Journal of Alzheimer's Disease 34 (2013) 835–839
DOI 10.3233/JAD-121798
IOS Press

Short Communication

Optimized Protocol for Amyloid-β Extraction from the Brain

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Accepted 18 December 2012

Abstract. Brain levels of amyloid-β (Aβ) are frequently assessed in transgenic mice models of Alzheimer's disease. The procedure involves tissue sample homogenization using different buffers in a sequential process. No attempt has been made to assess if these procedures are able to extract the total amount of Aβ present in the samples. Here we present data suggesting that standard protocols can lead to a dramatic underestimation of the Aβ content. Results show that higher extraction buffer volumes and at least two repetitions of the soluble and membrane-bound extraction steps are necessary for a more accurate estimation of the Aβ content in brain tissues.

Keywords: Alzheimer's disease, amyloid-β peptides, animal models, ELISA, tissue extraction, tissue homogenization

Brain levels of amyloid-β (Aβ) peptides are frequently assessed in transgenic mice models of Alzheimer's disease (AD). Currently, a considerable number of sequential Aβ extraction protocols exist [1–5], which differ mainly in the buffers used for homogenization and the number of extraction steps. In general, the procedure involves, at each step, homogenization of the tissue sample in an appropriate buffer followed by centrifugation, removal of supernatant, and re-homogenization of the pellet in other buffer used in the next step of the sequential process. However, only one previous attempt has been made to ascertain if these procedures are able to extract the whole amount of Aβ peptides present in these different fractions obtained from a brain tissue sample [6]. Indeed, it has been shown that the relative amounts of Aβ extracted from transgenic mouse and human brains are influenced by the extraction protocol [2].

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The present project was prompted by the finding that increasing the volume of homogenization buffer in our former extraction protocol produced substantial increments in the amount of peptide extracted from a given sample. This finding suggested that the extraction capacity of the buffers, at the usual 10% weight/volume proportion, could became saturated, leading to a dramatic underestimation of the real amount of Aβ peptide present in the sample. It could imply large differences in the estimations of total Aβ levels determined on identical samples depending on the protocol used and cause an apparent lack of effects for potential therapies aimed to reduce brain amyloid burden. In this context, several studies have reported reductions in the immunohistochemical amyloid loads without simultaneous changes in the biochemical loads measured by ELISA [7–11]. For these reasons, additional work on this issue was necessary.

Thus, we hypothesized that greater volumes of buffer for homogenization and eventual repetition of each extraction step could result in a more complete and accurate extraction of Aβ from brain tissue. Therefore, we carried out two series of experiments to

ISSN 1387-2877/13/$27.50

Table 1
Effects of homogenization buffer volumes on brain Aβ levels and Aβ concentrations

Volumes (mL)	Aβ	TBS (pg)	TX (pg)	AF (pg)	Total brain (pg Aβ)	TBS (pg/mL)	TX (pg/mL)	AF (pg/mL)	Total concentration (pg/mL)
0.6	$A\beta_{40}$	10.3 ± 1.30	10.2 ± 3.65	192.4 ± 18.05	212.9	17.1 ± 2.2	17.0 ± 6.1	321 ± 30.1	354.8
	$A\beta_{42}$	28 ± 4.96	9.7 ± 1.50	160.8 ± 14.33	197.7	46.7 ± 8.3	16.1 ± 2.5	268 ± 23.9	329.5
1	$A\beta_{40}$	21.8 ± 1.47	33.6 ± 2.17	299.8 ± 4.88	355.2	21.8 ± 1.5	33.6 ± 2.2	300 ± 4.9	355.2
	$A\beta_{42}$	42.1 ± 5.00	17.2 ± 2.63	289.8 ± 32.30	349.1	42.1 ± 5.0	17.2 ± 2.6	290 ± 32.3	349.1
2	$A\beta_{40}$	72.6 ± 1.09	115.1 ± 19.06	392.2 ± 22.20	579.9	36.3 ± 0.5	57.6 ± 9.5	196 ± 11.1	290.0
	$A\beta_{42}$	88.7 ± 5.5	38.7 ± 3.58	377.2 ± 58.09	504.6	44.4 ± 2.8	19.4 ± 1.8	188 ± 29.0	252.3
4	$A\beta_{40}$	69.7 ± 0.70	203.6 ± 31.5	364.1 ± 1.85	637.4	17.4 ± 0.2	50.9 ± 1.9	91.1 ± 0.5	159.4
	$A\beta_{42}$	206.9 ± 18.4	99.7 ± 9.00	614.4 ± 15.70	921.0	51.7 ± 4.6	24.9 ± 2.3	154 ± 3.9	230.3
6	$A\beta_{40}$	96.8 ± 11.95	260.7 ± 29.34	340.6 ± 41.65	698.1	16.1 ± 2.0	43.4 ± 4.9	56.8 ± 7.0	116.4
	$A\beta_{42}$	328.8 ± 12.8	158.4 ± 33.55	1285.7 ± 246.05	1772.9	54.8 ± 2.1	26.4 ± 6.0	214 ± 41.0	295.5

