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Research

The Influence of Sildenafil Versus Resveratrol on the Healing of Colonic Anastomosis: An Experimental Study

Sildenafil ve Resveratrolün Sol Kolon Anastomozunun İyileşmesi Üzerine Etkisi

Mehmet Kemal Yener[1], Enver Reyhan[2], Abit Yaman[3], Hüseyin Kılavuz[4], Alper Sözütek[1], Zeynel Abidin Taş[5], Atilla Yoldaş[6], Oktay Irkörücü[7]

[1]University of Health Sciences Türkiye, Adana City Training and Research Hospital, Clinic of General Surgery, Adana, Türkiye
[2]Mersin University Faculty of Medicine, Department of General Surgery, Mersin, Türkiye
[3]Çukurova University Faculty of Medicine, Department of Oncological Surgery, Adana, Türkiye
[4]University of Health Sciences Türkiye, Başakşehir Çam and Sakura City Hospital, Clinic of General Surgery, İstanbul, Türkiye
[5]University of Health Sciences Türkiye, Adana City Training and Research Hospital, Clinic of Pathology, Adana, Türkiye
[6]Kahramanmaraş Sütçü İmam University Faculty of Medicine, Department of Anatomy, Kahramanmaraş, Türkiye
[7]University of Sharjah College of Medicine, Department of Clinical Sciences, Sharjah, United Arab Emirates

ABSTRACT

Objective: The prevention of colonic anastomosis leakage is a subject of ongoing surgical investigation. The present experimental study was performed to investigate whether the administration of resveratrol (R) and sildenafil (S) to rats could improve the strength of intestinal anastomosis without obvious interference with other vital components of the repair process.

Methods: Thirty-six rats were randomly divided into four groups as follows: control (C), S, R, and combined (S&R). All rats underwent left colon resection plus anastomosis. After surgery, the C group received 10 mg/kg of tap water, while the S and R groups received S (10 mg/kg) and R (10 mg/kg), respectively, via an orogastric tube for 7 days. The S&R group received both S and R in the same dosages. All rats were euthanized on postoperative day 7. Anastomotic bursting pressure (ABP) was measured, and tissue samples were taken for histopathological examination and hydroxyproline (T.H.) level assessment.

Results: The ABP was significantly higher in the S&R group than in the C group (p=0.021). When evaluated histopathologically in terms of edema, the significant difference was due to the S&R group. Tissue edema was lower in the S&R group than in the C, S, and R groups (p=0.004). There were no significant differences between the groups when analyzed for polymorphonuclear lymphocyte increase, lymphocyte increase, macrophage increase, mucosa-epithelial status, and submucosal bridge. There were also no significant differences in hydroxyproline levels between the groups (p=0.222).

Conclusion: The results suggest that the combination of R and S significantly increased the bursting pressure in rats with left-sided colonic anastomosis, with S potentially being the key driver in this outcome. The current results are encouraging; however, additional studies will be needed for confirmation.

Keywords: Resveratrol, sildenafil, colon, anastomosis, wound healing

ÖZ

Amaç: Kolon anastomoz kaçağının önlenmesi halen araştırmaları devam eden önemli bir cerrahi problemdir. Bu deneysel çalışma resveratrol (R) ve sildenafilin (S) sıçanlarda uygulanmasının anastomoz iyileşme sürecinin diğer etkenler ile belirgin etkileşim olmaksızın anastomoz sağlamlığına katkısını araştırmak için gerçekleştirilmiştir.

Gereç ve Yöntem: Otuz altı sıçan rastgele olarak aşağıdaki şekilde dört gruba ayrıldı: Kontrol (K), S, R ve kombine (S&R). Tüm sıçanlara sol kolon rezeksiyonu ve sonrasında kolokolonik anatomoz uç uca anastomoz yapıldı. Ameliyattan sonra K grubuna 7 gün boyunca orogastrik tüp yoluyla sırasıyla S (10 mg/kg) ve R (10 mg/kg) verilirken K grubuna 10 mg/kg musluk suyu verildi. S&R grup, aynı dozajlarda hem S hem de R aldı. Tüm

Address for Correspondence: Mehmet Kemal Yener, University of Health Sciences Türkiye, Adana City Training and Research Hospital, Clinic of General Surgery, Adana, Türkiye
Phone: +90 544 367 04 11 E-mail: mkyener@gmail.com ORCID ID: orcid.org/0000-0001-7044-8060

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sıçanlara postoperatif 7. günde ötenazi yapıldı. Anastomoz patlama basıncı (APB) ölçüldü ve histopatolojik inceleme ve hidroksiprolin (T.H.) seviyesi değerlendirmesi için doku örnekleri alındı.

