Evaluation of diaphragmatic mobility following intra-abdominal sub-diaphragmatic fixation of a double-layered mesh in rats[1]

Tzanoglou Ioannis[I], Sakorafas George[II], Kostomitsopoulos Nikolaos[III], Mantziaras George[IV], Patraleksis Charalampos[V], Danias Nikolaos[VI], Stergiopoulos Spyridon[VI], Safioleas Michael[VII]

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[I]MD, General Surgeon Consultant, 4th Department of Surgery , University of Athens Medical School, "Attikon" General Hospital of Athens, Greece. Technical procedures, acquisition of data, statistics analysis, manuscript writing.
[II]MD, PhD, Head Director, 2nd Surgical Department of "Agios Savvas" Anticancer-Oncology Hospital of Athens, Greece. Design of the study, technical procedures, interpretation of data, final approval.
[III]PhD, Head Director, Laboratory Animal Facilities, Biomedical Research Foundation, Academy of Athens, Greece. Design of the study, interpretation of data, critical revision, final approval.
[IV]PhD, Hellenic Military Air Force, Air force General Hospital of Athens, Greece. Technical procedures, sonographic measurements of diaphragmatic mobility, veterinary care.
[V]MD, Pathologist, Pathologic-Anatomical Laboratory, "REA" Maternity Hospital of Athens, Greece. Pathological examination of tissue samples.
[VI]MD, PhD, Assistant Professor, 4th Department of Surgery, University of Athens Medical School, "Attikon" General Hospital of Athens, Greece. Study supervision, critical revision, final approval.
[VII]MD, PhD, Professor of Surgery, 4th Department of Surgery, University of Athens Medical School, "Attikon" General Hospital of Athens, Greece. Design of the study, critical revision, final approval.

ABSTRACT

PURPOSE: To evaluate the tissue integration of a double-sided mesh after fixation in diaphragm and to study the diaphragmatic mobility by ultrasound.

METHODS: Twenty male Wistar rats were used. The animals were assigned into two equal groups according to the day of euthanasia. The animals were anesthetized and a 1.5 x 1.5 cm of double-layer mesh was inserted between the diaphragm and the liver. For the evaluation of the diaphragm mobility a sonographic method was used. Measurements on specific breathing parameters were taking place. Pathological evaluation took place after the animal's euthanasia.

RESULTS: Extra-hepatic granuloma was not differentiated overtime, ($\chi 2=0.04$, $p>0.05$). Neither fibrosis was significantly differentiated, ($\chi 2=0.04$, $p>0.05$). Intra-hepatic granuloma was significantly differentiated overtime, ($\chi 2=10.21$, $p<0.05$). Concerning Te parameter, means were significantly differentiated over time, $F (3, 30) = 5.12$, ($p<0.01$). Ttot parameter, it was differentiated over time, $F (3, 8)=4.79$, ($p<0.05$). IR parameter was also longitudinally differentiated, $F (3, 30)=3.73$, ($p<0.05$).

CONCLUSION: The measurements suggest a transient malfunction of diaphragmatic mobility despite the fact that inflammatory reaction, fibrosis and extra-hepatic granuloma were not significantly differentiated with the passage of time.

Key words: Surgical Mesh. Diaphragm. Fibrosis. Foreign-Body Reaction. Rats.

Introduction

Surgical meshes today represent a group of implants mainly used for hernia repair. In general, the ideal mesh is characterized by a variety of requirements such as economic aspects, functionality and operative handling, sterility or even anti-infective and optimized biocompatibility[1].

The basic mechanism which explains the broad use is the fibrotic reaction. The ideal mesh should be effective in preventing hernia recurrence; this can be achieved not only by the mechanical action of the mesh, but also by the fibrotic reaction caused by it. Unfortunately, this fibrotic reaction led to pain and restriction of movement, as well as other clinical complications such as fistula formation and adhesions[2].

This realization led to the concept of a dual-sided mesh to prevent or minimize visceral adhesions on one side while maximizing fibroblast ingrowth and tissue incorporation on the other side. Bilaminar mesh types and composite materials that include a temporary tissue separating layer are now available[3].