Data are expressed as mean ± SEM. $n = 3$ mice per volume group.

determine, firstly, the optimal buffer volume for brain homogenization and, secondly, the number of repetitions of each step in order to achieve complete Aβ extraction.

In the first experiment, we used three 8-month-old Tg2576 transgenic mice per assayed extraction volumes of 0.6, 1, 2, 4, and 6 mL. Aβ peptides were sequentially extracted from frozen hemi-brains (159.97 ± 10.03 mg) in a three-step protocol. Brains were homogenized in TBS buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.4) containing a cocktail of protease inhibitors (Complete, Roche Diagnostics). Homogenates were centrifuged (175,000 × *g*, 30 min, 4°C) and supernatants (soluble fraction) were collected. The pellets were then re-homogenized in TBS plus 1% Triton X-100, centrifuged as above, and the resultant supernatants (membrane-bound fraction) were collected. Insoluble, plaque-bound Aβ was extracted re-suspending the remaining pellets in 70% formic acid; the resultant supernatants (insoluble fraction) were collected. Concentrations of brain Aβ peptides were quantified using a β-amyloid ELISA kit (ABtest Kit, Araclon Biotech, Zaragoza, Spain) as described elsewhere [12]. In brief, an anti-Aβ N-terminal monoclonal antibody was used as capture antibody and two highly specific anti C-terminal polyclonal antibody, pAB002 and pAB031 (Araclon Biotech, Zaragoza, Spain), were used as detection antibody for $A\beta_{40}$ and $A\beta_{42}$. The concentrations of $A\beta_{40}$ and $A\beta_{42}$ were calculated using standard curves for both peptides by comparing the sample's absorbance with the absorbance of known standard concentrations. Data obtained in brain homogenates were expressed as picograms (pg) of Aβ peptides.

Due to the low *n*, no attempt was made to calculate the *p* value for the comparisons between different extraction volumes. Only the statistical variable "effect size" (ES) was calculated, as the mean difference in peptide extracted between the two volume groups, divided by the pooled standard deviation. An ES of 0.2 was considered small; 0.5, medium; and 0.8 or greater, large.

The amount of Aβ peptides in the supernatants produced by the three-step (TBS, TX, and FA fractions) extraction procedure using different buffer volumes are summarized in the Table 1. The total ELISA measurable brain Aβ levels were higher as brain tissues were homogenized in higher buffer volumes. In particular, about a 6-fold higher level (in pg) in total $A\beta_{40} + A\beta_{42}$ occurred in the higher extraction volume (6 mL) when compared with the lower one (0.6 mL). In statistical terms, the ES of the homogenization volume on brain Aβ levels between these two extraction volume groups was 9.9. However, it is worth mentioning that there were differences between the two Aβ peptides. Thus, while $A\beta_{42}$ levels (in pg) were higher as brain tissues were homogenized in higher volumes (ES: 0.6 versus 1 mL, 5.42; 1 versus 2 mL, 3.37; 2 versus 4 mL, 9.59; and 4 versus 6 mL, 5.3), $A\beta_{40}$ levels were only higher in the homogenization volume of 2 mL, from which peptide levels were much smaller (ES: 0.6 versus 1 mL, 6.21; 1 versus 2 mL, 4.13; 2 versus 4 mL, 0.9; and 4 versus 6 mL, 1.5).

We must emphasize that the concentration of $A\beta_{42}$ (in pg/mL) remained approximately the same for all extraction volumes tested, which strongly suggests a saturation of the buffer solution. The concentration of $A\beta_{40}$ in TBS and TX fractions were also approximately the same for all the extraction volumes. However, the concentration of $A\beta_{40}$ in the FA fraction was substantially lower in the 4 mL volume, suggesting that a near complete extraction of the insoluble $A\beta_{40}$ was achieved with 2 mL (Table 1).

