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Effects of Cold Storage in Krebs-Henseleit Solution at +4 °C on Vasoreactivity of the Rat Thoracic Aorta

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ABSTRACT

Objective: This study aimed to investigate whether endothelium-dependent and -independent relaxation responses can be preserved intact in the rat thoracic aorta after storage for 3, 6, and 18 hours in Krebs-Henseleit solution at +4 °C. **Materials and Methods:** Isolated organ bath model and 10-12 weeks old male Wistar rats were used to perform the experiments. To investigate the effect of endothelium-dependent relaxation factors, the cyclooxygenase inhibitor indomethacin was added to the Krebs-Henseleit solution to inhibit endogenous prostanoid synthesis. Submaximal contraction response was obtained with a single dose of phenylephrine and then acetylcholine was administered cumulatively (10^{-9}-10^{-4} M) to induce endothelium-dependent relaxation responses. Besides, smooth muscle-dependent relaxation responses were obtained by applying sodium nitroprusside cumulatively (10^{-9}-10^{-5} M) following precontraction induced by phenylephrine. The statistical significance level was considered as $p<0.05$. **Results:** Thoracic aorta segments (0 hours, control) exhibited a mean contraction response of 3123 mg against 10^{-5} M phenylephrine. Although the responses slightly reduced in vascular rings stored for 3, 6, and 18 hours, no significant difference was observed ($p>0.05$). Besides, cumulatively administered acetylcholine did not cause a significant change in endothelium-dependent relaxation responses ($p>0.05$). Similarly, sodium nitroprusside did not modulate the endothelium-independent relaxation responses in aortic segments after storage for 3, 6, or 18 hours ($p>0.05$). **Conclusion:** In the present study, the first physiological findings have been obtained that the endothelium-dependent and -independent contraction-relaxation responses of rat thoracic aortas can be preserved intact after storage periods of 3, 6, or 18 hours in Krebs-Henseleit solution at +4°C.

Keywords: Cold Storage, Rat Thoracic Aorta, Vascular Smooth Muscle, Vasoreactivity.

+4 °C'de Krebs-Henseleit Solüsyonunda Soğuk Depolamanın Sıçan Torasik Aortunun Vazoreaktivitesi Üzerindeki Etkileri

ÖZ

Amaç: Bu çalışma, Krebs-Henseleit solüsyonunda +4 °C'de 3, 6 ve 18 saat süreyle saklanan sıçan torasik aortlarında endotel-bağımlı ve -bağımsız gevşeme yanıtlarının bozulmadan korunup korunamayacağını araştırmayı amaçlamıştır. **Gereç ve Yöntem:** Deneyler izole organ banyosu modeli ve 10-12 haftalık erkek Wistar sıçanlar kullanılarak gerçekleştirilmiştir. Endotel-bağımlı gevşeme faktörlerinin etkisini araştırmak için, endojen prostanoid sentezini inhibe etmek üzere Krebs-Henseleit çözeltisine siklooksijenaz inhibitörü indometazin eklenmiştir. Tek doz fenilefrin ile submaksimal kasılma yanıtı elde edilmiş ve ardından endotel-bağımlı gevşeme yanıtlarını indüklemek üzere kümülatif olarak (10^{-9}-10^{-4} M) asetilkolin uygulanmıştır. Ayrıca, fenilefrin ile indüklenen prekontraksiyonu takiben sodyum nitroprussid kümülatif (10^{-9}-10^{-5} M) uygulanarak düz kasa bağlı gevşeme yanıtları elde edilmiştir. İstatistiksel anlamlılık düzeyi $p<0.05$ olarak belirlenmiştir. **Bulgular:** Torasik aort segmentleri (0 saat/kontrol), 10^{-5} M fenilefrine karşı 3123 mg'lık ortalama kasılma yanıtı göstermiştir. 3, 6 ve 18 saat saklanan vasküler halkalarda yanıtlar biraz azalmasına rağmen, anlamlı bir fark gözlenmemiştir ($p>0.05$). Ayrıca, kümülatif uygulanan asetilkolin, endotel-bağımlı gevşeme yanıtlarında anlamlı bir değişikliğe neden olmamıştır ($p>0.05$). Benzer şekilde, sodyum nitroprussid 3, 6 veya 18 saatlik saklamadan sonra aort segmentlerinde endotel-bağımsız gevşeme yanıtlarını modüle etmemiştir ($p>0.05$). **Sonuç:** Bu çalışmada, Krebs-Henseleit solüsyonunda +4 °C'de 3, 6 veya 18 saatlik saklama sürelerinden sonra sıçan torasik aortlarının endotel-bağımlı ve -bağımsız kasılma-gevşeme yanıtlarının bozulmadan korunabildiğine dair ilk fizyolojik bulgular elde edilmiştir.