The use of a dual mesh placed intraperitoneally has increased in recent years to treat various hernias forms of the abdominal wall, including hiatal hernia. The intraperitoneal placement is clearly superior than the one on the Myoserosal layer placement due to the lower probability of recurrence (for mechanical reasons), while the problem of creating adhesions and fistulas in the bowel appears to have been addressed through the use of dual grids (with biodegradable internal membrane)[4].

The purpose of this study is to evaluate mainly the effects of intra-abdominal sub-diaphragmatic fixation of a double layered mesh on the mobility of the diaphragm, by using ultrasonography. To the best of our knowledge, this effect has not been investigated in the literature up to date. The second aim of this study was to investigate tissue integration of the mesh.

Methods

The study was performed at the Biomedical Research Foundation of the Academy of Athens (BRFAA). The experimental protocol was approved by the Veterinary Service of the Athens Prefecture according to the Presidential Decree 160/91-2010 covering the ethical experimentation on animals.

Twenty male Wistar rats (*Rattus Norvegicus Albinus*) were used, weighing an average of 275 gr. (250-300 gr). All animals kept at constant temperature conditions with controlled light/dark cycles, and handled according to the rules established in the BRFAA.

Prosthetic material

We have used the PROCEED surgical mesh (Ethicon, Somerville, NJ) which is a sterile multi-layered, thin, flexible, laminate mesh comprised of an oxidized regenerated cellulose (ORC) fabric and PROLENE soft mesh, a no absorbable polypropylene mesh which is encapsulated by a Polydioxanone polymer. The polypropylene mesh side of the product allows for tissue ingrowth, while the ORC side provides a bioresorbable layer that physically separates the polypropylene mesh from underlying tissue and organ surfaces during the wound-healing period to minimize tissue attachment to the mesh. The Polydioxanone provides a bond to the ORC layer[5].

Surgical technique

The animals were anesthetized and surgical anesthesia was maintained throughout the experiment with inhaled Isoflurane (0.5% to 3.0%). The hair was clipped thoroughly immediately before the surgical procedure, followed by antisepsis with Iodopovidone scrub. A 7 cm midline incision was made, caudal to the xyphoid and the peritoneal cavity was entered. A 1.5 x 1.5 cm of the double-layer mesh was inserted in a mostly intact peritoneum between the diaphragm (the nonabsorbable layer) and the liver (the ORC layer) and fixed in the diaphragm using four interrupted 5-0 Prolene suture (Ethicon Inc., Somerville, NJ) placed in each corner so that it would remain fully stretched and smooth and in constant contact with the diaphragm. The abdominal wall and the skin were sewn with a 3-0 polyglactine (Vicryl, Ethicon Inc., Somerville, NJ) simple continuous pattern. All the animals recovered from surgery uneventfully and were included in the study. A single dose of Cefuroxime (25 mg/Kg I.M) was given to the animals postoperatively.

Evaluation of diaphragmatic mobility using ultra-sonography

For the sonographic evaluation of the diaphragm's mobility, a standardized method was used[6-7]. We used the GE Vivid I ultrasound (GE Medical Systems Israel Ltd, Tirat Carmel, Israel) with a microconvex probe (8C RS, GE Yokogawa Medical Systems, Ltd, Tokyo, Japan), of a variable frequency of 5 to 13 MHz. In order to achieve a more detailed imaging we used a frequency of 12 MHz, with one focal zone set at a depth of 1.25 – 1.75 cm. Two-dimensional mode was used to find the best approach and to select the exploration line of diaphragm. In the transverse plane, images were obtained from the midline, just inferior to the xiphoid process, and perpendicular to the abdomen. The liver was used as an acoustic window. All examinations were recorded on a personal computer for subsequent blind analysis. The probe was placed in the xyphoid appendix of the anesthetized animal in the subcostal area and was directed medially, cranial and dorsally so that the ultrasound beam reached the vault of the diaphragm that wraps the liver mass. Thus, the inspiratory and expiratory cranial-caudal displacement of the diaphragm respectively shortened and lengthened the probe-diaphragm range. Consequently, the bright line formed by echoes originating from the diaphragm successively moved upwards and downwards on the M-mode graph. The M-mode sonogram was displayed on the video screen with a horizontal sweep speed of 50 mm/sec and was continuously recorded.

