THE CHOICE OF ANESTHETIC TYPE AND CONDITIONS FOR 2,3,5-TRIPHENYLTETRAZOLIUM CHLORIDE STAINING OF BRAIN SLICES IS IMPORTANT IN THE ASSESSMENT OF ISCHEMIC INJURY IN RATS IN THE EARLY STAGES OF PATHOLOGY

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Studies of ischemic brain injury are an important area of modern biomedical research. So far, a lot of ischemic stroke models have been proposed, along with different imaging and staining modalities aimed to visualize the damaged tissue. In this work we use a rat model to investigate how the experimental setup affects the interpretation of experimental data obtained in the acute phase of ischemic stroke (5 hours after the occlusion of the middle cerebral artery). We show the association between the choice of the type of anesthesia and the severity of ischemic injury: in our experiments brain damage was the most pronounced in the animals anesthetized with a combination of chloral hydrate and Rometar; the least damage was observed for isoflurane. Staining was performed using the popular dye 2,3,5-triphenyltetrazolium chloride (TTC). We demonstrate that parameters of brain slices incubation in TTC also need to be accounted for when interpreting the results obtained during the acute phase of stroke, the optimum incubation time being 30 min and temperature 37 °C.

Keywords: stroke, ischemic injury, brain slices, 2,3,5-triphenyltetrazolium chloride, staining, anesthesia, rats

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ВЛИЯНИЕ ТИПА АНЕСТЕЗИИ И УСЛОВИЙ ПРОКРАШИВАНИЯ ТКАНЕЙ МОЗГА КРАСИТЕЛЕМ 2,3,5-ТРИФЕНИЛТЕТРАЗОЛИЕМ ХЛОРИСТЫМ (ТТХ) НА ОЦЕНКУ ИШЕМИЧЕСКОГО ПОВРЕЖДЕНИЯ МОЗГА КРЫС НА РАННИХ СТАДИЯХ ПАТОГЕНЕЗА

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Изучение ишемического повреждения головного мозга является важным направлением современных медико-биологических исследований. К настоящему моменту разработано множество моделей ишемического инсульта, а также предложены различные способы визуализации поврежденных тканей мозга. В данной работе мы исследовали, как различные условия проведения эксперимента, моделирующего ишемический инсульт у крыс, влияют на интерпретацию результатов в острой фазе заболевания (5 ч с момента окклюзии средней мозговой артерии крыс). Мы показали, что на ранней стадии развития патологии существенное влияние оказывает выбор используемой анестезии животных. В наибольшей степени повреждение мозга было выражено при использовании для анестезии смеси хлоралгидрат/Рометар, в наименьшей — при использовании изофлурана. Для визуализации повреждения мозга животных мы использовали наиболее популярный краситель 2,3,5-трифенилтетразолий хлористый (ТТХ). Мы установили, что температура и время инкубации срезов мозга в растворе ТТХ также значительно влияют на интерпретацию результатов при оценке ишемического повреждения в острой фазе патологии. Оптимальными условиями окрашивания срезов мозга в растворе ТТХ являются 30-минутная инкубация срезов при 37 °C.

Ключевые слова: инсульт, ишемическое повреждение, срезы мозга, 2,3,5-трифенилтетразолий хлористый, окрашивание, анестезия, крысы

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Ischemic stroke is one of the most serious neurological conditions and the second leading cause of death and disabilities worldwide after cardiovascular diseases [1–4]. So far, no effective treatment strategies have been proposed for this disease, and its pathogenesis remains understudied.

Of all currently existing models of ischemic stroke [5–12], monofilament occlusion of the middle cerebral artery stands out as the most common. First described by Koizumi et al. [13], it has been improved and adapted for use in different laboratory animals, such as rats [14] and mice [15].

