

Assessment of cerebral circulation in rats during euthanasia with a mixture of urethane and alpha-chloralose: Possibilities of imaging photoplethysmography

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Background. Imaging photoplethysmography (IPPG) is a non-contact optical method for studying the blood supply to biological tissues. It is successfully used in various studies and diagnostic procedures as a tool for monitoring changes in local hemodynamics in response to various types of interventions or under any pathological conditions. In experimental biomedicine, urethane (Ur) and alpha-chloralose (Ch) have long been used to anesthetize laboratory animals, but according to the American Veterinary Medical Association, these drugs are not acceptable for euthanasia. **Aim.** To evaluate the latency period of cessation of cerebral circulation in rats anesthetized with a mixture of Ur-Ch after intravenous bolus injection of Ur-Ch at a lethal dose by means of the IPPG parameters: Amplitude of Pulsatile Component (APC) and Optical Intrinsic Signal (OIS), and to compare the results obtained with a similar indicator of fatal changes in systemic hemodynamics and gas exchange function. **Methods.** Studies were performed on 15 anesthetized, curarized Wistar stock rats, which were subject to routine euthanasia after completion of acute experiments. IPPG waveforms were recorded through thinned parietal bones, allowing visualization of intracranial vessels. Parameters APC, OIS, electrocardiogram (ECG), blood pressure (BP), CO₂ at the end of exhalation (EtCO₂) and heart rate (HR) were recorded before, during and after infusion of a lethal dose (no less than 1000 mg/kg of Ur + 100 mg/kg of Ch); the duration of the infusion was 10-15 seconds. The main estimated magnitude was the time of extinction of life support functions (TELSF), determined from the moment of the start of Ur-Ch administration until the moment of death based on the achievement of a composite point consisting of three points, namely: cardiac arrest (HR is zero), a drop in mean BP below 10 mmHg, and a decrease in EtCO₂ below 1%. **Results.** Bolus administration of Ur-Ch in a lethal dose to an anesthetized rat caused irreversible loss of life support functions already during the infusion, namely, cardiac arrest (TELSF = 5.6 [4.0–7.0 sec]), critical drop in BP (TELSF = 10.2 [8.2–11.7] sec), and cessation of gas exchange (TELSF = 13.8 [12.9–15.5] sec). At the same time, characteristic changes in the patterns of IPPG signals were observed: cessation of APC (TELSF = 5.5 [4.5–7.5] sec) and break of the OIS (TELSF = 10.4 [8.3–12.3] sec), which indicate fatal disorders of the blood supply to the brain. **Conclusion.** Ur-Ch infusion at a dose of at least 1000+100 mg/kg appears to be a reliable and humane method of euthanasia of rats under general anesthesia. The use of IPPG made it possible to identify optical markers of cessation of cerebral circulation caused by Ur-Ch.

Keywords: euthanasia, imaging photoplethysmography, urethane, alpha-chloralose.

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Introduction

Imaging photoplethysmography (iPPG) is a non-contact optical technique used to study spatial and time distribution of blood flow parameters by measuring optical radiation intensity after interaction with biological tissue. The main test parameters of iPPG signal include an amplitude of pulsatile component (APC) calculated as a ratio of a fast varying component that reflects tissue blood filling fluctuations at the heart rate to a slowly varying component giving an idea of the total blood volume in the region of interest [1], and an optical intrinsic signal (OIS) governed by a slowly varying component [1]. iPPG is successfully used in experimental clinical studies and diagnostic procedures as

a simple and precise tool for monitoring local hemodynamic changes in response to various interventions or in any disease states [2–6].