Bulgular: APB, S&R grupta K grubuna göre anlamlı olarak daha yüksekti (p=0,021). Histopatolojik olarak ödem açısından değerlendirildiğinde aradaki anlamlı fark S&R gruba bağlıydı. Doku ödemi S&R grupta K, S ve R gruplarına göre daha düşüktü (p=0,004). Polimorfonükleer lenfosit artışı, lenfosit artışı, makrofaj artışı, mukoza-epitelyal durum ve submukozal köprüleşme için analiz edildiğinde gruplar arasında anlamlı fark yoktu. Gruplar arasında hidroksiprolin seviyelerinde de anlamlı farklılıklar yoktu (p=0,222).

Sonuç: Sonuçlar, R ve S kombinasyonunun, sol kolonik anastomozlu sıçanlarda patlama basıncını önemli ölçüde artırdığını ve S'nin bu sonuçta potansiyel olarak anahtar rol oynadığını göstermektedir. Mevcut sonuçlar umut vericidir, ancak doğrulama için ek çalışmalara ihtiyaç duyulacaktır.

Anahtar Kelimeler: Resveratrol, sildenafil, kolon, anastomoz, yara iyileşmesi

INTRODUCTION

Colorectal surgery is a common occurrence in the modern surgical era, but the long-standing problem of anastomotic leakage (AL) remains a challenging complication for surgeons to overcome. The incidence of this complication is reported at 3-19% and is still the leading cause of high morbidity and mortality among patients undergoing this procedure (1-3).

Although AL has multifactorial aetiology, adherence to surgical principles plays a crucial role in constructing a safe colonic anastomosis. Among these principles, the perfusion of the anastomosis is probably the most significant and overlooked condition. It is well documented that the healing process of colonic anastomosis is significantly enhanced by improving the microcirculation, thereby regulating tissue oxygenation (1-5).

Sildenafil is a selective phosphodiesterase type 5 (PDE-5) inhibitor. It is a water-soluble compound and is found in several parts of the body. Sildenafil protects cyclic guanosine monophosphate (cGMP) from degradation by cGMP-specific PDE-5. Nitric oxide (NO) is a well-known mechanism that binds to guanylate cyclase receptors, resulting in increased levels of cGMP, leading to muscle relaxation and vasodilatation of vessels, and thereby improving microcirculation (4,5).

Resveratrol (3, 5, 40-trihydroxytrans-stilbene) is a natural polyphenolic compound known from ancient times that is present in grapes and red wine and possesses anti-inflammatory, cardioprotective and neuroprotective properties. It has been shown that resveratrol reduces the damage in experimentally induced colitis by alleviating oxidative stress and stimulating apoptosis (6). Its chemopreventive efficacy through inhibition of cyclooxygenase (COX) and ornithine decarboxylase (ODC) enzymes have been well researched. Resveratrol has demonstrated decreases in oxidative stress while inducing NO synthesis in ischemia-reperfusion (6-8).

The effects of sildenafil and resveratrol individually were previously researched in ischemic colon anastomosis, ulcerative colitis, and ischemic colitis models by the present authors and other researchers (6,9-14). The combined effects of the two compounds on left-sided colon anastomosis have not been previously studied. Considering the pharmacological effects mentioned above, the present experimental study was performed to investigate whether administering a combination of resveratrol and sildenafil to rats could improve the strength of colon anastomosis without obvious interference with other vital components of the repair process.

METHODS

Animal Ethical Considerations

Ethical committee approval of the Kahramanmaraş Sütçü İmam University Faculty Medicine (decision no: 06, date: 08.05.2019) was received and then this study was carried out at the Experimental Animal Research Center of Kahramanmaraş Sütçü İmam University Faculty Medicine, Kahramanmaras, Türkiye. This research was also conducted following the Guide for the Care and Use of Laboratory Animals (NIH, 1985).

