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Abstract: Thrombolysis with recombinant tissue plasminogen activator (rt-PA) is the only pharmacological approved treatment for ischemic stroke, despite its associated increasing risk of hemorrhagic transformation. Since many of rt-PA effects in blood-brain barrier (BBB) are not well characterized, the study of protein changes in BBB cells after rt-PA administration may help to understand its adverse effects. Our aim was to analyze protein levels of four commonly used housekeeping proteins: β-Actin, α-Tubulin, GAPDH and HPRT in bEnd.3 endothelial cell line subjected to oxygen and glucose deprivation (OGD) conditions and rt-PA treatment to determine their reliability as Western blot loading controls. bEnd.3 monolayers were subjected to 2.5 hours of OGD and reperfusion with/without 20 µg/ml of rt-PA. At 3, 6, 24 and 72 hours post-OGD, protein levels were analyzed by Western blot using Stain-Free technology. OGD significantly decreased β-Actin, α-Tubulin, GAPDH and HPRT protein levels at 3, 6, 24 and 72 hours post-OGD without significant rt-PA treatment effects except for the GAPDH levels increase in control condition at 3 hours post-OGD. The present study clearly demonstrated that β-Actin, α-Tubulin, GAPDH and HPRT proteins are not suitable as loading controls for Western Blot analysis in bEnd.3 cells after OGD.

Cover Letter

Journal of Proteomics Editorial Board
Editor-in-Chief
June 12th, 2018

Dear Dr. Juan J. J. Calvete

Enclosed you will find the revision of our manuscript JPROT-D-18-00063R1 named "Evaluation of common housekeeping proteins under ischemic conditions and/or rt-PA treatment in bEnd.3 cells" by P Comajoan, C Gubern, G Huguet, J Serena, E Kadar and M Castellanos that we submit to your consideration. We are grateful for the reviewer's corrections and as requested, we have addressed point by point the specific comments in the attached file "Detailed responses to reviewers".

We hope that you find this new version of the manuscript to be acceptable for publication in the Journal of Proteomics

Thank you in advance for your time and consideration.

Yours sincerely,

Elisabet Kádár Garcia

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Reviewer #1: This is a review on a revised paper entitled "Evaluation of common housekeeping proteins under ischemic conditions and/or rt-PA treatment in bEnd.3 cells" by Comajoan et al.

I think you need to correct some issues in the revision.

Issues that need to be addressed:

Minor points

R1-1) As you replied, OGD duration is quite variable. You should revise to "OGD of 2.5 hours" in the line 9 of Abstract.

As suggested, OGD duration have been incorporated in the line 9 of Abstract.

R1-2) As you changed the title, "BBB in vitro model" in Graphical Abstract would be better phrased as "mimic primary culture of endothelial cells".

We agree with the observation and we have modified the sentence “BBB in vitro model” to “bEnd.3 endothelial cell culture” in Graphical Abstract. We agree with the idea that the reviewer suggests and for that reason we have introduced it in the revised manuscript answering the R1-3 question. However, we have considered more appropriate as a methodological title the “bEnd.3 endothelial cell culture” sentence.

R1-3) In the line of 58,
You should explain bEnd.3 cells in more detail.
For example, --- bEnd.3 cells which appear to be a suitable model for the BBB and mimic primary culture of BBB endothelial cells.

As suggested, we have introduced a more detailed explanation of the use of bEnd.3 cell line as a suitable model for the BBB presenting similar barrier characteristics to the primary brain microvascular endothelial cells into the Introduction section and we have included an additional reference (line 58-60).

R1-4) In the line of 66,
"In vitro BBB model" would be also better phrased as "Cell culture".

This change has been made (line 68).

R1-5) In the line of 24 and figure legends, There are still some typographical errors. Would you correct decimal character to period from comma?

These changes have now been made.

R1-6) In Fig. 2,

OGD line is difficult to distinguish from line for OGD+rt-PA. Would you change distinguishable lines?

We agree with this observation and have modified the Figure 2 accordingly.

Significance

We reported altered levels of β-Actin, α-Tubulin, GAPDH and HPRT housekeeping proteins in bEnd.3 endothelial cell line after an ischemic insult. Therefore, we demonstrated that these proteins are not suitable as loading controls for Western Blot analysis in our experimental conditions and we recommended the use of Stain-Free gels as an alternative to traditional housekeeping proteins normalization.

