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High performance collection of cerebrospinal fluid in rats: Evaluation of erythropoietin penetration after osmotic opening of the blood–brain barrier

Natalia Ceaglio[a,*], Gustavo Orozco[b], Marina Etcheverrigaray[a], Mónica Mattio[a], Ricardo Kratje[a], Norma Perotti[a], Marcos Oggero[a]

[a] Cell Culture Laboratory, School of Biochemistry and Biological Sciences, Universidad Nacional del Litoral, Ciudad Universitaria, Paraje "El Pozo", C.C. 242, S3000ZAA Santa Fe, Argentina
[b] Zelltek S.A., PTLC, RN168, S3000ZAA Santa Fe, Argentina

HIGHLIGHTS

- An improved method to sample CSF from the cisterna magna of rats was developed.
- The procedure renders blood-free CSF with a high percentage of animal survival.
- The method is simple, fast and avoids the use of stereotaxic instruments.
- The success of the method lays in the use of topically injected ephinephrine/lidocaine.
- The blood-to-brain influx of rhEPO after osmotic opening of the BBB was analyzed.

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ABSTRACT

An important issue to be considered when studying a new drug for treatment of central nervous system (CNS) diseases is its ability to cross the blood–brain barrier (BBB) and distribute throughout the brain. As cerebrospinal fluid (CSF) has demonstrated to be an invaluable reservoir to study CNS availability of therapeutic proteins, we have developed an improved method for CSF sampling from the cisterna magna of rats. The technique enables the simple and rapid collection of adequate quantities (50–75 μl) of blood-free CSF, rendering a high percentage of animal survival (99%) without clinic or neurological consequences. Its success in avoiding blood contamination of CSF lays in the use of a mixture of lidocaine/ephinephrine topically injected in the rat's suboccipital area and neck. Another relevant feature of the methodology is its low cost, since the puncture device can be easily assembled with cheap and available materials and, more importantly, neither expensive stereotaxic equipment nor frame is required. The present method is demonstrated by studying the CSF pharmacokinetics of recombinant human erythropoietin (rhEPO), a well-studied therapeutic candidate for neurological diseases. Moreover, we applied this technique to evaluate a strategy of osmotic disruption of the BBB to achieve a faster delivery of rhEPO into the CNS.

1. Introduction

Delivery to the central nervous system (CNS) remains a main concern in the development of drugs intended for the treatment of neurological diseases. Thus, the ability of a therapeutic candidate to achieve CNS entry needs to be studied in early stages of the development process.

The complexity of CNS nature makes brain penetration assessment of drugs an intricate task that cannot be analyzed on the basis of a single parameter. The existence of separate compartments with different pharmacokinetic properties in the brain demands the integration of data from distinct assays that can give raise to a coherent model of brain penetration and distribution in drug discovery. Novel concepts to accomplish this rely on the determination and combined interpretation of the rate of permeation across the blood–brain barrier (BBB), the extent of brain penetration and the intra-brain distribution in terms of total and unbound drug levels through different *in vitro* and *in vivo* assays (Di et al., 2008).

Evaluation of permeability of a drug is a measure of the rate of permeation across the tight layer of brain endothelial cells, and can be performed *in vitro* using culture models which intend to reconstitute the BBB. However, permeability data only give information

* Corresponding author. Tel.: +54 342 4564397; fax: +54 342 4564397.
E-mail address: nceaglio@fbcb.unl.edu.ar (N. Ceaglio).

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about one aspect of CNS penetration, i.e. rate, and the extent of brain penetration needs to be determined *in vivo*. The most common method for evaluating CNS penetration is the measurement of the whole brain homogenate/plasma ratio in rodents. However, homogenization of the brain tissue destroys all compartments and so these data do not provide reliable information on the concentration at the site of action. Moreover, total brain concentrations can be confused with high non-specific binding or sequestration of a compound in the brain tissue (Reichel, 2009). Discrimination of the free fraction of the drug, which eventually elicits the therapeutic action, can be performed by relatively new *in vitro* studies based on the equilibrium dialysis of the compound between buffer and brain homogenate (Maurer et al., 2005).

