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original article

The effect of peritoneal dialysis with alkaline dialysate in peritonitis carcinomatosis: an experimental study in mice

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SUMMARY: **The effect of peritoneal dialysis with alkaline dialysate in peritonitis carcinomatosis: an experimental study in mice.**

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Objective. *The aim of this study was to neutralize acidic pH using an alkaline dialysate for continuous ambulatory peritoneal dialysis (CAPD) in mice with peritoneal carcinomatosis (PC) and to investigate the change of the pH level in the acidic fluid along with its effects on liver oxidative stress, liver and kidney histopathology and the lifespan of the body.*

Materials and methods. *A total of 38 mice were randomly divided into 4 groups.PC development was inhibited by intraperitoneal injection of Ehrlich tumor cells in all mice in each group.*

Results. *In the group-1 receiving CAPD, the pH levels of acidic liquid were higher; and the levels of liver TBARS were lower with higher reduced glutathione levels. Histopathological damage in group-1 was less than in group-2. In Group 3 receiving CAPD, the average lifespan extended by 10.4%. The average lifespan extended by 26.1%.*

Conclusion. *This study indicated that applying CAPD with alkaline dialysate in PC contributed to the neutralization of acidosis of the intraperitoneal acid structure;had favorable effects on oxidative stress markers in liver tissue; prevented histopathological injury in liver and kidney tissues, and extended the life span of the body in mice.As this is a simple, inexpensive, and easily available method, larger studies are warranted to evaluate its effects.*

KEY WORDS: Peritonitis carcinomatosis - Peritoneal dialysis - Alkaline - Cancer - Survival.

Introduction

The accumulation of fluid in the peritoneal cavity may reach pathological levels in some cases. While liver disease is the most common reason for pathological fluid accumulation, called ascites, 10% of the cases is associated with cancer and can be a clinical manifestation for peritoneal carcinomatosis (PC) (1). While PC may arise from a disorder in the gastrointestinal (GI) tract, it may also originate from ovarian malignancies. As ovarian malignancy, which is common among women, typically remains asymptomatic in its early stages, it is highly possible to encounter PC when the diagnosis is ultimately made (2, 3) in the later stages of the disease. However, since a great majority of the patients are diagnosed in the advanced stages, they are considered to be "incurable".

While homeostasis continues in the body, the elemental composition and pH of body fluids may alter in pathophysiological conditions. Cancer tissues and acidity forming around the microenvironment are examples of such alterations.

The Walburg effect is an important metabolic process for cancer metabolism and microenvironment of a cancerous cell (4). As a result of the Walburg effect, lactate and proton accumulate in the tumor tissue forming a hypoxic area around the tumor (4, 5). Studies demonstrated that the pH level can decrease by

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6.0 in a cancer tissue and its microenvironment (6-8).

Acidosis has a potential influence on tumor metastasis and cancer progression including cancer cell metabolism (9, 10) and survival (11, 12), chromosomal instability (13, 14), and tumor angiogenesis (15, 16). Several studies have emphasized the importance of correcting acidosis for treatment of cancer (17, 18). The acidification of local tissues can be caused by dysregulated cell metabolism and/or defective blood perfusion to remove acidic metabolic by products. A myriad of studies show that localized interstitial acidosis is a biochemical hallmark in inflammatory tissues, ischemic organs and solid tumors (10, 19). Interstitial acidosis has been shown to cause tissue injury and aggravate disease progression (20, 22).

Continuous ambulatory peritoneal dialysis (CAPD) has been in use for years in renal replacement therapy. While CAPD is performed to remove toxic substances from the body, the substances needed by the body, such as amino acids and bicarbonate, can be administered via the peritoneal dialysis fluid (23, 24). In this way, the diffusion of solid structures including microglobulin is limited across the peritoneal pores; however, small components can transmit bilaterally through the peritoneum faster (25). A study using computed tomography indicated the surface area of the peritoneal membrane in contact with dialysate to be 0.55±0.04m^2 (26). Flessner et al. (27) reported that the ratio of the peritoneal surface area in contact with dialysate to the entire anatomical surface area of the peritoneal membrane is less than 50%; however, this ratio can increase up to 100% if the dialysate is sustained more than 24 hours.

As our first plan, we wanted to bring this information to the alkaline medium such as the original interstitial fluid property of the peritoneal cavity where cancer cells are common. We tried to prevent the formation of tumor cells with the alkaline character of this field. Our second plan was to reduce the tumor burden by dialysis and removal of the cancer cells in this area from the body.

