ORIGINAL ARTICLE

Substantial decrease in SARS-CoV-2 RNA after fixation of cadavers intended for anatomical dissection

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Abstract
With the onset of the COVID-19 pandemic, a problem arose with classic body donation programmes for obtaining cadavers for anatomical dissections, science and research. The question has emerged whether bodies of individuals who died of COVID-19 or were infected by SARS-CoV-2 could be admitted to Departments of Anatomy. To determine the risk of SARS-CoV-2 transmission to employees or students, the presence and stability of SARS-CoV-2 RNA in cadavers after fixation agents' application and subsequent post-fixation baths over time were examined. The presence of viral RNA in swabs from selected tissues was assessed by the standardized routine RNA isolation protocol and subsequent real-time PCR analysis. To support the results obtained from the tissue swabs, samples of RNA were exposed in vitro to short and long-term exposure to the components of the injection and fixation solutions used for the bodies' conservation. Substantial removal of SARS-CoV-2 RNA was observed in post-mortem tissue following perfusion with 3.5% phenol, 2.2% formaldehyde, 11.8% glycerol and 55% ethanol, and subsequent post-fixation in an ethanol bath. In vitro experiments showed significant effects of formaldehyde on SARS-CoV-2 RNA, while phenol and ethanol showed only negligible effects. We conclude that cadavers subjected to fixation protocols as described here should not pose a considerable risk of SARS-CoV-2 infection while being handled by students and staff and are, therefore, suitable for routine anatomical dissections and teaching.

Keywords Embalming · Dissection · Infections · Coronavirus · Polymerase chain reaction

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Introduction

The SARS-CoV-2 virus caused the COVID-19 pandemic, which affected the world in 2020 and persists in the population to this day. It also intervened in the teaching of Anatomy at medical faculties, particularly in anatomical dissection, which forms an integral part of Anatomy teaching and depends on the supply of donor bodies. Since the beginning of March 2020, when COVID-19 broke out in the Czech Republic, the number of cadavers positive for SARS-CoV-2 began to increase. But at the same time, there has been raised concern about the increased risk of COVID-19 infection for students and laboratory dissection assistants. Different countries have different legal guidance regarding dissections of SARS-CoV-2-positive cadavers. These are mainly influenced by concerns about possible virus survival in dead bodies and the potential risk of infection (Shchegolev and Tumanova 2021). Therefore, several institutions had suspended the body donation program (Brassett et al. 2020). For teaching purposes, this problem could be solved by embalming the dead bodies.

Embalmed bodies play an irreplaceable role in the teaching of Anatomy to students. They are used not only for studying Anatomy, but also for practicing new surgical approaches and for the preclinical testing of new instruments and devices (Eisma and Wilkinson 2014). Preservation is defined as an action for keeping something safe from harm, destruction or decomposition (Brenner 2014). It can be achieved by treating the deceased with special chemicals (embalming) or cooled to −20 °C (Balta et al. 2015; Brenner 2014). Formaldehyde, ethyl alcohol and glycerin are among the most commonly used fixatives (Balta et al. 2015; Brenner 2014). In addition to vascular injection, post-fixation can also be used. This is important, particularly in the case of the poor condition of blood vessels, preventing a sufficiently high-quality injection, and thus contributes significantly to increasing the quality of cadaver fixation (Hammer et al. 2012).

It is known that SARS-CoV-2 can be detected in tissues and organs of deceased patients who were tested positive during their lifetime (Aquila et al. 2021; Beltempo et al. 2021; Plenzig et al. 2021; Bonelli et al. 2022). Therefore, precautions including the wearing of goggles, double gloves, shields or water-resistant aprons are recommended when dissecting COVID-19-positive bodies (ECDC 2020). Generally, embalming methods lead to inactivation of various bacteria, viruses and fungi (Correia et al. 2014; Gupta 2013). In the past, the effectivity of chemicals routinely used for embalming was also proven on the viability of the SARS-CoV virus (Darnell et al. 2004; Kariwa et al. 2006; Rabenau et al. 2005a). However, as fixatives can act as dehydrants, heat effectors, cross-linkers and combinations of these, their effect on RNA utility and integrity can be detrimental (Srinivasan et al. 2002). Despite this possibly leading to a reduction of SARS-CoV-2 virus in embalmed tissues, it could negatively affect, in another way, the results of SARS-CoV-2 detection in embalmed bodies, which are based on RNA detection.