Approaches to circumvent the limitation of the crude measurement of total levels in brain include microdialysis of ISF and CSF sampling. The latter is the most practical approach in terms of effort, cost and throughput. The use of CSF as an appropriate CNS sampling site relies in the assumption that CSF concentration represents the unbound concentration in the brain when steady-state equilibration of freely diffusible drug concentration is achieved throughout the brain, regardless of whether it exists at a single or multiple sites (Shen et al., 2004). This hypothesis considers that the brain exhibits kinetic characteristics of a homogeneous compartment at steady-state. Consequently, the critical issue to consider CSF sampling as a reference for assessing the extent of brain delivery is whether CSF concentration is in kinetic synchrony with the biophase concentration, i.e. the compartment where the drug effectively drives the pharmacologic effect. Many studies have provided proof-of-concept that CSF concentration can be an appropriate indicator of drug availability to the CNS for the hydrophilic or large molecular mass compounds with poor to moderate permeability (Shen et al., 2004), such as therapeutic proteins. However, these studies are not frequently performed in rat models due to the complexity to obtain CSF samples and low sample volume yields.

Collection of CSF from rats can be achieved by direct lumbar puncture into the intrathecal space (De la Calle and Paino, 2002) but it has the disadvantage that the lumbar canal of rats is very narrow for perpendicular puncture, so that CSF sampling has a low success rate (Wang et al., 2005). Besides, for studies in the cranial region this sample is not useful because its biochemical composition may not fully reflect the neurological function that is fulfilled in situ.

The preferred origin of CSF in rats is the cisterna magna. Many extraction methods involve the implantation of a permanently positioned long cannula or catheter, a procedure that inevitably becomes associated with bleedings in variable frequency and injury to the nervous tissue (Consiglio and Lucion, 2000; Huang et al., 1995; Swartz and Stenberg, 1980). Other methodologies are based on the surgical insertion of a tube epidurally via a hole in the calvarium or affixed to the posterior atlanto-occipital membrane (Huang et al., 1996), with the disadvantage of exposing the rat to a trauma with consequent injury. In all of these cases, a disruption of the cisterna magna is performed previously to the collection. More recently, methods that involve cisterna puncture at the moment of sampling have been developed (Pegg et al., 2010). However, all these established techniques are subjected to a variable degree of blood contamination in the CSF. Moreover, they employ a stereotaxic device or frame that confers complexity to the methodology and increments its cost.

We have developed a simple technique for the collection of CSF from the cisterna magna of Wistar rats with the following distinguishable features: (1) very low risk of blood contamination due to local injection of a mixture of lidocaine–ephinephrine that gives rise to a simultaneous in situ vasoconstriction and anesthesia; (2) complete elimination of complex equipment such as stereotaxic instruments; and (3) the potential of being performed by a trained operator in a very short time.

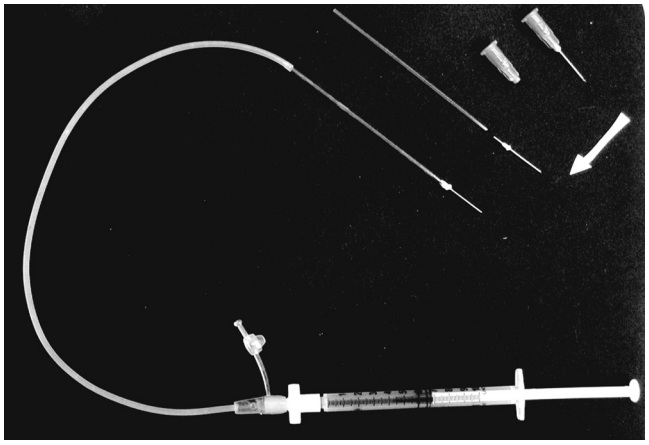

Fig. 1. Construction of puncture device.