Thirty-six Wistar-albino male rats weighing 191 to 310 g were used in this study. The animals were kept in polycarbonic cages at a room temperature of 20±2 °C with 50±10% humidity in a 12-hour (h) light and dark cycle. The rats were allowed to take rat food (Purina®) in the form of standardized dry pellets.

Study Design, Anesthesia and Surgical Procedure

The thirty-six rats were divided into four groups as follows:

Control group (C): Left colon resection plus end-to-end anastomosis created; 10 cc of tap water per day was given via an orogastric tube for postoperative 7 days.

Sildenafil group (S): Left colon resection plus end- to-end anastomosis created; sildenafil was given at a dose of 10 mg/kg per day via an orogastric tube for postoperative 7 days.

Resveratrol group (R): Left colon resection plus end- to-end anastomosis created; resveratrol was given at a dose of 10 mg/kg per day via an orogastric tube for postoperative 7 days.

Combined resveratrol and sildenafil group: Left colon resection plus end- to-end anastomosis was created; both sildenafil and resveratrol were given at a dose of 10 mg/kg per day via an orogastric tube for postoperative 7 days.

Feeding of rats was discontinued 12 h before surgery. Sedation was administered by intramuscular injection of ketamine 50 mg/kg (Ketalar: Parke Davis, Eczacıbaşı, Istanbul, Türkiye) and xylazine 10 mg/kg (Rompun: Bayer AG, Leverkusen, Germany). Animals were left to breathe unassisted, and their body temperature was fixed at 37 °C by a heater during the operation (10-14).

All operations were performed by the same surgeon for the standard technique. After shaving the abdominal skin of the animals, it was stained with povidone-iodine. The table was set at a 30-degree angle to prevent the risk of aspiration. Subsequently, a 4 cm upper midline incision was rendered, and this was the entry point of the abdomen in all groups. The full-thickness colon segment was accessed 3 cm proximal to the peritoneal reflection. The transected colon segments were individually anastomosed end-to-end with 5/0 prolene sutures. The anterior abdominal wall was closed with a single-layer continuous absorbable 3/0 suture (Vicryl, Johnson & Johnson), and the skin was closed with an interrupted 3/0 silk suture (Figure 1) (11-13).

The postoperative condition was assessed 6 h within the first 48 h and every 10 h thereafter for 7 days. Diclofenac sodium (2 mg/kg, intravenous) was used twice, just after surgery and on postoperative day 1 to reduce pain in the wound area. Esomeprazole was concomitantly administered by intragastric gavage at a dose of 50 micromol/kg to prevent gastropathy (9-15).

All rats were euthanized on postoperative day 7 by administering 2 mL pentobarbital sodium (200 mg/mL, K.U. Life, Copenhagen) intraperitoneally. Anastomotic burst pressure was measured, and then the anastomosis site was resected leaving at least 3 cm space from the proximal and distal edges to evaluate tissue hydroxyproline levels and perform histopathological examination (10-14).

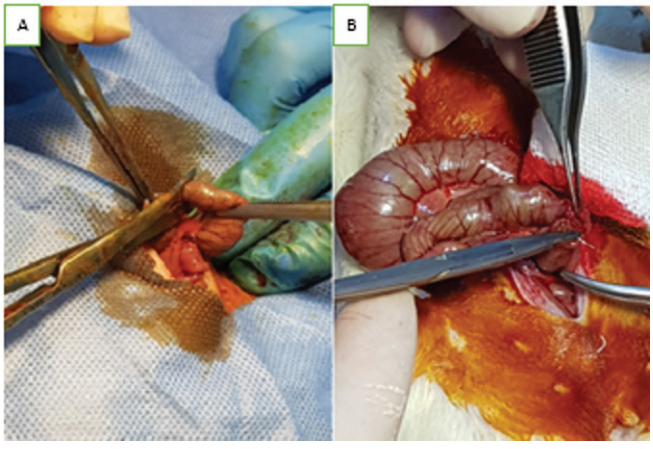

Figure 1. A) Left colon transection, B) End-to-end anastomosis of transected segments

Anastomotic Bursting Pressure (ABP) Measurement

Evaluation of burst pressure, tissue hydroxyproline levels, and histopathological examination were performed using the same methods as previous similar studies (10-22). Intestinal pressure was measured using a pressure monitoring system. After cleaning the lumen of the anastomosis line resected by relaparotomy, the manometer (Pressure Gauge with Erka Profi Cuff) was connected with an airtight 3/0 silk suture at a distance of 3 cm from the proximal end of the anastomosis. The distal end of the anastomosis was also connected with an airtight 3/0 silk suture. An anastomosis line was placed in a bowl filled with water (Figure 2). Progressively higher pressure was applied and the pressure at which the air bubbles emerged was recorded as bursting pressure in millimeters of mercury (mmHg) (10-22).

