

Schlüsselwörter Fettembolie · interaktives Bildanalysen-System, I.B.A.S. · Osmiumtetroxid

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Introduction

The vast improvement in the survival of severely and multiply injured patients has once more focused medical awareness on the contribution of the 'fat embolism syndrome' to both early and delayed post-trauma mortality (Schien & Saadia 1990). It has been suggested that although in most traumatised patients fat emboli do occur, particularly when there is a combination of extensive soft tissue injury coupled with long bone and pelvic fractures (Weisz & Steiner 1971), it is the number and size of such emboli that are of major importance: the more abundant the number of such emboli and the bigger their size, the more severe is their expected impact on the patient's potential for survival (Goris & Draaisma 1982). The clinical importance of fat emboli was highlighted even further with the recently increasing trend of active orthopaedic intervention as part of the emergency treatment of trauma patients (Meek et al. 1981). Treatment protocols nowadays emphasise the requirement for an early internal fixation of unstable long bone fractures with the further potential for fat embolisation intra-operatively, but the theoretical prevention of delayed fatal embolisation (Riska et al. 1976).

To assess objectively any of these hypotheses and to alter surgical intervention regimes appropriately and soundly, it would be useful to be able to assess accurately the degree and spread of fat emboli in patients who have succumbed to their injuries and to relate these to the time elapsed after their infliction, and to the type of injuries sustained. Such an assessment by light microscopy is a painstakingly laborious process, fraught with observer er-

ror and a potential for numerous inaccuracies. Previous attempts at semi-quantitative evaluation of pulmary fat emboli carried out in parallel with chemical analyses by gas chromatography (Holczabek 1964; Falzi et al. 1964; Holczabek & Machata 1971) have not gained widespread usage. Another method was to estimate the total fat content of the lungs after chloroform extraction and to correlate this to the histological identification of fat emboli (Brinkmann et al. 1976).

Our aim was to devise a semi-automated morphometric microscopical method for assessing fat emboli in tissues that could be used to assess a larger series of cases in which it is desired to assess the distribution of fat emboli in a number of organs. Although the system was designed with the hardware and equipment specially available in our own laboratory, variation in the hardware and software can be easily incorporated in other versions of this system.

Material and methods

The tissue samples which were used in the development of this inter-active system were taken from archival material and chosen at random. Histological sections obtained from the victims of the Lockerbie air disaster in December 1988 were available. These samples had been examined microscopically and shown to exhibit a high incidence of fat emboli with a distribution ranging from zero to very extensive and diffuse embolisation. These former sections acted as the in-built control.

In all the cases studied, sections from the lungs and the liver were available. These autopsy blocks had been fixed in formalin for several days and were washed in osmium tetroxide (OsO_4) for 2 hours followed by rinsing in sodium tricocodylate buffer. The blocks were embedded in paraffin wax and sectioned at 8–10 μ in standard fashion. All sections had been treated, stained and prepared in an identical manner. As a counterstain a light haematoxylin and eosin procedure followed. The fat emboli were readily identifiable as uniformly dense black, rounded globules within blood vessels of various sizes.

The slides were systematically examined using a Leitz Ortholux 2 microscope with a×10 objective lens, coupled to a black and white Sony CCD XC-77CE video camera. The video image was inputted to a Kontron image analysis system (Interactives Bild Analysen System – IBAS) coupled with a Kontron Vidas Plus keyboard and monitor. Images were digitised into 512×512 pixels using a grey scale ranging from 0–255 (black to white). Analysis and processing of the digitised images were carried out by a sequence of macro routines fashioned into a program specifically tailored to assess the parameters indicated (see Appendix 1).

Each slide was systematically examined using the Vernier scale on the microscope stage to sequentially advance each television video (TV) image. The slide was scanned in a grid fashion and the total area covered by the tissue section was scanned. Although measurements of the area of the tissue sample were taken from the sample edge to sample edge, no measurements were made from the periphery of the sections, the reason for this being that in a number of sections diffuse and irregular osmium deposits were non-specifically and artefactually attached to the edge of the sections. Within the other areas of the sections examined it could be confirmed in all instances that the osmium stained globular fatty masses. Measurements were therefore only taken once the tissue had been advanced a minimum of one full field of view from the periphery.