Therefore, two main issues have arisen. The central question of the paper was whether the conservation and fixation procedure utilized in this study is effective in the elimination of the SARS-CoV-2 virus (specifically its RNA) in cadavers and whether these do not present an increased risk of transmission and can be used for medical students' education. In addition, to determine the level of SARS-CoV-2 RNA degradation over time, RNA of the virus was exposed in the short and long term in vitro to the chemical substances used for cadavers' conservation.

Materials and methods

SARS-CoV-2 testing in cadavers

The bodies of three persons (female, age 94 years; male, age 71 years; male, age 80 years) registered in the Anatomy Bequest Programme of Masaryk University, according to the valid legislation of the Czech Republic, were used for the study. The first two persons were sampled in 2021, the third person was sampled at the end of 2022.

For comparison purposes, "control body" was included into the study as well. The "control body" was a patient (72-year-old man) of the Institute of Forensic Medicine, St. Anne's Faculty Hospital in Brno, who died at home in 2021 and was found 10 days after his death. The body was subjected to a standard autopsy to determine the cause of death, including analyses of various parts of the body for the presence of SARS-CoV-2 RNA virus, as it was standardly performed at this institute. Samples of nasopharyngeal swabs, oropharyngeal swabs, lung tissue swabs, aqueous humour from the eye were among the samples taken during the autopsy. The "control body" was not intended for fixation and it was already in an advanced stage of decomposition at the time of the autopsy performed at the Institute of Forensic Medicine.

The procedure of fixing the cadaver for anatomical dissection at our workplace proceeded as follows (the composition of the fixation solution is original to the Department of Anatomy). Firstly, a primary simultaneous arteriovenous injection was performed by gravity *per sinus sagittalis superior* and *arteria femoralis dextra,* using 8.5 l of a solution containing 3.5% phenol, 2.2% formaldehyde, 11.8% glycerol and 55% ethanol, denatured with 4% gasoline (anhydrous solution to increase the sanitizing effect was used). Then the injection of a solution of the same composition in the

umbilical region and the gluteal region was added. Subsequently, the body was stored in a stainless fixation tank for 8 weeks in a solution of 70% ethanol (post-fixation). After this period, the body was removed from the tank, placed in a plastic bag and sealed. The body was stored at 2–4 °C for at least eight months before being prepared for anatomical dissection.

Samples were represented by the swabs (FLOQSwabs Genetics, Copan, Italy) from the surface of tissues that were taken (1) before perfusion by fixative solution, (2) after perfusion and (3) after 8 weeks post-fixation in 70% alcohol (Table 1). The swabs were immediately placed in 600 µl of the Lysis buffer (EliGene® Viral DNA/RNA FAST Isolation Kit, Elisabeth Pharmacon, Brno, Czech Republic) diluted in the ratio 1:1 with water. 200 µl of aqueous humour from anterior chamber of eye was collected using disposable needle with small syringe. 500 µl of blood was collected from *vena femoralis*. Both liquid samples were added to the 500 µl of Lysis buffer. Ten ml of 70% ethanol solution (in triplicate) from stainless fixation tank were collected, mixed with sodium acetate, pH 5.2 (3 M; Merck, Rathway, NJ, USA) in ratio 9:1 and centrifuged at 13 000 g/10 min. The pellet was resuspended in 500 µl of Lysis buffer. The analysis of samples from all four bodies was performed at the Laboratory of Neurobiology and Pathological Physiology, Institute of Animal Physiology and Genetics, Academy of Sciences of the Czech Republic.

The RNA from swabs, liquid samples were isolated, according to the original protocol of EliGene® Viral DNA/RNA FAST Isolation Kit (Elisabeth Pharmacon, Brno, Czech Republic). 200 µl of the suspension with the swab or liquid suspension was mixed with the Lysis Buffer with the Solution M added. In this step, 5 µl of internal amplification control (IAC) from the EliGene® COVID-19 UKV/SAV RT (Elisabeth Pharmacon) was pipetted to the suspension. The whole volume was loaded on to the column, washed, and eluted to 50 µl of Elution buffer.

The qPCR analysis was performed by the EliGene® COVID-19 UKV/SAV RT (CE IVD kit with defined sensitivity and specificity; Elisabeth Pharmacon) in a total volume of 20 µl with 5 µl of the extracted RNA added. Besides the detection of SARS-CoV-2 virus, the kit also allows differentiation between the alpha and beta variants of the virus. The qPCR protocol comprised RT step at 55 °C for 15 min, followed by the initial denaturation at 95 °C for 3 min and 45 cycles of amplification at 95 °C for 5 s, 55 °C for 15 s and 67 °C for 15 s. The experiments were performed in CFX Touch qPCR cycler and the analysis of the results was performed by CFX Maestro 2.0 software (version 5.0.021.0616).

