Evaluation of the Effects of Remifentanil and Dexmedetomidine on Crush Injury and Renal Functions in an Experimental Model

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Study Protocol with Statistical Analysis Plan

Ethical approval for this study was obtained from the Local Animal Ethics Committee of the University of Health Sciences, Ankara Training and Research Hospital, under protocol number 0741 dated 10.08.2023. The experimental phase of the study was conducted at the Experimental and Clinical Research Laboratories of Ankara Training and Research Hospital. Blood samples were analyzed at the Department of Medical Biochemistry, Faculty of Medicine, Gazi University, and tissue samples were evaluated at the Pathology Department of Ankara Bilkent City Hospital. The principal investigator and all participating researchers possessed valid animal use certificates and had received the necessary formal training.

The study was conducted in accordance with the standards of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (ETS 123), using 28 male Wistar-Albino rats with an average weight of approximately 250 grams. Prior to the experiment, the rats were kept in a controlled environment with a temperature of 22–25°C and a 12-hour light / 12-hour dark cycle. They were fed ad libitum with standard rat chow (containing 23% protein, 5% fat, 15% fiber, and 50% carbohydrates) and had access to tap water.

On the day of the experiment, the rats were randomly divided into four groups of seven animals each: Control group (C), Sham group (S), Dexmedetomidine group (D), and Remifentanil group (R). Each rat was assigned a group label and an identification number (e.g., “Rat 1-C” indicates rat number 1 in the control group). The interventions for each group were applied as outlined in Table 1.

Before any procedures, all rats received intramuscular anesthesia with ketamine (50 mg/kg) and xylazine (5 mg/kg). They were positioned on a flat surface and secured by all four limbs to prevent movement. Venous access was established via the tail vein, and the first blood sample (baseline, 0 hour) was collected in a volume of 0.5–0.8 mL. Blood was drawn into Greiner Bio-One MiniCollect® Complete 0.5 mL/0.8 mL CAT Serum Sep Clot Activator tubes. The samples were centrifuged at 4000 rpm for 15 minutes to separate the serum from blood cells. The supernatant serum was then transferred into 1.5 mL Eppendorf tubes in aliquots of 500–1000 µL. The serum samples were stored at −80°C until the day of analysis.

Table 1. Animal groups and administered interventions

Group	**Control (C)**	**Sham (S)**	**Dexmedetomidine (D)**	**Remifentanil (R)**
n	**7**	**7**	**7**	**7**
Bilateral gastrocnemius compression	No	Yes	Yes	Yes
Abdominal incision/closure	No	Yes	Yes	Yes
Drug administration	No	No	Yes	Yes
Blood sampling (0/2/6 h)	Yes	Yes	Yes	Yes
Bilateral renal tissue collection	Yes	Yes	Yes	Yes

To simulate crush injury, rats in the Sham (S), Dexmedetomidine (D), and Remifentanil (R) groups underwent bilateral compression of the gastrocnemius muscles under ketamine-xylazine anesthesia using a metal clamp that applied equal pressure. This compression was maintained for a duration of two hours. Peripheral cooling and changes in coloration were monitored throughout the procedure. After the two-hour compression period, the clamps were removed from both gastrocnemius muscles, and second-hour blood samples were collected via tail vein access.

Following the release of compression, intravenous drug infusions were administered to the respective groups through the tail vein. The Dexmedetomidine group received Sedadomid (dexmedetomidine, Koçak Farma) at a dose of 3 µg/kg as an initial bolus followed by continuous infusion at 3 µg/kg/hour. The Remifentanil group received Opiva (remifentanil, Tüm–Ekip İlaç) at a continuous infusion rate of 2 µg/kg/min. Drug administration lasted for one hour.

Simultaneously, in order to simulate a one-hour surgical procedure following crush injury, midline laparotomy was performed in the Sham, Dexmedetomidine, and Remifentanil groups. The abdominal cavity was opened and subsequently closed at the end of one hour.

After the two-hour muscle compression, the one-hour simulated surgery, and the drug infusion period (i.e., three hours in total, corresponding to the 6th hour from baseline), blood samples were collected from the inferior vena cava. Subsequently, the rats were euthanized, and both kidneys were harvested. Right and left kidneys were stored separately in 10% formalin for histopathological evaluation. Blood sampling was performed at three different time points: 0, 2, and 6 hours. One rat in the Sham group died during the experiment.