In this work, we also demonstrate the application of this procedure for the assessment of recombinant human erythropoietin (rhEPO) pharmacokinetics in CSF after osmotic opening of the blood–brain barrier, which stands as a CNS delivery strategy for drugs with low passage through this selective barrier.

2. Materials and methods

2.1. Animals

Two-month-old female Wistar rats (Comisión Nacional de Energía Atómica, Argentina) with an average body weight of 250 g were housed in a temperature-controlled room at 23 °C with a 12 h light/dark cycle and free access to food and water. Animal use was carried out in accordance with the "Guide for the Care and Use of Laboratory Animals" (National Research Council, USA, 2010).

2.2. Anatomy study of rats

The X-ray images of the skull and neck of rats in front and profile position were taken from fresh cadavers, using KODAK-MIN R2000 films with fine grain emulsion (Kodak, Argentina).

2.3. CSF sampling method

2.3.1. Construction of puncture device

A 25 G × 5/8 in. needle (Neojet, China) was removed from its hub and attached to a non-heparinized glass capillary tube (75 mm long, external diameter 1.50 mm, Parawall, Argentina). This tube was attached to a silicon catheter with a 2 mm diameter (nasogastric tube K-33, Laboratorio Rivero, Argentina) connected to a syringe (TJ, Argentina) (Fig. 1).

2.3.2. Collection of CSF from cisterna magna

Rats were anesthetized with a mixture of ketamine (80 mg/kg) and xylacine (10 mg/kg) via intramuscular injection and placed in a surgery table. The fur from the back of the neck was shaved and the area was disinfected with chloroxylenol. Then, the skin, the subcutaneous tissue and the deep soft tissue from the back of the suboccipital area and neck were infiltrated with a solution of lidocaine 20 mg/ml and epinephrine 5 μg/ml (Scott-Cassara, Argentina). A midline incision of the skin and subcutaneous tissue was made in cephalo-caudal direction with a No. 15 blade scalpel. The superficial muscles were separated by blunt dissection until visualization of the prevertebral aponeurotic plane. Following hemostasis control, the rats were held manually in order to achieve head hyperflexion for a maximum opening of the suboccipital space. To avoid