*Graphical Abstract
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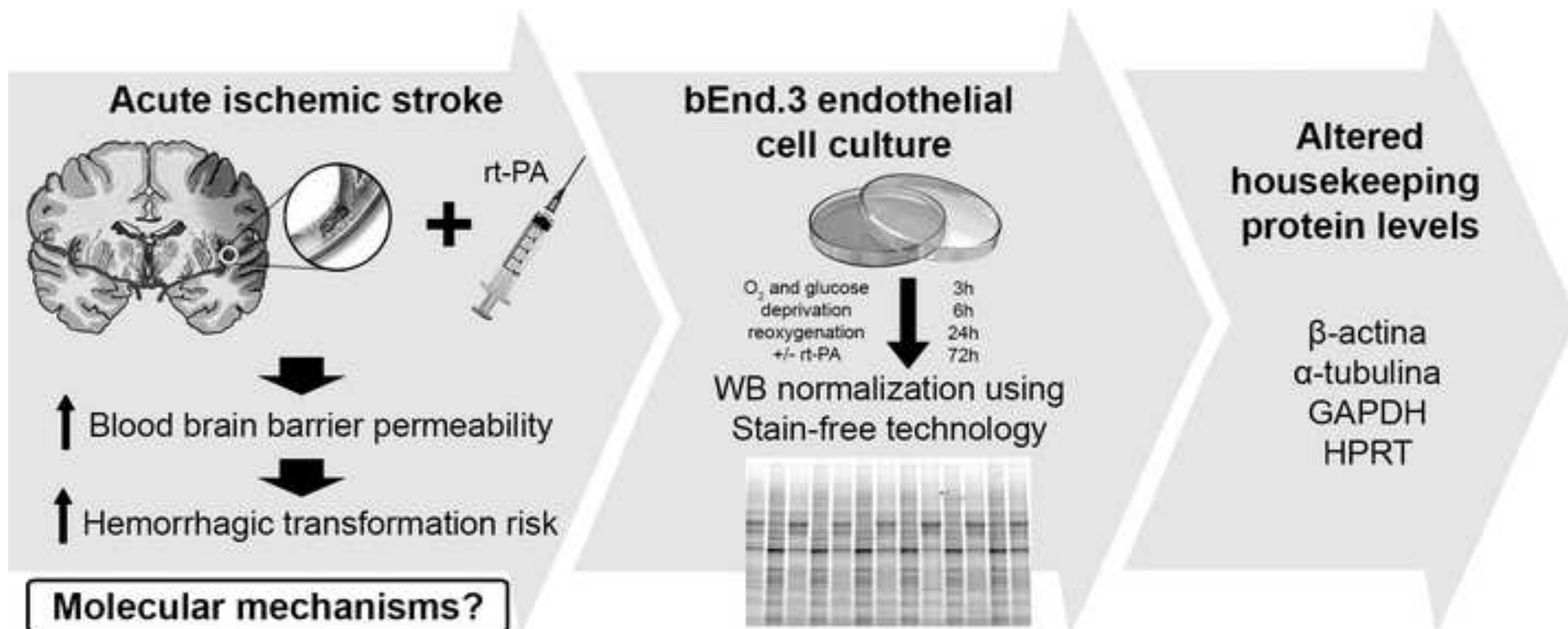

Highlights

- Time profile analysis of four common housekeeping proteins after ischemic insult and/or rt-PA administration in bEnd.3 cell line
- We reported β-Actin, α-Tubulin, GAPDH and HPRT protein levels decreased after 2,5 hours of oxygen-glucose deprivation and subsequent reperfusion
- We demonstrated that these proteins are not suitable as loading controls for Western Blot in our OGD experimental conditions
- We observed rt-PA treatment did not modify β-Actin, α-Tubulin and HPRT protein levels at any analyzed time after reperfusion.
- We demonstrated rt-PA treatment induced a significant temporal increase of GAPDH levels in normoxic conditions

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Evaluation of common housekeeping proteins under ischemic conditions and/or rt-PA treatment in bEnd.3 cells.