To estimate an approximate number of viral particles in the samples, a simplified calibration curve was included in each run. According to the declaration of the manufacturer of the kit, the positive control from the detection kit contains 10^3 of synthetic DNA molecules of the SARS-CoV-2 targets per microliter. The undiluted and 10× and 100× diluted positive control was used for the construction of the calibration curve in a range $10^3 – 10^1$ DNA molecules/µl, i.e., $5 \times 10^3 – 5 \times 10^1$/qPCR reaction. Each isolated RNA was processed as a technical triplicate. Samples positive at least in one repeat were considered positive.

For determination of others than alpha and beta variants, sequencing of partial sequence of spike protein including S1 subunit with the RBD domain and part of the S2 subunit was performed according to the Illumina library construction and sequencing protocol (Artic v3; dx.doi.org/ 10.17504 /protocols.io.bh4zj8x6). After purification of PCR products with 10 U of Exonuclease I and 5 U of Antarctic Phosphatase (both NEB, Ipswich, MA, USA) at 37 °C for 30 min and deactivation of the enzymes at 95 °C for 10 min, PCR products were sequenced using Mix2Seq kit (Eurofins Genomics, Germany). The alignment and further identification of sequences was done with Staden Package software and GISAID (https://www.gisaid.org/) database, respectively.

Table 1 Samples' description and quantity of SARS-CoV-2 RNA in embalmed bodies over time

Sample origin	Proband 1 (female, age 94)			Proband 2 (male, age 71)			Proband 3 (male, age 80)		
	1[*]	2[*]	3[*]	1[*]	2[*]	3[*]	1[*]	2[*]	3[*]
Nasopharyngeal swab	1 064 000	16 250	196	1 484 000	1 320 000	1 555	83 490	3 789	Negative
Swab from root of tongue and palate	Negative	Negative	16	40 840	42 790	850	24 480	Negative	Negative
Aqueous humour	Negative	Negative	Negative	Negative	N/A	Negative	Negative	Negative	Negative
Swab from lung tissue	7 351 000	6 436	1	222 400	248 600	356	17 990	15	Negative
Blood from femoral vein	Negative	N/A	N/A	Negative	N/A	N/A	Negative	Negative	Negative
anal swab	Negative	Negative	Negative	37 550	Negative	4	17	Negative	Negative

Numbers reflect the estimated quantity of the RNA per qPCR reaction

*1 – samples collected before any manipulations just after receipt of the cadavers; 2 – samples collected after the arteriovenous injection; (from 1 to 5 days after) 3 – samples collected after 8 weeks' conservation in ethanol bath

In vitro SARS-CoV-2 resistance to fixative compounds

To assess the short- and long-term effect on the stability of RNA in time in vitro, the RNA isolated from a SARS-CoV-2-positive patient in concentration 4×10^4 copies/µl was mixed with the conservation and fixation chemicals used for embalming the deceased. RNA in a total amount 4×10^7 was mixed to obtain the solution of 3.5% phenol, 2.2% formaldehyde, both phenol and formaldehyde at once and 60% ethanol in a total amount of 4 ml. The quantity of viral RNA was determined by the qPCR directly after the fixative addition and then after 24 h, 3 days, 7, 10 and 15 days of incubation in a conservation solution with the particular chemical added and stored at 4 °C for the whole course of monitoring. A non-treated aliquot of RNA was used for the comparison. The isolation of RNA and qPCR as described above was applied in the analysis of the samples. Each sample was treated in biological triplicate; each sample was run as technical duplicate in qPCR.

Results

SARS-CoV-2 positivity in cadavers

The first proband (female, age 94) was tested positive for the SARS-CoV-2 antigen test two days before death. Swabs from the nasopharynx and lung tissue contained a high quantity of SARS-CoV-2 virus before injection and fixation; others were negative (Table 1). Additional samples taken after 5 h of cadaver fixation showed a decrease of viral RNA load in the nasopharyngeal and pulmonary swabs. An additional great decrease was recorded by the third sampling after 8 weeks. Nevertheless, residual viral RNA remained in the nasopharynx and lung tissue, albeit to a small extent.