Along with the variety of ischemic stroke models, there are different techniques allowing visualization of stroke-induced tissue damage. Infarcted zones of brain sections can be made visible using histological stains, such as traditional hematoxylin and eosin [16, 17], or Nissl staining and its modifications [18, 19]. Impregnation of nervous tissue with silver is reported to be helpful in detecting neuronal degeneration in the early stages of stroke [20, 21]. The same is true for Fluoro-Jade stains [22–24], but the exact mechanism of their action is still unknown. One of the simplest techniques to visualize ischemic lesions in brain slices is 2,3,5-triphenyltetrazolium chloride (TTC) staining [25]. Enzymes with dehydrogenase activity found in living cells reduce TTC to formazan, which stains healthy tissue deep red, whereas damaged tissue lacking healthy mitochondrial activity resists staining. Immunohistochemistry also has something to offer and can be employed to observe apoptotic cells in the lesion [26, 27]. Non-invasive techniques for stroke diagnosis include magnetic resonance imaging [28], positron emission tomography [29] and single-photon emission computed tomography [30]. The list of approaches to ischemic injury visualization is not limited to these modalities; detailed information is available in themed reviews [31].

Because approaches to studying stroke pathogenesis and developing treatment strategies are so different, the Stroke Therapy Academic Industry Roundtable (STAIR) has prepared a series of guidelines on ischemic stroke modeling [32–35], describing, in particular, a number of factors affecting its results and their interpretation, such as the selected model itself, the animal's breed, the type of an anesthetic, the visualization technique, etc.

Even protocols for standard interventions may vary greatly. For example, TTC staining, which is now the most common technique used to visualize ischemic areas in brain slices, was originally performed on rats' brain sections 24 hours after induced occlusion (the brain sections were incubated for 30 min at 37 °C) [25]. However, some authors were able to visualize infarcted tissue using TTC staining just a few hours after occlusion [21, 36–43]. Incubation time of brain slices in the TTC solution may vary from 5 min [44] to standard 30 min [25]. Some protocols warn that TTC is unstable when heated, therefore, staining should be performed at room temperature [45]. TTC is mainly used for staining brain slices, but sometimes animals are perfused with TTC transcardially [38, 46].

In this work we show that effective visualization of damaged tissue obtained from rats with acute ischemia depends largely on temperature and duration of incubation of brain slices in the TTC solution. These two factors can skew interpretation of the results. We also demonstrate that the type of an anesthetic affects the scope of ischemic injury in the early stage of stroke (5 hours after the occlusion), while in the later stages (24 hours after the occlusion) its role is insignificant.

METHODS

Experiments involving animals were carried out in compliance with the Directive 2010/63/EU of the European Parliament and the European Council, dated September 22, 2010. The study protocol was approved by the Animal Care and Use Committee of the Institute of Bioorganic Chemistry, RAS.

The study was carried out in male Wistar rats (weight ranging from 280 g to 330 g) purchased from Pushchino breeding facility. The rats were kept in the animal house of the Institute of Bioorganic Chemistry in plastic cages, 3 animals per cage. The animals had free access to water and food.

