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Simple methods for cerebrospinal fluid collection in fetal, neonatal, and adult rat

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ABSTRACT

Background: Cerebrospinal fluid (CSF) collection and its analysis are common medical practices useful in the diagnosis, therapy, and prevention of central nervous system (CNS) disorders. In recent years, several types of research have improved our insight into CSF and its role in health and disease. Yet, many characteristics of this fluid remain to be fully understood.

New methods: Here, we describe how to collect CSF from embryonic, postnatal, and adult stages of the rat. In adults, CSF can be collected through simple stereotaxic surgery to expose the membrane overlying the cisterna magna (CM) of an anesthetized rat and collection of CSF through micropipette puncture through the membrane. In embryos and pups, CSF is aspirated, using a fire-polished micro-capillary pipette, from the CM of animals.

Results: Application of these methods provides the maximum volume of pure, uncontaminated CSF (embryonic day 19: 10–15 microliter, postnatal day 5: 20–30 microliter, adults: 100–200 microliter) with a success rate of approximately 95% in every age.

Comparison with existing methods: Compared to the existing protocols, these methods obtain considerable volumes of CSF, which may accelerate the measurement of biological markers in this fluid. Also, these techniques do not require surgical skills and according to the practical points mentioned during sampling, the procedures can be performed in rapid fashion.

Conclusion: We describe simple methods for collecting CSF in live rats. These protocols provide clean, uncontaminated CSF for experiments to understand the exact role of this fluid in the development and maintenance of the CNS health.

1. Introduction

Cerebrospinal fluid (CSF) is a vital body fluid that is colourless and watery in appearance. It is produced by the choroid plexus (Jacobs et al., 2011) and is secreted into the lateral, third and fourth ventricles of the brain. From there, it flows into the subarachnoid space, which surrounds the brain and spinal cord, as well as the cisterna magna (CM). CSF is an isotonic, iso-osmotic, and balanced ionic solution that contains a range of essential substances, including cholesterol, proteins, cytokines, growth factors, and nutrients (Odland et al., 2011; Shapey et al., 2019; Atchley et al., 2022). This makes it an important physiological fluid that supports the central nervous system (CNS) (Bueno et al., 2020; Gato et al., 2020; Miyan et al., 2020) While many anatomy and medical textbooks describe CSF as merely filling the space of the ventricles and serving as a cushion for the brain or a waste disposal system, this ignores the crucial role of CSF in signalling and supporting the growth and development of the CNS. It contains signalling molecules including SHH, OTX, WNT, FGF, and other growth factors and morphogens, as well as specific molecules and proteins synthesized by the choroid plexus for CSF (Arner et al., 2003; Sivakumar et al., 2008; Requena-Jimenez et al., 2021; Yang et al., 2021; Pandamooz et al., 2022) that are modified in different neurological conditions, shown recently for different

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aetiologies of human neonatal hydrocephalus (Naureen et al., 2013, 2014). Additionally, it acts as a growth medium and supportive environment for the developing and adult brain, stimulating stem cells and neuron migration and its pathways are required for many of its functions (Miyan et al., 2020). During early brain development, the CSF appears to be the major route of entry for nutrients into the brain as the brain vasculature is not well developed at this stage. Metabolic support for the cerebral cortex is an important function of CSF and recent research demonstrates that CSF is critical to cerebral folate metabolism and that changes in CSF can result in profound changes in folate metabolism in different conditions including hydrocephalus and Alzheimer's disease (Miyan et al., 2022; Jimenez et al., 2019). CSF also plays a role in various body states such as sleeping, appetite, and brain restoration after trauma, where it can contain associated factors.