In the second proband (male, age 71), SARS-CoV-2 virus was detected in the laboratory before death. In this case, samples from the nasopharynx, the tongue, lung tissue and also the anal swab were positive for SARS-CoV-2 presence (Table 1). Samples taken after 24 h (the vessels were clogged and the fixative fluid could not flow ideally, so a longer interval) after the fixation did not show a substantial drop in the quantity of viral RNA, except for the anal swab sample. Nevertheless, similarly to Proband 1, a considerable decrease in the SARS-CoV-2 quantity was recorded after 8 weeks of conservation. First two donors were infected by the alpha variant of SARS-CoV-2.

In the proband 3 (male, age 80 years), SARS-CoV-2 RNA was found in respiratory tract and anal swab during first sampling. Decrease of viral RNA quantity was observed after second sampling. Samples from third collection was

negative at all (Table 1). This proband was infected by the omicron variant of SARS-CoV-2.

A "control proband" (male, age 72 years) was sampled 11 days after death without any fixation. His body laid at home at room temperature for 10 days. Above mentioned PCR method was used to detect the concentration of viral RNA in the "control body". We found 778 000 copies of viral RNA in nasopharyngeal swab, 3 319 copies in oropharyngeal swab, 141 000 copies in lung tissue swab, and 22 680 copies in aqueous humor. This body was infected by the alpha variant of SARS-CoV-2.

Testing of the liquid (70% ethanol) from the stainless fixation tank was completely negative.

SARS-CoV-2 in vitro resistance to fixatives

In vitro test of fixatives showed an immediate detrimental effect of 2.2% formaldehyde and a mix of 2.2% formaldehyde and 3.5% phenol on SARS-CoV-2 RNA. In both of them, a 2 log decrease (from 4×10^4 copies of RNA/µl of non-treated sample to 6×10^2 and 2×10^2 copies of RNA/µl of formaldehyde and formaldehyde/phenol solution treated samples, respectively) was observed immediately after their addition. This decrease was followed by another 2 log decline observed at day 3 (9×10^1 and zero copies of RNA/µl for formaldehyde and formaldehyde/phenol solution, respectively) and no signal until the end of the experiment (Fig. 1). On the contrary, neither in 3.5% phenol nor in 70% ethanol a considerable decline was observed. The amount of RNA was approximately the same throughout the whole experiment (maximally 1 log decline in the case of ethanol; Fig. 1). A non-treated aliquot of RNA (control sample) was stable throughout the whole experiment (Fig. 1).

Discussion

A number of cases of contagion and death due to the transmission of infection from the cadaver to the dissector are known from the history of anatomical dissection (Shoja et al. 2013). The risk of infection transmission from human cadaveric material is also highlighted by other authors (Demiryurek et al. 2002; Tabaac et al. 2013). *Mycobacterium tuberculosis*, hepatitis B and C, HIV and prions causing transmissible spongiform encephalopathies, such as Creutzfeldt-Jakob disease or Gerstmann-Straissler-Scheinker syndrome, are mentioned due to the potential risk of infection. Some pathogens, e.g. *Mycobacterium tuberculosis* or intact *orthopox* virus can survive for years in infected tissues (Healing et al. 1995). Compared to embalmed bodies, a higher risk is associated with frozen cadavers, due to the ability of some infectious agents to withstand low
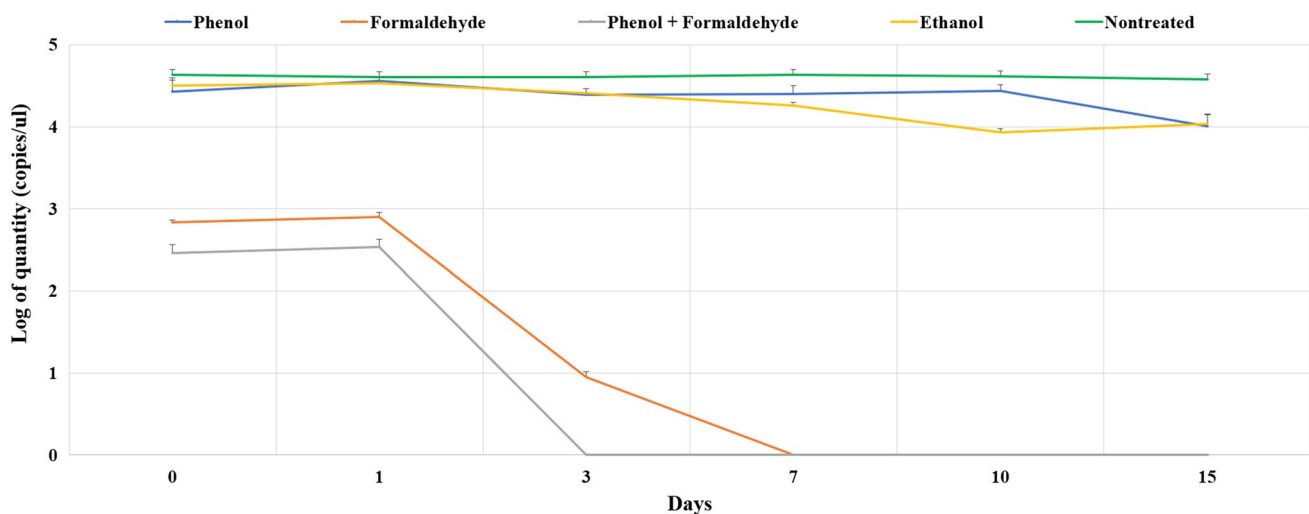
Fig. 1 Effect of fixatives used for embalming dead bodies on SARS-CoV-2 RNA in vitro. Amount of RNA is expressed as number of viral genome per 1 µl