Biochemical analyses of creatinine (mg/dL), urea (mg/dL), total antioxidant capacity (TAC, mmol/L), and total oxidant status (TOS, µmol/L) were performed using the MINDRAY-BS400 device with an enzymatic spectrophotometric method.

For creatinine measurement, the OttoBC139 kit was used, employing an immunoturbidimetric method. The formation of antigen/antibody complexes in the samples was analyzed turbidimetrically. Polyethylene glycol was added to enhance sensitivity and to prevent false-negative results in samples with excessive antigen concentration.

For urea measurement, the OttoBC157 kit was used, based on the urease-GLDH method. In this method, urea was hydrolyzed by the urease enzyme, producing ammonia and carbon dioxide. The resulting ammonia then reacted with glutamate dehydrogenase to form glutamate and NAD^+, and the consumption of NADH during this reaction was measured spectrophotometrically.

TAC and TOS levels were measured to assess oxidant and antioxidant status. TAC was determined using the RL0017 kit, based on the ABTS radical cation decolorization method. As antioxidant capacity increased, the degree of color fading was measured spectrophotometrically. The measurement precision was below 3%, and results were expressed as Trolox equivalents (mmol/L).

TOS was analyzed using the RL0024 kit via the oxidation of the ferrous ion–o-dianisidine complex. Oxidants in the sample oxidized ferrous ions to ferric ions, which then formed a colored complex with xylenol orange in an acidic environment. This color change was measured spectrophotometrically, and results were expressed in micromolar hydrogen peroxide equivalents (µmol H_2O_2/L).

Serum levels of TIMP-2, IGFBP7, KIM-1, and NGAL were measured by qualified personnel using a solid-phase sandwich ELISA method on the BIO-TEK EL system. The following kits were used according to the manufacturer's instructions: Rat TIMP-2 ELISA Kit (BT LAB, Lot: E0323Ra), Rat IGFBP7 ELISA Kit (BT LAB, Lot: E1785Ra), Rat KIM-1 ELISA Kit (BT LAB, Lot: E0549Ra), and Rat NGAL ELISA Kit (BT LAB, Lot: E0762Ra). The general working principle of the kits is as follows:

Each ELISA plate consists of 96 wells pre-coated with immobilized antibodies specific to Rat TIMP-2, KIM-1, NGAL, or IGFBP7, which bind the corresponding standards or samples. Prepared samples and standards are pipetted into the wells, where the target molecules present in the sample bind to the immobilized antibodies. The plate is then washed to remove unbound substances.

Next, biotin-conjugated anti-Rat TIMP-2, KIM-1, NGAL, or IGFBP7 antibodies are added to each well. These secondary antibodies bind to the captured target proteins. Following incubation, another washing step is performed to eliminate unbound biotin-conjugated antibodies.

Horseradish peroxidase (HRP)-conjugated streptavidin is then added to each well, binding to the biotin-labeled antibodies. After a final wash to remove excess streptavidin-HRP, a substrate solution is added, which reacts with the HRP enzyme to produce a colorimetric signal.

The intensity of the color produced is proportional to the concentration of TIMP-2, KIM-1, NGAL, or IGFBP7 in the sample or standard. The reaction is terminated with the addition of a stop solution, and the absorbance is measured at 450 nm. The concentrations of TIMP-2 (ng/mL), IGFBP7 (ng/L), KIM-1 (ng/mL), and NGAL (ng/mL) in the samples are then calculated based on the standard calibration curves.

In our study, a total of 54 kidney specimens—right and left kidneys from 27 rats—were evaluated. The excised kidney tissues were examined both macroscopically and microscopically at the Pathology Department of Ankara Bilkent City Hospital. The tissues were fixed in 10% formalin for 24 to 48 hours. After measuring the macroscopic dimensions of the tissues, samples were placed into individual cassettes in a manner that allowed visibility of the cut surfaces. These samples were then processed in the Sakura Tissue-Tek VIP 6AI fully enclosed vacuum tissue processor and embedded in paraffin using the Leica EG1150 C modular embedding system. Subsequently, 4-micrometer-thick unstained sections were obtained in duplicate from each paraffin block using the Leica RM2125RT rotary microtome. The sections were stained with Hematoxylin and Eosin (H&E) and Periodic Acid–Schiff (PAS).