Investigating CSF in terms of volume, protein, and molecular content is vital in elucidating the role of CSF in normal physiology of the brain as well as pathophysiology and pathogenesis of neurological conditions such as schizophrenia (Murphy et al., 2020) and autism spectrum disorder (Runge et al., 2020), as well as neurodegenerative diseases including Alzheimer's (Naz et al., 2022), hydrocephalus (Requena-Jimenez et al., 2021), autoimmune diseases such as multiple sclerosis, and brain cancer (Reichel et al., 2011). Many questions remained unresolved regarding the mechanisms underlying these diseases including the role of noted alterations in the CP-CSF system and CSF composition changes (Fame et al., 2020; Cousins et al., 2022).

In order to have a comprehensive insight into the role of CSF in development, and CNS homeostasis, further studies are necessary. To do this, it is vital to be able to collect a considerable amounts of pure, uncontaminated CSF for analysis. Although, CSF collection in humans is accessible and obtained through a lumbar puncture, CSF collection in rats is challenging due to limited volume, especially in the embryonic stage, and the high risk of blood contamination. In the adult rats, different procedures have been tested for collecting uncontaminated CSF including cisterna magna cannulation (Consiglio and Lucion, 2000), collection from the lumbar region via a lumbar puncture (Wang et al., 2005), percutaneous methods without any incision (Nirogi et al., 2009), and recently Barthel et al. described a modified method for CSF collection from visible cisterna magna (Barthel et al., 2021). Although CSF collection in embryos and pups gives less fluid for analysis owing to their small sizes, it is relatively easier than in adults using effective techniques. In 2015, Rodríguez-Fanjul and colleagues described direct puncture of the cisterna magna as a simple method for CSF collection in rat pups that doesn't require complex surgical experience (Rodríguez-Fanjul et al., 2015). Also, Zappaterra et al. introduced ventricular embryonic CSF collection through direct puncture of the lateral ventricles (Zappaterra et al., 2013). All these approaches have advantages and disadvantages. Therefore, in this study, we aimed to test applicable methods for collecting embryonic, postnatal, and adult CSF in rats. The described protocols provide clean, uncontaminated CSF for experiments to better understand the exact role of CSF in the development and maintenance of CNS health, and for developing new diagnostic tools and therapeutic strategies.

2. Material and methods

2.1. Animals

Animal care and protocols were followed as approved by Animal Care Committee of Shiraz University of Medical Sciences that follow internationally agreed ARRIVE guidelines. This study has been approved by Research Ethics Committees of Laboratory Animals - Shiraz University of Medical Sciences (Approval ID: IR.SUMS.AEC.1402.002).

In the present study, 15 adult Sprague-Dawley male rats (230–280 g), 15 females (180–210 g), and 15 pregnant rats (300–350 g) were used. The rats were housed in a standard pathogen-free environment with ad libitum access to food and water. Housing conditions included constant temperature control between 20 and 22 °C, relative humidity 50–60%, and 12/12 h reversed light/dark cycle.

2.2. Adult CSF

2.2.1. Anesthesia

The rats were anesthetized with a mixture of 100 mg/kg Ketamine (NARKAMON, Bioveta, Czech Republic) and 5 mg/kg Xylazine (XYLASED, Bioveta, Czech Republic) which were administered subcutaneously behind the neck. Furthermore, Lidocaine (Lidonaline, NASR, Iran) as a local analgesic was injected subcutaneously into the operation area.

2.2.2. Preparing suction syringe

To prepare a suction syringe for collecting CSF in adult rats; first, a 27 G dental needle was connected to a polyethylene-10 (PE-10) tube and the other end of the tube was attached to a 1 mL syringe for inducing negative pressure. Due to the possibility of creating a large negative pressure that can induce rupture of surface blood vessels on the brainstem, choosing an appropriate size of syringe is important (Fig. 1A). All materials used in this procedure were sterile and prepared inside a laminar flow hood Class I.