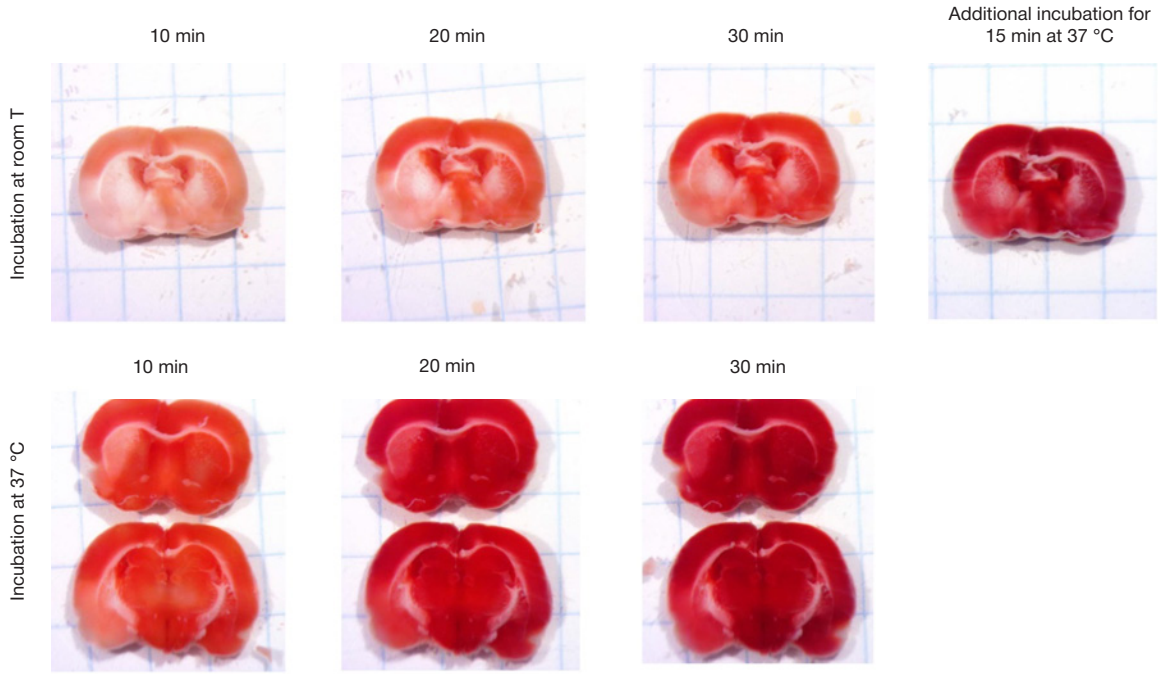

Fig. 1. Effects of different temperatures and duration of incubation of rat brain slices in 1 % TTC solution on visualization of ischemic injury 5 hours after the occlusion. The pictures show brain slices obtained from a Wistar rat with the occluded middle cerebral artery. One slice was stained at room temperature, another — at 37 °C. Samples were photographed at set time intervals. Anesthetic used: Zoletil/Rometar

Occlusion of the middle cerebral artery was induced according to the protocol [14]. We used three types of anesthetics:

1. isoflurane (marketed as Aerrane by Baxter, USA): a 5 % concentration for general anesthesia induction and a 1.5 % concentration for anesthesia maintenance.

2. tiletamine hydrochloride/zolazepam hydrochloride (Zoletil by Virbac Sante Animale, France; 40 mg/kg) + xylazine hydrochloride (Rometar by Bioveta, Czech Republic; 10 mg/kg), injected intraperitoneally;

3. chloral hydrate (Dia-M, Russia, 400 mg/kg).

The animals were analgesized with 5 mg/kg ketoprofen (Ketonal by Sandoz, Switzerland) administered subcutaneously; local analgesia was induced by administering 2 % Novocain.

In our study we used commercial middle cerebral artery sutures by Doccol (USA; catalog number 403756PK10Re) 0.185 mm in diameter.

The rats were decapitated after set time intervals, their brains removed and cut into 2 mm thick frontal sections, which were then placed in 1 % TTC solution (Sigma-Aldrich, USA). Staining was done at different temperatures (20 °C and 37 °C).

RESULTS

In an attempt to investigate how different TTC staining conditions affect visualization of ischemic lesions, we modeled middle cerebral artery occlusion in rats [14]. The occlusion was permanent, i. e. the vessel remained blocked throughout the experiment. The animals were anesthetized with a mixture of Zoletil and Rometar injected intraperitoneally. Five hours after the occlusion the brains were removed and cut into 2 mm thick frontal sections. Then, some slices were incubated in 1 % TTC solution at room temperature, while other were placed into TTC preheated to 37 °C. Photos of brain sections were taken at equal time intervals to assess how different temperatures and duration of incubation in the TTC solution affected visualization of ischemic tissue. Lesions became visible after 10 min of incubation at both temperatures: unlike the intact areas, they were weakly stained (Fig. 1). Further incubation in TTC at 37 °C produced a more intense color; after 20 min of incubation the color contrast between the healthy and ischemic tissues became less pronounced, as the damaged tissue developed an intermediate pink color. However, at room temperature the color contrast between the damaged and healthy tissues increased. Longer incubation at 37 °C produced a well-developed color throughout ischemic areas (Fig. 1). It is very important to control TTC staining conditions when only a short time has elapsed after occlusion induction, because damaged tissue may still contain living cells affecting color development. Twenty-four hours after the occlusion, the injury was clearly visible, and the color contrast between the lesion and the healthy tissue did not lose its intensity even after 2 hours of incubation at 37 °C.