Experimental biomedicine often needs to remove an animal from experiment humanely, for which various euthanasia methods are used. Over at least 20 years, our research team has performed multiple experimental series using rats anesthetized by a mixture of two well-known non-inhalation general anesthetics — urethane and alpha-chloralose (Ur-Ch), while euthanasia of animals at surgical stage of anesthesia was performed via intravenous injection of this drug combination or urethane at lethal doses [4–6]. However, the American Veterinary Medicine Association Guidelines don't address either urethane or alpha-chloralose

as „acceptable agents for euthanasia“ of small rodents due to a „lack of data“ or specifics of pharmacokinetics, respectively, at least when they are used separately, though both drugs are recommended as „anesthetics before application of approved additional methods“ [7]. Nevertheless, many years of our experience definitely confirm that bolus infusion of Ur-Ch in an animal initially anesthetized by the specified mixture causes cardiac and respiratory arrest as early as at the time of administration. To verify our observations, we have performed the study for the following purpose: use the APC and OIS behavior analysis to evaluate the latent period of cerebral circulation arrest after intravenous injection of a lethal Ur-Ch dose and to compare the obtained results with the same fatal change variable in system hemodynamics and pulmonary function.

Materials and methods

Laboratory animals: compliance to ethical standards and preparing for experiment

The study involved 15 male Wistar rats with a weight of 423 ± 82 g which were subject to scheduled removal from acute experiments under a research project devoted to iPPG examination of sumatriptan effects on intracranial perfusion changes induced by intraperitoneal (I.P.) nitroglycerin administration, results of which were published in Scientific Reports [6]. Animal care and preparation for experiments are described in detail in the above-mentioned paper and corresponded to ethical standards of the International Association for the Study of Pain and Directive 2010/63/EU of the European Parliament; a research protocol was approved by the local ethics committee of Pavlov First Saint Petersburg State Medical University (No. 100_IF2_122023/26_210). Briefly, rats anesthetized using I.P. Ur-Ch injection (induction dose of 800 mg/kg urethane + 60 mg/kg alpha-chloralose (Sigma, St. Louis, MO, USA), additional titration by 100 + 7.5 mg/kg I.P. boluses, respectively, in the regime „upon request“ until the surgical stage of anesthesia was achieved) then animals were subjected to tracheostomy, cannulation of femoral vein and artery, I.P. catheter insertion, and formation of two closed cranial windows (CCW) by means of layer-by-layer thinning of parietal bones.

Life support and physiological parameter monitoring hardware

After completion of surgical preparation, the animal was placed into a stereotaxic apparatus (Stoelting Co., Wood Dale, IL, USA), then muscle relaxation (pipecuronium bromide (Arduan, Gedeon Richter, Budapest, Hungary), I.V., 1 mg/kg — induction, 0.4 mg/kg — maintenance) and artificial pulmonary ventilation (APV) (SAR-830 ventilator, CWE, Inc., Ardmore, Pennsylvania, USA) were performed, and equipment was connected to ensure continuous monitoring of arterial blood pressure (ABP), heart rate (HR) and

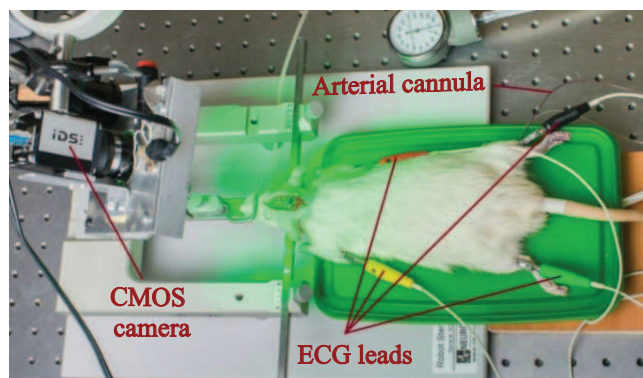


Figure 1. General experimental setup view. Designations: CMOS camera — a CMOS camera with a ring illuminator of iPPG system, ECG leads — ECG needle electrodes, Arterial cannula — a plastic cannula in the right femoral artery for arterial pressure control.