Anahtar Kelimeler: Soğuk Depolama, Sıçan Torasik Aortu, Vasküler Düz Kas, Vazoreaktivite.

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INTRODUCTION

The limitation of isolated tissue bath experiments, in which physiological and pharmacological responses of vascular tissues can be examined, is the necessity of using cellular functions without disruption. Many studies are reporting that the vascular endothelium may be damaged during isolation and storage (Kristek et al., 1993; Török et al., 1993). Therefore, the vascular preparations should be hung in tissue baths rapidly after isolation. This requirement limits the number of studies to be carried out with vascular segments isolated from the same animal and allows an animal to be used for only one vessel type in experimental animal models where cardiovascular studies are performed. This issue causes a greater constraint for physiological and pharmacological research where human vessels are used, as these vessels are more difficult to obtain (Cupitra et al., 2021; Wise et al., 2015).

Preservation of vascular tissues has an important role not only in facilitating experimental research but also in terms of organ transplantation success. The vascular endothelium is a dynamic structure responsible for the synthesis and release of many vasoactive substances necessary for the physiological functions of the vessel (Yetik-Anacak & Catravas, 2006). The preservation of endothelial integrity is of great importance in terms of both the integrity of the organ and the transplantation of the vascular graft. In cases where vascular tissues are not well protected and endothelial integrity is impaired, decreased nitric oxide synthesis, vascular spasm, and dysfunction of the transplanted tissue due to increased thrombogenicity may be observed (Chong et al., 2001; Dragun et al., 2001). In addition, it is important to preserve the viability of vascular tissues to evaluate the long-term effects of drugs.

In previous studies, researchers have used refrigerators (Kristek et al., 1993; Shibata, 1969; Török et al., 1993) or freezing at lower temperatures to preserve vessels intact. However, conditions in which endothelium- and vascular smooth muscle-related responses could remain intact could not be provided in these studies (Ku et al., 1994). The preservation of the functions of vascular tissues mainly depends on the preferred storage solution and duration (Jahania et al., 1999). It has been reported that contraction and endothelium-dependent relaxation responses are impaired in isolated vascular rings stored in the cold for a few days (Kristek et al., 1993). Tissues that were stored cold overnight had better results, although there were differences depending on the storage solutions used. As for the preservation solution, the most used in physiology is Krebs-Henseleit at 4 °C, because the arteries placed in it show just a tendency to decrease vascular reactivity after 24 h (Cupitra et al., 2021). This study aimed to investigate whether endothelium-dependent and -independent relaxation responses can be preserved intact in the rat thoracic aorta after storage for 3, 6, and 18 hours in Krebs-Henseleit solution at +4 °C.

MATERIALS AND METHODS

Test animals

The study was carried out using 10-12 weeks old male Wistar Albino rats obtained from Bursa Uludag University Experimental Animal Breeding Application and Research Center.

Chemicals and reagents

Phenylephrine (PE), acetylcholine (ACh), sodium nitroprusside (SNP), indomethacin (INDO), and chemicals in Krebs-Henseleit solution were purchased from Sigma Aldrich (St Louis, MO, USA). PE, ACh, SNP, and INDO were dissolved in distilled water.

Isolated organ bath experiments

Animals were sacrificed by decapitation without anesthesia. The thoracic cavity of the animals was rapidly opened. Then, the thoracic aorta was removed delicately and placed in a 0-4 °C Krebs-Henseleit solution in a Petri dish. The Krebs-Henseleit solution consisted of 119.0 mM NaCl, 4.7 mM KCl, 1.5 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 25.0 mM $NaHCO_3$, and 11.0 mM glucose. The isolated aortas were cleared of adherent tissues and separated into 2-3 mm segments. Thus, 3-4 aortic segments were obtained from each animal. Aortic segments were hung immediately (0 hours, control) or after stored for 3 hours, 6 hours, or 18 hours in Krebs-Henseleit solution at +4 °C to the isometric force transducer (BIOPAC Systems, 42 Aero Camino-Goleta, USA) of the four-chamber tissue bath (Commat, Ankara, Turkey) via stainless steel wires to measure the isometric contraction-relaxation responses. The temperature of the baths was adjusted to 37 °C (±0.5 °C) by employing a thermocirculator. The tissue baths were continuously aerated with a gas mixture of 95% O_2-5% CO_2. The Krebs-Henseleit solution, containing the tissues, was refreshed every 10 min during the resting period. After the equilibration period, the active agents were precisely administered into the baths using an adjustable automatic pipette (Jespersen et al., 2015). In preliminary experiments, the submaximal contraction dose for PE was determined as 10^{-5} M.