temperatures for a relatively long time (Douceron et al. 1993; Song et al. 2010).

When handling cadavers of individuals who have died as positive for SARS-CoV-2, the increased risk of transmission of infection exists, especially if droplets or aerosols are formed during handling. The risk of infection is potentially highest among the staff performing embalming procedures. For body donation programs during the COVID-19 pandemic, recommendations of the International Federation of Associations of Anatomists were published (Kramer 2020). In deceased, SARS-CoV-2 RNA was detected several days after death (Heinrich et al. 2021; Musso et al. 2021) and even multiplication of the virus (Pfefferle 2020) was demonstrated, which both highlight the necessity to ensure the safety of corpses, specifically for educational purposes. It is known that, after fixation of cadavers with formalin (aqueous formaldehyde solution), the pathogens present in the tissues should be destroyed (Burke and Sheffner 1976). The negative effect on SARS-CoV-2 of disinfectants and chemicals, including those applied in fixing cadavers, was described (Darnell et al. 2004; Kariwa et al. 2006; Rabenau et al. 2005a). Recently, Quondamatteo et al. (2021) demonstrated the neutralization of SARS-CoV-2 using the Vero E6 cell system, after addition of various embalming solutions in vitro (Quondamatteo et al. 2021).

The use of the fixation procedures applied in our study showed a decrease of SARS-CoV-2 RNA quantity after intravenous injection and subsequent post-fixation in the bodies. In Proband 2, the decrease of viral RNA quantity was low (except for the anal sample) after arteriovenous injection. This can probably be explained by atherosclerotic arteries and insufficient penetration of fixatives into the body. Subsequent immersion of the body in the fixative solution

considerably reduced the number of SARS-CoV-2 detected (Table 1), which can be explained by the fixative solutions directly entering into the respiratory tract, including the lungs, and thus justifying the importance of a post-fixation step. Concurrently, the influence of the fixative solution injected into the bodies before the ethanol bath still persists. We are aware, that some degree of RNA degradation in deceased not exposed to fixatives can occur. However, it is known from the literature that SARS-CoV-2 RNA is able to persist in the dead bodies for long periods in high concentrations (Beltempo et al. 2021; Musso et al. 2021; Plenzig et al. 2021; Bonelli et al. 2022) and the ability of RNA to persist in environment has been also commonly used in forensics practice (Sirker et al. 2016). The capability of SARS-CoV-2 or its RNA to persist and to be detected in human tissues several days after the death was also confirmed in "control" deceased. More than 1×10^5 copies of viral RNA in his nasopharyngeal and lung swab, 1×10^4 in aqueous humour and more than 1×10^3 copies of viral RNA in oropharyngeal swab were detected in this COVID-19 positive person, who died at home and was found more than ten days after his dead. With respect to the abovementioned papers (Beltempo et al. 2021; Bonelli et al. 2022; Musso et al. 2021; Plenzig et al. 2021), high concentration of viral RNA in the "control body" several days after the death (without action of fixatives) and considerable drop in RNA concentration in our experiment (especially in Proband 1 and 3), the effect of fixation procedures on SARS-CoV-2 RNA is more than likely. Detrimental effect of fixatives on SARS-CoV-2 RNA was also confirmed by negative results of solution from fixation tank.