2.2.3. Surgery

After shaving the fur of the neck region, the anesthetized rat was placed on a stereotaxic frame (TSE Systems, Germany) and fixed with ear bars. The head's angle relative to its body is nearly 45–90° (Barthel et al., 2021) because itis of great importance to make the distance between the first vertebrae and the foramen magnum more palpable for touching the occipital bone and precisely under this bone the thin dura is visible (Zarghami et al., 2013). It is essential to regulate this position, because excessive bending of the neck also decreases the distance between the dura and the surface of the brainstem (medulla). Thus increases the risk of penetrating into the brainstem. Each surgical tool and stereotaxic frame were disinfected with 70% ethanol. Before making any incision, we disinfected the neck's skin with povidone-iodine and 70% ethanol using sterile cotton swabs. Next, a 2 cm incision along the midline and between the ears was made by scalpel. Lidocaine was injected subcutaneously as a local analgesic and also to decrease the probability of bleeding during surgery. Besides, the area was cleaned with cold PBS. For accurate localisation of the CM, the surface muscles and fascia were dissected with scissors and micro forceps. It is worth noting that, there are 3 major layers of neck muscles over the dura of CM. The first layer was detached with scissors and forceps, and no bleeding occurred at this stage. Then, the next layer consisted of two longitudinal blocks of muscle, again dissected with forceps. Finally, the third layer, a thin sheet sitting directly on the dura, was detached with fine forceps and this step should be done carefully, because there is a possibility of damage to the thin and delicate membrane of the dura by the forceps. Thus, the occipital bone was palpable and exactly under this area, the soft tissue and triangular shape of CM was visible (Fig. 1 B-D). It is worth noting that at the base of skull in the occipital bone, there is a large oval opening, the foramen magnum and just above the level of this structure, there is the CM. The area was cleaned with sterile cold PBS to avoid contamination, the needle was inserted gently into the CM with the aid of a stereotaxic guide, making it necessary to insert the needle vertically. The colourless CSF enters the tube immediately the needle is inserted. Here, a negative pressure, induced by pulling on the syringe, can increase the volume of collected CSF. This must be done carefully as blood contamination occurs if any cisternal blood vessels are damaged (the diameter of blood vessels on the brainstem visibly increases as suction is applied). If any blood enters into tube, the PE tube can be cut a distance ahead of the blood/CSF interface to maintain purity of the