The measurements in each animal were performed by the same experienced investigator.

Both preoperative and postoperative measurements were performed under the same anesthesia protocol.

Parameters of diaphragmatic function/mobility which were measured are the following:

Parameters of respiratory function

- **DIA** (Diaphragm inspiratory amplitude)
- **Ti** (Diaphragm inspiratory time)
- **DIV** (Diaphragm inspiratory motion velocity) = DIA/Ti
- **DEA** (Diaphragm expiratory amplitude). Same with DIA
- **Te** (Diaphragm expiratory time)
- **DEV** (Diaphragm expiratory motion velocity)=DEA/Te
- **Ttot** (Total breathing time)
- **DMT** (Diaphragm Motion Time). The duration of one breathing curve
- **DRT** (Diaphragm Resting Time) = Ttot-DMT. It represents the calm period of a respiratory cycle
- **IR** (Ti/Ttot) represents the rate of T inspiratory time/ Total breathing time.

The results concerning the diaphragmatic mobility were analyzed with semi-computerized techniques using the obtained sonographic measurements and the parameters were analyzed as follows (Figure 1).

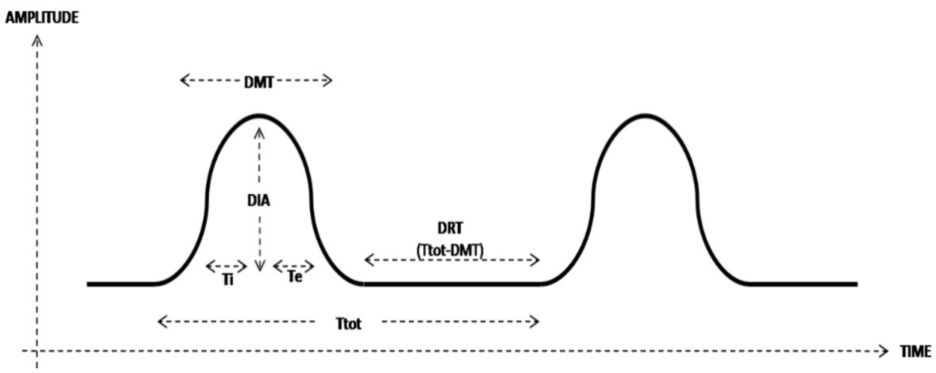

FIGURE 1 - Parameters of diaphragm mobility and respiratory function of the animals.

Study groups and observation periods

The animals (n=20) were assigned into two equal groups according to the predefined day of euthanasia:

Ten were sacrificed on 28th postoperative day (group 1) and ten were sacrificed on 90th postoperative day (group 2). Sonographic measurements were performed to all animals immediately before the operation took place (1st). Postoperatively, the animal's diaphragmatic mobility was reassessed by sonographic evaluation at 4th (group 1 and 2) 8th and 12th weeks (group 2). At the 4th week (group 1) and at the 12th week (group 2) the animals again were anesthetized and euthanized. A U shape laparotomy was performed and the whole peritoneal cavity was exposed. Liver and diaphragm including the implanted mesh were removed en bloc and fixated for microscopic evaluation. No dissection of the site of implantation was performed to avoid alteration of subsequent microscopic findings. However, no adhesion or seromas nor hematomas were found in the rest of the abdomen.

Histological examination

Five sections of 0.5 cm of every sample were taken including all the structures and tissues and soaked in 10% formalin and then in paraffin blocks. Fine sections (5 μm) were stained with hematoxylin – eosin (HE) protocol. Using a blind trial, a pathologist evaluated all of the specimens. As we mentioned before, a macroscopic evaluation of the specimens was not performed. Thus, based on similar experimental research, three parameters were evaluated, affecting mesh/tissue incorporation adhesion formation and scar formation: the chronic inflammatory response, the fibrosis degree and the foreign body reaction. The slides were scored for inflammatory cell infiltrates (neutrophils, eosinophils, macrophages, lymphocytes and giant cells), fibrosis, granulomatous foreign body reaction [separated to intra-hepatic and extra- hepatic (Figure 2(a&b)], seromas and hematomas as well. One animal of group 1 was excluded from the statistical analysis of the histological evaluation as the sample was considered to be inappropriate (Figure 2).