The program parameters, that is, the scale conversion (given the objective lens used), object measurement i.e. the emboli, and field measurement i.e. the tissue area, were carried out prior to the input of the initial TV image (see Appendix 2). Once inputted, the captured image was first digitised and then normalised [NORMIM]: this function causes an increase in the image contrast by linearly scaling the grey levels of the image into the full dynamic range of the system's image memory. The image was subsequently medianated [MEDIAN]: this is a smoothing operation designed to conduct a rank operation resulting in an averaging of the grey levels within a matrix of pixels thus giving all objects present in the image a unique, uniform grey level. This was followed by a delineation [DELIN] routine causing the boundaries between the different medianated objects to be given a sharp transition thus sharpening each object's edges.

An interactive discrimination [DISZLEV] facility was used whereby the operator could discriminate objects from the image background and cause the background to be uniformly set to white (255 on the grey scale). The image then undergoes an automatic identification procedure whereby all the pixels that are a part of the same object become connected into a unified entity. Neighbouring objects are also displayed in contrasting colours in order to facilitate later operator interaction.

An automatic gating function at this point [SCRAP] rejects objects not within a pre-established pixel area (40–5000) thus rejecting vesicles, e.g. intracytoplasmic vacuoles, lipofuscin-containing lysosomes in the liver, carbon particles in the lung, and nuclei which routinely acquire the same grey-scale as that of the desired fat emboli. A further, interactive facility becomes available [REJECTOBJ] allowing the operator the choice of rejecting any undesirable objects still in the image subsequent to its automatic measurement [MEASOBJ] by the program. Measurement data are loaded onto a database for storage.

Using the already normalised, medianated and delineated image, the program again allows the interactive discrimination of the image, this time in order to select the image background, i.e. the tissue itself. Once delineated, the program automatically measures the tissue area, storing it in a second database. At this point the program has looped full circle and is primed for the next image input. Once all the data has been acquired and the program's main loop halted, a statistics program is run to give total and mean tissue and embolic area, the standard deviation of both and the maximum and minimum embolic areas.

Results

Each field takes an average of 23 s to process and given a standard histological slide, each tissue scan can be completed in approximately 3 hours.

Because the distribution of emboli within a histological specimen is random throughout the tissue, any measurement of the number of individual fat embolisations within a given tissue area would render the measurement invalid. Instead, it was decided that the total area of the emboli present would be used and coupled with an estimation of the total tissue area, a method would be available for determining the degree of embolisations in a given tissue.

The following simple equation:

$$\frac{\text{TOTAL AREA OF EMBOLI}}{\text{TOTAL AREA OF TISSUE}}$$

which was called the Fat Embolism Index (FEI) (see Fig. 1) was derived for each examination.

Because the system is capable of detecting variations in the number and size of emboli between different tissue types, the FEI allows the performance of cross correlations of the degree of embolisation between, for example,

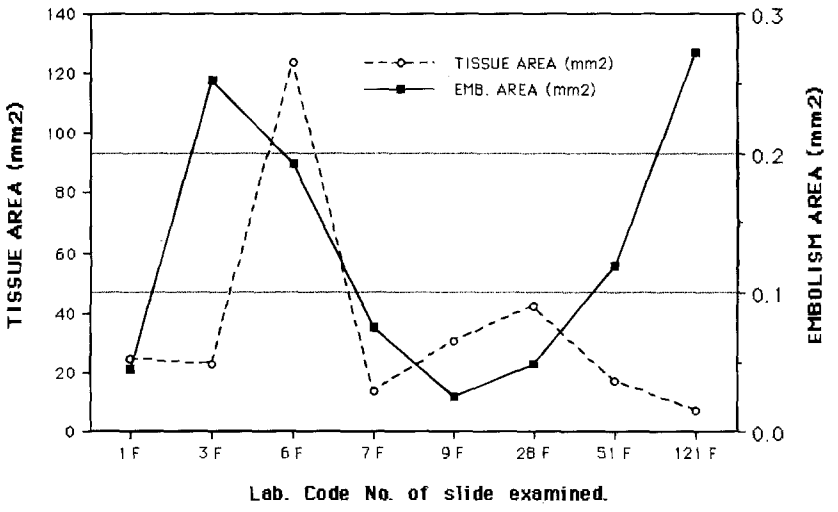

Fig. 1 Graph showing total area of tissue on slide and area covered by fat emboli