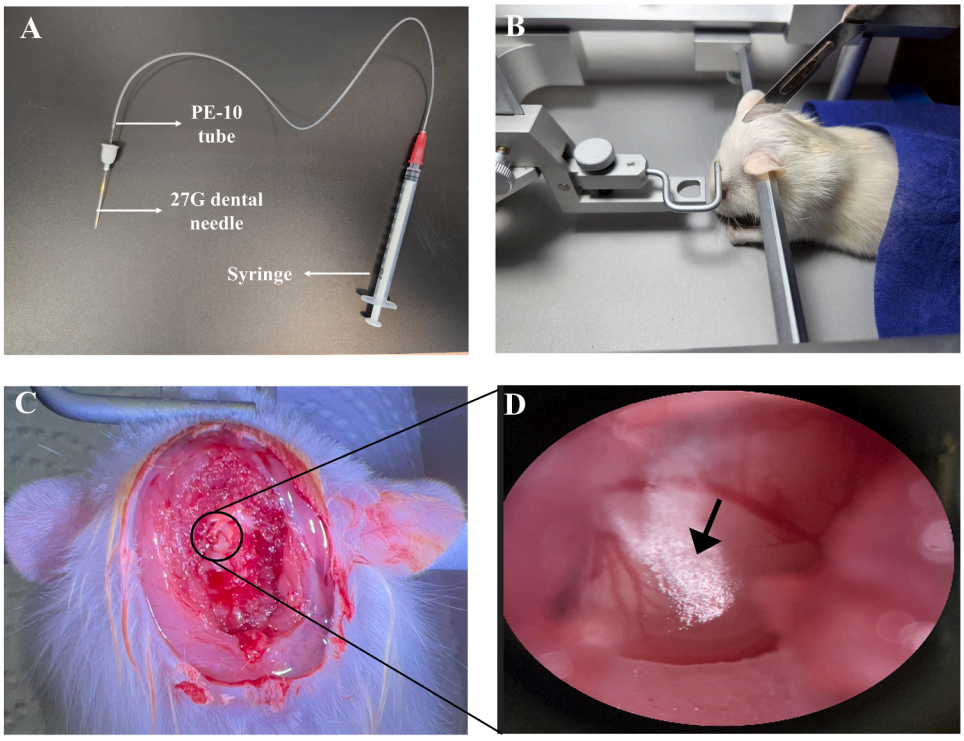

Fig. 1. Adult rat CSF collection. (A) 27 G needle connected to a PE-10 tube is used for CSF sampling. (B) Lateral view: fixing the animal in the stereotaxic frame with ear bars and the angle of head to its body must be nearly 90°. Immediately after fixing the head in the device, the midline incision was made between ears with scalpel. (C) Dorsal view: through dissection of muscles and fascia, the occipital bone was palpable and visible cisterna magna is shown. (D) Enlarged view of cisterna magna under the microscope. Arrow shows the position of needle insertion.

previously collected CSF from mixture of plasma and CSF.

Samples were ejected into sterile microtubes and centrifuged at 10,000 G at 4 °C for 10 min to remove any contaminating cells (it must be noted that CSF was collected from open and visible CM, that reduce the possibility of contamination with interstitial fluid and surrounding tissue) The supernatant was collected and stored at − 80 °C until further analysis (supplementary video 1).

Supplementary material related to this article can be found online at doi:10.1016/j.jneumeth.2023.109971.

2.3. Postnatal CSF

2.3.1. Preparing needle. A custom needle was made of a fire-polished microcapillary pipette connected to a 100 µl tip, to collect small amounts of CSF in postnatal stages (Fig. 2 A). Before use, the needles were sterilized by autoclave. The capillary tube sharpness is of great importance as a blunt end results in tissue destruction and sample contamination.

2.3.2. CSF collection. Rat pups on the fifth day after birth (P5) were used in this experiment. Hypothermia was used to provide a short reversible period of anaesthesia during which the CSF sample can be collected. A short acting anaesthetic (isoflurane 4%) could be used as an alternative to hypothermia. First, pups were washed with sterile PBS, and each pup was put in a container and immersed in ice water for 10-20 min. Once the pup was anesthetized, the head and neck areas were disinfected with 70% ethanol, and the head was flexed down onto the chest at angle of 90° to its body to have a clear view of CM, as shown in Fig. 2 B. Under bright light, the pipette was moved on the skull between ears and exactly under this hard part of skull, called occipital bone, the soft tissue and the diamond shape of the CM can be distinguished. The needle was inserted vertically into this area very slowly, in order to decrease the possibility of any damage (Fig. 2 C). Due to the capillary properties of the pipette, and the inherent pressure in CM, CSF was observed readily flowing into the pipette negating the need to apply any suction pressure. Clear, blood-free CSF samples collected, centrifuged and frozen as described for adult samples. All procedure was performed