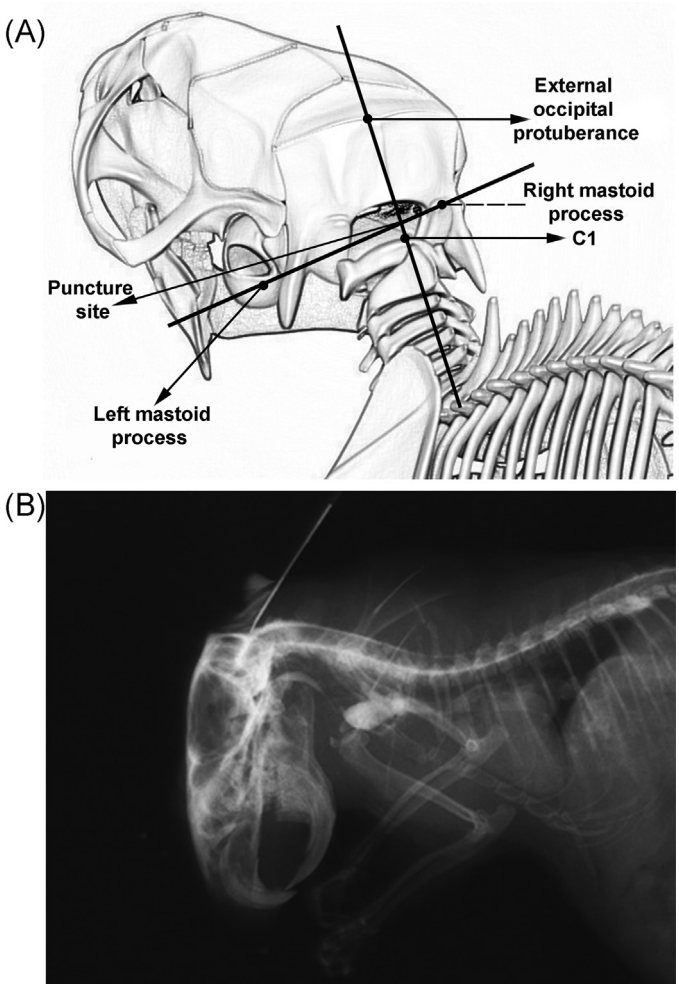

Fig. 2. Site of puncture to access the cisterna magna for CSF sampling. (A) Schematic illustration of a rat's skull and neck showing the anatomic landmark for puncture as the intersection of an imaginary vertical line extended from the external occipital protuberance to the spinal process C1 and a horizontal line between both mastoid processes. (B) X-ray image of a rat where the operator has introduced the needle in order to indicate the site for puncture. The syringe of the puncture device was filled with an iodinated contrast medium.

compression of the neck, the operator's fingers were put below the branch of the jaw and the base of the mouth while the thumb rested on the occipital bone to attain head hyperflexion. The rhomboid depressed area corresponding to the atlanto-occipital space was localized by palpation. The intersection of an imaginary vertical line extended from the external occipital protuberance to the spinal process C1 and a horizontal line between both mastoid processes was defined as the anatomic landmark to puncture the suboccipital region in order to access to the cisterna magna (Fig. 2). Once identified, the puncture device (Fig. 1) was inserted and CSF was collected by gentle aspiration until the glass capillary tube was full (approximately 50–75 μl). The colorless CSF sample was drawn into an eppendorf tube and frozen at −20 °C. The displaced muscle planes of the rats were restituted and reconstructed by suturing with absorbable suture material Dexon 3/0 using an atraumatic curved needle. Animals were left in cages with wood shavings for recovery.

2.4. CSF analysis

2.4.1. Physical analysis

CFS samples were described in terms of their macroscopical aspect, indicating the turbidity degree, their color and the presence or absence of blood clot.

2.4.2. Total cell count

Both erythrocytes and total nucleated cells were counted using a hemacytometer and expressed as number of cells per mm^3. Counts were performed before centrifugation and within 60 min after CSF collection.

2.4.3. Chemical evaluation

After centrifugation, chemical analyses of the supernatants were performed. Protein content was measured by the Bradford method. The presence of globulins was evaluated using the Pandy's test. This assay is performed by adding a drop of CSF to 1 ml of Pandy reagent (phenol-saturated aqueous solution). Turbidity indicates the presence of globulins, with a detection limit of about 50 mg/dl. Glucose levels were determined by the GOD/POD enzymatic method using the Reflectoquant® glucose test strips in a reflectometer (RQflex 2, Merck, USA).

2.5. Pharmacokinetics of rhEPO after the osmotic opening of the BBB

2.5.1. Drug

rhEPO was provided by Zelltek SA (Santa Fe, Argentina) as a 1.939 mg/ml solution in 100 mM NaCl, 20 mM citrate buffer (pH 7.0).

2.5.2. Intravenous administration

Rats were divided into two groups of 30 animals each. One group was injected intravenously with a single dose of 500 μg of rhEPO in phosphate-buffered saline (PBS). The other group received an intravenous injection of 500 μg of rhEPO diluted in 2 ml of a solution of mannitol 1.1 M. Using the method described in this work, CSF samples were collected at 5, 15, 30 min and 1, 2, 4, 6, 8, 18 and 24 h post-injection ($n = 3$ per timepoint) and stored at −20 °C. rhEPO concentrations in CSF were determined using a sandwich ELISA as described previously (Mattio et al., 2011).

2.5.3. Pharmacokinetic data analysis

The concentrations of rhEPO in rat-derived CSF samples, in which PBS or mannitol was used as vehicle, were plotted against the corresponding time and the area under curve (AUC) was calculated using Microcal Origin software version 5.0 (Microcal Software, USA). Comparison between treatments was performed using Student's t test. Differences were considered significant when $p < 0.05$.