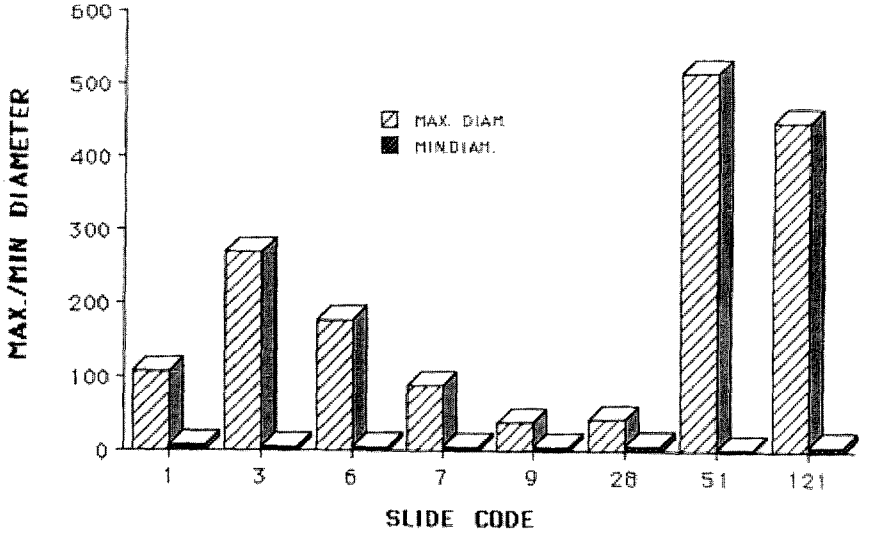

Fig. 2 Range of embolic diameter in each lung tissue sample

the lung of one patient and the lung of a second patient, or between an individual patient's liver and lung tissue, in cases where all the tissues under correlation have been prepared identically. The formation of the FEI thereby allows surveys to be carried out with reproducible accuracy and with ease.

The result, as expected, showed that there is no correlation or trend between the histological tissue area and the total quantity of fat emboli that it contains (see Fig. 2). The quantity of fat emboli present in a tissue specimen may indeed be determined by some physical or histological factor, but at this juncture this determining factor still remains unknown.

The variation in embolic diameter within a single tissue and between different samples can be seen in Figs. 3–5 where, in one case, there is a 600-fold difference in the range of diameters. These results were achieved by having the image analysis program take diameter measurements at every 5° of arc (i.e. 72 separate measurements) and selecting the maximum and minimum diameter from each tissue.

Discussion

This system still requires the presence of an operator in order to advance the sub- stage so that a systematic scanning of the slide can be performed. With the addition of a motorised sub-stage and a minor program addition, the operator was no longer required to sit throughout the analysis of each histological section, resulting in the technique taking much less time to execute; other large surveys can be carried out much more promptly and efficiently, and a high degree of accuracy of observation and measurement can be maintained throughout.

The accuracy of the program, as shown in Fig. 6, is an almost perfect correlation with that of the operator count, any discrepancies between the two being due to the presence of the gating procedure rejecting any object outside the specified area limits. The accuracy of the program, as compared to that of the operator, would improve even further over any significant period of time as the operator is subject to both fatigue and tediousness, unlike the program system.