These results suggested that homogenization of brains in a low volume of buffer was inadequate and led to dramatic underestimation of brain Aβ levels. It

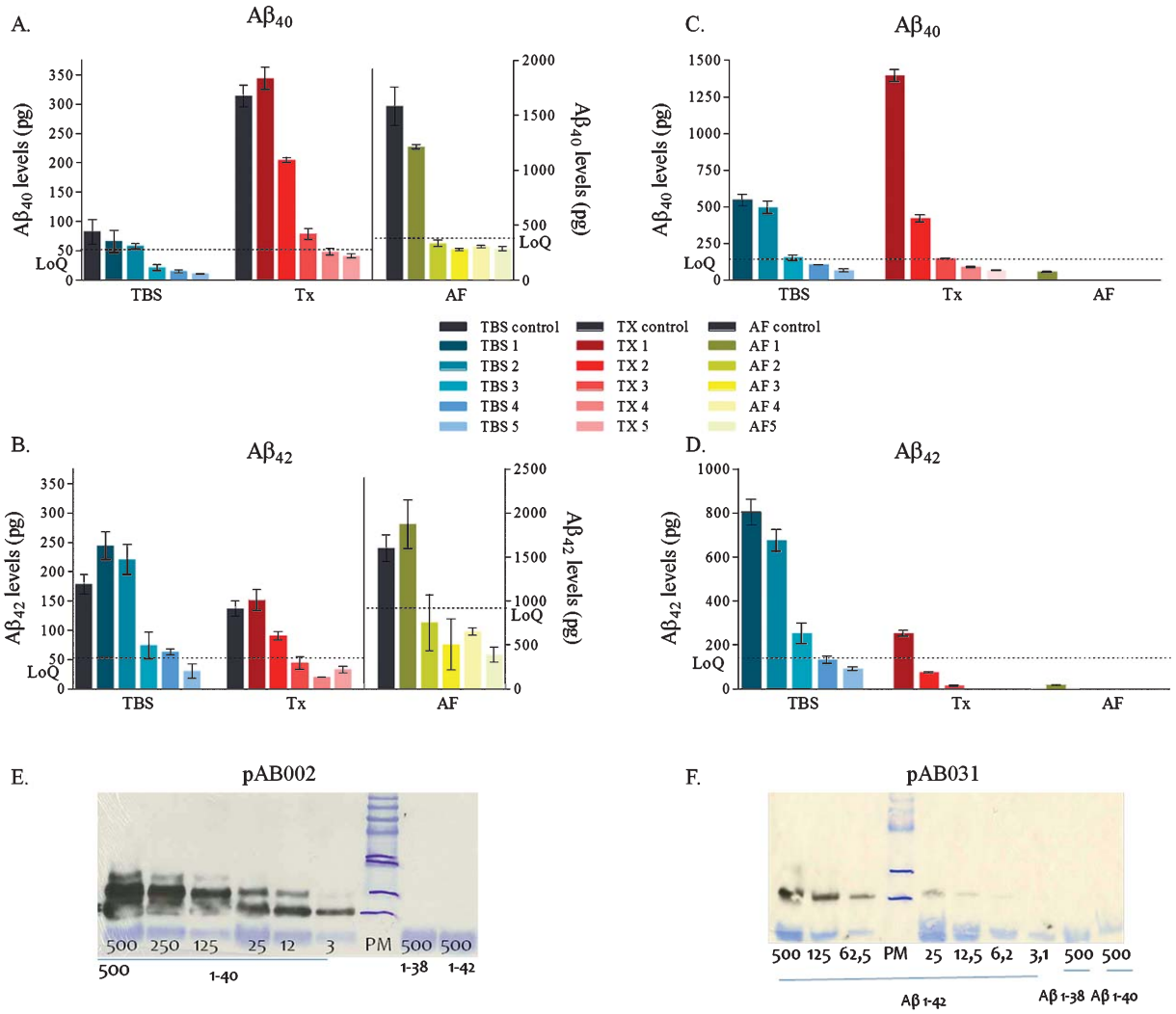

Fig. 1. Levels of $A\beta_{40}$ (A and C) and $A\beta_{42}$ (B and D) from mouse brain obtained by a three-step (TBS, TX, and FA) extraction protocol. Concerning older animals (A and B), black bars represent brains from three male transgenic mice homogenized only once with 6 mL of each buffer which served as control group. Colored bars represent brains from other three male transgenic mice homogenized five consecutive times in 6 mL of each homogenization buffer before going into the next extraction step. Substantial amount of peptides were recovered from the three first repeats of the experimental protocol for the TBS and TX fractions but not for the FA fraction. These same results in TBS and TX fraction were observed in brains from young Tg2576 mice devoid of amyloid plaques (C and D). Data are expressed as mean ± SEM. Scale in the right vertical axe applies for TBS and TX fractions and scale in the left vertical axe applies for FA fraction. LoQ, limit of quantification. Volume/tissue ratio = 40 mL/g. Specificity of the anti-$A\beta_{40}$ (E) and anti-$A\beta_{42}$ antibodies (F) were controlled by western blot which resulted in non-observable cross-reactivity with $A\beta_{42}$ and $A\beta_{40}$, respectively, as well as with $A\beta_{38}$.

was apparent from our results (Table 1) that the use of higher volumes of homogenization buffer resulted in the measurement of higher amounts of brain Aβ. However, as a plateau was not reached for the extracted peptide with any volumes used in this experiment, it could not be assured that a complete extraction was achieved by homogenizing the brain tissue in 6 mL of homogenization buffer, particularly for $A\beta_{42}$. Then, a complementary approach was necessary to achieve a more complete peptide extraction since the use of extraction buffer volumes over 6 mL is unadvised for technical reasons. Thus, in our second series of experiments we repeated each extraction step five times with the same buffer before going onto the next step in the extraction protocol.

For this experiment, we used another three 8-month-old Tg2576 mice. Brain tissues were homogenized in 6 mL of TBS and centrifuged as mentioned before. The supernatants were removed (fraction TBS1) and the pellets re-homogenized again in 6 mL of the same TBS

to obtain the supernatant (fraction TBS2) and so on up to obtain five TBS fractions from each brain. The same procedure was followed with TX and FA buffers to obtain five TX fractions and five FA fractions from each brain. All supernatants were assayed for Aβ.

