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The Impact of 0.9% NaCl on Mesothelial Cells After Intraperitoneal Lavage During Surgical Procedures*

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A – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation; **D** – writing the article; **E** – critical revision of the article; **F** – final approval of article

Abstract

Background. Normal saline gained wide popularity in abdominal surgery as a basic compound used in intraoperative drainage of the peritoneal cavity. However, recent studies have revealed that saline solution is not quite biocompatible with the intraperitoneal enviroment and may promote peritoneal adhesions.
Objectives. The aim of the study was to evaluate the function and viability of human mesothelial cells cultured *in vitro* in 0.9% NaCl solution from intraperitoneal lavage carried out during laparoscopic cholecytectomies.
Material and Methods. The study included 40 consecutive patients suffering from gallstones who underwent laparoscopic cholecystectomy. Fluid was collected after intraperitoneal lavage during the surgical procedures. The samples obtained were used as a medium for *in vitro* incubation of primary human mesothelial cells. After 24 h the synthesis of interleukin 6 (IL-6), plasminogen activator inhibitor (PAI) and tissue plasminogen activator (tPA), as well as the index of cell proliferation were assessed in all the experimental groups.
Results. All the mesothelium cell cultures treated with fluid samples obtained *ex vivo* were characterized by elevated levels of IL-6. The highest concentrations of PAI-1 were found in groups of cells exposed to fluid with bile; similarly, tPA synthesis was extremely elevated in groups treaded with fluid containing bile and small amounts of hemolyzed blood. In contrast, cell proliferation was exceedingly high in 2 groups of cells placed in a standard culture medium and in 0.9% NaCl solution.
Conclusions. Normal saline introduced into the abdominal cavity modifies the biological and physicochemical conditions of the intraperitoneal environment. The impact of 0.9% NaCl on mesothelial cells is manifested in destabilized tissue regeneration, which supposedly initiates adhesion formation (**Adv Clin Exp Med 2016, 25, 6, 1193–1198**).

Key words: oxidative stress, peritoneal adhesions, intraperitoneal lavage, tPA/PAI-1 ratio, fibrinolytic disability.

Peritoneal adhesions can affect as many as 93–100% of patients treated surgically [1]. Most of them appear in the first few days after operation and their progression can cause severe postoperative complications. Adhesions are responsible for chronic abdominal and pelvic pain, chronic constipation, female infertility and even small bowel obstruction [2–4].

Regardless of surgical practice, the initiation of adhesiogenesis results from local injury of peritoneal membranes, requiring subsequent regeneration. Local damage to mesothelial cells decreases

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fibrinolytic activity and promotes free radical generation [5]. These in turn induce suppression of mesothelial cells, which is manifested in reduced ability to proliferate, and consequently in destruction via the apoptosis pathway. It should be noted that in addition to the mesothelium, surrounding tissues are also damaged, which further impairs the healing process and increases the risk of peritoneal adhesions [6, 7].

A solution of 0.9% NaCl, called normal saline, is one of the most common crystalloids used in medical practice [8]. It has also gained wide popularity in abdominal surgery as a basic compound used in intraoperative drainage of the peritoneal cavity to remove cells, tissue debris, blood, clots and other pollutants generated during an operation [9, 10]. However, recent studies have revealed that saline solution is not quite biocompatible with human cells and may have a destructive effect on them. In terms of its chemical properties, the composition of normal saline differs from human body fluids. Its osmolality (308 mOsm/L) is slightly higher than that of human body fluids, and the concentrations of both sodium and chloride in normal saline are in equal molar ratio, respectively [Na^+] 154 mmoL/L and [Cl^-] 154 mmoL/L [11]. Moreover 0.9% NaCl solution is devoid of any buffers, so the pH remains acidic with an average value of 5.5–6. It is suspected that normal saline may cause a variety of adverse reactions, e.g. depression of fibrinolytic activity, oxygen free radical generation and proinflammatory cytokine synthesis [12, 13]. The results of previous research published by the present authors and others suggested that use of 0.9% NaCl solution in an intraabdominal lavage may lead to the formation of peritoneal adhesions [12, 13].