3. Results

3.1. Procedure for CSF collection: general comments and practical considerations

We have developed a simple method for CSF sampling that allowed us to collect volumes ranging from 50 to 75 μl with a 100% success rate, meaning that from every rat that was punctured, a CSF sample was obtained. Ninety-three CSF samples were collected, 93% of which had a clear and colorless aspect, indicating absence of blood contamination. The procedure could be completed in about 5 min. Rats survived in 99% of the cases and they could even be punctured at least two more times with an interval of about 10 days from the previous aspiration (data not shown). This period of time allowed the inflammation recovery of the punctured area, so that the consecutive punctures in the same rat also rendered normal clear CSF samples. This is an important issue to be considered when approaching longitudinal studies, such as serial CSF sampling to investigate specific biomarkers in the progression of Alzheimer's disease in rat models (Kester et al., 2012), or determination of myelin basic protein or its fragments in CSF as a marker of demyelination in models of multiple sclerosis (Ohta et al., 2000),

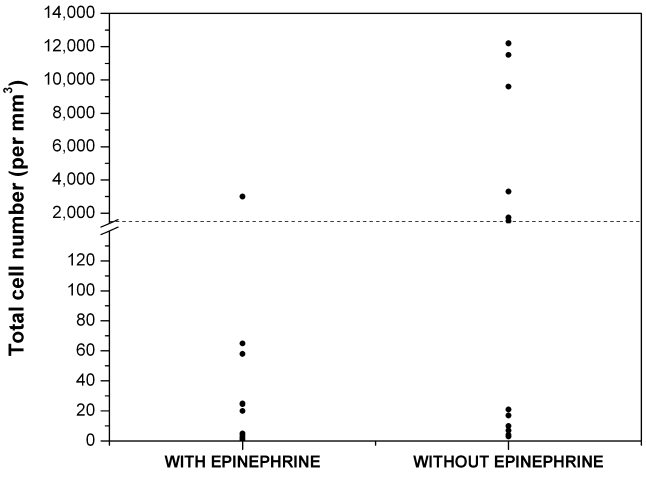

Fig. 3. Incidence of blood contamination in CSF samples collected in the presence or in the absence of epinephrine using the described methodology. Total cell number per mm^3 was determined for each CSF sample obtained using both approaches ($n = 12$ per group). The dashed line indicates the threshold above which samples were visibly contaminated with blood (1500 cells/mm^3).

among many other applications that involve repeated sampling of the same animals over long periods of time.

Some critical issues have to be taken into account to successfully reproduce this methodology. The manual handling of animals to achieve head hyperflexion is vital for a maximum opening of the suboccipital space and, thus, to get clean CSF samples. Previous attempts for CSF collection without performing any incision in animals placed in a surgery table with the head lying at a 45° angle to the rest of the body rendered CSF samples in 95% of the cases. However, only 40% of this fluid presented the regular colorless and clear aspect. The remaining 60% was stained with blood in different degrees, constituting a hematic fluid due to a traumatic puncture. The improved posture helped increase the percentage of clear CSF collection to 80%. Importantly, this posture also protected the respiratory function of the animal by avoiding the compression of the upper airway and prevented acute ischemia by neck vascular package compression.

Another key point to achieve successful punctures is the surgical incision of the skin and soft tissue dissection which includes divulsion of muscle planes across the midline. This practice provides a better perspective about the appropriate site where the needle should be placed and simplifies the access to the spinal canal.

The use of a solution of lidocaine 20 mg/ml and epinephrine 5 μg/ml locally infiltrated in the skin, subcutaneous tissue and deep soft tissue from the back of the suboccipital area and neck represents the major novelty of the procedure. Lidocaine just acts as a local anesthesic and as a post-surgery analgesic. Epinephrin gives sustained vasoconstriction of local tissues, reducing peripherial circulation in the area and lowering the risk of accidental puncture of vessels. Consequently, infiltration with epinephrine prior to CSF sampling increases the probability of obtaining higher proportion of colorless, blood-free fluids. Indeed, as shown in Fig. 3, 50% of the samples ($n = 12$) collected using the described methodology but in the absence of this vasoconstrictor were macroscopically contaminated with blood, with total cell counts above 1500/mm^3. Contrarily, 92% of samples collected using epinephrine were clear and with total cell counts below 100/mm^3 ($n = 12$).