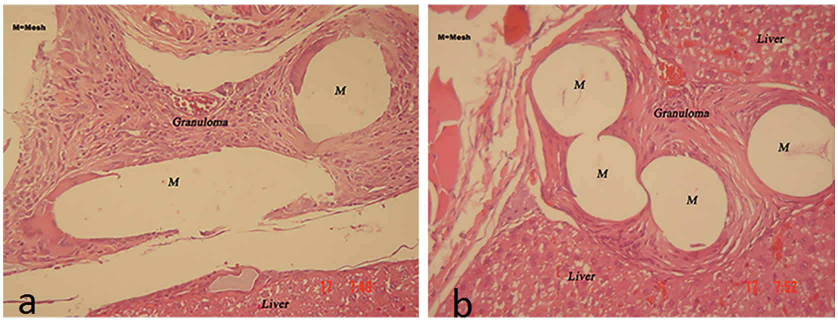

FIGURE 2 - Extra-hepatic (**a**) and intra-hepatic (**b**) granuloma (HE, x 200).

Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS 14.0). For the statistical analysis of the diaphragms mobility the means of the repeated assessments of the respiratory curve were compared through a series of subject design models. The Mauchly sphericity test was found to be statistically significant, the differences when assessed through the multivariate test while the aforementioned test was insignificant, the univariate test was used. The effect size was estimated by the η2 coefficient. Chi square test was used to compare percentages. For the histological measurements χ2 was used.

Results

Respiratory curve parameter longitudinal means comparisons: 1st, 4th, 8th and 12th week.

As shown in Table 1 only four respiratory curve parameters were significantly differentiated over time.

TABLE 1 - Respiratory curve parameter means and standard deviations by the 1st, 4th, 8th and 12th week.

	Week									
	1st		4th		8th		12th		F	η^2
	M	SD	M	SD	M	SD	M	SD		
DIA	0.28	0.08	0.27	0.06	0.30	0.06	0.32	0.10	1.08	
Ti	0.32	0.04	0.34	0.07	0.34	0.05	0.32	0.05	1.02	
DIV	0.94	0.25	0.82	0.16	0.94	0.32	1.04	0.29	1.56	
DEA	0.28	0.08	0.27	0.06	0.30	0.06	0.32	0.10	1.10	
Te	0.93_{ab}	0.25	$\mathbf{0.79}_{a}$	**0.12**	0.94_{ab}	0.22	0.94_{b}	0.22	**5.12****	**0.34**
DEV	0.32	0.10	0.33	0.04	0.32	0.08	0.29	0.09	0.61	
Ttot^{+}	1.25_{ab}	0.27	$\mathbf{1.15}_{a}$	**0.17**	1.20_{ab}	0.42	1.27_{b}	0.28	**4.49***	**0.18**
DMT	0.52	0.06	0.52	0.09	0.53	0.06	0.53	0.07	0.12	
DRT	0.73_{ab}	0.24	$\mathbf{0.63}_{a}$	**0.11**	0.75_{ab}	0.24	0.77_{b}	0.24	**3.53***	**0.26**
IR	0.26_{ab}	0.06	$\mathbf{0.30}_{a}$	**0.04**	0.28_{ab}	0.06	0.26_{b}	0.06	**3.73***	**0.27**

Statistical result assessed using the multivariate test as Mauchly W test was statistically significant.
*p<0.05, **p<0.01. Means not sharing the same indicator are significantly differentiated according to Bonferroni test, p<0.05.

