**Abstract**

**Background/Aim:** There have been different experimental conditions for *in vitro* studies on human umbilical arteries (HUA) in tissue bath system. This diversity was mainly reflected in variables such as stretching tension, incubation period and initial constriction challenging with potassium (KCl). The aim of the study was to establish optimal experimental conditions which will provide better responsiveness of HUA preparations, as well as to examine the impact of 24 h cold storage on viability and responsiveness of HUA to KCl and serotonin.

**Methods:** The KCl-induced constrictions at different stretching tensions (0.5 g, 1.0 g, 2.0 g, 4.0 g), incubation times (30 min, 60 min, 120 min), and after multiple initial constriction challenging were compared. Dose response curves for serotonin were obtained under different conditions (1.0 g and 60 min vs. 2.0 g and 120 min). The influence of 24 h cold storage on KCl- and serotonin-induced vasoconstriction of HUA preparations was examined as well.

**Results:** The strongest constrictions induced by serotonin or KCl were obtained when preparations were adjusted at 2.0 g and incubated for 120 min. The KCl-induced constrictions observed after 120 min were statistically higher (p < 0.05) when preparations were challenged three times (30 min, 60 min, 120 min), compared to those challenged only once. The preparations that were stored at 4 °C for 24 h showed significantly stronger serotonin-induced constrictions (p < 0.01). The cold storage had no influence on KCl-induced constriction.

**Conclusion:** For performing *in vitro* studies on HUA preparations in tissue bath, we propose stretching tension of 2.0 g, incubation period of 120 min and multiple initial constriction challenging with KCl as optimal experimental condition. We also showed that HUA preparations retained functional viability even after 24 h of cold storage.

**Key words:** Human umbilical artery; Stretching tension; Incubation time; Constriction; Serotonin.

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**Introduction**

In addition to numerous animal models of pregnancy and sporadic human clinical trials, studies on isolated umbilical blood vessels have a special place in research related to pregnancy complications. The *in vitro* studies on umbilical arteries and veins allow direct assessment of vascular reactivity of utero-placental and foeto-placental circulation. However, studies on animal blood vessel preparations cannot completely replace human preparations and their pathophysiological models. Therefore, human umbilical blood vessels are of particular importance, since they...
can provide the closest possible image of the \textit{in vivo} system. The importance of the HUA has long been recognised, and its use in research dates back to the 1950s.\textsuperscript{3} Umbilical cord is one of the few human tissues that is widely available and provides a high yield of HUA preparations.

Majority of the research studies on HUA are focused on elucidating the vascular dysfunction that occurs in preeclampsia, which is associated with an increase in utero-placental vascular resistance.\textsuperscript{4} It is well known that HUA are not innervated blood vessels,\textsuperscript{5} and that their vasoactivity is regulated exclusively by autacoids from blood circulation, such as serotonin, histamine, bradykinin, angiotensin, oxytocin, as well as by different eicosanoids.\textsuperscript{6} Due to the described specificity of umbilical circulation, it is assumed that these vasoactive agents may play an important role in the pathogenesis of preeclampsia.

Since it was found that mechanical properties and reactivity of umbilical blood vessels are changed in preeclampsia,\textsuperscript{6, 7} it is important to establish optimal experimental conditions for physiological models that will allow for the interpretation of these changes. The length and stretch of HUA preparations, the duration of incubation time, the composition of the solution to maintain the viability of the preparations and the initial constriction challenging procedures are some of the key determinants in defining the conditions that must be achieved before performing experiments in the tissue bath system. The majority of authors apply the same conditions regarding the form and length of the HUA preparation (rings, 3-4 cm long), aeration of the HUA preparation (mixture of 95 \% O\textsubscript{2} and 5 \% CO\textsubscript{2}) and composition of the nutrient solution (Krebs-Ringer bicarbonate solution),\textsuperscript{6, 8-10} whereas the differences are reflected mainly in passive tension, incubation period and initial constriction challenging. Given the existence of great diversity in the application of physical properties for testing HUA vasoactivity, it is of particular importance to establish an optimisation of the tissue bath methodology that will allow better responsiveness of HUA preparations.

The aim of the study was to analyse the effects of different physical variables such as stretching tension, incubation time, initial constriction challenging and cold storage period to determine the optimal conditions for measuring the vasoactivity of isolated HUA.