3.2. Characterization of CSF samples

From the 93 CSF samples that were collected using the method finally described in this work, 86 were transparent and colorless without the presence of blood clots, rendering the typical "rock crystal" appearance. The rest was visibly contaminated with blood or had a slightly yellowish aspect.

Table 1
Total cell counts, protein and glucose concentrations, and presence of globulins in CSF samples.

Rat	Total cell count (per mm^3)	Proteins (mg%)	Presence of globulins	Glucose (g/l)
1	1	15.8	(–)	0.6
2	65	22.2	(–)	0.8
3	>3000	n/d	n/d	n/d
4	20	19.7	(–)	1.1
5	2	13.5	(–)	0.6
6	5	15.6	(–)	1.3
7	1.8	17.9	(–)	1.1
8	24.5	17.9	(–)	0.9
9	2.5	16.3	(–)	1.3
10	4.0	17.1	(–)	1.1

n/d: non determined (traumatic puncture).

Ten rats that received no treatment were used to obtain CSF samples for their characterization in terms of cells, proteins, globulins and glucose content (Table 1). Total red (RBC) and white blood cells (WBC) never exceeded 100/mm^3, with a mean value of 14 total cells/mm^3 ($n = 9$), i.e. less than 1% of the RBC contamination corresponding to the macroscopic threshold for blood contamination (1500–3000 cells/μl, Patten, 1968; Ylitalo et al., 1976). The exception was the CSF obtained from rat 3, which moved during the sampling procedure and evidenced a blood clot indicative of a traumatic puncture.

Total protein concentrations were within the range found in normal CSF (15–30 mg%, Frankmann, 1986), with a mean value of 17.3 ± 2.5 mg% ($n = 9$). These results confirmed that there was no significant contamination with serum, whose normal protein values range from 6 to 8 g%. Moreover, all samples gave a negative result when analyzed qualitatively for the presence of globulins using Pandy reaction, indicating that there were no lesions in cellular membranes that could increment this protein fraction. Finally, glucose concentrations were slightly higher than those expected for an animal that has fasted (mean value 0.96 ± 0.29 g/l, $n = 9$), probably because they were not deprived from food the hours previous to CSF collection.

3.3. CSF pharmacokinetics of rhEPO in rats. Mannitol-induced permeabilization of the BBB

We applied this method to study the pharmacokinetics of rhEPO in CSF of Wistar rats. In addition, we were interested in evaluating a strategy for the permeabilization of the BBB in order to achieve an increased passage of this molecule to the central nervous system. For this, an osmotic disruption using mannitol 1.1 M was performed simultaneously to the injection of rhEPO. Fig. 4 shows the CSF concentration of the cytokine versus time (between 0 and 24 h) following a 500 μg intravenous dose of rhEPO diluted in saline or in mannitol. In both cases, the maximum concentrations were achieved after 2 h post-administration (17.5 and 17.0 ng/ml, respectively). However, the use of mannitol enhanced the delivery of rhEPO to the CNS in early time points, so that it was detected in the CSF after just 5 min post-dose (inset of Fig. 4, where the pharmacokinetic data between 0 and 4 h are shown). When the injection was performed without mannitol, rhEPO could be detected in CSF only after 30 min and in low concentration. Though using both methodologies rhEPO concentrations were detectable up to 24 h post-injection, rhEPO levels in later time points were higher in the CSF of rats which were subjected to BBB permeabilization. The area under the curve (AUC_{0-24h}) of the pharmacokinetic profile of these rats was consequently higher (153.2 ± 2.4 versus 126.2 ± 8.3 for rats injected with EPO in saline, $p = 0.045$). These