Materials and methods

Animals

We have used 38 male Swiss albino mice which were 25 - 30 g in weight and 10 weeks old. Mice were from Adana Veterinary Control and Research Center and were kept 12 h under light and 12 h under dark cycles at 25°C for 5 days before study. Mice were fed with standard pellet diet and tap water ad libitum. The Ethical Approval of the research was obtained from the Adana Veterinary Control and Research Center Ethics Committee (2016/3554). Every mouse has cancer. The subjects were divided into 4 groups. Biochemical and histopathological examination was performed in Group 1 and Group 2. Peritoneal dialysis was applied to Group-1(n=9). Group-2(n=9) treatment was not performed. Group-3 and Group-4 survival times were followed. Peritoneal dialysis was applied to Group-3(n=10). Group-4(n=10) treatment was not performed. Experimental groups were designed as seen in Table 1.

The formation of PC

Stock mouse with PC was obtained from experimental animals laboratory, Cerrahpaşa Medical Faculty, Istanbul University. Erlich ascites carcinoma (EAC) formed by injected intraperitoneally 0.2ml (1X10^6 cells) ascites fluid doing paracentesis form peritonitis of stock mouse (28). Cells in the injected ascites liquid stained with Tryphan Blue and counted with hematocytometer.

TABLE 1 - EXPERIMENTAL GROUPS OF MICE.

Experimental group	Group-1	Group-2	Group-3	Group-4
TREATMENT	During the first 7 days, 1 cc (once a day) dialysate was administered through the intraperitoneal route. On the 8th day, the dialysate was administered in the amount of acid taken via paracentesis.	No therapy was applied.	During the first 7 days, 1 cc (once a day) dialysate was administered through the intraperitoneal route. On following days until the date of death, the dialysis solution was given once a day in the amount of acid taken through paracentesis.	No therapy was applied.

SD: Standard Deviation

Preparation of the dialysis solution

A 100 ml dialysate was prepared by isotonic sodium chloride (Laurus, Istanbul, Turkey) with sodium bicarbonate (8.4%) (Onfarma, Samsun, Turkey), magnesium sulfate (15%), calcium picken (10%) (ADEKA, Istanbul, Turkey) and distilled water composition. This dialysis solution was protected from sunlight till the administration time. The pH value of the prepared dialysate was 7.56. Considering its composition, our dialysate was compatible with the interstitial fluid with a pH value of 7.56 containing 141mEq/L Na,111 mEq/L Cl, 30 mEq/L HCO3, 1.83 mEq/L Mg, 3 mEq/L Ca, and 1.83 sulfate.

The CAPD management method

For the mice in Group 1 and Group 3, a 0.03lt/kg dialysis solution was gently administered into the intraperitoneal area through the right quadrant once a day during the first 7 days of the experiment. The first dose of dialysate was given 24 hours after the intraperitoneal injection of EAC. A 26 gauge injector was used for administration. Prior to each application, skin was disinfected with 95% isopropyl alcohol. Following the formation of acidic fluid after the 7th day, the acidic fluid was removed through the left lower quadrant by paracentesis, and a dialysate in the same amount with the removed acidic fluid was given intraperitoneally through the right lower quadrant. In order to create the same stress in Group 2 and Group 4, a 26 gauge needle was inserted into the intraperitoneal through the right lower quadrant, but no substance was injected.

Measuring the pH value in the acidic fluid

After euthanasia, the 1 ml acidic fluid removed from each subject's body was stored in a sterile glass tube and transferred to the laboratory in a +4°C coolpack. The acidity of the fluid was analyzed with a pH meter (Hanna Instruments PH 211) at the laboratory.

The preparation of liver tissue

After cervical decapitation of mice, whose abdomen was dissected by median incision, approximately 1gr liver tissue to measure GSH and TBARS (thiobarbituric acid reactive substances) was sampled and washed with 0.9% NaCl solution. Livers were immediately stored in tubes until at -20°C biochemical analysis. Tissues were homogenized for 3 min with 10 (v/w) volumes of ice cold PBS (phosphate buffered saline) pH 7.4 in 2.5mM ATP using a homogenizer (Ultra turaxt-18). Sample were centrifuged (Nüve NF 800R) at 14000 rpm for 30 min at +4°C. Supernatants were used to determine protein, GSH, TBARS contents.