Published data on SARS-CoV-2 RNA degradation are mostly related to the effect of disinfectants or components

used to preserve tissue samples (e.g. paraffin-embedded tissues). Formaldehyde is the main prefixation agent in paraffin-embedded tissue preparations; it is an excellent preservative of tissue morphology. On the other hand, it causes fragmentation of nucleic acid, modification of nucleotides and crosslinking between nucleic acid strands, which all lead to fragmentation of RNA, low yield of extracted RNA and subsequently less effective PCR (Malnar and Rezen 2019). The conditions of prefixation are crucial, which explain different RNA yields and integrity in different studies using paraffin-embedded tissues (Matsuda et al. 2011; Su et al. 2004). Formaldehyde, a disinfectant used for fomites or excreta, shows a detrimental effect on coronavirus within seconds, even at concentrations below 1% (Rabenau et al. 2005a, b). Similarly, ethanol employed as a disinfectant or storage medium, displays a negative impact both on coronavirus (Kratzel et al. 2020; Rabenau et al. 2005a). SARS-CoV-2 is an enveloped virus that contains a lipid layer on its surface, which is important for its infectivity (Palacios-Rapalo et al. 2021). Ethanol as non-polar solvent dissolve the lipid surfaces of enveloped viruses including coronaviruses (Basak and Deb 2021).

Our data obtained from the embalmed bodies were confirmed by the in vitro experiment using SARS-CoV-2 RNA (Fig. 1). A destructive effect was observed either in formaldehyde itself or in its combination with phenol. This supports the previous studies showing the negative effect of formaldehyde on SARS-CoV virus multiplication (Darnell et al. 2004; Rabenau et al. 2005a, b) and the detectability of RNA (Malnar and Rezen 2019). According to Malnar and Rezen (2019), degradation of RNA by formaldehyde is fast and a major reduction of extractable RNA occurs immediately after its addition. This is also consistent with our results. It is evident from our in vitro experiment that formaldehyde plays a fundamental role in RNA degradation, as the impact of phenol itself is insignificant. The same applies to ethanol. Both phenol and ethanol are used in DNA/RNA extraction protocols and thus their negative effect on nucleic acids should not be distinctive (Su et al. 2004). The anti-viral effect of alcohols (ethanol, phenol) consists of denaturation of proteins, which will probably be the main mechanism of their action on SARS-CoV-2 during embalming procedure. The detection of low levels of SARS-CoV-2 RNA in probands No. 1 and 2 was, therefore most likely due to the remaining RNA fragments and their detection by highly sensitive qPCR methods, rather than the presence of viruses alone (Calabrese et al. 2020; Malnar and Rezen 2019). Despite the limitations of our study in omitting SARS-CoV-2 viability assays and the relatively small number of cadavers tested, based on our in vitro experiments, results retrieved from the embalmed cadavers, and data published previously we believe that the fixative and post-fixative procedures used in this study ensure that the cadavers are free of infection and are therefore suitable and safe for educational purposes.

Conclusions

Embalming treatment of deceased with a solution of phenol, formaldehyde, glycerol and ethanol, and subsequent immersion in 60 -70% ethanol led to considerable drop of SARS-CoV-2 RNA. It is evident that perfusion by fixatives itself is not ideal, so the subsequent placement of the body in the fixative solution (post-fixation) is important and justified. The results of the in vitro experiment with fixative compounds showed the major and detrimental effect of formaldehyde on RNA, with only a negligible effect of phenol and ethanol. Deceased treated with the procedures outlined in this study should not pose an increased risk of SARS-CoV-2 infection during their handling and are therefore suitable for anatomical dissection and student teaching. However, it is advisable to take a cautious approach in accepting donors with known covid infection, especially based on the fact that, perfusion may not be efficient enough due to vascular problems which would reduce the efficiency of the anti-viral activity of the embalming process. As precautionary measures, strict hygiene measures should be observed when handling a body.

Data availability Data available upon request from the corresponding author.

Declarations

Conflict of interest The authors declare that there are no conflicts of interest regarding the publication of this paper.

Ethical approval We declare that the study has been approved by the Ethics Committee of the Faculty of Medicine of the Masaryk University and has therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and all subsequent Revisions.

Informed consent Used cadavers of the study were registered in the Masaryk University Anatomical Remains Program. We declare that we have obtained informed consent from the donors.

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