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1 Abstract

2 Thrombolysis with recombinant tissue plasminogen activator (rt-PA) is the only pharmacological
3 approved treatment for ischemic stroke, despite its associated increasing risk of hemorrhagic
4 transformation. Since many of rt-PA effects in blood-brain barrier (BBB) are not well characterized,
5 the study of protein changes in BBB cells after rt-PA administration may help to understand its
6 adverse effects. Our aim was to analyze protein levels of four commonly used housekeeping
7 proteins: β-Actin, α-Tubulin, GAPDH and HPRT in bEnd.3 endothelial cell line subjected to oxygen
8 and glucose deprivation (OGD) conditions and rt-PA treatment to determine their reliability as
9 Western blot loading controls. bEnd.3 monolayers were subjected to 2.5 hours of OGD and
10 reperfusion with/without 20 µg/ml of rt-PA. At 3, 6, 24 and 72 hours post-OGD, protein levels were
11 analyzed by Western blot using Stain-Free technology. OGD significantly decreased β-Actin, α-
12 Tubulin, GAPDH and HPRT protein levels at 3, 6, 24 and 72 hours post-OGD without significant rt-PA
13 treatment effects except for the GAPDH levels increase in control condition at 3 hours post-OGD.
14 The present study clearly demonstrated that β-Actin, α-Tubulin, GAPDH and HPRT proteins are not
15 suitable as loading controls for Western Blot analysis in bEnd.3 cells after OGD.

16

17 Keywords

18 Housekeeping proteins; OGD; rt-PA; Blood brain barrier; Endothelial cells; Ischemic stroke;

19

20 INTRODUCTION

21 According to the World Health Organization, stroke is a leading cause of death and disability in the
22 world (2012). Despite all clinical trials carried out in recent years, thrombolytic therapy with
23 recombinant tissue plasminogen activator (rt-PA) has been the only drug shown to be effective for
24 the treatment of ischemic stroke when administered within the first 4.5 hours of stroke onset [1].
25 However, its administration is associated with an increasing risk (8%) of symptomatic hemorrhagic
26 transformation (HT) of the ischemic brain. This adverse effect constitutes an important limitation for
27 the generalization of rt-PA therapy which, at the present time, is given to less than 5% of patients
28 with ischemic stroke [2,3].

29 HT is mostly caused by the disruption of the blood-brain barrier (BBB) after the ischemic insult as a
30 result of the increase of permeability and the subsequent pass of erythrocytes through the
31 structure. BBB is composed of astrocyte end-feet, pericytes, specialized brain capillary endothelial
32 cells (EC) and the extracellular matrix (ECM) components [4,5]. At the molecular level, it has been
33 shown that rt-PA could affect directly to EC through triggering the degradation of tight junction
34 proteins [6] which are essential for maintaining cerebrovascular homeostasis and regulating the
35 vascular endothelial growth factor (VEGF) expression and the subsequent stimulation of endocytosis,
36 transcytosis and angiogenesis [7]. rt-PA has also been implicated in the degradation of basement
37 membrane components such as collagen IV, laminin and fibronectin through plasmin activation, low

38 density lipoprotein receptor associated protein-1 (LRP-1) stimulation, and matrix metalloprotease
39 (MMPs) induction [8].

40 Since many of rt-PA's pleiotropic interactions are not well characterized, the identification and time
41 profile analysis of endothelial protein levels after ischemic conditions and rt-PA administration may
42 shed light on the molecular mechanisms underlying rt-PA's adverse effects in this important element
43 of neurovascular unit of BBB [9].

44 The Western Blot or Immunoblot is a widely accepted technic used to determine the expression
45 changes of particular proteins under specific experimental conditions such as ischemia. The most
46 common way to validate the detected differences is performing a loading normalization using high-
47 abundance housekeeping proteins expressed at a constant level and theoretically not affected by
48 experimental conditions. β-Actin, α-Tubulin and glyceraldehyde 3-phosphate dehydrogenase
49 (GAPDH), among other housekeeping proteins, are usually chosen as loading controls [10]. Different
50 studies have used these housekeeping proteins to normalize results after *in vitro* and *in vivo*
51 ischemia models [11,12] but, other studies have reported altered expressions of actin, tubulin and
52 GAPDH due to the effects of the ischemia [13–15]. These controversial results make questionable
53 the use of these loading controls for western blot normalization purposes in ischemic conditions. In
54 this sense, different papers have recently confirmed Stain-Free technology as a more reliable
55 method than housekeeping's labeling, avoiding saturate signals and housekeeping variations due to
56 experimental conditions [16–19]. Moreover, there are no published data analyzing the effect of rt-
57 PA in normoxic and ischemic conditions on expression of these putative housekeeping proteins in
58 bEnd.3 cells. This immortalized mouse cerebral endothelial cell line appears to be a suitable *in vitro*
59 BBB model presenting similar barrier characteristics to the primary brain microvascular endothelial
60 cells [20,21].