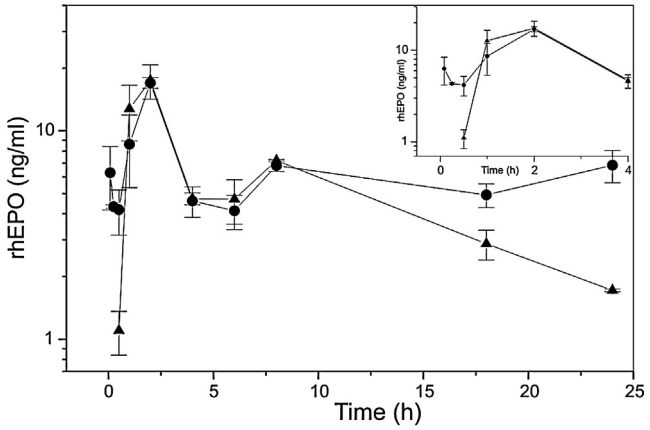

Fig. 4. Pharmacokinetic profile of rhEPO in rat CSF following intravenous administration of the cytokine in saline (▲) or in 1.1 M mannitol (●) between 0 and 24 h. Inset shows data between 0 and 4 h.

results not only indicate the success of the osmotic opening of the BBB for large drug penetration but also demonstrate the usefulness of a reliable CSF sampling method, as the one developed in this work.

4. Discussion

CSF sampling is a frequently used strategy to estimate CNS availability of drugs. Therefore, when designing a new centrally acting drug it is important to count on a reliable method for collection of blood-free CSF samples in order to get an unambiguous interpretation of its passage through the BBB.

The method described in this study is simple, easy to perform and takes no more than 5 min after training of a single operator. Blood contamination is virtually avoided because of the in situ injection of a mixture of lidocaine/epinephrine. Lidocaine relieves pain and also acts as a post-surgery analgesic, contributing to the welfare of animals. Ephinephrine produces vasoconstriction of local tissues, reducing the probability of accidental puncture of the vasculature and preventing blood contamination at the source of collection. The puncture device is easy to assemble and the material is cheap and readily available. The use of a glass capillary tube placed between the needle and the aspirating system in the puncture device helps control and standardize the volume of CSF sampled, preventing the rat's death caused by depletion of such fluid. Thus, the procedure results in a high rate of animal survival (99%), in which neither clinic nor neurological consequences were observed. The volume obtained (50–75 μl) is adequate for many techniques that are used to determine drug concentrations, which generally exhibit high detectability. More importantly, the use of any stereotaxic equipment is completely avoided, resulting in a low cost method. This is particularly beneficial for those labs which may need sampling CSF for a particular purpose but only as a complementary experiment to its principal research activity.

In comparison with techniques that involve cannulation (Bouman and Van Wimersma Greidanus, 1979) or surgical implantation of a tube into the cisterna magna (Huang et al., 1995) our methodology is much less invasive and less time consuming, as the preparation of cannulae system and surgical procedure demands some days of recovery before the procedure can continue (Consiglio and Lucion, 2000). As it can be inferred from the pharmacokinetic experiments of rhEPO in CSF, our procedure is fast enough to allow injection of the drug and collect a CSF sample within 5 min post-administration, after completing the following steps: skin disinfection, lidocaine/epinephrine infiltration, surgery procedure and CSF sampling. The risk of infection and neuronal damage is remarkably reduced, since the cisterna magna is only disrupted at the time of the extraction.