The ease of use of the system is such that an initially unskilled operator can be trained to use it within a

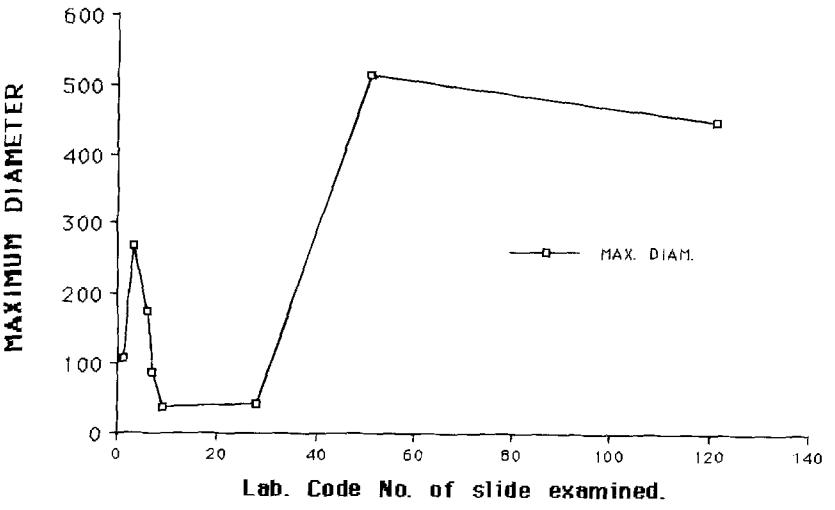

Fig. 3 Graph showing the spread of maximum diameters of emboli in different lung specimens

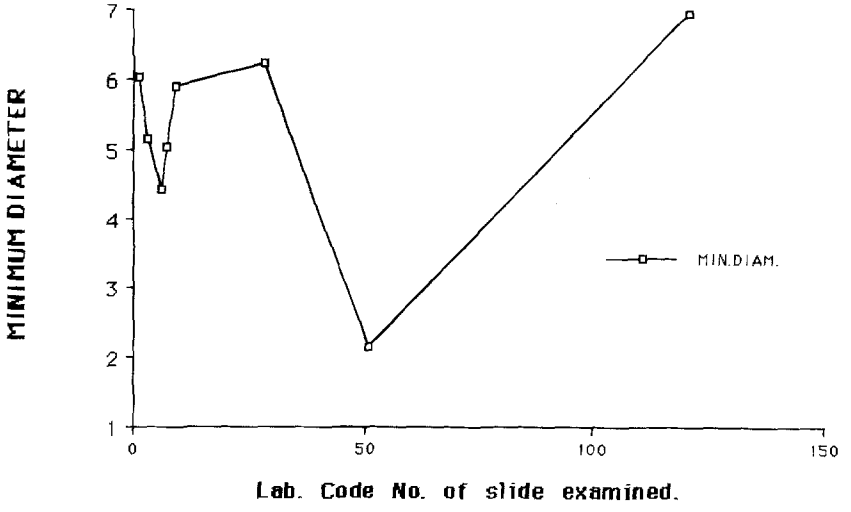

Fig. 4 Graph showing the spread of minimum diameter of emboli in different lung specimens

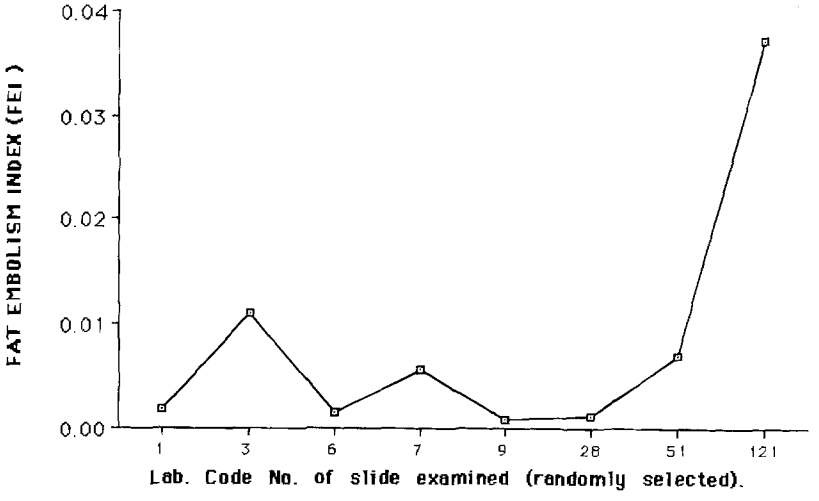

Fig. 5 Comparison between histological slides, prepared identically, using FEI

mere matter of hours. As the program routine contains "prompts" and "flags"; the operator's attention cannot become lost due to distraction or as a result of having to leave the system in mid-routine and he will have to respond to the demands and queries of the program before he can proceed any further.