To investigate the effect of endothelium-dependent relaxation factors, the cyclooxygenase inhibitor INDO (1 mM) was added to the Krebs-Henseleit solution to inhibit endogenous prostanoid synthesis. Submaximal contraction response was obtained with a single dose of PE and then ACh was administered cumulatively (10^{-9}-10^{-4} M) to induce endothelium-dependent relaxation responses. Besides, smooth muscle-dependent relaxation responses were obtained by applying SNP cumulatively (10^{-9}-10^{-5} M) following precontraction induced by PE.The tissues were rested for 45 min between the concentration-response curves and the Krebs-Henseleit solution was refreshed every 10 min during this time.

Eight aortic rings were used for each group (n=8).

Statistical analysis

SPSS 23.0 program (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. All data are expressed as mean±SD (standard deviation). Statistical significance between two independent groups was analyzed by independent sample t-test; a one-way analysis of variance

(one-way ANOVA) test was used for multiple comparisons. Dunnett's T3 post hoc test was used to determine the differences between the groups. The statistical significance level was considered as $p<0.05$.

Ethical Consideration

This study was approved by the decision of Bursa Uludag University Animal Experiments Local Ethics Committee (BUÜ HADYEK) dated 05/04/2022 and numbered 2021-05/05. All experiments were conducted following the legislation on the Welfare and Protection of Animals Used for Experimental and Other Scientific Purposes.

RESULTS

In preliminary experiments, the submaximal contraction dose for PE was determined as 10^{-5} M.

Rat thoracic aorta segments hanged rapidly after isolation (0 hours, control) exhibited a mean contraction response of 3123 mg against 10^{-5} M PE. Although the responses slightly reduced in vascular rings stored for 3 hours, 6 hours, and 18 hours in Krebs-Henseleit solution at +4°C, no statistically significant difference was observed ($p=0.245$, $p=0.179$, and $p=0.134$, respectively) (Figure 1).

Cumulatively administered (10^{-9}-10^{-5} M) ACh did not cause a statistically significant change in endothelium-dependent relaxation responses in rat thoracic aortas precontracted by submaximal (10^{-5} M) PE dose after storage in Krebs-Henseleit solution at +4 °C for 3 hours ($p=0.884$ for 10^{-9} M; $p=0.835$ for 10^{-8} M; $p=0.571$ for 10^{-7} M; $p=0.610$ for 10^{-6} M; $p=0.619$ for 10^{-5} M), 6 hours ($p=0.591$ for 10^{-9} M; $p=0.532$ for 10^{-8} M; $p=0.346$ for 10^{-7} M; $p=0.377$ for 10^{-6} M; $p=0.424$ for 10^{-5} M), or 18 hours ($p=0.589$ for 10^{-9} M; $p=0.541$ for 10^{-8} M; $p=0.264$ for 10^{-7} M; $p=0.195$ for 10^{-6} M; $p=0.288$ for 10^{-5} M) (Figure 2).