Concerning the Te parameter (diaphragm expiratory time), means were significantly differentiated over time, $F(3, 30) = 5.12$, $p < 0.01$, with an effect size equal to 0.34. According to the polynomial comparisons, linear function was statistically significant, $F(1,10) = 5.11$, $p < 0.05$, indicating a decrease between the first and the second measure with an effect size of the differentiation equal to 0.34 (Figure 3a). The quadratic function was also significant, $F(1, 10) = 7.80$, $p < 0.05$, according to which an increase could be observed with an effect size of 0.44. There was no further differentiation of the polynomial comparisons. As far as the pairwise multiple comparisons are concerned, the 4th week's mean ($M = 0.79$ $SD = 0.12$) was significantly lower than the 12th week's ($M = 1.14$ $SD = 0.28$) one while there were no other significant pair differentiations.

Ttot parameter (Total breathing time), was differentiated over time, $F(3, 8) = 4.79$, $p < 0.05$, according to the multivariate test. The effect size, according to η^2 coefficient, was equal to 0, 18. While the polynomial comparisons were insignificant for all levels, $F(1, 10) = 2.09$, $p < 0.05$, $F(1, 10) = 3.76$, $p < 0.05$ and $F(1, 10) = 0.02$, $p > 0.05$ respectively (see graph 2), according to multiple pairwise comparisons, 4th week's mean ($M = 1.15$ $SD = 0.17$) was significantly lower than the 12th one's ($M = 1.45$ $SD = 0.31$). No other comparisons concluded in any significant results (Figure 3b).

DRT parameter [(Ttot-DMT) It represents the calm period of a respiratory cycle] was also differentiated longitudinally, $F(3, 30) = 3.53$, $p < 0.05$, with an effect size equal to 0.26. Polynomial comparisons were not statistically significant, $F(1, 10) = 3.89$, $p > 0.05$, $F(1, 10) = 4.36$, $p > 0.05$ and $F(1, 10) = 0.86$, $p > 0.05$ for the three degrees functions respectively (see graph 3). Concerning multiple pairwise comparisons, the 4th week's mean ($M = 0.63$ $SD = 0.11$) was significantly lower than the 12th's ($M = 0.92$ $SD = 0.27$), while no other pairs were significantly differentiated (Figure 3c).

In conclusion, the IR parameter [(Ti/Ttot) It represents the rate of T inspiratory/Total breathing time] was also longitudinally differentiated, $F(3, 30) = 3.73$, $p < 0.05$, with an effect size equal to 0.27. According to the polynomial comparisons, the first degree function was insignificant, $F(1, 10) = 3.95$, $p > 0.05$. The Second degree function on the other hand was significant, $F(1, 10) = 7.80$, $p < 0.05$, signifying a mean decrease. The effect size of this differentiation was equal to 0.44. There was no other significant longitudinal differentiation, as the third degrees function was insignificant, $F(1, 10) = 0.17$, $p > 0.05$. As far as the multiple pairwise comparisons are concerned, the 4th week's mean ($M = 0.30$ $SD = 0.04$) was significantly higher than the 12th's ($M = 0.23$ $SD = 0.03$) while no other comparisons were statistically significant (Figure 3d).