CO₂ at the end of exhalation (EtCO₂) (MLT844 amplifier with pressure sensor (AD Instruments Inc., Colorado Springs, USA) and the Capstar-100 gas detector (CWE, Inc., Ardmore PA, USA) combined with the Power1401-3 analog-to-digital converter with Spike2.v.8 (Cambridge Electronic Design, Cambridge, UK)), electrocardiogram (ECG) (KAP-01-Kardiotehnika-EKG, TD INKART, Saint Petersburg, Russia) and body temperature (Bio-TC1 temperature controller, Biotekhnologii, Saint Petersburg, Russia).

An iPPG system consisting of a digital CMOS camera (8 bit, GigE uEye UI-5220SE, Imaging Development Systems GmbH, Germany) and ring illuminator with 8 LEDs (TDS-P003L4F07, TDS Lighting Co., China) operating at a wavelength of 525 ± 25 nm, light flux of 100 lm and power of 3 W each was mounted above the rat's head (Figure 1). A lens with a focal distance of 25 mm (M1214-MP2, Computar, Japan) was used for imaging. Object illumination and image recording were performed through a linearly polarizing film in orthogonally crossed polarization directions to remove surface reflection effects [8]. Video frames were recorded with a frequency of 100 frames per 1 s, a frame size was 752–480 px, frames were sent to a PC via Ethernet and saved as PNG files. ECG signal in two leads was recorded together with video recording, where clock pulses from the video camera were sent to one of their ECG channels, thus ensuring synchronization of video recording and ECG with a time accuracy of at least 1 ms. ECG data were sent to the PC via USB 2.0. Meningeal vessel imaging was performed through the CCW, surface of which was coated with paraffin oil (VEST Ltd., Russia) to improve transparency.

Euthanasia

According to the main study design [6], nitroglycerin (I.P., 10 mg/kg, Perlinganit, EVER Pharma Jena, GmbH, Germany) was administered to all animals before removal, from which 8 and 6 animals additionally received I.V.

sumatriptan (Tokyo Chemical Industry Co., Ltd., Japan) at doses of 4 mg/kg and 10 mg/kg, respectively, and an equivalent volume (0.8 ml) of isotonic sodium chloride solution was I.V. administered to 1 rat. iPPG parameters and main physiological variables were recorded in all animals during 2.5 h, and then euthanasia was performed. The total time spent by the rats in the experiment from the moment of induction of anesthesia to experiment completion time was at least 6 h.

Immediately before euthanasia, all animals were under surgical anesthesia, mechanically ventilated and with stable indicators of cardiovascular function, body temperature and respiratory exchange. Animals were removed from experiment by I.V. bolus injection of at least 1000+100 mg/kg Ur-Ch dissolved in at least 1.5 ml isotonic sodium chloride solution; infusion time was 10–15 s. Recording of all hemodynamic variables and iPPG signals started 60 s before the Ur-Ch injection start and continued for 3 min. Death was certified based on the achievement of a composite point consisting of three conditions as follows: cardiac arrest (heart rate equal to zero), drop of mean ABP (mABP) below 10 mm Hg and reduction of EtCO₂ below 1%. Selection of nonzero mABP was based on the fact that mABP of < 10 mm Hg, especially when it continues decreasing, indicates full system circulation failure, absence of effective tissue perfusion and shows only residual hydrostatic pressure in the artery–cannula–sensor system. Whereas in clinical practice, EtCO₂ < 1% is the basis for stopping resuscitation due to its obvious hopelessness [9]. The ventilator was switched off when EtCO₂=0% was displayed.

Data processing

Life support function failure time (LSFF) determined from the lethal Ur-Ch dose injection start time to the death certification time according to criteria listed in Section 3 was the main assessed parameter. LSFF was calculated separately for mABP, HR, EtCO₂ and iPPG indices, while sharp changes in normal APC patterns (oscillation failure) and OIS (signal level breakdown) were considered as indicative of cerebral circulation arrest.