The aim of the present study was to evaluate the function and viability of human mesothelial cells cultured *in vitro* in 0.9% NaCl solution obtained during routine intraperitoneal lavage during laparoscopic cholecytectomies.

Material and Methods

Fluid samples were obtained from 40 consecutive patients (34 women and 6 men), who had been suffering from gallstones and underwent laparoscopic cholecystectomies. Each fluid sample was collected after intraperitoneal lavage performed during the surgical procedure. The cholecystectomies were elective in 31 cases, whereas nine patients underwent emergency surgery due to acute cholecystitis or exacerbation of chronic cholecystitis.

During a single procedure about 675 mL (± 541 mL) of normal saline was instilled intraperitoneally, and after its removal an average volume of 211 mL (± 190 mL) still remained within the abdominal cavity, representing 33.8% (± 18.2%) of the amount initially introduced. After two rounds of centrifugation (150 g, 10 min/10°C), the collected liquids were frozen in aliquots at −20°C and stored for further analysis.

The samples were divided by macroscopic evaluation into four groups: 1) clear to transcluscent ($n_1 = 18$); 2) a low degree of hemolysis ($n_2 = 9$); 3) a high degree of hemolysis ($n_3 = 9$); and 4) samples with bilirubin ($n_4 = 4$). The samples were then used as a medium for incubating primary human mesothelial cells prepared according to the authors' own protocol described in a previous publication [14].

The synthesis of interleukin-6 (IL-6), plasminogen activator inhibitor (PAI) and tissue plasminogen activator (tPA) was assessed after exposing the mesothelial cells to the following solutions: a culture medium, the same culture medium mixed 1 : 1 (v/v) with 0.9% NaCl, and the culture medium mixed 1 : 1 (v/v) with fluid samples collected after abdominal lavage (the pH of all the samples was adjusted to 7.4). Next, a 24 h incubation was started in a 5% CO_2 atmosphere at a temperature of 37°C. At the end of the incubation the supernatant was obtained from the wells and stored at −80°C until cytokine measurement. The cells were lysed with 0.1N NaOH, and the total protein concentration in the lysate was measured using the Bradford method. The concentration of cytokines in the supernatants was measured with commercially available ELISA kits (Bender Medsystems GmbH, Vienna, Austria and R&D Systems, Memphis, USA). The release of the analyzed cytokines was expressed per amount of cell protein.

For the growth experiment mesothelial cells were seeded into 48-well clusters at a density of 5×10^4/well in standard culture medium After 12 h the culture medium was removed from the wells and replaced either with standard culture medium or with culture medium mixed 1 : 1 (v/v) with 0.9% NaCl, or with culture medium mixed 1 : 1 (v/v) with tested fluid samples (the pH of all the samples was adjusted to 7.4). Additionally, ^{3}H-methylthymidine was added to each well to obtain a final concentration of 1μCi/mL.

After 24 h of incubation the supernatant was removed and the cells were harvested from the wells with trypsin-EDTA solution (0.05% trypsin, 0.02% EDTA) and then precipitated twice with 20% trichloroacetic acid (TCA). The cells' precipitate was washed twice with TCA and afterwards lysed in 0.1 NaOH. The radioactivity of the lysate was measured in a β-scintillation counter (LKB Wallac, Perkin Elmer Life Sciences, Turku, Finland). Incorporation of radiolabelled ^{3}H-methyl-

thymidine into the DNA of the growing cells was used as an index of proliferation. The results were expressed as counts per minute (CPM).

All the results are presented as mean ± SD. Graphpad Prism 5.0 software (GraphPad Software Inc., San Diego, USA) was used for the statistical analysis. Data from multiple comparisons were verified using the one-way analysis of variance (ANOVA), complemented by Tukey's test. The relationship between clinical features and laboratory results was analyzed using the unpaired Student's t-test and Fisher's exact test. A p value of 0.05 was considered statistically significant.