Our next step was to find out how a choice of an anesthetic influences the scope of ischemic brain injury. Damaged tissue was visualized using TTC staining. In this series of experiments

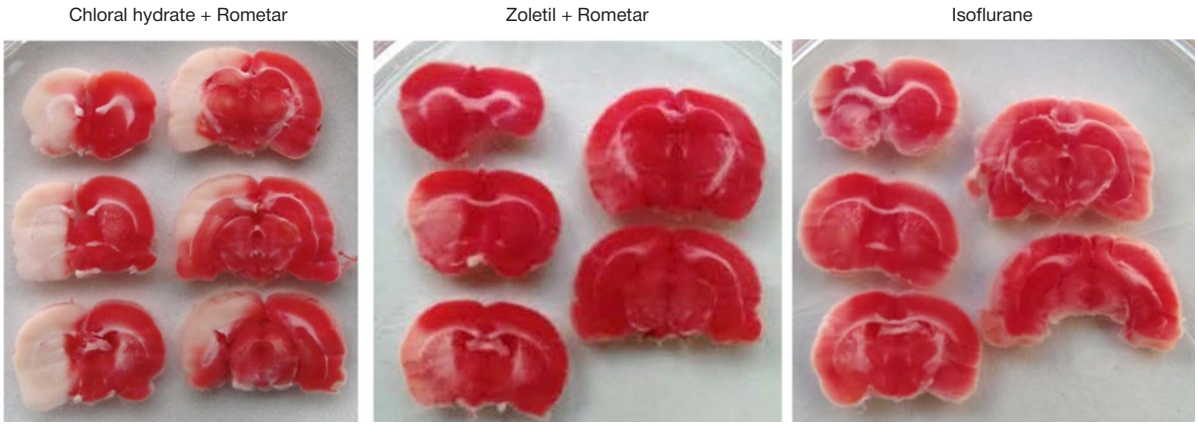

Fig. 2. Effects of different anesthetics on the scope of ischemic injury in rats with the permanently occluded middle cerebral artery (5 hours after the occlusion). Brain slices were incubated under identical conditions in 1 % TTC solution for 30 min at 37 °C

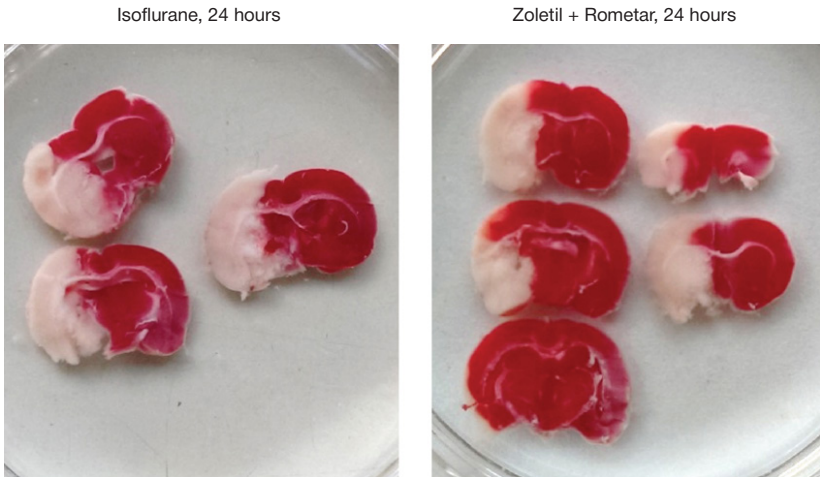

Fig. 3. Brain slices of rats anesthetized with different drugs 24 hours after the induced permanent occlusion of the middle cerebral artery. The slices were incubated under identical conditions in 1 % TTC solution for 30 min at 37 °C