The measurement of GSH

We the method proposed by Beutler et al. (29) to measure the hepatic glutathione (GSH). Dithionitrobenzoic acid (DTNB) was used as the substrate, and the amount of GSH was determined. GSH of the liver as non protein was measured at 412 nm. The results were expressed as μmol/mg protein.

The measurement of TBARS

TBARS assay was determined by using colorimetric method with changes in absorbance read at 532 nm (Optizen 3220 UV) against standards (0.5–25 nmol ml–1 1,1,3,3-tetraethoxypropane). TBARS was calculated using the formula as nmol/mg protein (30, 31).

Histopathologic evaluation

Each liver sample and left kidney was stored in a separate box containing 10% formaldehyde until histopathologic evaluation. Tissue samples were embedded in paraffin blocks and slices (0.4 microns) were obtained. The slices were deparaffinized in incubator at 75ºC for 45 minutes. The pathologist did not know the groups of mice. Evaluation of histopathologic and renal injury was performed in hematoxylin-eosin–stained sections using some parameters. Liver tissues were evaluated histologically in terms of increased connective tissue, granular degeneration, necrotic cells and vascular congestion and elevated mononuclear cell infiltration. Additionally, the kidneys were examined considering the expansion of mesangial matrix in glomerular capillaries, tubular dilatation and degeneration and severity of adhesion in the Bowman's capsule. All parameters were assessed as 0, absent; 1, mild; 2, moderate; and 3, severe.

Survival analysis

Group-3 and group-4 were followed for mean survival time (MST) and average survival time (AST). MST and AST were calculated according to these formulas:

MST = (the first death day + the last death day) / 2
AST = the total death days / total mice number
The percentage of Increasing Mean Standard Life (IMSL) and The Percentage of Increasing Average Standard Life (IASL) were calculated with these formulas.
IMSL (%) = (experimental group MST- control group MST) × 100/control group MST
IASL (%) = (experimental group AST- control group AST) × 100 / control group AST (28, 32, 33). Statistical evaluation was compared with the Mann Whitney U test.

Statistical analysis

The statistical analysis of data was performed with SPSS 20.0 (IBM, Hong Kong) package program. Student t-test was used for normal dispersion data and Mann-Whitney U test for abnormal dispersion data. The limit of statistical significance was set at $p< 0.05$.

Results

Biochemical test results

While the mean pH value was 6.395 in Group 1, it was found to be 6.301 in Group 2($p<0.05$) (Table 2).

The mean GSH level of the liver tissue was 0.064µmol/mg in Group 1, it was 0.027µmol/mg in Group 2 ($p=0.03$). The average TBARS value was found to be 2.941 nmol/mg in Group 1 whereas it was 5.584 nmol/mg in Group 2 ($p=0.007$) (Table 2).

Histopathological results

The histopathological examination showed that the increase in connective tissue, granulation tissue, necrotic cells and vascular congestion were statistically significantly lower in Group 1 as compared to Group 2. However, although mononuclear cell infiltration was lower in the 1st group, the difference was not statistically significant. Group 1 indicated less mesangial matrix accumulation in the glomeruli, and tubular damage with dilatation in comparison with Group 2, and the difference between the groups was statistically significant. Although the 1st group had less adhesions between the Bowman's capsule, the difference was not statistically significant (Table 3) (Figures 1, 2) (Liver and kidney tissue samples).

Survival time

Accordingly the first death date was 11th day and the last death was 18th inoculation EAC in group-3 and the first death date was 10th day and the last death was 13th inoculation EAC in group-4. Considering the survival time, the MST was 12.7 and AST was 14.5 in Group 3; whereas they were found to be 11.5 and 11.5 in Group 4, respectively. Thus, there was a statistically significant difference between Group 3 and Group 4 regarding the MST ($p<0.001$). In the light of these results, the IMSL and IASL were calculated to be 10.4% and 26.1%, respectively (Figure 3).

Discussion

Preventing the spread of tumor cells during surgery for intra-abdominal cancer without peritonitis carcinomatosa is a crucial part of the surgical procedure as in the no-touch technique. However, it is still not possible to identify and indicate the spread of the cancer cells into the intraperitoneal cavity during a surgical intervention.

There are limited strategies to be applied to achieve tumor regression and extend the survival time for the cases diagnosed with PC. Patients with early stage PC are treated with aggressive cytoreduction and hy-

TABLE 2 - BIOCHEMICAL TEST RESULTS.