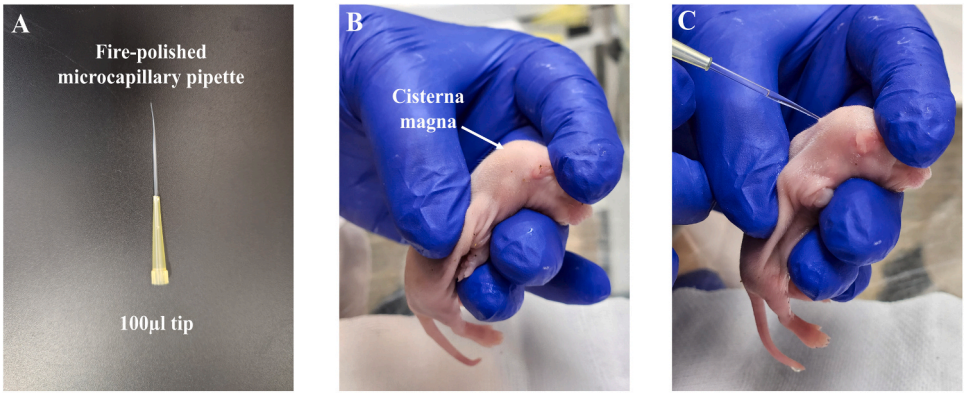

Fig. 2. Rat pup CSF collection method. (A) The picture shows a fire-polished microcapillary pipette attached to 100 µl tip that is used for CSF sampling. (B) Once the pup was numb, it was held between our fingers, and its head flexed downward at an angle of 90° to its body for distinguishing the exact location of the cisterna magna that is shown in the picture. (C) After finding CM, the needle was inserted vertically and the colourless fluid flows out automatically.

inside a laminar flow hood to minimize the risk of contamination (supplementary video 2).

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2.4. Embryonic CSF

Male and female rats were mated and gestational day 0 was determined by the detection of a vaginal plug. On embryonic day 19, pregnant rats were anesthetized with Ketamine (50 mg/kg) and Xylazine (10 mg/kg), a C-section was performed rapidly, and fetuses were placed in cold PBS in a sterile petri-dish. Each fetus was cleaned from any extra-embryonic tissues and blood contamination by sterile cold PBS wash.

2.4.1. CSF collection. To collect CSF from rat embryos; custom-made needles were used as described for postnatal CSF collection. Fetal head and neck were disinfected with 70% ethanol and sterile cotton swabs. The experiment was performed inside the laminar flow hood. The CM area was found under the bright light, then, the needle was inserted into this region to obtain the maximum volume of CSF. The lateral ventricles were distinguished by transillumination through the head and CSF collection was performed from these sites as well. After transferring samples into sterile microtubes, centrifuging and storing conditions were similar to the previous described CSF collection methods (Fig. 3 A-B) (supplementary video 3).

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It is worth noting that, all above mentioned methods were carried out on live animals and following collection, the animals were sacrificed.

2.5. CSF analysis

In order to measure the quality of CSF samples, two techniques were carried out: protein assay, and cell counting. For these methods, it was necessary to pool samples of each stage individually before freezing. Total protein concentration was determined by the BCA assay kit (23228, Thermofisher, USA) with absorbance measured at 570 nm wavelength. In addition, the presence of white blood cells (WBCs) and red blood cells (RBCs) were defined with cell counting that was performed manually using a haemocytometer.

3. Results

For adult CSF collection, 20 male and female adult Sprague Dawley rats were used. The average time required from first incision to collect CSF was 8–10 min. Here, 19 uncontaminated samples were collected by visual control, and just one sample was assumed to be contaminated due to the yellow appearance. The average amount of collected CSF was in the range of 100–200 µl per individual rat at adult stage. In embryonic and postnatal stages, generally 10 pregnant rats were used in which C-section was performed on four of them at 19th day of gestation, and the remaining pregnancies were taken to term giving birth to litters that were used for CSF collection on the 5th postnatal day. CSF was collected with a success rate of 96%, with an average volume of 10–15 µl on E19 and 20–30 µl on P5. This technique is fast and it only takes 1 min per embryos or pup. No sign of blood contamination was detected with microscopic examination and few cells were seen (WBCs; 2–4, RBCs; 0–2). Detailed information is given in Table 1.