Behavior of the test physiological variables was analyzed in Spike2, v. 8 (Cambridge Electronic Design, Cambridge, UK), MATLAB version R2024a (MathWorks, Natick, Massachusetts, USA) and GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA).

To calculate APC, the slowly varying component (DC) of the iPPG signal was calculated using a low frequency filter (4th order Butterworth filter) using the `filtfilt` function in MATLAB to exclude phase shift. Then, the fast varying component (AC) of iPPG signal was calculated as

$$AC = - \left(\frac{\text{row_PPG}}{DC} - 1 \right) 100\%, \quad (1)$$

where `row_PPG` is the initial („raw“) iPPG signal. Subtraction of 1 is caused by the need for signal reduction into the [-1;1]

range, signal was inverted in order to make a larger blood content corresponding to higher signal values. After this, for APC calculation, ACs plotted from the next R peak of ECG were averaged over 12 cardiac cycles ($\overline{AC}$) as shown in [10] and the amplitude of the averaged wave was calculated:

$$APC = \max(\overline{AC}) - \min(\overline{AC}). \quad (2)$$

OIS was calculated using an algorithm described in [5]:

$$OIS_i(x, y) = \frac{I_0(x, y) - I_i(x, y)}{I_0(x, y)} 100\%, \quad (3)$$

where $I_0(x, y)$ is the spatial distribution of pixel value in the first video frame of the brain recorded for each animal, and $I_i(x, y)$ is the distribution pixel value in the next recorded frames, i is a frame number, x, y are the spatial coordinates of a pixel in images.

Nonlinear conversions of the obtained electric signal were performed during ECG recording and processing, therefore values were normalized to the [0; 1] range. Such deviation from traditional measurement of cardiac electrical activity in physical quantities didn't serve as a constraint for this study.

To find significant differences between LSFF values for different parameters, the Wilcoxon signed-rank test was used; the nonparametric method was selected due to non-Gaussian data distribution according to the Shapiro–Wilks test. In tables and curves, results are shown as Me[Q1-Q3].

Results

Initial (before lethal Ur-Ch dose injection) values of system hemodynamic, respiratory exchange and iPPG indices are shown in Table 1.

I.V. bolus injection of Ur-Ch had an „avalanche“ effect on all assessed parameters that started developing „at the tip of a needle“, i.e. directly during infusion (Figure 2); LSFF values (in seconds) are listed in Table 2.

Comparison of iPPG LSFF indices and system parameters has found that LSFF (APC) was the same as LSFF (HR) ($P = 0.4651$), but differed significantly from LSFF (mABP) ($P = 0.0017$, Wilcoxon matched-pairs signed rank test). Whereas significant differences in LSFF were found in the OIS-HR pair ($P = 0.0065$), but not when comparing OIS and mABP ($P = 0.9460$, Wilcoxon matched-pairs signed rank test).

Discussion

This study has first shown that I.V. bolus injection of a lethal Ur-Ch dose in an anesthetized rat caused irreversible loss of life support functions as early as during infusion, i.e. heart arrest, critical ABP drop and respiratory exchange cessation, which is accompanied by typical changes of iPPG signals indicating fatal cerebral circulation disorders. The obtained results indicate high power, speed of action and reliability of Ur-Ch as a combined drug for euthanasia,