Biochemical test	Group-1 Mean ± SD	Group-2 Mean ± SD	p
pH	6.396±0.143	6.301±0.067	**p<0.001**
TBARS	2.941±1.601	5.584 ±2.196	**0.007**
GSH	0.064±0.049	0.027±0.014	**0.03**

SD: Standard Deviation, TBARS: Thiobarbituric acid reactive substances, GSH: Glutathione

TABLE 3 - HISTOPATHOLOGICAL RESULTS.

Histopathological results		Group-1 Mean± SD	Group-2 Mean± SD	p value
LIVER	Connective tissue	0.33±0.50	1.11±0.93	**0.001**
	Granulation tissue	0.78±0.44	1.22±0.44	**0.048**
	Necrotic cells	0.44±0.53	1.11±0.93	**0.031**
	Vascular congestion	0.44±0.52	1.22±0.83	**0.041**
	MNL cell infiltration	0.67±0.50	1.11±0.33	0.079
KIDNEY	Mesangial matrix accumulation	1.33±0.50	2.33±0.71	**0.003**
	Tubular damage	1.33±0.50	2.22±0.83	**0.014**
	Adhesion between the Bowman's capsule	1.44±0.53	2.00±0.86	0.12

SD: Standard Deviation

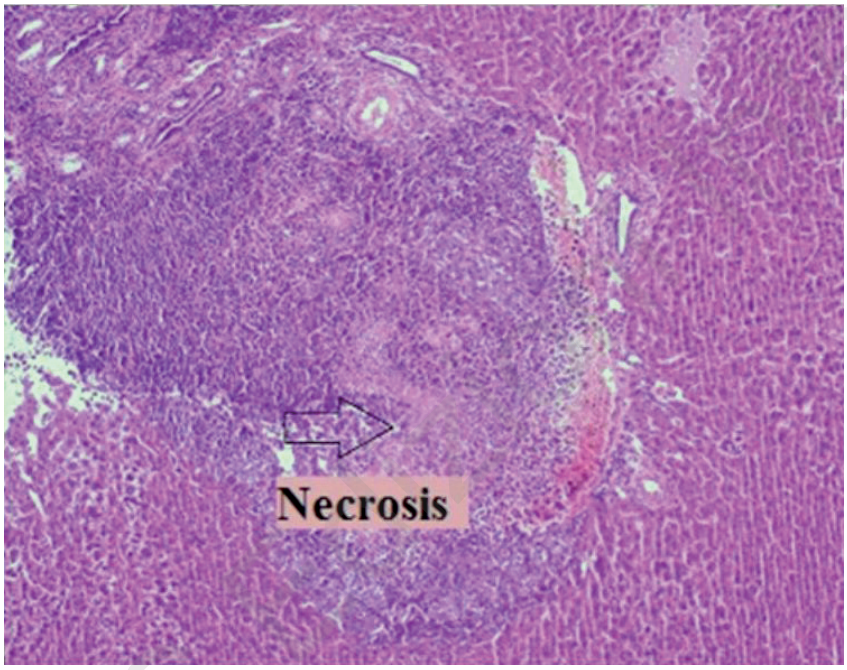

Figure 1 - Necrosis example in the liver.

pertensive intraperitoneal chemotherapy (32).

In case of ovarian PC, the greater the initial tumor burden is, the longer the exposure to chemotherapeutic agents is and the higher the risk of cancer cells to acquire resistance treatment becomes (35). Formation of abnormal tumor vessels on solid tumors affects both the delivery of the chemotherapeutic agents to the cells in the center and the success of treatment (36). Large tumor masses have a low growth fraction and are resistant to chemotherapeutic agents since they are composed of mostly non-dividing cells or the cells in the G0 phase. It has been indicated in several studies addressing ovarian cancer that optimal cytoreduction enhance the response rate to chemotherapy.

However, despite the application of optimal cytoreduction, the Walburg effect continues on the tumor tissues, which are newly forming or could not be excised to prevent a risk, and on those, which are too small to be seen. As a result, the acidity generated by the tumor cells damages the intact tissues, which eventually influence the patient's health negatively.