Results are shown in Fig. 1. They indicate that substantial additional soluble and membrane-bound Aβ was extracted from the first three fractions obtained by re-homogenizing the remaining pellets in the same buffer. The levels of soluble and membrane-bound Aβ in the fourth and fifth fractions were below the limit of quantification by the ELISA method and were considered negligible. Insoluble Aβ required only one homogenization step with FA to be practically entirely extracted.

This "extra" soluble Aβ extracted in the successive repetitions with the same buffer is unlikely to come from the membrane-bound Aβ since quantification of the TX1 fraction resulted in Aβ levels similar to those obtained in the control protocol, in which the five previous re-homogenizations in TBS were not performed (TX-control, Fig. 1). The same held for the "extra" membrane-bound Aβ, which is unlikely to come from insoluble Aβ (FA1 fraction) as levels obtained in the first repetition were also very similar to those obtained with the control protocol (FA1 versus FA control, Fig. 1). Moreover, these results support the idea that the extraction capacity of the TBS and TX buffers becomes saturated in the former protocol, leading to a dramatic underestimation of brain peptide content. In addition, it seemed reasonable to assume that with the control protocol, the TX and FA fractions could contain considerable soluble and membrane-bound Aβ, respectively, which had not been extracted in the previous step. This could additionally lead to a misinterpretation of the relative distribution of peptide amounts among the different fractions.

In conclusion, the total Aβ extraction from brain tissue requires: 1) a high volume of homogenization buffer and 2) repeated re-homogenization with the same buffer before continuing to the next step in the extraction protocol. Our results suggested that a three buffer extraction protocol with three repetitions of re-homogenization with TBS and TX and one of FA using a volume of homogenization buffer of 6 mL should be adequate for the near complete ELISA measurable Aβ extraction from the brain. Nevertheless, because of the possible complex peptide-matrix interactions, the ideal homogenization volume and number of repetitions should be empirically adjusted for every set of buffers and characteristics of the tissue (species, age, stage of disease).

ACKNOWLEDGMENTS

The authors would like to acknowledge Ian Sherriff for the English language revision of the manuscript.

All work with mice followed the University Policies on the Use and Care of Animals and was approved by the ethical committee of the Center of Biomedical Research of La Rioja (CIBIR).

Authors' disclosures available online (http://www.j-alz.com/disclosures/view.php?id=1618).

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