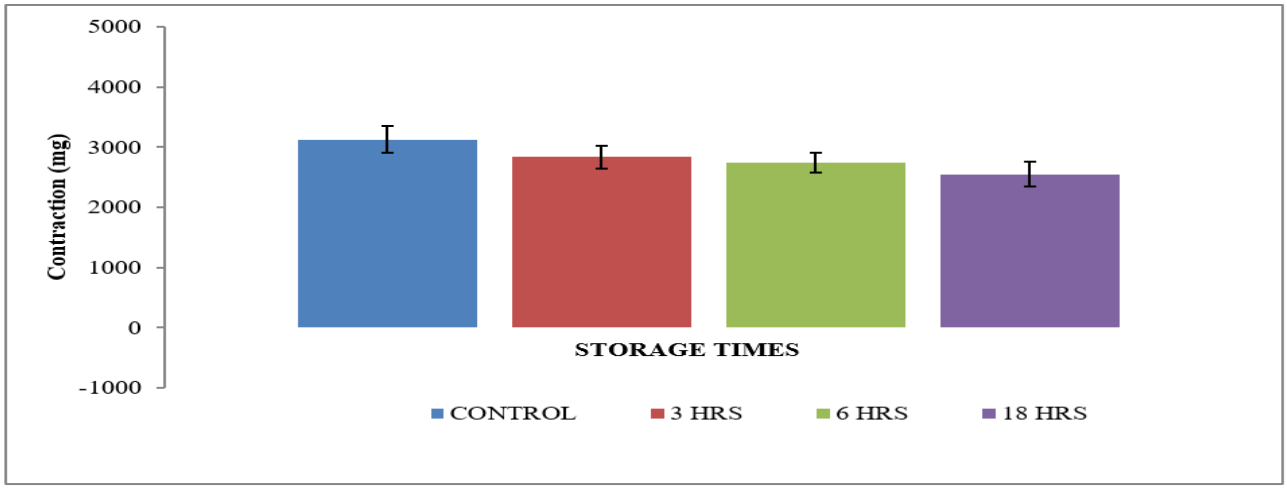

Figure 1. Comparison of maximal absolute (milligram) contractions in response to 10^{-5} M PE in rat thoracic aortas stored for 0 hours (control), 3 hours, 6 hours, and 18 hours in Krebs-Henseleit solution at +4 °C. The data were expressed as the mean±SD. n=8 in each group.

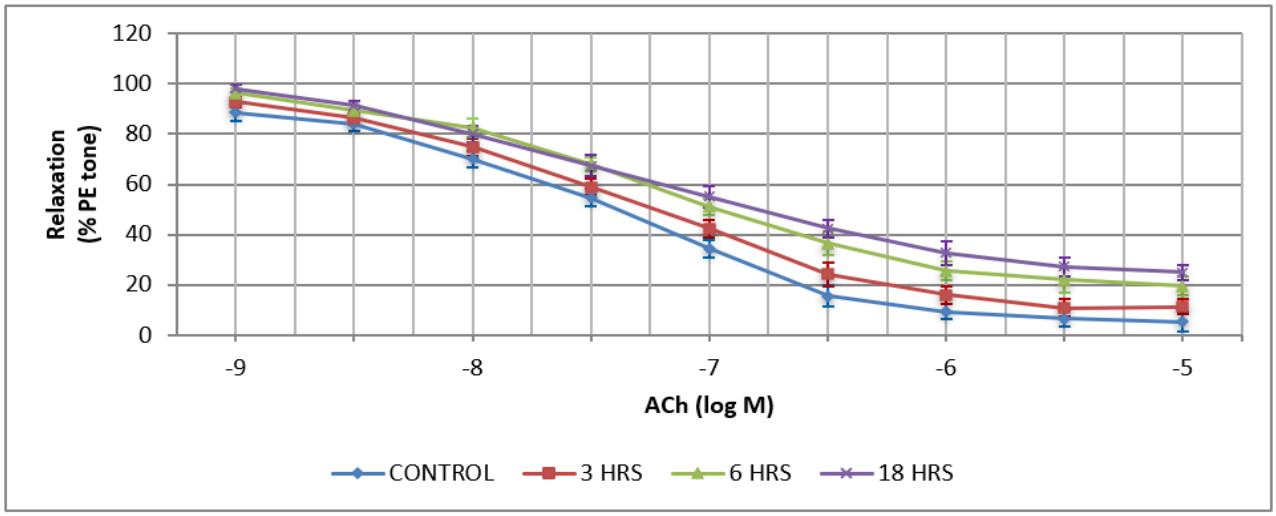

Figure 2. Endothelium-dependent relaxation responses induced by cumulatively administered (10^{-9}-10^{-5} M) ACh in rat thoracic aortas. Aortic segments stored for 0 hours (control), 3 hours, 6 hours, and 18 hours in Krebs-Henseleit solution at +4 °C were precontracted with submaximal (10^{-5} M) PE dose. The relaxant effects were assessed as a percentage of the contraction in response to 10^{-5} M PE. The data were expressed as the mean±SD. n=8 in each group. PE: phenylephrine. ACh: acetylcholine.