Results

All mesothelium cultures treated with fluid samples obtained *ex vivo* were characterized by elevated secretion of interleukin-6. However, only in the group grown in the fluid with a high degree of hemolysis was the release of IL-6 significantly higher than in the two control groups (medium, NaCl) and in the group exposed to clear liquid. Mesothelium placed in pure 0.9% NaCl solution demonstrated the lowest IL-6 synthesis compared with the fluid samples collected *ex vivo* and the standard culture medium (Table 1).

Moreover, the highest concentration of PAI-1 was found in the groups of cells exposed to the fluid with bile; similarly, tPA synthesis was extremely elevated in the groups treated with samples containing bile and having a high degree of hemolysis. Mesothelium incubated in pure 0.9% NaCl and clear lavage fluid demonstrated the lowest synthesis of tPA among all the cell groups tested. However, these differences were not statistically significant (Table 1).

In contrast, cell proliferation was highly stimulated in the two groups of cells growing in the standard culture medium and in 0.9% NaCl alone. Mesothelium showed symptoms of decreased cell proliferation when exposed to any of the fluid samples obtained from abdominal lavage, except for the clear fluid. The lowest growth was recorded in the cultures in the liquid containing bile, and only slightly higher growth was observed in the two groups with hemolysis (Table 2).

Finally, a relationship was discovered between clinical features and laboratory results. Samples

Table 1. Synthesis of Interleukin 6 (IL-6), PAI-1 and tPA in mesothelial cells exposed to culture medium (medium), culture medium mixed with 0.9% NaCl (NaCl), clear lavage fluid (Clear), lavage fluid with a low degree of hemolysis (hem+), lavage fluid with a high degree of hemolysis (hem+++), and lavage fluid with bilirubin (Bile)

	Medium	**0.9% NaCl**	**Bile**	**Clear**	**hem+**	**hem+++**
IL-6 pg/mL (SD)	434.1 (55.6)	401 (56.9)	477.5 (176.9)	644.4 (186.9)	597.4 (235.6)	931.2 (862.5)
p				p < 0.01 vs. medium p < 0.001 vs. NaCl	p < 0.05 vs. NaCl	p < 0.001 vs. medium p < 0.001 vs. NaCl
PAI1 ng/mL (SD)	6.5 (1.2)	5.8 (0.5)	7.7 (0.8)	5.2 (1.1)	5.3 (1.8)	5.6 (1.1)
p			p < 0.01 vs. NaCl	p < 0.05 vs. medium p < 0.001 vs. bile	p < 0.05 vs. medium p < 0.001 vs. bile	p < 0.001 vs. bile
tPA pg/mL (SD)	163.5 (36.9)	145 (49.3)	182.9 (123.6)	151.5 (59.5)	180.7 (80.2)	273.8 (117.1)
p						p < 0.05 vs. NaCl p < 0.001 vs. clear

Table 2. Proliferation of mesothelial cells (CPM) exposed to culture medium (medium), culture medium mixed with 0.9% NaCl (NaCl), clear lavage fluid (Clear), lavage fluid with a low degree of hemolysis (hem+), lavage fluid with a high degree of hemolysis (hem+++), and lavage fluid with bilirubin (Bile)

	Medium	**0.9% NaCl**	**Bile**	**Clear**	**hem+**	**hem+++**
CPM pg/mL (SD)	13900 (6504.2)	14858.3 (5719.6)	2090.4 (3764.9)	9970.5 (4774.8)	4107.5 (4377.3)	5888.5 (4946.6)
p			p < 0.001 vs. medium p < 0.001 vs. NaCl		p < 0.001 vs. medium p < 0.001 vs. NaCl	p < 0.01 vs. medium p < 0.01 vs. NaCl