4. Discussion

CSF is attracting greater attention in recent research as an active signalling pathway in different brain/body states, and in development and neurogenesis. Research has identified regulatory roles of CSF in healthy and diseased conditions due to the presence of different proteins and molecules in this fluid (Requena-Jimenez et al., 2021; Cousins et al., 2022; Lehtinen et al., 2011). The biochemical and cellular content of CSF can reflect the brain responses to diseases and injury more accurately owing to direct contact of this fluid with extracellular space in CNS and through its role in removing toxins and waste products from the brain, partly through the glymphatic pathways (Reddy and van der Werf, 2020; Reeves et al., 2020; Tice et al., 2020). Thus, CSF analysis is of great importance as the exact functions of its components need to be elucidated in driving development, supporting healthy function, or resulting from changes leading to neurological conditions and diseases, as has been found for hydrocephalus. CSF may present novel therapeutic methods including manipulation of choroid plexus gene expression to modify CSF composition. In addition, investigating the CSF composition

Table 1
The summary of the animal information used for CSF collection and results.

	Embryonic	Postnatal	Adult
Average weight (g)	2.3–3.5	8–12	235–267
Animal numbers	42	58	20
Sample volume (µl)	5–10	20–25	125–203
RBCs/µl	0–2	1–2	0–1
WBCs/µl	3–4	2–4	0–2
Proteins (µg/mL)	3242.43	1777.33	341.98

CSF, cerebrospinal fluid; RBCs, red blood cells; WBCs, white blood cells.

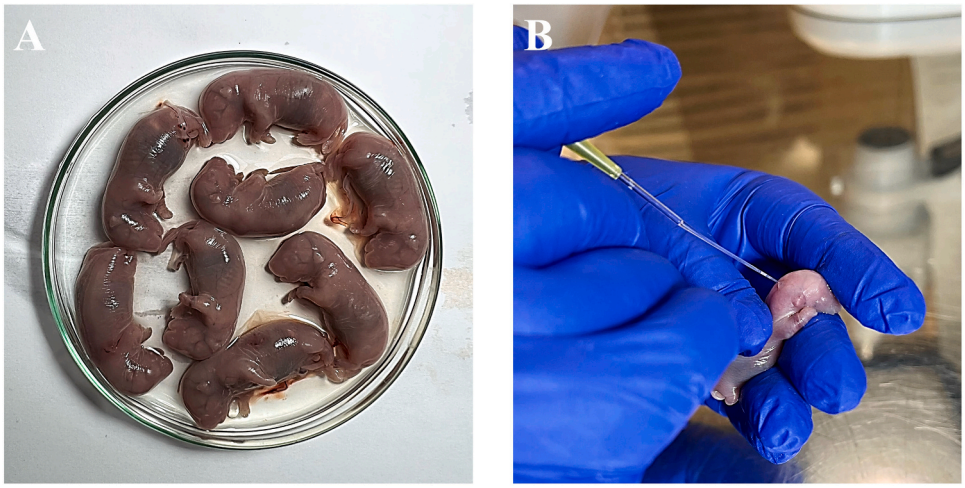

Fig. 3. Rat embryo CSF collection method. (A) Cleaning litters from any extra embryonic tissues and blood contamination with cold PBS. (B) Placing the fetus between our fingers and under the bright light, CM is visible and needle can be inserted.

by age is invaluable as recent studies have reported distinct proliferation potential in adult CSF in comparison with the embryonic ones (Mashayekhi and Salehi, 2006; Miyan et al., 2006; Buddensiek et al., 2010). Hence, the ability to collect CSF in different ages is profitable for future research. In this study, we describe easy to perform methods to collect CSF samples in different stages of rat development that give clean, uncontaminated CSF.