61 The aim of this study was to analyze the levels of four common housekeeping proteins, β-Actin, α-
62 Tubulin, GAPDH and Hypoxanthine-guanine phosphoribosyltransferase (HPRT) in the bEnd.3
63 endothelial cell line subjected to oxygen and glucose deprivation (OGD) conditions and/or treatment
64 with rt-PA to determine whether they are reliable as loading controls to Western blot or not in these
65 experimental conditions during a time period from 3 to 72 hours post-OGD.

66

67 MATERIAL & METHODS

68 *Cell culture*. Immortalized mouse brain endothelial cell line (bEnd.3) were purchased from
69 ATCC (CRL-2299), seeded in 60mm Petri dishes (Corning, USA) and grown in DMEM high glucose
70 (HG) medium with 1% glutamine (Gibco, USA), 10% fetal bovine serum (Gibco, USA) and 1%
71 Penicillin/Streptomycin (HyClone Laboratories, USA). All bEnd.3 cells used for these experiments
72 were cultured between 25-30 passages, which have been shown to maintain excellent BBB
73 characteristics *in vitro* [20].

74 *OGD performance and rt-PA treatment*. After overnight starvation in DMEM HG with 1% fetal
75 bovine, bEnd.3 monolayers were subjected to OGD. Briefly, the medium was replaced with glucose-
76 free DMEM without FBS (Gibco, USA) previously perfused with N_2 to purge the oxygen. Then, the
77 cells were placed into a 37ºC humidified hypoxic chamber with a constant N_2 flow of 1 L/min and

78 0.15 bar pressure for 2.5 hours. This OGD period was selected after confirm that a viable cell
79 population remains until 72 hours though with ionic and paracellular permeability as well as tight
80 junction protein levels differences between control, OGD and OGD + rt-PA treated cells
81 (unpublished results). Regarding the control (CTR) group, the same procedure was carried out with
82 the difference that the glucose-free medium was supplemented with glucose (5.5mM) and
83 incubated at 37ºC with 5% of CO_2. At the end of the OGD period, media were removed and replaced
84 with DMEM HG medium containing 10% FBS and with or without rt-PA at a concentration of 20
85 µg/ml. As reported in previous publications, we used a dose of 20 µg/mL of tPA, based on the finding
86 that such a concentration can be observed in blood [22].
87
88 *Cell viability.* At 0, 24, 48 and 72 hours of reperfusion, with or without rt-PA treatment, cell
89 viability was assessed with 3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide (MTT) assay
90 (5mg/ml, Sigma). Absorbance was measured at 570 nm using a SpectraMax 340PC384 Microplate
91 Reader and results were expressed as a percentage of the value in the control group.
92
93 *Western Blot analysis.* At 3, 6, 24 and 72 hours post-OGD, cells were collected and protein
94 was isolated using Lysis Buffer (Cell Signaling, The Netherlands). Protein concentration was
95 measured using BCA method (Thermo fisher scientific, USA). Protein samples (10 µg) were loaded
96 and separated by electrophoresis on Criterion™ TGX Stain-Free™ Precast Gels (Bio-Rad) at 120 V for
97 80-90 minutes. Then, proteins were transferred to PVDF membranes at 30 V overnight at 4 ºC. After
98 1 hour of blocking with TBST 5% BSA (EMD Millipore, USA), membranes were incubated 1 hour at
99 room temperature with primary antibodies: anti-β-Actin (sc-47778), 1:1000; anti-α-Tubulin (sc-
100 5286), 1:1000 from Santa Cruz Biotechnology and anti GAPDH, 1:20000 (MAB374) from EMD
101 Millipore, USA and overnight at 4 ºC with anti-HPRT (sc-376938), 1:100 from Santa Cruz
102 Biotechnology, in TBST 3% BSA. HRP-conjugated secondary antibodies were used for 1 hour at room
103 temperature. Protein bands were revealed using Immobilon Western Chemiluminescent HRP
104 Substrate (EMD Millipore, USA) and quantified with Alpha Innotech software (AlphaEaseFC™).
105
106 *Stain-Free total protein staining and quantification.* The Criterion Stain-Free gels (BioRad)
107 were used as a loading control in Western Blot since they contained a trihalo compounds that
108 allowed rapid fluorescent detection of total protein loaded in each lane and after the transfer in
109 PVDF membranes. Once the gel had run, it was placed to the transilluminator and exposed 5
110 minutes with UV light in order to activate the fluorescence signal. Then, each lane was quantified
111 using Alpha Innotech software (AlphaEaseFC™). The space between lanes was taken as a background
112 according to Aldridge GM *et al.* [10] as shows in Figure 1.