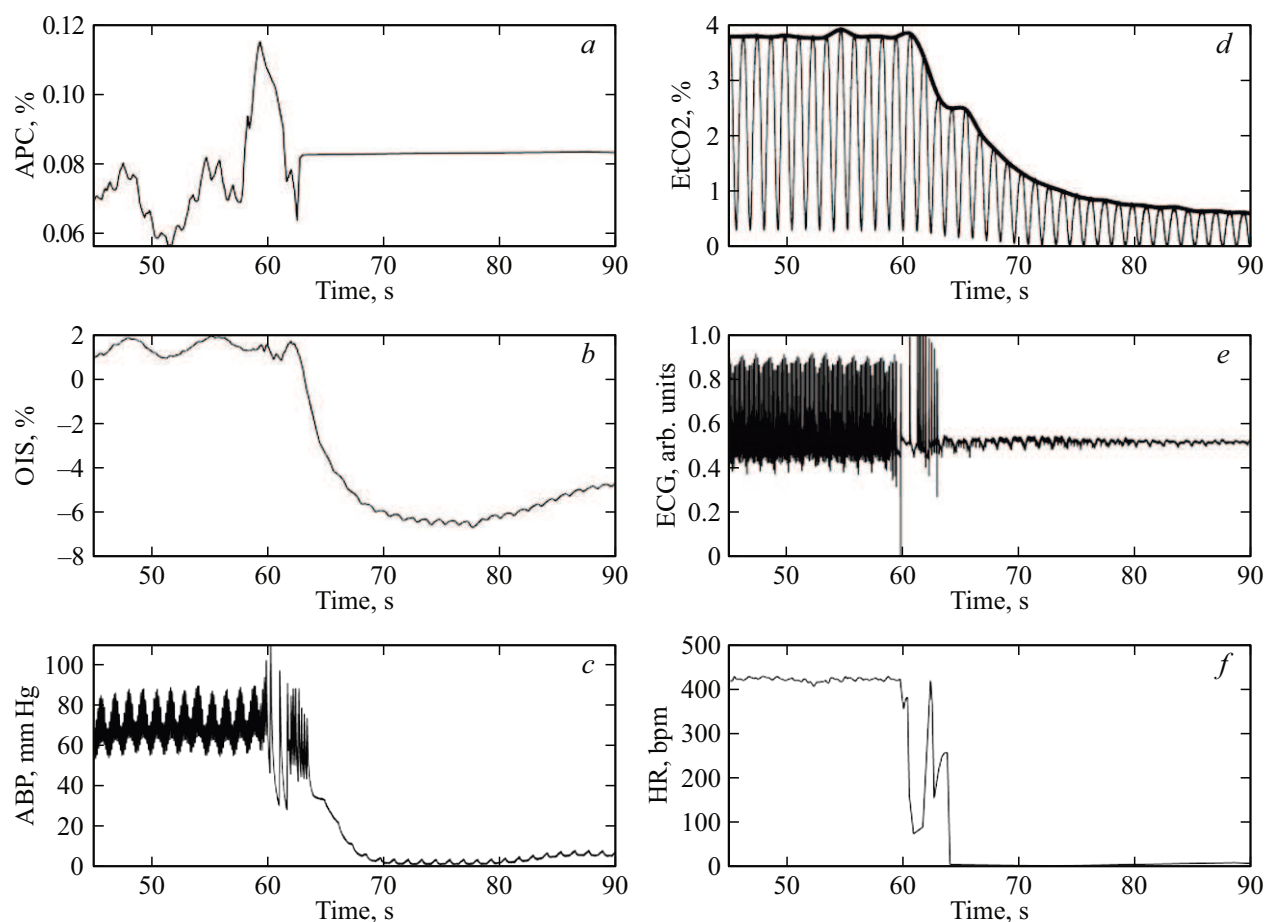


Figure 2. Example of typical changes in native records of the assessed physiological parameters caused by I.V. Ur-Ch injection at a lethal dose (infusion start at 60 s).

Table 1. Initial values of the assessed physiological parameters

iPPG parameters		System indices		
APC, %	OIS, %	mABP, mm Hg	HR, bpm	EtCO ₂ , %
0.08 [0.05-0.1]	1.03 [0.67-1.4]	58 [46-69]	390 [327-421]	3.65 [3.4-3.7]

Table 2. LSFF values by the assessed physiological parameters

iPPG indices, s		System indices, s		
APC, %	OIS, %	mABP, mm Hg	HR, bpm	EtCO ₂ , %
5.5 [4.5–7.5]	10.4 [8.3–12.3]	10.2 [8.2–11.7]	5.6 [4.0–7.0]	13.8 [12.9–15.5]

at least when it is administered intravenously to small rodents under general anesthesia and once again confirm high sensitivity of the iPPG method as an intracranial hemodynamics evaluation tool.