Up until now, several procedures have been carried out for adult CSF collection. In 2000, the cisterna magna cannulation method was published by Consiglio and Lucion (2000). which had the advantage of multiple CSF collection in the same rat but was a time consuming method with high risk of infection due to the presence of implanted cannula. Five years later, lumbar CSF collection was used that was a modified version of De la Calle et al. method, which addressed sample volume and risk of blood contamination, but sample volume remains low with high rates of contamination in comparison with our method described here (Wang et al., 2005; De la Calle and Paíno, 2002). Another study demonstrated a percutaneous technique which does not involve any incision, was less invasive but the amount of CSF was comparatively low (Nirogi et al., 2009). More recently, a visible CM method was described, which is similar to our method (Consiglio and Lucion, 2000). Although, this method was applicable, we have amended it for easier and more accessible sampling. While all of the above-mentioned methods are applicable in rats, they need special surgical skills and precision. Hence, we established methods, which can provide large amounts of CSF (125–203 μl) per rat that do not require experienced operator for collection. The important advantage of this method over others is the visibility of the CM after dissection of overlaying muscles. This is the most important route to uncontaminated CSF samples as it leads to a direct view of the vasculature. Sampling CSF is done with the aid of a stereotaxic guide which accelerated the operation and provides minimal needle movement that prevents contamination.

The macroscopic examination of collected CSF from our methods showed no sign of yellow or red colour indicating samples were not contaminated with blood. Furthermore, the number of RBCs counted microscopically was very low, and this is a vital consideration for any method as blood contamination can completely alter biological markers in CSF. We measured protein concentration in CSF samples of different stages. Based on our results, the total protein concentration of CSF in embryos was higher in comparison to adult and postnatal stages. The data of our study was consistent with those from previous research, that was reported 284 ± 13 mg/dl in embryos (day 19), 103 ± 11 mg/dl in newborns (day 10), and 24 ± 8 mg/dl in adult rats (Dziegielewska et al., 1981). Since the CSF protein levels reported in this study are very similar to those detected by Dziegielewska and colleagues, this indicates no evidence of significant plasma contamination of the samples.

In recent years, a few methods have been published for embryonic and postnatal CSF collection. Due to the small size of rat embryos, collecting colorless and pure CSF is challenging. Zappaterra et al. in 2013, described a method to isolate uncontaminated samples of ventricular CSF and it was efficient but special equipment was required (Zappaterra et al., 2013). A couple of years later, a postnatal CSF collection technique was described by Rodríguez-Fanjul et al. that was similar to an embryonic sampling method (Rodríguez-Fanjul et al., 2015). The main differences were the region of CSF sampling and the type of needle. We have described a reproducible method for collecting CSF in fetuses and neonates. The great advantage is the simplicity of the method that does not require any special equipment or skill that makes it easy to use. We can collect the maximum volume of CSF in these stages from the cisterna magna without blood contamination. The use of fire-polished microcapillary pipettes allows the operator to detect blood contamination and to stop the sampling procedure to preserve the purity of already collected CSF. Flexing the head downward with the angle of 90° allows to find the exact location of CM for perfect insertion of the collecting microcapillary tube. Due to ease, advantages and practicality of the methods described in this study, it is expected that these methods could motivate scientists for more investigation on CSF due to feasible access.

5. Conclusion

In summary, we present suitable and simple methods for CSF collection in three stages of rat development that provide sufficient volumes of uncontaminated samples for analyses. These techniques do not require surgical skills and according to the practical points mentioned during sampling, the procedures can be performed in rapid fashion. Compared to the existing protocols, these methods obtain considerable volumes of pure uncontaminated CSF, which may accelerate the measurement of biological markers in this fluid.

CRediT authorship contribution statement

Mohammad Saied Salehi, Mehdi Dianatpour, Sareh Pandamooz, Jaleel A. Miyan: Conceptualization. **Zahra Tavakkoli, Mohammad Saied Salehi, Sareh Pandamooz, Fatemeh Jameie, Moosa Rahimi, Farhad Koohpeyma**: Methodology. **Zahra Tavakkoli**: Writing – original draft. **Sareh Pandamooz, Jaleel A. Miyan**: Writing – review & editing. **Mohammad Saied Salehi, Mehdi Dianatpour, Sareh Pandamooz, Jaleel A. Miyan**: Supervision. **Sareh Pandamooz**: Funding acquisition.

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Declaration of Competing Interest

The authors declare that they have no competing interests.

Data availability

Data will be made available on request.

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