Histopathological Examination

After measuring the anastomotic burst pressure, the 1 cm portion containing the anastomosis region was excised to be 1 cm proximal and 1 cm distal. Some tissue samples were used for the biochemical examination while the remainder were fixed in 10% formaldehyde. The samples were then parafinized, and 5 µm sections were cut and stained with hematoxylin-eosin to determine the collagen content. The samples were assessed blindly by an experienced pathologist. The results were obtained using the Verhofstad scale (15). Parameters including necrosis, edema, polymorphonuclear lymphocytes (PMNL) increase, lymphocyte increase, macrophage increase, mucosa-

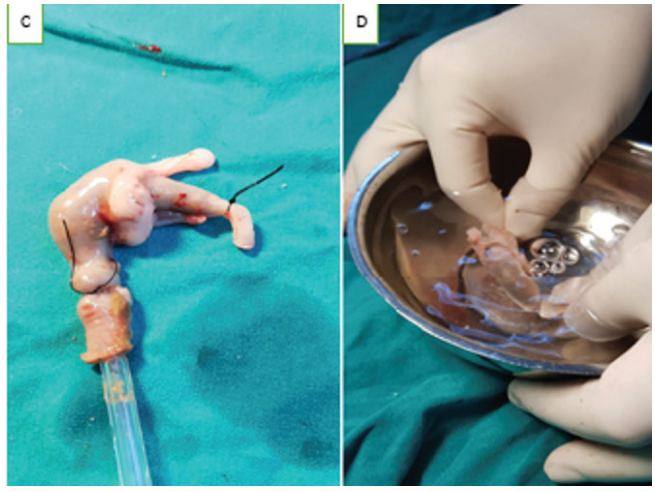

Figure 2. C) Measurement of burst pressure, D) Bursting moment of anastomosis line in pressure measurement

epithelial status, and submucosal bridge at the anastomosis line were examined with the help of this scale (15,22).

Hydroxyproline Level Measurement

The hydroxyproline level was measured as previously described. After measuring ABP, the anastomosis line was removed from the adherent tissue. 1 cm of the anastomotic line was excised. The sample harvested from the anastomosis line was submitted to the biochemistry laboratory. Acid digestion was performed in the oven for approximately 48 h by adding 1 mL of distilled water and 1 mL of 12 N hydrochloric acid (HCL) per 100 mg of tissue. It was vortexed using 0.6 mL of 50% isopropyl alcohol and dissolved by adding a further 0.6 mL of 50% isopropyl alcohol. Additionally, 0.2 mL of chloramine-T was added and then the solution was allowed to rest for 10 minutes (min). Subsequently, 1 mL of Erlich reagent was added and vortexed. It was kept in a 50 °C water bath for 90 min and was scanned against water at 560 nm wavelength. Tissue hydroxyproline values were calculated as micrograms/gram (µg/g) dry tissue (16). Hydroxyproline measurements were completed blindly by a biochemist who was unaware of the groupings.

Statistical Analysis

All statistical analyses were performed using the IBM SPSS 21.0 package program. Values were presented as mean ± standard deviation. The normality of burst pressure and hydroxyproline level measurements according to groups were evaluated with Kolmogorov-Smirnov test statistics (Table 1).

The data for ABP and tissue hydroxyproline were evaluated using One-Way analysis of variance (ANOVA). The data for histological score were evaluated using the chi-square test. Post-hoc analyses were performed with Tukey's HSD. The values were accepted as statistically significant when $p<0.05$.

RESULTS

No complications occurred that would affect the results of the study. No local or systemic side effects were observed after the administration of resveratrol or sildenafil. Anastomosis burst pressures were measured individually and compared between groups. Regarding the group means, the anastomotic burst pressure was 192.22±84.07 mmHg in the C, 247.77±53.80 mmHg in the S, 208.88±81.46 mmHg in the R, and 312.22±112 mmHg in the combined group. The results showed that there was a significant difference between the burst pressures of the control and combined groups (p=0.021). There was also a significant difference in burst pressures between the resveratrol and combined groups (p=0.004). However, no statistically significant difference existed between the sildenafil and combined groups (Table 2).