## Methods

### Ethical principles

The present study was conducted with the approval of the Ethics Committee for Research on Humans and Biological Materials, at the Faculty of Medicine, University of Banja Luka, the Republic of Srpska, Bosnia & Herzegovina (B&H).

### Tissue preparation

Umbilical cords were obtained from healthy pregnant women after full-term vaginal delivery or Caesarean section at the Clinics for Gynaecology and Obstetrics, University Clinical Centre of the Republic of Srpska (Banja Luka, B&H). After delivery, the segments of umbilical cords (5-10 cm in length) were immediately placed into the modified Krebs-Ringer bicarbonate solution and transported on the ice to the Laboratory of Pharmacology and Toxicology, Centre for Biomedical Research, Faculty of Medicine, University of Banja Luka. For this purpose, the modified Krebs-Ringer solution with less calcium ions than standard solution (CaCl\textsubscript{2} 0.16 mmol/L) was used.

The umbilical artery was cleaned of Wharton jell and the connective tissue was carefully removed. The cleaning was performed in Petri dishes filled with modified Krebs-Ringer solution on the flat ice pack. The preparation was cut into rings of 3-4 mm in length and each ring was suspended between two stainless steel wires in a jacketed tissue bath containing 20 mL Krebs-Ringer bicarbonate solution (37 °C, pH 7.4), aerated with a mixture of 95 \% O\textsubscript{2} and 5 \% CO\textsubscript{2}.

One of the wire hooks was attached to a transducer (CH1-SN: IT) connected to the amplifier (SN:BS 007) with recording system (Fast Acquisition, Elunit Group, Serbia) that recorded changes in isometric tension, using an e-Lab software. The second wire hook was attached to a displacement unit allowing fine adjustments of passive tension (g). During equilibration time, the organ bath solution was changed every 10 minutes and tension was adjusted when necessary.

### Experimental protocols

#### Resting tension and incubation time

In order to evaluate the optimal resting tension and incubation period for HUA preparations, the different stretching conditions and incubation times were performed to obtain maximal con-
striction induced by potassium chloride (KCl). At the beginning of experiment the rings were equilibrated unstretched in organ bath at 37°C for 30 minutes. After that, the preparations were divided in four groups and each preparation was stretched to a different resting tension: 0.5 g, 1.0 g, 2.0 g and 4.0 g. When rings reached the determined tension, KCl (40 mM) was applied in precisely defined time points: 30 min, 60 min and 120 min. Between each KCl addition, rings were washed for several times and then adjusted to a determined resting tension.

KCl-induced constriction challenge
The intention was to assess the influence of the repeated initial constriction with KCl when preparations were set on optimal resting tension of 2.0 g. After 30 min of incubation time, the preparations were stretched to a passive force of 2.0 g. One group of preparations were allowed to equilibrate for 120 min and then were exposed to KCl initial challenge (40 mM), while on the other group of preparations three initial KCl challenges (40 mM) were performed, at 30 min, 60 min and 120 min, as previously described.

Serotonin dose-response curve
The aim of this protocol was to compare the dose-response curves for serotonin in HUA preparations under different passive tension and incubation time variables. When the first incubation period of 30 min ended, the preparations were stretched and equilibrated at different conditions. One group of preparations was stretched at 1.0 g and incubated for 60 min, while the other group was stretched at 2.0 g and incubated for 120 min. All preparations were exposed to KCl (40 mM) in order to obtain a reference constriction (100%). After several wash-out periods, when preparations achieved the stable resting tension, a cumulative concentration response curve for serotonin (10^8 – 3 x 10^5 M) was obtained. At the end of each experiment, the viability of preparation was tested by challenging it with KCl-induced constriction.

Cold storage
Within this protocol the intention was to examine the effect of cold storage on HUA vasoreactivity. Optimal conditions (incubation period of 2 h and passive tension of 2.0 g) established in previous protocols were applied at HUA preparations after 24 h of cold storage at 4 °C. At the beginning of experiment, KCl-induced constrictions (40 mM) were established and after several washing-out periods, when basal line was stable, a cumulative concentration response curves for serotonin (10^8 – 3 x 10^5 M) were obtained.

Drugs and solutions
A Krebs-Ringer bicarbonate solution of the following composition was used (mmol/L): NaCl 118.3, KCl 4.7, CaCl2 2.5, MgSO4 1.2, NaHCO3 25, KH2PO4 1.2, and glucose 5.6. All stock solutions and serial dilutions were made in distilled water and were prepared shortly before the start of each of the experiments. Serotonin was obtained from Sigma Chemical Co. (St. Louis, MO, USA) and potassium chloride (KCl) was obtained from Lach: Ner (Zagreb, Croatia).