Urethane is classified as a long-acting non-inhalation anesthetic that is used exclusively for acute experiments, which do not involve the animal's recovery and restoration of consciousness („non-recovery“ procedures). Such constraint

is associated with carcinogenic and mutagenic properties of the drug, and also with its ability to provoke complications in the postoperative period [11–13]

Meanwhile, urethane is positioned as an anesthetic that minimally suppresses neural activity in some areas of the central nervous system and maintains some autonomic vegetative reflexes, which makes it a drug of choice for neurophysiological studies and examination of respiratory

function, cardiovascular reactions, motor activity of urinary tract and gastrointestinal tract [14–16]. It was proposed to use the urethane anesthesia state as a sleep model [17], though there are alternative opinions on the subject [18,19]. In general, urethane is by some properties as good as more advanced and commonly known anesthetics used in clinical medicine such as ketamine [20], propofol [21] and isoflurane [22,23].

Alpha-chloralose is a general long-acting non-inhalation anesthetic, powerful hypnotic with a weak analgesic activity, resembles urethane by some properties and, similar to urethane, is used only for non-recovery experiments [11,13]. The drug can induce convulsions, acidosis, respiratory depressions and arrhythmia, and is used in some countries as rodenticide, causing accidental poisoning of domestic animals [24] and humans [25]. Combined anesthesia by the Ur-Ch mixture enhances the narcotic effect and minimizes adverse reaction risks by reducing the dose of each of the components, especially urethane.

Changes in APC and OIS signals found via iPPG, which occur in connection with lethal Ur-Ch dose administration indicate a dramatically fast irreversible cerebral circulation arrest. When comparing LSFF for system hemodynamic and cerebral circulation indices, note a high match level of HR and APC values as well as OIS and mABP values, which can be adequately explained by the nature of iPPG signals. Actually, APC reflects arterial pulsations, therefore the starting point of APC „levelling“ in the record almost coincides with the heart arrest time. At the same time OIS depends on the total cerebral circulation volume, maintenance of which is directly associated with the ABP level, therefore, OIS „collapse“ occurs almost simultaneously with ABP drop. Thus, it can be said that Ur-Ch quickly induces a kind of „pharmacological cerebral exsanguination“ leading to brain death.

In connection with our findings, the opinion of the American Veterinary Medicine Association mentioned in „Introduction“ concerning urethane and alpha-chloralose as unacceptable drugs for euthanasia of rodents causes some bewilderment, especially taking into account that such methods as CO inhalation or cervical dislocation are classified as allowed methods [7]. However, the authors of the above-mentioned document probably had in mind euthanasia of awake animals that are naive with respect to preliminary administration of any consciousness and/or nociception inhibiting drugs. At the same time, according to other documents, for example, guidelines of the Animal Care and Use Committee of the University of North Texas, urethane at a dose of 1.5 g/kg (I.P.) is seen as an euthanasia drug „in experiments with rats initially anesthetized by a volatile anesthetic“, for example, isoflurane; it is particularly emphasized that „I.V. bolus infusion of urethane leads to immediate death“ [12]. In our experiments, all animals subject to removal, as mentioned above, are in the surgical anesthesia state and additional Ur-Ch administration is essentially an intentional anesthetic overdosage causing quick death.

Conclusion

I.V. bolus infusion of Ur-Ch at a dose of at least 1000+100 mg/kg is a reliable and humane method of euthanasia of rats under general anesthesia that ensures quick failure of all life support functions. iPPG made it possible to identify optical markers of Ur-Ch-induced cerebral circulation cessation.

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Conflict of interest

The authors declare no conflict of interest.

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