In the present study, we aimed to eliminate all intraabdominal tumor cells, from the largest to the

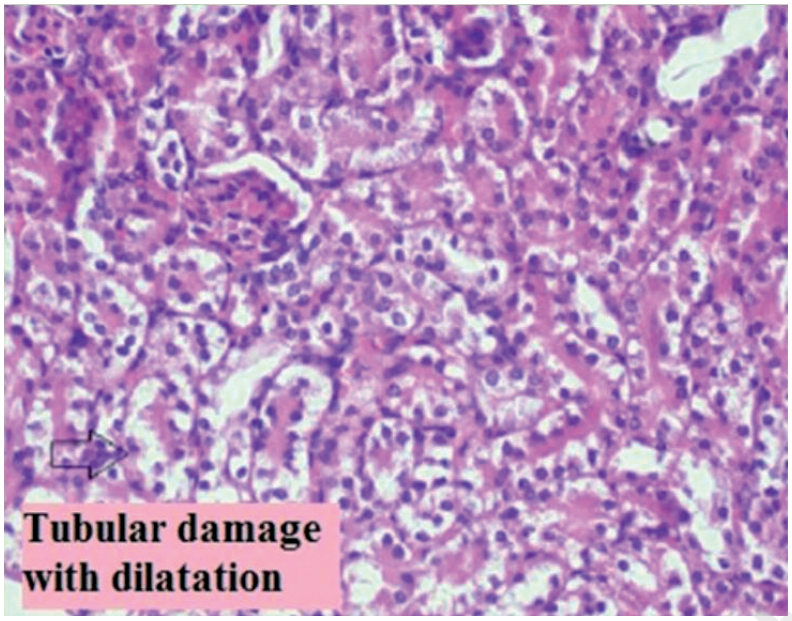

Figure 2 - Example of tubular damage and dilatation in kidney tissue.

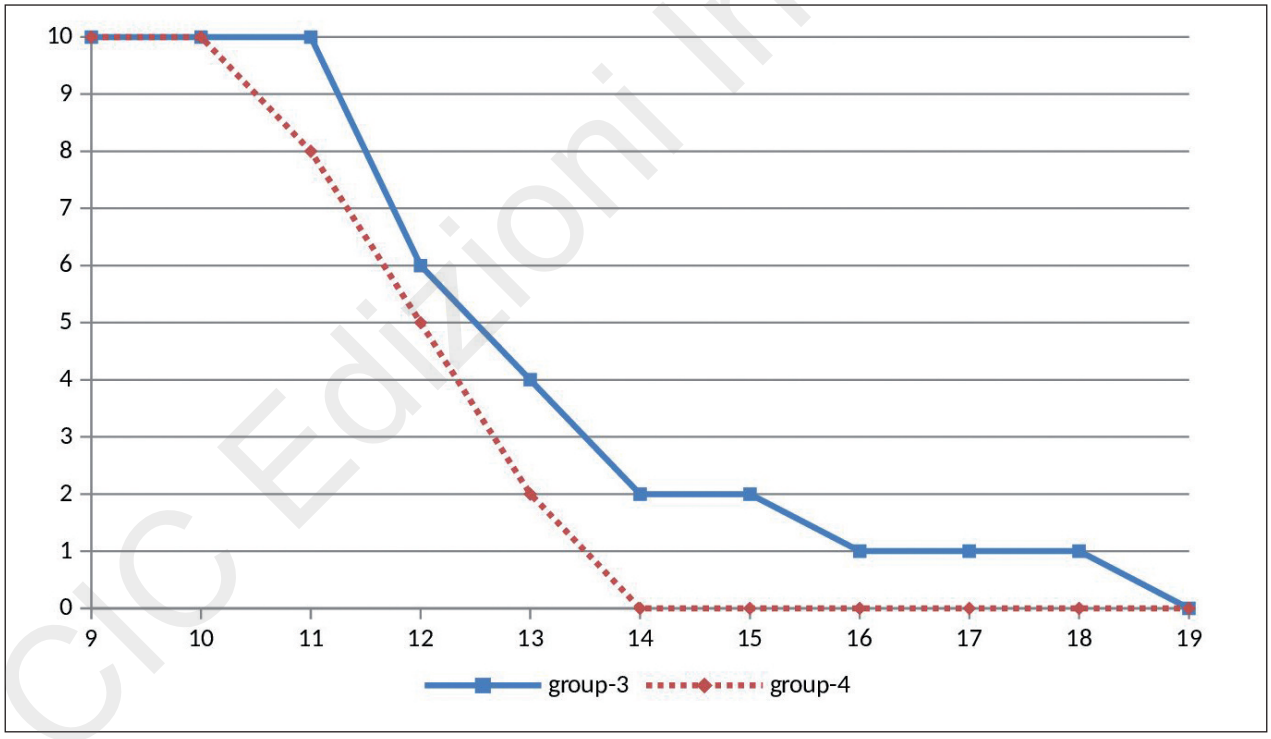

Figure 3 - Survival by days in group-3 and group-4.

smallest, that contact the intraperitoneal fluid. While preparing a peritoneal dialysis fluid, one should consider both the target of the therapy and its biocompatibility.