Some approaches for CSF collection by percutaneous needle puncture have been developed recently. Nirogi et al. (2009) described a method in which no incision is made in the skin to introduce the needle but they require a stereotaxic frame in which the animal is fixed with ear bars. Pegg et al. (2010) developed a procedure in which dura is exposed after incision and retraction of superficial and underlying muscles, and then CSF collection is made through a stereotaxic needle puncture, so that the complete procedure is performed with the anesthetized rat mounted in a stereotaxic device. As CSF study is frequently used to investigate metabolism and neurotransmitters in the brain and for long-term evaluation of the effects of a drug on the CNS, it is important to consider that fixation in a stereotaxic frame may alter the release of neurotransmitters. For example, Takasugi et al. (2005) demonstrated that fixation of the animal in such equipment induces noxious stimuli which results in increased levels of amino acids. Also, anatomic differences in rat strains can require changes of the stereotaxic parameters. In some cases, adjustments in the method of subjection to the frame are necessary to avoid scratching wounds, as it was reported by van den Berg et al. (2002). Such adjustments are also necessary to avoid the asphyxia of the animal held in such equipment, as described by Mahat et al. (2012), which increases even more the complexity and, consequently, the cost of the methodology.

In summary, complete elimination of stereotaxic equipment replaced by a well-trained operator represents a very attractive alternative offered by our CSF sampling methodology in order to prevent the above-mentioned drawbacks of this fixation instrument.

To achieve CNS entry in the presence of an intact BBB, a molecule must bear adequate molecular weight and sufficient liposolubility, among other properties. Unfortunately, very few therapeutic agents meet these characteristics. For example, though rhEPO represents a promising agent for the treatment of a broad field of neurological and psychiatric diseases (Sargin et al., 2010; Siren et al., 2009), its passage through the BBB is very slow, reaching a maximum CSF concentration of 0.02% of the higher plasma level (Mattio et al., 2011). Therefore, in this study, we employed an intravenous infusion of a hyperosmolar solution of mannitol containing rhEPO to increase the penetration of the cytokine into the CNS of Wistar rats and simultaneously to show the potentialities of our CSF sampling procedure. This approach allowed rhEPO to reach CSF faster than using a conventional injection in which rhEPO was administered in saline, so that the cytokine could be detected just after 5 min post-administration. The traditional approach for osmotic BBB disruption involves the use of intra-arterial carotid infusion of 20% mannitol at a rate of 0.15 ml/seg (Bellavance et al., 2008), with the disadvantage of being a very invasive method. The use of intravenous mannitol has been investigated less, but recent studies document its ability to allow the CNS delivery of neurotrophic factors, viral vectors and cells (McCarty et al., 2009; Seyfried et al., 2008; Yang et al., 2011). It is well established that hypertonic solutions not only of mannitol but also of arabinose, lactamide, saline and urea increase BBB permeability by inducing the shrinkage of cerebrovascular endothelial cells, thus producing a disruption of inter-endothelial tight junctions (Bellavance et al., 2008; Rapoport, 2000). The pathway that rhEPO uses for blood-to-brain influx is still controversial. Banks et al. (2004) reported that rhEPO penetrates BBB very slowly by means of extracellular pathways. Thus, osmotic opening of the BBB may enhance this transport mechanism. However, Brines et al. (2000) have shown evidence that rhEPO crosses the BBB through binding to receptors in the cerebral

capillary endothelium, beginning endocytosis followed by translocation into the brain. In the latter case, hypertonic mannitol would widen the interendothelial tight junctions, allowing the simultaneous extracellular and receptor-mediated rhEPO entry into the CSF.

It has been demonstrated that increased BBB permeability after hyperosmolar mannitol infusion is essentially reversed within 10 min (Rapoport, 2000). Thus, the therapeutic application of this strategy might acquire importance when rhEPO is used for the treatment of acute CNS diseases such as stroke, where time seems to be a determinant factor for the success of treatment.

5. Conclusion

In summary, the method described here for CSF sampling from the cisterna magna of rats distinguishes from other existing techniques in that it is simple, fast and completely avoids the use of any expensive stereotaxic instrument. It represents a reliable methodology for evaluating drug candidates' passage through the BBB, for studying approaches to gain increased access to the CNS, and for many other applications in which traces of blood in the CSF may dramatically alter the results and hence lead to the misinterpretation of the biomarkers measured.

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