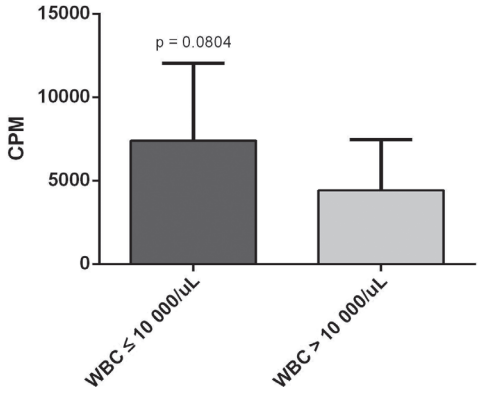

Fig. 1. The intensity of mesothelial proliferation (CPM) depended on whether the fluid samples were collected from patients with normal white blood cell counts (WBC ≤ 10000/uL) or elevated white blood cells (WBC > 10000/uL)

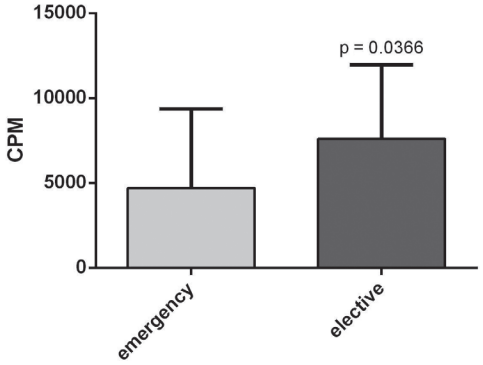

Fig. 2. The intensity of mesothelial proliferation (CPM) depended on whether the fluid samples were collected from patients who had undergone emergency surgery or elective surgical treatment

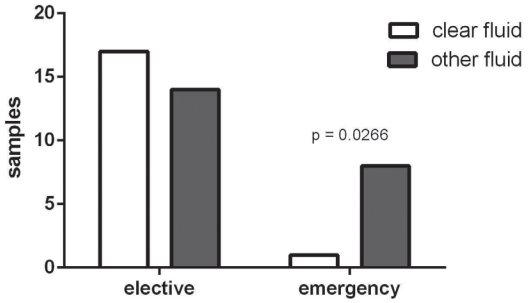

Fig. 3. The number of clear fluid samples (clear fluid) and samples with blood or bile (other fluid) obtained during elective or emergency procedures

obtained from patients with elevated blood leukocytes and fluids form patients who had undergone emergency surgery tended to be more suppressive to mesothelial cells cultured *in vitro* (Fig. 1, 2). Furthermore, clear samples with small amount of debris were obtained mainly from patients qualified for elective surgery. Samples with blood or bile were generally collected during procedures performed due to severe clinical symptoms (Fig. 3).

Discussion

The 0.9% NaCl solution called normal saline is one of the most common substances used in clinical routine. Among the many advantages of this crystalloid, one attribute remains indisputable: normal saline is still considered a biocompatible and biofriendly solution. However, recent reports, particularly studies published in the last decade, have shown the devastating effects of saline on human cells destroying the myth of its absolute biocompatibility. It has been found that the composition of 0.9% NaCl significantly differs from body fluids and has a direct impact on electrochemical gradients, including ligand-receptor interactions [15, 16].

Every mesothelial tissue injury decreases cell viability and disturbs the biochemical balance of the peritoneal membrane. Intraabdominal damage due to reductions in peritoneal fibrinolytic activity are among the main triggers of adhesion formation [6, 17]. As a consequence of peritoneal lesions the production of tPA, the main fibrinolytic stimulator, decreases rapidly and the release of PAI-1 is simultaneously stimulated [5, 18]. Recent reports have confirmed that the relationship between tPA and PAI-1 activities plays an important role in adhesiogenesis [12, 19]. In the present study lavage fluid containing bile intensified PAI-1 synthesis. Bile, mainly consisted of bilirubin and bile acids, may cause multiple negative changes within the intraperitoneal environment manifested in fibrinolytic disability and proinflammatory cytokine secretion initiating leukocyte activation. Through its chemical properties bile can also act as a hypertonic agent, promoting the mesothelial stress response and oxygen free radical synthesis [20].