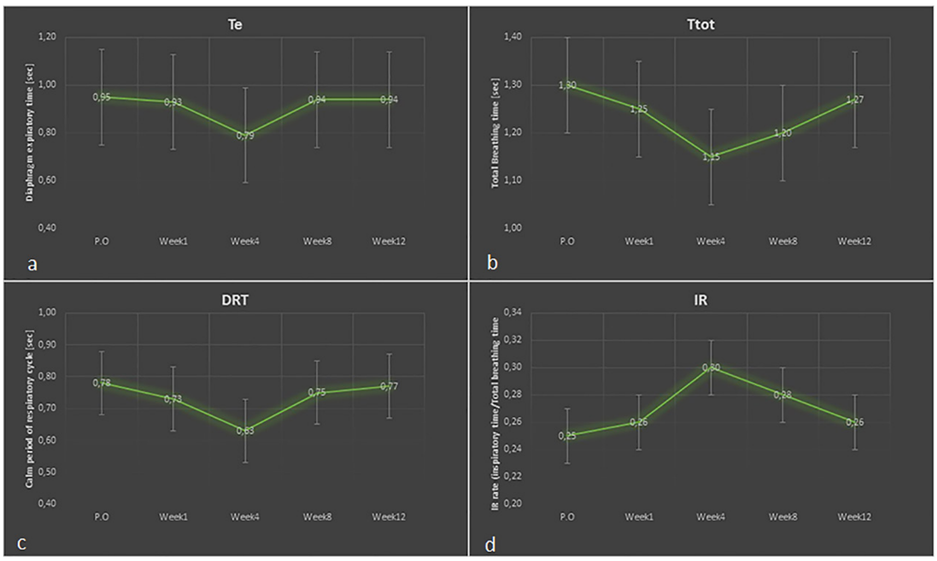

FIGURE 3 - Te(**a**), Ttot(**b**), DRT(**c**) and IR(**d**) longitudinal means.

Histological finding percentage comparisons by the 4th and 12th week after the operation

Concerning percentage differentiation, extra-hepatic granuloma was not differentiated with the passage of time, χ^2 ($df = 2$ $N = 19$) $= 0.04$, $p > 0.05$. Similarly, inflammatory reaction was also not significantly differentiated between the two groups, χ^2 ($df = 2$ $N = 19$) $= 2.82$, $p > 0.05$. Neither fibrosis was significantly differentiated, χ^2 ($df = 2$ $N = 19$) $= 0.04$, $p > 0.05$

Intra-hepatic granuloma, on the other hand, was significantly differentiated over time, χ2 (df = 3 N = 19) = 10.21, $p < 0.05$. More analytically, cases that exhibited no intra-hepatic granuloma (6) all belonged to the ones that were sacrificed at the 4th week. Of the ones that exhibited minimal intra-hepatic granuloma, 33,3% (1) belonged to the group that was sacrificed at the 4th week and 66,7% (2) belonged to the group sacrificed at the 12th week. Similarly, of the cases that exhibited minimal to moderate intra-hepatic granuloma, 33,3% (1) belonged to the group sacrificed at the 4th week versus 66,7% (2) that belonged to the group sacrificed at the 12th week. Of the cases that exhibited moderate intra-hepatic granuloma, 14,3% (1) belonged to the group sacrificed at the 4th week versus 85,7% (6) that belonged to the group sacrificed at the 12th. No seromas nor hematomas were found during the histological examination on both groups of samples (Table 2).

TABLE 2 - Frequency distribution of intra-hepatic granuloma 4 and 12 weeks after the operation.

Time of sacrifice	Intra-hepatic granuloma								Total	
	None		Minimal		Minimal-moderate		Moderate			
	f	%	f	%	f	%	f	%	f	%
4th week	6	100	1	33.3	1	33.3	1	14.3	9	47.4
12th week	0	0	2	66.7	2	66.7	6	85.7	10	52.6
Total	6	31.6	3	15.8	3	15.8	7	36.8	19	100

Note: Relevant frequencies are calculated by column (intra-hepatic granuloma).

Discussion

The use of mesh is not uncommon for the surgical treatment of diaphragmatic hernia. Therefore, it would be of practical interest to know what the changes are regarding diaphragmatic mobility following mesh implantation, given its mechanical actions on the diaphragm and the action of inflammatory changes induced by the mesh.

During the present study the 20 rats proved to be excellent experimental animals. They had a favorable postoperative evolution without any evidence of complications, despite the presence of a foreign body. None presented seromas, hematomas or any surgical site infection. No deaths occurred. All experimental animals maintained their normal habits, were fed well and presented adequate healing progress. Based on other similar studies[8-10] the number of rats needed to obtain a significant result would be approximately nine in each group. One more rat was used in order to maintain an adequate sample size until the last day in case one of the rats died or presented a serious complication. Another aspect worth mentioning was the duration of the experimental protocol. The total duration of nighty days was an appropriate time to obtain a significant result.