113 *Statistical analysis*. SPSS software (IBM SPSS Statistics 22) was used to perform the statistical
114 analysis. Since variables were not normally distributed, non-parametric Mann-Whitney test was used
115 to perform the analysis. *P* value <0.05 was considered as significant.

116

117 # RESULTS

118 OGD decreased significantly the viability of bEnd.3 cells from 0 to 72 hours while rt-PA
119 administration at reperfusion only induced a significant decrease of viability at 72 hours in the OGD
120 rt-PA group (Figure 2).

121 Figure 3 shows a representative gel with total protein staining of bEnd.3 murine endothelial cells
122 subjected to 2.5 hours of OGD and the subsequent reperfusion, in which it is possible to observe
123 differences in protein profile between OGD and CTR samples. The most remarkable differences were
124 seen between 3 and 24 hours post-reperfusion, while at 72 hours the profiles were similar again.
125 Regarding rt-PA treatment, the possible differences between groups were much more attenuated
126 and therefore, practically unobservable.

127 As can be seen in Figure 4, protein levels of β-Actin, α-Tubulin, GAPDH and HPRT in bEnd.3 were
128 significantly decreased after 2.5 hours of OGD at 3, 6, 24 and 72 hours of reperfusion compared to
129 CTR condition. Moreover, rt-PA treatment had no effects on β-Actin, α-Tubulin and HPRT protein
130 levels since no differences were observed neither between CTR and CTR + rt-PA nor OGD and OGD +
131 rt-PA groups at 3, 6, 24 and 72 hours of reperfusion (Figure 4B, C and E). However, GAPDH levels
132 significantly increased after rt-PA treatment in the CTR condition ($p = 0.05$; CTR vs CTR + rt-PA) at 3
133 hours but not at 6, 24 and 72 hours post-reperfusion (Figure 4D).

134

135 DISCUSSION

136 The aim of the present study was to evaluate the suitability of β-Actin, α-Tubulin, GAPDH and HPRT
137 proteins as loading controls for Western Blot analysis in an *in vitro* model of ischemia and
138 reperfusion, with or without rt-PA administration, using the murine brain endothelial bEnd.3 cell
139 line. Our results showed that none of them seems to be appropriate for normalization purposes
140 when OGD is applied to bEnd.3 cells since the levels of these proteins significantly diminished from 3
141 to 72 hours post-reperfusion, independently of rt-PA administration.

142 It has been previously reported that common housekeeping proteins are not always reliable loading
143 controls and it is necessary to test them in each experimental condition before normalizing the
144 results [10,17,19,23]. Stain-Free gels appears as a good option to test the suitability of candidate
145 housekeeping protein [18,19]. Total protein staining also allowed to observe a differential protein
146 profile due to OGD in bEnd.3 endothelial cell line. In concordance with this, Andreev *et al*. described
147 a translation alteration of about 3,000 genes during 1 hour of OGD in a PC12 neural cell line [24].
148 Furthermore, it has been demonstrated that hypoxia-inducible factor (HIF)-1 was increased in
149 bEnd.3 cells after 18 hours post-6 hours of OGD [25]. In concordance, a similar HIF-1 response to
150 hypoxia was described in neuroblastoma cells and associated with protein profile alterations as a
151 result of an induction of genes involved in anaerobic metabolism, oxygen transport and angiogenesis
152 [26].