Reductions in PAI-1 activity observed in the other experimental groups in the present study resulted from various circumstances. One was the decreased viability of mesothelial cells as effect of CO_2 insufflation during the laparoscopic cholecystectomy procedures. Elevated intra-abdominal pressure restrains blood perfusion and initiates intraperitoneal acidosis [21, 22]. However, an excess of carbon dioxide is potentially harmful to human cells. Some studies suggest that the intraabdominal presence of CO_2 reduces cell metabolism and consequently decreases the inflammatory response [21–23]. Locally diffused CO_2 suppresses adhesiogenesis and probably helps to protect the mesothelium. In the present study the level of PAI-1 was lowered in all cell cultures exposed to fluids collected *in vivo* except for the samples containing bile. This result may confirm the protective effect of CO_2 [23].

The high concentrations of tPA and relatively low levels of PAI-1 observed in the samples with

hemolyzed blood suggest that dissolved blood cellular components, presumably macrophages, have a profibrinolytic impact [24, 25]. The mesothelium incubated in lavage fluids with hemolysis was characterized by an extremely elevated tPA/PAI-1 ratio compared to the other groups, suggesting that limited intraperitoneal bleeding with leukocyte infiltration initiates proinflamatory cytokine activity, leading to more efficient peritoneal regeneration [18, 26]. It can be posited that samples with hemolyzed blood contain more heme and iron ions, potentiating free radical generation and oxidative stress response [27]. On the other hand, the mesothelium cells incubated in 0.9% NaCl alone and in clear lavage fluid displayed the lowest level of tPA and PAI-1. As mentioned earlier, normal saline, the main component of these samples, demonstrates hyperosmotic properties. The slightly higher osmolality of the samples lowered the viability of the mesothelial cells incubated in them, resulting in decreases in tPA and PAI-1 synthesis.

Recent reports have revealed that tissue regeneration following peritoneal damage is under certain conditions responsible for adhesion formation [7, 19]. The present study has shown that except for one group of cells cultured in clear lavage fluid, exposure to samples collected *ex vivo* reduced the rate of mesothelial proliferation. In all likelihood the presence of proinflammatory compounds, including bile acids, morphotic elements (especially leukocytes) and high concentrations of tissue enzymes or cytokines, destabilizes cell replication [26, 28]. Simultaneously, there were no significant differences between the proliferation levels in a standard culture medium and in 0.9% NaCl solution. It can be concluded that mesothelial cells, despite the hyperosmotic impact of 0.9% NaCl solution, are able to adapt and function even in unfavorable conditions [29, 30].

The findings of the present study also demonstrate a relationship between clinical symptoms and the laboratory tests performed *in vitro*. Fluid samples obtained from patients with clinical signs of acute cholecystitis and blood leukocytosis presented a tendency to depress cell proliferation. Presumably, elevated inflammatory factors circulating in the blood intensify the local peritoneal response following tissue damage during the surgical procedure.

In the present study the impact of pure 0.9% NaCl on mesothelial cells was characterized by a relatively low tPA/PAI-1 ratio, a slight reduction in IL-6 activity and a significant increase in cell proliferation. The authors suspect that as a result of inadequate fibrinolytic activity, normal saline solution promotes proliferation of mesothelial tissue, which in turn initiates adhesion formation. On the other hand, the presence of peritoneal bleeding and local inflammation related to surgical trauma contribute to increases in IL-6 activity and the tPA/PAI-1 ratio. The data obtained showed that despite operative damage to the peritoneum, mesothelial cells are able to maintain proper fibrinolysis, with sufficient ability to proliferate and regenerate.

The study showed that normal saline introduced into the abdominal cavity modifies the biological and physicochemical conditions of intraperitoneal environment and therefore it should not be used for peritoneal lavage.

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