When hydroxyproline levels were analysed, the mean values were 1566.17±126.77 µg/g in the C, 1554.60±96.99 µg/g in the S, 1634.15±132.2 µg/g in the R and 1527.86±68.68 µg/g in the combined group. There were no significant differences found in hydroxyproline levels during group comparisons (p=0.222) (Table 3).

Table 1. Normality evaluation

	Burst pressure			Hydroxyproline level		
	K-S	df	p	K-S	Df	p
Control group	0.197	9	0.200*	0.202	9	0.200*
Sildenafil group	0.170	9	0.200*	0.230	9	0.185
Resveratrol group	0.249	9	0.115	0.204	9	0.200*
Combined group	0.221	9	0.200*	0.242	9	0.135

p: Kolmogorov-Smirnov (K-S). *This is a lower bound of the true significance

Table 2. Mean difference evaluation of anastomotic burst pressures (mmHg)

	Mean ± SD	Min-max		F	Df	p
Control group	192.22±84.07	80-300	28.02			
Sildenafil group	247.77±53.80	140-300	17.93	3.511	35	0.021
Resveratrol group	208.88±81.46	100-300	27.15			
Combined group	312.22±112	160-480	37.33			

p: One-Way analysis of variance (ANOVA). Min-max: Minimum-maximum, SD: Standard deviation

Histopathological examination was performed using the Verhofstad scale (15) to evaluate the inflammatory process and anastomotic healing. When all groups were evaluated in terms of edema, a statistically significant difference was found (p=0.004). When comparing the groups in pairs, this difference was found to be due to the combined group in which the level of edema was lowest. A second result after comparing the groups in pairs is that there were significant differences between the combined and Cs, between the combined and Ss, and between the combined and Rs. When all groups were evaluated in terms of necrosis, a statistically significant difference was found (p=0.037) When the groups were analyzed according to PMNL increase, lymphocyte increase, macrophage increase, mucosa–epithelial status, and submucosal bridge, there were no significant differences found (Table 4) (Figure 3).

Table 3. Mean difference evaluation of anastomotic tissue hydroxyproline levels (µg/g)

	Mean ± SD	Min-max	F	Df	p
Control group	1566.17±126.77	1338.10±1715.08			
Sildenafil group	1554.60±96.99	1370.30±1670.42	1.545	35	0.222
Resveratrol group	1634.15±132.2	1504.99±1916.38			
Combined group	1527.86±68.68	1397.39±1657.98			

p: One-Way analysis of variance (ANOVA). Min-max: Minimum-maximum, SD: Standard deviation

Table 4. Relationship evaluation of groups with histopathological findings

		Control group		Sildenafil group		Resveratrol group		Combined group		Total		Pearson chi-square	Df	p
		n	%	n	%	n	%	n	%	n	%			
Necrose	Low	7	77.8	5	55.6	1	11.1	6	66.7	19	52.8	13.426	6	0.037
	Prominent	0	0.0	1	11.1	3	33.3	3	33.3	7	19.4			
	Serious	2	22.2	3	33.3	5	55.6	0	0.0	10	27.8			
Oedema	Low	1	11.1	3	33.3	4	44.4	9	100.0	17	47.2	19.176	6	0.004
	Prominent	5	55.6	5	55.6	5	55.6	0	0.0	15	41.7			
	Serious	3	33.3	1	11.1	0	0.0	0	0.0	4	11.1			
PMNL	Little increase	1	11.1	2	22.2	1	11.1	3	33.3	7	19.4	3.200	6	0.783
	Significant increase	4	44.4	4	44.4	3	33.3	4	44.4	15	41.7			
	Diffuse infiltration	4	44.4	3	33.3	5	55.6	2	22.2	14	38.9			
Lymphocytes	Little increase	4	44.4	5	55.6	6	66.7	8	88.9	23	63.9	4.214	3	0.239
	Significant increase	5	55.6	4	44.4	3	33.3	1	11.1	13	36.1			
Macrophages	Little increase	3	33.3	5	55.6	3	33.3	6	66.7	17	47.2	3.588	6	0.732
	Significant increase	5	55.6	3	33.3	5	55.6	3	33.3	16	44.4			
	Diffuse infiltration	1	11.1	1	11.1	1	11.1	0	0.0	3	8.3			
Mucosa-epithelium	Normal glandular	0	0.0	0	0.0	3	33.3	0	0.0	3	8.3	13.600	9	0.137
	Normal cubic	3	33.3	1	11.1	1	11.1	3	33.3	8	22.2			
	Incomplete cubic	5	55.6	6	66.7	5	55.6	4	44.4	20	55.6			
	No	1	11.1	2	22.2	0	0.0	2	22.2	5	13.9			
Submucosal bridge	Good bridge	0	0.0	1	11.1	0	0.0	0	0.0	1	2.8	5.004	9	0.834
	Moderate bridge	4	44.4	2	22.2	3	33.3	4	44.4	13	36.1			
	Poor bridge	5	55.6	5	55.6	5	55.6	4	44.4	19	52.8			
	No bridge	0	0.0	1	11.1	1	11.1	1	11.1	3	8.3			