Statistical analysis
Results were presented as mean ± standard error (mean ± SEM) for number of preparations studied. Figures and statistical analysis were performed using SigmaPlot 14.0 (Systat Software Inc.). Two-way ANOVA with repeated measures was used to compare concentration-response curves for serotonin. Statistical significance between two groups was determined by Student’s t-test and the p values less than 0.05 were considered to be significant.

Results
Resting tension and incubation time
Preparations of HUA showed different responses to KCl at various stretching tensions and incubation periods. Preparations that were stretched at 0.5 g and incubated for 30 min did not show any response to KCl, while other preparations that were stretched at tensions of 1.0, 2.0, 4.0 g showed very weak constrictions. By prolonging the incubation time to 60 or 120 min, all preparations showed stronger constrictions than those obtained at 30 min. When preparations were stretched at tension of 2.0 and 4.0 g the constrictions were stronger than those stretched at 0.5 g, but the significance was confirmed only for 2.0 g. Further prolongation of incubation time to 120 min resulted in significant increase in constrictions for preparations stretched at tension of 2.0 and 4.0 g. However, additional increase in stretching tension of 4.0 g was not followed by stronger constriction response, as it would be expected (Figure 1).
Potassium-induced constriction challenge
KCl-induced vasoconstriction (40 mM) was significantly stronger in HUA preparations that were exposed to three initial challenges (separate, non-cumulative additions of KCl at 30 min, 60 min and 120 min), compared to the preparations that were exposed to only one initial challenge (at 120 min) (Figure 2).

Serotonin dose-response curve
Concentration-response curve for serotonin, with the stretching tension of 2.0 g and incubation pe-

Cold storage
Preparations that were stored at the temperature of 4 °C for 24 h did not show any significant difference in the magnitude of the KCl-induced constriction compared to the preparations of HUA that were used as fresh preparations, without cold storage (Figure 4).

Serotonin-induced constrictions were significantly stronger (p < 0.01) in cold-stored HUA preparations. Concentration-response curves for serotonin indicated higher efficacy of serotonin
Discussion

The main purpose of this study was to determine the optimal stretching tension and incubation period for researches that use HUA in tissue bath, as well as to examine the effect of multiple initial challenging and cold storage on HUA vasoactivity.

Bertrand et al reported that for reliable measurement of pharmacological properties it is necessary to stretch the HUA preparations to 5.0 or 6.0 g, while other investigators in previous studies established the resting tension of 2.0 g as an optimal tension for HUA preparations. In addition, there are reports that applied a stretching tension of 4.0 g is optimal during 4 h of incubation, although in most studies the incubation time was 2 h.

Results of these experiments have shown that the response of HUA to KCI was stronger after increasing stretching tension and incubating period. The maximal response was obtained when preparations were stretched at 2.0 g and incubated for 120 min. Despite the fact that after 120 min there was no significant difference between responses of arteries stretched at 2.0 g and 4.0 g, the stretching tension of 4.0 g was harder to achieve, thus, 2.0 g and 120 min are suggested as optimal experimental conditions. The sensitivity of HUA to KCI was not affected by stretching tension since no significant difference in responses was found between the HUA preparations stretched at 1.0, 2.0 or 4.0 g, although the differences in constrictions were significant when the mentioned tensions were compared with the lowest one applied, 0.5 g. This is in accordance with the intact sensitivity of chorionic vessels to KCl. However, in HUA preparations stretched at minimal passive tension (0.5 g), the incubation time might have an effect on the sensitivity to KCI, since constrictions were absent after 30 min of incubation, while prolongation of incubation time enhanced responsiveness.