Cumulatively administered (10^{-9}-10^{-5} M) SNP did not cause a statistically significant change in endothelium-independent relaxation responses in rat thoracic aortas precontracted by submaximal (10^{-5} M) PE dose after storage in Krebs-Henseleit solution at +4 °C for 3 hours (p=1.000 for 10^{-9} M; p=1.000 for 10^{-8} M; p=1.000 for 10^{-7} M; p=1.000 for 10^{-6} M; p=1.000 for 10^{-5} M), 6 hours (p=0.579 for 10^{-9} M; p=0.591 for 10^{-8} M; p=0.604 for 10^{-7} M; p=0.345 for 10^{-6} M; p=0.339 for 10^{-5} M), or 18 hours (p=0.574 for 10^{-9} M; p=0.531 for 10^{-8} M; p=0.523 for 10^{-7} M; p=0.310 for 10^{-6} M; p=0.408 for 10^{-5} M) (Figure 3).

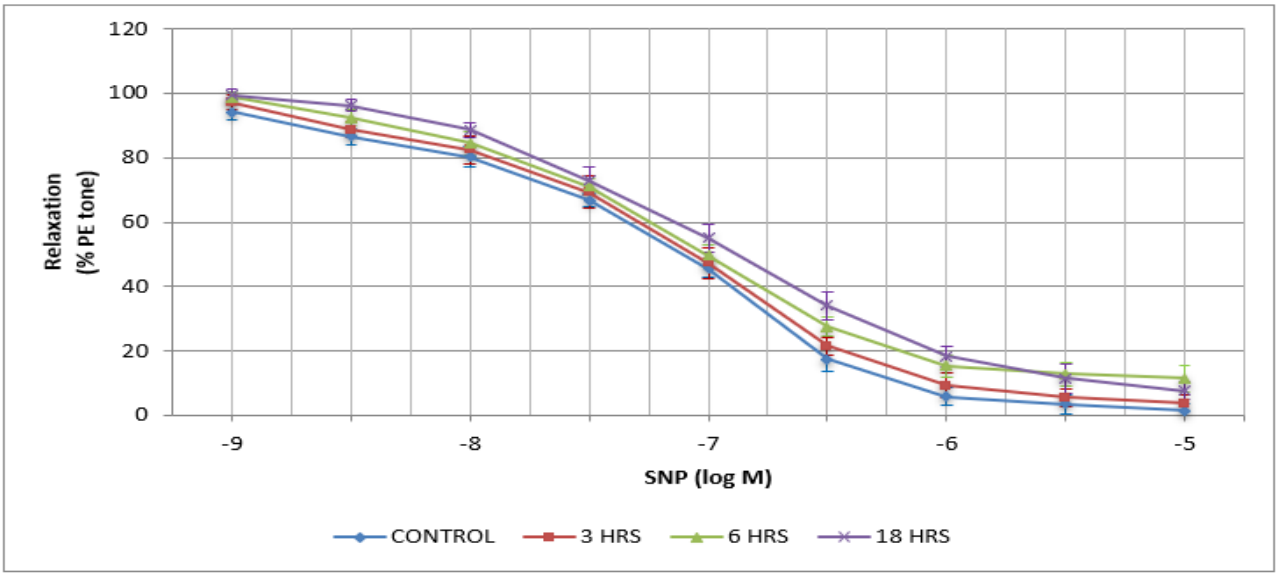

Figure 3. Endothelium-dependent relaxation responses induced by cumulatively administered (10^{-9}-10^{-5} M) SNP in rat thoracic aortas. Aortic segments stored for 0 hours (control), 3 hours, 6 hours, and 18 hours in Krebs-Henseleit solution at +4 °C were precontracted with submaximal (10^{-5} M) PE dose. The relaxant effects were assessed as a percentage of the contraction in response to 10^{-5} M PE. The data were expressed as the mean±SD. n=8 in each group. PE: phenylephrine. SNP: sodium nitroprusside.

DISCUSSION

This study aimed to investigate the effects of cold storage on the vascular activity of rat thoracic aorta. In this regard, it was shown for the first time that both endothelium-dependent and -independent relaxation responses can be preserved intact in rat thoracic aorta after storage for 3, 6, and 18 hours at +4 °C in Krebs-Henseleit solution.