Polypropylene incites an intense inflammatory reaction and often forms a dense scar plate around the material. This can translate into clinical patient complaints of discomfort, foreign body sensation, or limited abdominal wall movement. Materials with less polypropylene may modify this host response without unduly sacrificing strength. The concept of a dual-sided mesh to prevent or minimize visceral adhesions on one side while maximizing peritoneal ingrowth has evolved. Bilaminar mesh types and composite materials that include a temporary tissue separating layer are now available[11-22].

Proceed Dual-mesh is a lightweight monofilament Polypropylene mesh with large pore sizes. Additionally, The ORC side provides a layer that separates the mesh from the underlying organs and that characteristic minimizes tissue attachment to the mesh. In this respect, although not used in same experimental studies, it was considered a very good choice for our protocol.

All meshes produce adhesions when placed adjacent to the bowel, but their extent is determined by their pore size, filament structure, surface area, and even by the patients' individual inflammatory reaction. The pathophysiological mechanism of adhesion formation in the presence of biomaterial in a simplified scheme result from fibrin exudate that follows trauma. The fibrin clots form temporary adhesions that last until the fibrinolytic system absorbs the fibrin with the help of a plasminogen activator. This absorption is delayed by inflammation, ischemia, and foreign bodies. The delay allows the fibrin clots to be invaded by fibroblasts, macrophages, and new blood vessels, thus allowing a maturation of a fibrin clot into tissue adhesions[24-30].

In our experimental study a lightweight large pore sized mesh was used to prevent contact with the neighboring granuloma leading to a bridging scar. Through statistical analysis, it was found that either inflammatory reaction, neither fibrosis nor extra-hepatic granuloma were significantly differentiated with the passage of time even though inflammatory response had a higher histological score at the 1st month in comparison with the animals sacrificed at the 3rd month. On the other hand intra-hepatic granuloma was significantly differentiated over time ($p<0.05$). Only three animals (33.3%) from the first group developed minimal to moderate intra-hepatic granuloma while the other six (66.6%) showed no development in contrast with the animals of group 2 that all ten (100%) developed intra-hepatic granuloma. These findings are a strong indication that a mesh related chronic foreign body reaction (responsible for mesh migration, adhesions and fistula formation) becomes stronger after the 1st month of mesh implantation and probably due to an anti-adhesive effect of the absorbable coating (ORC).

Diaphragmatic motion can be evaluated with the use of m-mode ultrasound. M-mode ultrasonography is a simple method, widely used in echocardiography and available in all modern ultrasound equipment. M-mode sonography offers direct visualization of diaphragmatic movement, providing a time-motion curve describing quantitatively diaphragm movements.

It is intuitive to assume that a given ventilatory output could result from a wide variation in the combined determinants of "air pumping" capacity such as inspiratory and expiratory time (Ti and Te, respectively) and the duty cycle, i.e. the fraction of the breathing cycle (Ttot) during which inspiration takes place (TI/Ttot)

The findings from statistical analysis of the diaphragms mobility data comes to reinforce the above hypothesis. Even though not all of the respiratory curve parameters were disrupted over time, four of them showed a significantly differentiated distribution. The measurements of Te (diaphragm expiratory time), Ttot (Total breathing time), DRT (calm period of respiratory cycle) showed a significantly lower 4th week's mean than the 12th week's mean (M=0.79 vs 1.14, M=1.15 vs 1.45, M= 0.63 vs 0.92 respectively) and p values ($p<0.01$, $p<0.05$, $p<0.05$ respectively). Additionally, the IR parameter was also longitudinally differentiated F=3. 73, $p<0.05$ with lower 12th week's mean.

Conclusions

The malfunction in the mobility of the diaphragm, which is transient and returns to normal levels after eight weeks. This probably indicates a positive correlation with the stage of the wound healing as it coincides with the third phase, that of collagen production, despite the fact that inflammatory reaction, fibrosis and extra-hepatic granuloma were not significantly differentiated with the passage of time. Further research may be needed to export distinct results regarding mesh migration over time and if that affects the mobility of the diaphragm.

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Correspondence:
Tzanoglou Ioannis
"Attikon" General Hospital of Athens
1 Rimini Str., 124 62, Chaidari, Greece
Phone: (0030)2105831364
Fax: (0030)2109590274
i_tzanoglou@msn.com

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