Likelihood chi-square test. PMNL: Polymorphonuclear lymphocytes

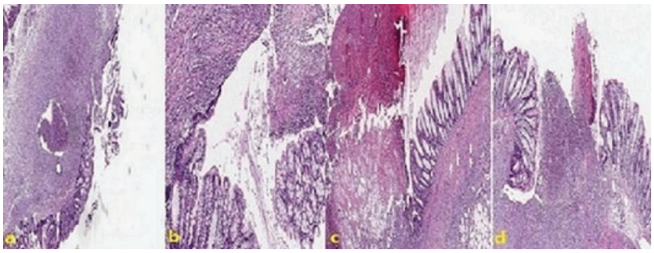

Figure 3. a. Control group, b. Sildenafil group, c. Resveratrol group, d. Combined group. Light microscopic examination (H&E staining) (400x magnification)

DISCUSSION

AL is a significant problem in terms of mortality and morbidity in patients undergoing colorectal surgery (1-3). Studies have shown that anastomotic leaks prolong hospital stays and increase medical costs (14-22). The individual effects of sildenafil and resveratrol were previously researched separately in ischemic colon anastomosis, ulcerative colitis, and ischemic colitis by some of the present authors and other researchers (6,9-14,20). The present experimental study was performed to investigate whether combined administration of resveratrol and sildenafil to rats could improve the strength of standard intestinal anastomosis without obvious interference with other vital components of the related healing process. To the authors' knowledge, this is the first study to investigate the effects of a combination of resveratrol and sildenafil on left-sided colonic anastomosis.

Before discussing the results, some important properties of sildenafil and resveratrol should be highlighted briefly to understand why anastomosis with these agents was chosen as the focus of the study. Sildenafil is a potent, selective PDE-5 inhibitor that was initially studied as an antianginal medication and is currently marketed to treat erectile dysfunction. Its effectiveness is due to the enhancement of vasodilatation by inducing muscle relaxation in the vessels and improving microcirculatory blood supply. Sildenafil also inhibits platelet aggregation, thus avoiding small-vessel obstruction (9-14). It is a water-soluble compound and is found in several parts of the body. It protects cGMP from degradation by PDE-5. NO binds to guanylate cyclase receptors, resulting in increased cGMP levels and leading to muscle relaxation in the vessels (9-12). The other agent resveratrol (3,5,40-trihydroxytrans-stilbene) is a naturally occurring polyphenolic possessing anti-inflammatory, cardioprotective and neuroprotective properties. Its preventative efficacy has recently been shown through inhibition of COX and ODC enzymes. Resveratrol reduces oxidative stress and induces apoptosis, thereby reducing damage in experimentally induced colitis (6,9-14,20). Some studies have shown that resveratrol decreased oxidative stress while inducing NO synthesis in ischemia-reperfusion (7,8,20).

Consistent with the above studies, the present study hypothesized that the synergistic effect of resveratrol and sildenafil and their antioxidant, antiplatelet, and anti-inflammatory effects could improve anastomotic healing due to its vasorelaxation, angiogenic, and antiapoptotic properties (6-15). The results showed that the C's mean anastomotic burst pressure was 192.22±84.07 mmHg. It was 247.77±53.80 mmHg in the S and 208.88±81.46 mmHg in the R. The mean burst pressure in the combined group was 312.22±112 mmHg. The results revealed a significant difference between the control and combined groups in terms of burst pressure (p=0.021). It was observed that the combination of sildenafil and resveratrol had a positive synergistic effect on the ABP and improved the strength of the intestinal anastomosis without adverse effects on other components of the associated healing process.