we also modeled permanent middle cerebral artery occlusion in Wistar rats. The animals were anesthetized using three types of anesthetics: isoflurane (Aerrane), a mixture of Zoletil and Rometar injected intraperitoneally and a mixture of chloral hydrate and Rometar also injected intraperitoneally. Five hours after the occlusion the brains were removed, sectioned, and incubated in 1 % TTC solution at 37 °C for 30 min. The lesion size was the smallest in the animals who had received isoflurane (this was reliably demonstrated in 6 animals), and the color contrast between the damaged and healthy TTC-stained tissues was minimal. The most severe damage was observed in the animals who had received a mixture of chloral hydrate and Rometar (this was reliably demonstrated in 5 animals). The Rometar/Zoletil mix produced interesting results. Of 7 animals, only 2 developed massive stroke; in 5 other animals the lesions did not develop a contrasting color during staining (Fig. 2). To sum up, the choice of an anesthetic is an important factor that must be accounted for when studying acute ischemia. The underlying cause of the contributions made by anesthetics is not clear, though. The neuroprotective effect of isoflurane has been reported by a number of authors [47–49], but its mechanism remains unexplained. Interestingly, 24 hours after the occlusion of the middle cerebral artery in rats, the size of the lesion did not depend on the type of an anesthetic (Fig. 3).

DISCUSSION

We have analyzed how different factors affect the results of TTC staining of brain sections obtained from rats with induced permanent ischemia. Our study demonstrates that visualization of damaged tissue in the early phases of stroke (5 hours after the occlusion) is particularly sensitive to TTC staining conditions (incubation temperature and duration) and the type of an anesthetic. Therefore, we do not recommend TTC staining for assessing the size of the lesion in the early stages of ischemic stroke, regardless of the opinion expressed in a number of academic works.

Besides, TTC staining does not provide unambiguous evidence about the viability of cells in the ischemic tissue during the acute stage. TTC is an indicator of mitochondrial dehydrogenase activity. A number of studies confirm that mitochondrial dysfunction is one of the major consequences of ischemia [50, 51]. However, an intermediate color developed by tissue during staining raises a question of interpretation. Normally, in healthy tissue TTC is enzymically reduced to formazan, which stains the tissue deep red. In dead tissue this reaction does not happen, and the tissue remains white. But in our experiments the ischemic tissue developed an intermediate pink color whose intensity was growing as the incubation time and temperature of the environment were increasing. In the study [52] the researchers calculated the proportion of intact mitochondria in the brain sections that were subject to TTC staining and developed or did not develop a color. The study showed that about 5 % of mitochondria were intact in the areas that did not stain. Intermediate pink meant that the proportion of functioning mitochondria in the lesion was higher.

It is known that permanent occlusion does not necessarily cause immediate damage to mitochondria, and the latter remain intact for a few hours or even days, while other cell organelles, such as the nucleus, have already been destroyed [52]. In this case TTC-based visualization will not show tissue damage and, therefore, the real picture of progressing pathology will be blurred. A more traumatizing ischemia-reperfusion injury causes more rapid damage to mitochondria, which also should be accounted for when working with certain stroke models. Besides, TTC staining is not recommended for longer than 24 hours following artery occlusion because the lesions can accumulate inflammatory cells with intact mitochondria [52].

CONCSLUIONS

Our study conducted in rats with the permanently occluded middle cerebral artery demonstrates that estimates of the ischemic injury size in the early stages of stroke are affected by a number of factors, including the type of an anesthetic and staining conditions. Five hours after the occlusion, the least damage was observed in rats anesthetized with isoflurane; the most severe damage was observed in the animals who had received the chloral hydrate/Rometar mix. The optimum conditions for TTC staining of brain slices are 30 min incubation at 37 °C. Protocols that recommend a shorter incubation time and lower temperatures can yield incorrect results for the samples obtained in the early stages of stroke. But 24 hours after the occlusion damaged areas can be effectively visualized using TTC staining, regardless of incubation time/temperature and the selected anesthetic. Therefore, 24 hours are optimal for qualitative and quantitative TTC-based analysis of ischemic brain injury.

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