For histochemical PAS staining, the following steps were performed: the slides were first deparaffinized and prepared for staining. Solution A (periodic acid) was applied and left for 5 minutes, followed by rinsing with distilled water. Then, solution B (Schiff reagent) was added, and the slides were incubated for 15 minutes. After that, the slides were placed in warm water for 5 minutes and rinsed again with distilled water. Solution C (Harris hematoxylin) was then applied for 4 minutes, followed by a rinse with distilled water. Solution D (bluing reagent) was applied and incubated for 2 minutes. The slides were washed under running water for 2 minutes and rinsed again with distilled water, followed by sequential dehydration in 96% and 99.9% alcohol. The slides were then cleared twice in xylene (2 minutes each) and finally coverslipped using mounting medium.

All slides were independently and blindly evaluated by two pathologists using a dual-headed light microscope. All fields in each slide were examined. Histological changes were assessed through quantitative evaluation of acute tubular necrosis (ATN), based on the extent of necrotic and apoptotic cells, brush border loss, tubular dilatation, apical bleb formation, and cast formation. Additionally, congestion, chronic inflammation, and calcium-phosphate crystal deposition were recorded for each group.

Apoptosis was defined as cell rounding, shrinkage, and nuclear chromatin condensation. In PAS-stained sections, brush border loss on the luminal side of the tubules was assessed. Calcium-phosphate crystal presence was scored as 0 (absent) or 1 (present). Chronic inflammation was scored as 0 (none), 1 (focal, if not filling a ×10 microscopic field), or 2 (diffuse, if filling the field). All other parameters were scored as follows:
0 = absent,
1 = 0–10%,
2 = 11–25%,
3 = 26–45%,
4 = 46–75%,
5 = 76–100%.

All statistical analyses were conducted using R Studio Version 2024.12.0+467. A comprehensive statistical analysis approach was employed to evaluate biomarker changes between experimental groups. Initially, descriptive statistics were calculated, and appropriate statistical tests such as the Wilcoxon rank sum exact test and others were applied based on variable type. Histograms and boxplots were generated to examine variable distributions and differences in means between groups.

To visualize the data, the LOESS (Locally Estimated Scatterplot Smoothing) method was applied to assess temporal changes in biomarker levels. The statistical power analysis was based on literature-referenced sample size calculations to determine whether the chosen sample size was adequate for group comparisons. However, due to the small sample size, although various analyses were feasible, insufficient statistical power was achieved for multilevel categorical comparisons of certain variables. Therefore, some variables were re-categorized to enhance interpretability and statistical robustness.

To assess differences in histopathological indicators and kidney function biomarkers between groups, variables were reclassified. Initially, multinomial logistic regression models were considered, but due to statistical instability and convergence issues, these models could not be applied. The primary reason for this was the insufficient power when analyzing four treatment groups along with variables containing four to five

subcategories each. To address this limitation and improve the interpretability of results, histopathological variables were simplified into two main categories: “Mild” (0–25%) and “Advanced” (26–100%). Percent-based variables were similarly reclassified. This strategy was adopted to strengthen the evaluation of inter-variable relationships and to reduce the risk of type II errors (false negatives).

To determine differences between treatment groups, one-way ANOVA tests were performed, followed by post-hoc Tukey tests for pairwise comparisons in cases of statistical significance. To examine individual variability and repeated measures, linear mixed-effects models were utilized. Interaction terms were initially included to assess the effect of time on variables but were later removed from the final model due to convergence issues.

Subgroup analyses were conducted to compare rats subjected to compression versus those not compressed, and to evaluate differences between groups that received only hypnotic agents. Correlation matrices were constructed to explore associations between kidney function biomarkers, and Pearson or Spearman correlation coefficients were calculated depending on data distribution. A p-value of < 0.05 was considered statistically significant in all analyses.