As a result, the answers to the following questions were highlighted by the authors. 1) Do both sildenafil and resveratrol have effects individually on anastomotic strength? Yes, but although bursting pressure increased by administering the agents individually, the results in this respect did not reach statistical significance. 2) Does the combination of these two potent drugs potentially have a synergistic effect on anastomotic strength? The answer is yes, and the results showed that when using the combination treatment, the increase in anastomotic strength was statistically significant compared with the C (p=0.021). The burst pressure was significantly higher in the combined group than in the control and Rs. Similarly, when the combined group was compared to the S, the bursting pressure was higher in the combined group. However, this difference did not reach statistical significance and may be due to the low sample size. These results showed that a combination of sildenafil and resveratrol was effective in increasing the ABP. 3) Does the combination treatment usage have obvious interference with other vital components of the repair process? The answer is no, and when the histopathological analysis was performed of PMNL increase, lymphocyte increase, macrophage increase, mucosa-epithelial status, and submucosal bridge, there were no significant differences between the groups. Tissue edema was lower in the combined group than in the control, sildenafil, and Rs (p=0.004). Necrosis was lower in the combined group than in the control, sildenafil, and Rs (p=0.004) When hydroxyproline levels were compared between groups, there were no significant differences found (p=0.222).

In summary, it is worth emphasizing that there were no adverse effects of the agents on anastomotic healing parameters neither individually nor in combined usage. Alternatively, although an increase in anastomotic strength was evident, no other positive histopathological effects were detected other than decreased edema. This may be because the animals were euthanized on day 7, which is late in the healing process. We chose the 7th postoperative day for euthanizing the rats as this was parallel to previous studies. Some of the literature regarding the effects of these agents on histopathological parameters and hydroxyproline levels is controversial. In some studies, the observed hydroxyproline levels were increased by the above-mentioned agents, while in others there was no change. Additionally, in some studies histopathological parameters improved, while in other studies there were no improvements (6,9-14,20). The key result, which is consistent with the literature, is that these two agents individually or in combined usage do not have negative effects on hydroxyproline levels and histopathological healing parameters, while their combined usage increases ABP with a synergistic effect.

In this study, a left colon anastomosis model was used in rats because experimental research on anastomotic healing has been exclusively performed in rats since the basic repair patterns are believed to be similar to those in humans. We constructed a left colonic anastomosis because lower colorectal surgery is more prone to complications due to fecal load and technical difficulties, and the effects of these complications are more problematic (6-23).

As in all studies, there were some weaknesses evident in this study. First, NO levels in the colonic tissue were not determined in this study. Second, although the sample size is enough for an experimental study, it is not a sufficient basis on which to generalise. Third, the precise cellular mechanisms by which these agents enhance anastomotic wound healing are not clear, and this warrants further research.

CONCLUSION

AL is a significant problem in terms of mortality and morbidity in patients undergoing colorectal surgery. The results of this experimental study show that a combination of sildenafil and resveratrol increased the ABP without obvious interference with other vital components of the repair process. Additionally, it is likely that sildenafil was the key agent in these outcomes. Although the results are encouraging, additional studies are needed.

ETHICS

Ethics Committee Approval: Ethical committee approval of the Kahramanmaraş Sütçü İmam University Faculty of Medicine (decision no: 06, date: 08.05.2019) was received and then this study was carried out at the Experimental Animal Research Center of Kahramanmaraş Sütçü İmam University Faculty Medicine, Kahramanmaraş, Türkiye.

Informed Consent: Experimental study.

Authorship Contributions

Surgical and Medical Practices: M.K.Y., A.Y., H.K., Concept: M.K.Y., E.R., A.S., Design: M.K.Y., E.R., A.S., A.Yo., O.I., Data Collection or Processing: M.K.Y., A.Y., H.K., Z.A.T., A.Yo., Analysis or Interpretation: M.K.Y., E.R., A.Y., Z.A.T., O.I., Literature Search: M.K.Y., A.Y., Writing: M.K.Y., A.Y., H.K., O.I.

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