It is important for the efficiency of experimental studies that vascular tissues can be stored for longer periods without disturbing their viability. The ability to preserve the functional effects of tissues without deterioration allows the use of different vessels/tissues of the same animal in isolated tissue bath studies. Thus, it can also be possible to investigate the long-term effects of drugs. Moreover, since the transplanted organs are usually transplanted together with the surrounding vascular beds, it is important for the success of the transplantation that the functions of the vessels can be preserved intact. In the literature, for this purpose, methods of culturing vascular tissues, freezing, or storing them in cold (+4 °C) have been tried. Many studies are showing that contraction responses differ in cultured vessels (Binko et al., 1999; Thyberg, 1996). Another method is to freeze the vessels in liquid nitrogen. However, it has been reported that relaxation responses and smooth muscle contractions related to endothelium and smooth muscle are impaired in this method (Langerak et al., 2001; Rendal et al., 2004; Rendal Vázquez et al., 2004a; Rendal Vázquez et al., 2004b).

Recently, studies have been conducted to minimize functional loss by adding preservative agents such as dimethyl sulfoxide (DMSO) and sucrose to the storage solutions of tissues during the freezing process or by gradually freezing and thawing them. However, none of these studies found that endothelium- or smooth muscle-derived relaxation responses of vascular tissues were impaired, as well as contraction responses (Langerak et al., 2001; Rendal et al., 2004). In studies using the cold storage method, vascular functions were tested in various storage solutions, periods, and temperatures (Kristek et al., 1993; Neil et al., 2002; Payne et al., 2002; Piepot et al., 2002; Rinaldi, 2001; Török et al., 1993). It has been reported that contraction responses, as well as endothelium-dependent relaxation responses, are impaired in isolated vascular preparations that are kept cold (for a long time) for days (Kristek et al., 1993; Török et al., 1993). In tissues that were stored for a shorter period, overnight, better results were obtained, depending on the storage solution used. For instance, with the University of Wisconsin (UW) solution, endothelium- or smooth muscle-dependent relaxation responses in canine coronary microvascular vessels were protected (Murphy et al., 1997).

It has been reported that the sensitivity to ACh did not change in rat aortas stored in Hepes-Krebs-Henseleit

solution for 24-48 hours, but the maximal response to ACh and contraction agents decreased (Stanke-Labesque et al., 1999). Similarly, in the present study, ACh sensitivity was shown to be unchanged in rat thoracic aortic rings stored in Krebs-Henseleit solution at +4°C for 3, 6, or 18 hours. This study also demonstrated that ACh-induced maximum relaxation response was not affected by cold storage for these times.

It is known that in cases where endothelial functions are impaired and therefore nitric oxide (NO) production decreases, contraction responses increase due to the decrease in basal relaxation provided by nitric oxide (Amerini et al., 1995; Mendizabal et al., 1999; Vo et al., 1992). Therefore, in the current study, the absence of any increase in PE-induced contractile responses indicates that endothelial function is preserved. Besides, the absence of a significant decrease despite a slight attenuation in PE-mediated contraction responses indicates that the dynamics in smooth muscle contraction physiology are still effective and vascular functions can continue without deterioration.

In this study, 3, 6, or 18 hours of cold storage did not cause a significant change in relaxation responses to SNP. These findings are consistent with studies showing that endothelium-independent relaxation responses do not change after cold storage in rat aortas (Stanke-Labesque et al., 1999). The fact that 3, 6, or 18 hours of cold storage did not affect the contraction-relaxation responses in rat thoracic aortas suggests that the characteristic properties of smooth muscle do not change.

The main difference in storage solutions is that the ion concentrations in the solutions mimic the extracellular ion content, such as Krebs-Henseleit solutions, or the intracellular ion content, such as Euro Collins or UW solutions (Mühlbacher et al., 1999). Preservation of vasoactive properties after storage at +4 °C indicates that Krebs-Henseleit solution is a suitable storage solution for cold storage of vessels.

In conclusion, this study shows that ideal conditions can be provided in the rat thoracic aorta by using the appropriate solution and time, in which vasoactive functions can be kept intact. In this regard, the first physiological findings have been obtained that the endothelium-dependent and -independent contraction-relaxation responses of rat thoracic aortas can be preserved intact after storage periods of 3, 6, or 18 hours in Krebs-Henseleit solution at +4°C. These results suggest that different types of vessels may also be isolated from an animal for later use in isolated organ bath experiments. Besides, the results of this experimental study may also provide a prediction regarding the viability of vascular tissues used in transplant surgeries. However, further studies using different animal models, vessel types, solutions, and temperatures are needed to obtain more precise results regarding storage times.

Conflict of Interest

The author declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Author Contributions

Plan, design: SD; **Materials and Methods:** SD; **Data analysis and interpretation:** SD; **Writing and corrections:** SD.

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