In this connection, we prepared a CAPD dialysate compatible with the interstitial fluid compartment in order to neutralize acidity within the intraperitoneal area that contains cancer cells and tissues. For this purpose, we created the entire buffer with bicarbonate. The peritoneal area and peritoneum microenviron-

ment through the peritoneal micropores were targeted for dialysate infusion.

We focused two factors in this study: (a) the neutralization of acidity in the intraperitoneal area; (b) the removal of the tumor cells in the acidic fluid generated in PC and the toxic substances passing through peritoneum from the body with CAPD.

The reason why we administered 0.030 l/kg per prior to the presence of intraperitoneal acidic fluid was to neutralize the minimal acidity generated by tumor cells and prevent tumor progression. After the formation of acid, we removed max. 4 ml acidic fluid from each patient and administer the same amount of dialysate into the intra-abdominal space just after paracentesis in order to maintain the hemodynamic stability.

In renal replacement therapy, the CAPD solution is administered with 6-8 h intervals depending on the targeted function type. However, considering our aim and the fact indicated by previous studies that the area of contact between the peritoneal membrane and dialysate becomes complete in 24 hours, we arranged the CAPD cycles with 24-hour intervals.

The pH value of the fluids collected from the mice after euthanasia on the 9th day of the trial was higher (more alcali) in Group 1 as compared to Group 2 which shows that we were able to achieve our first (a) objective.

According to the biochemical examination of liver tissues, TBARS (a lipid peroxidation product resulting from the damage by reactive oxygen species) was lower in Group 1 than in Group 2 and the reductant glutathione reserve (a defense mechanism) was higher in Group 1 as compared to Group 2. It can be stated in the light of these results that our CAPD therapy was useful in reducing tumor acidosis and functional damages on the interstitial tissue, which were identified in previous studies on the intraperitoneal area and the microenvironment around the peritoneum. Additionally, the rate of connective tissue, granular degeneration, mononuclear cell infiltration, necrotic cells and vascular congestion was lower in Group 1 in comparison with Group 2. Furthermore, the rate of the expansion of mesangial matrix in glomerular capillaries, tubular dilatation and degeneration, and the severity of adhesion in the Bowman's capsule was lower in Group 1 than in Group 2. Considering these findings, it can be argued that preventing acidification in PC ensured the protection of the cellular structure.

As compared to Group 4, the mean survival time showed an increase of 10.4% ($p<0.001$) and the average survival time increased 26% in Group 3. Our results are consistent with the studies (7-9) arguing that preventing acidification can improve survival in cancer patients. These results support the hypothesis our study built upon.

In this study, performing CAPD with a dialysate compatible with the interstitial fluid decreased the acidification of the peritoneal fluid, ensured the protection of the organism from harmful reactive oxygen substrates, preserved the GSH reserve as well as the histopathological structure of the hepatic and renal tissues in adjacent to the peritoneum, and therefore, extended the survival time of the organism with cancer. Also, we believe that removing the cancer cells and the toxins in the acidic fluid out of the body may have contributed to the alkalization, and thus, worked in favor of the organism's well-being.

In consideration of these results, it may be possible to increase the response to chemotherapy by (a) eliminating the cancerous cells to prevent the microspread of tumor cells during CAPD application performed after curative surgery for intra-abdominal carcinomas and (b) decreasing the total number of tumor cells through the implementation of CAPD with alkaline dialysate either as adjunctive therapy to cytoreductive surgery or as the main therapy for the patients with PC who cannot undergo cytoreductive surgery.

Conclusion

Due to its favorable contribution to mortality and morbidity for patients both with resectable and unresectable metastases, applying CAPD alkaline dialysate during or after surgical intervention can be a new therapeutic strategy for intra-abdominal gastrointestinal, gynecological, urological carcinomas and especially for peritoneal carcinomatosis.

Limitation- We do not know the consequences of intraperitoneal administration of different dialysate solutions because no similar studies have been done before. We are thinking of preparing a hypothetical peritoneal dialysis solution. There is a need for more different works.

Conflict of interest

There is no conflict of interest between the workers and another company.

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