In order to assess the functional integrity of HUA preparations, most authors use KCl as a reference contraction activator, applying different protocols to achieve initial challenge. The concentration of KCl that caused the maximal constriction for HUA preparations was found to be 80 mM, while the submaximal concentrations ranged from 20 mM to 60 mM. However, Bertrand et al applied initial challenge with 100 mM of KCl, usually with repetitions of 3 to 4 times, while Tufan et al used 40 mM KCl for contractile response and repeated it twice, the second constriction of which was taken as a reference one. Estañ et al asserted that 40 mM KCl was the optimal concentration that would reach the submaximal constriction in foeto-placental blood vessels. Considering the all above mentioned findings, it was decided to use KCl as a reference contractile agent at a concentration of 40 mM. Our results have shown that greater constrictions were obtained after multiple challenging with 40 mM KCl. The assumption for presence of greater constrictions and thereby increase of intracellular calcium ions (Ca$^{2+}$) could be that repeated KCl-induced membrane depolarisation lead to prolonged activation of Ca$^{2+}$ voltage channels and release from intracellular depots. It is also possible that during multiple challenging a higher amount of Ca$^{2+}$ remains in the cell which is caused by inappropriate Ca$^{2+}$ displacement after every wash-out period.

It has long been known that serotonin is one of the most potent vasoconstrictors of HUA. Finding that concentration of serotonin in umbilical...
Conflict of interest

None.

Acknowledgements

Results from the present study indicate that both KCl- and serotonin-induced constrictions were higher when HUA preparations were adjusted at 2.0 g stretching force and 120 min incubation period and that multiple initial challenging enhance vascular response to KCl. It was also shown that HUA preparations preserved functional sensitivity during 24 h cold storage for both KCl- and serotonin-induced constrictions. Considering all the above, mentioned experimental conditions are proposed as optimal to perform studies on HUA in isolated tissue bath, even 24 h after sampling.

Conclusion

None.

Conflict of interest

None.

vessels at birth was $10^{-7}$ M determined the range of serotonin concentrations most commonly used for in vitro studies. In the present study the dose-response curves for serotonin ($10^{-8}$ - $3 \times 10^{-5}$ M), obtained after different stretching tensions and incubation periods, showed force- and time-dependent constrictions. It was found that the efficacy of serotonin was significantly higher under the optimal experimental conditions established in this study (2.0 g and 120 min). However, sensitivity to serotonin was not affected when preparations were stretched either at 1.0 g for 60 min or at 2.0 g for 120 min. It has been previously reported by Tufan et al that maximal serotonin constriction for HUA, expressed as percentage of KCl-induced constriction, was approximately 130 %. Dayigolu et al applied the same experimental conditions (4.0 g and 4 h) and achieved a maximal constriction of approximately 145 %. In the present experiment, higher maximal constriction for serotonin (approximately 180 %) were reached, using lower stretching tension (2.0 g) and shorter incubation time (120 min). Therefore, the advantages of this approach are reflected on savings of time and efforts in settings of HUA preparations for experiments.

In HUA preparations that were exposed to 24 h cold storage at 4 °C, the KCl produced qualitatively less constrictions compared to those in non-cold storage HUA preparations, but this difference was not statistically significant. Nevertheless, Sinanović and Chiba reported that KCl-induced constrictions were significantly decreased in dog and monkey skeletal muscle arteries after 3-5 days storage at 4 °C. They suggested that hypothermic conditions might have led to the change in sensitivity of Ca²⁺ voltage channels and thus Ca²⁺ influx. Moreover, it has been shown that cold storage (24-72 h at 4 °C) of rat thoracic aorta also diminished KCl-induced constrictions and that reactive oxygen species may be responsible for storage-induced changes in vascular reactivity.

On the other hand, serotonin showed significantly higher efficacy, but not sensitivity in HUA preparations that were exposed to 24 h cold storage, compared to the control HUA preparations. Although the percentage of achieved serotonin constriction was almost twice as high in the preparations exposed to cooling, it still does not represent a realistic image of the increase in serotonin efficacy, taking into account that the reference KCl constrictions were lower in these preparations. The effects of cold storage on serotonin vasoactivity have been investigated in various blood vessel preparations, but the results were not consistent. There are reports that show that cold storage did not modify vascular response of serotonin on dog and monkey muscle arteries, whereas other reported that serotonin-induced constriction of rat aorta was almost completely diminished after 72 h of cooling at 4 °C. Interestingly, Kevelaitis et al. found that endothelial dysfunction during cold preservation enhanced the sensitivity of serotonin on rat coronary arteries. Since the results of the present experiments showed that cold storage increased serotonin constrictions, further studies are needed in order to examine the role of endothelial dysfunction on this phenomenon.

Like in the optimisations already performed for isolated human pulmonary arteries, as well as for isolated human chorionic arteries, the results presented here represent an optimisation of the methodology for isolated HUA.

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