Although this system was designed primarily for the detection of fat emboli, it can easily be adapted to allow the selection and analysis of other histological entities e.g. applications exist within the fields of fibres analysis, soil analysis, etc.

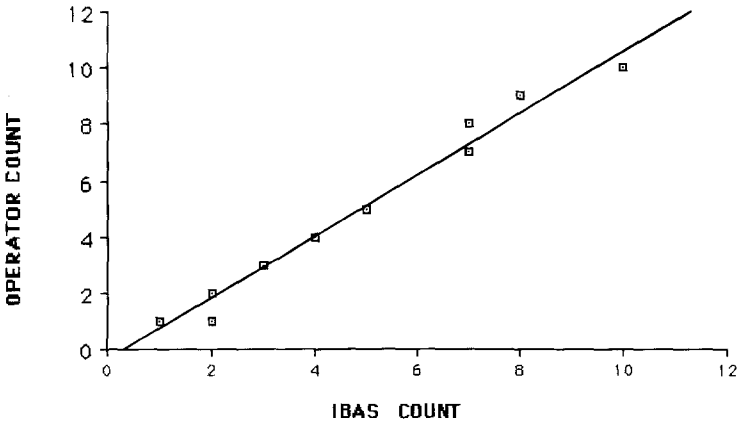

Fig. 6 Comparison of computer accuracy against operator accuracy; IABS is an acronym for: Interaktives Bild Analysen System

This method does not permit an assessment of the pathogenesis of the fat emboli, in particular whether they are associated with bone marrow emboli: it simply assesses their presence and measures their size and distribution in tissue sections.

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Appendix 1

The following is the IBAS interpreter which guides the Kontron system. Kontron uses the language 'C'.

```
resetpar
scalgeom 6,"x1 0leitzb/w",_OFF,_OFF
Initobj AREA, DMAX, DMIN
InitField AREAP, TOTALAREA
append=_OFF
while 1
loadlut "grey"
tvon
pause
tvinp 1
write " "
write "EMBOLISM DATABASE"
normin 1,2,1
median 2,3,5
write "SHARPEN EMBOLI"
delin 3,4,111,5,_OFF
dis2lev 4,5,0,0,_ON,_ON,1
identify 5,6,_ON,OFF
write "REJECT OBJECT"
scrap 6,6,_OFF,0,40,_OFF,_OFF
rejectobj 6,2,0
measobj 6,"embol",append
write "END EMBOLI"
write "TISSUE DATABASE"
write "HIGHLIGHT TISSUE"
dis2lev 4,5,0,28,_ON,_ON,1
identify 5,6,_ON,_OFF
measfield 6,"tissue",append
outlist "embol",_OFF
outlist "tissue",_OFF
append=_ON
write "END TISSUE"
write " "
write " "
write "SELECT NEW IMAGE"
endwhile
while 2
Amin=0.0
Amax=100000000.0
statistic[32]:=0.0
measdbst "embol","AREA",15,_OFF,_ON,0.00,100.00,
100.00,"statistic","*"
write "total embolism area is ",statistic[4]
write "embolism mean area is ",statistic[6]
write "embolism S.D. is ",statistic[7]
write "individual max. area is ",statistic[3]
write "individual min. area is ",statistic[2]
pause
measdbst "embol","DMAX",15,_OFF,_ON,0.00,100,00,100.00,
"statistic","*"
write "embolism max. diameter is ",statistic[3]
measdbst "emboli","DMIN",15,_OFF,_ON,0.00,100.00,100.00,"
statistic","*"
write "embolism min. diameter is ",statistic[2]
measdbst"tissue","TOTALAREA",15,_OFF,_ON,0.00,100.00,
100.00,
"statistic","*"
write "total tissue area is ",statistic[4]
stop
endwhile
stop
```

Appendix 2

The following is a flow diagram outlining the procedure sequence for the IBAS Interpreter which guides the Kontron system.