153 β-Actin, α-Tubulin, GAPDH and HPRT proteins have been used as housekeeping proteins in many
154 experimental conditions, including ischemic *in vivo* and *in vitro* models [27,28]. However, other
155 studies have reported altered expressions of actin, tubulin and GAPDH as a result of the ischemia
156 [13–15], which is in agreement with our results. According to this, Maneen MJ *et al.* [29]
157 demonstrated a reduction of actin levels in rats subjected to 1 hour of right middle cerebral artery
158 occlusion (MCAO) and 30 minutes of reperfusion, and proposed that the actin polymerization is
159 particularly susceptible to oxidative stress after stroke. Moreover, a significant decrease of actin
160 expression after exposing bovine brain microvessel endothelial cells to 6 hours of hypoxia and

161 aglycaemia was also reported by Brown RC *et al.,* suggesting that maintenance of the actin
162 cytoskeleton is energy-dependent and requires a constant supply of ATP [13]. In concordance, Torii
163 H *et al.* and Coucha M *et al.* concluded that hypoxia/reoxygenation produced a cytoskeletal
164 reorganization in which filamentous actin decreased [30,31]. Similar to actin, α- and β-Tubulin are
165 important structural proteins of the cytoskeleton and alterations in their expression and distribution
166 were also observed after ischemic conditions [32]. Particularly, Ma XL *et al.* reported a significant
167 decrease in α-Tubulin expression in rats after 60 minutes of cerebral ischemia and 1 hour of
168 reperfusion [15]. Similarly, Kumar K *et al.* and Hatakeyama T *et al.* described a significant tubulin
169 mRNA and immunoreactivity decrease, after 10 minutes of transient ischemia and reperfusion at 6
170 and 48 hours [33,34]. Regarding GAPDH, a critical enzyme involved in glycolysis for energy
171 production [35], Tanaka R *et al.* described different GAPDH levels depending on the analyzed area
172 and post-ischemia time point in an *in vivo* model of cerebral ischemia. Specifically, they reported a
173 nuclear accumulation of GAPDH in the ischemic core area after 2 hours of MCAO without
174 reperfusion that decreased in a time-dependent manner from 3 to 48 hours after reperfusion [14].
175 Finally, HPRT is an important enzyme that catalyzes the conversion of hypoxanthine to inosine
176 monophosphate in a rescue pathway to obtain ATP. Although some articles reported unchanged
177 mRNA HPRT expression and it was accepted as housekeeping control for real time-PCR analysis in *in*
178 *vivo* models of permanent MCAO (pMCAO) [36,37], other studies have reported reduced HPRT
179 mRNA levels under ischemic conditions [38]. In accordance, our results have showed a decrease of
180 HPRT protein levels after ischemia. To the best of our knowledge, this is the first study analyzing
181 HPRT protein levels after an ischemic insult and particularly showing that ischemia results in a
182 decrease in the levels of this protein in bEnd.3 cells.

183 Although with many limitations, *in vitro* OGD models reproduce part of the cascade of molecular
184 events resulting after cerebral ischemia highlighting the subsequent energy failure and metabolic
185 disturbances. These disturbances included changes in the oxygen levels, in glucose metabolism and
186 the depletion of energy metabolites including ATP, phosphocreatine, lactate and N-acetyl aspartate
187 [39]. Therefore, proteins involved in energy-dependent processes, which required a constant supply
188 of ATP such as actin and tubulin polymerization, or related with metabolic pathways to obtain ATP
189 such as GADPH and HPRT, may be altered in our experimental conditions. Contrary to our results
190 some studies carried out in the bEnd.3 model presented β-Actin as unchanging housekeeping
191 protein. Jie Liu *et al.* and Haoming Song *et al.* exposed bEnd.3 cells to 2 hours of OGD, without or
192 with 6 hours of reoxygenation, respectively [11,27] and Soonmi Won *et al.* performed a 6-hours of
193 OGD and 3-hours of reoxygenation model [40]. We consider that the discrepancies could be due to
194 methodologic differences including the analyzed time points after OGD. In fact, our study presents
195 an extensive time profile analysis (from 3 to 72 hours after OGD) of β-Actin, α-Tubulin, GADPH and
196 HPRT protein levels in bEnd.3 cells.

197 To the best of our knowledge, this is the first study evaluating the effects of rt-PA on the levels of the
198 analyzed housekeeping proteins. Our results demonstrate that rt-PA administration did not modify
199 β-Actin, α-Tubulin and HPRT levels, neither under normal conditions nor after OGD at the different
200 time point analysis. On the other hand, the addition of rt-PA to bEnd.3 cultures resulted in an
201 increase in the GAPDH levels at CTR condition at 3 hours post-rt-PA administration. Although similar
202 results had not been described in CTR conditions, it has been previously reported that GAPDH
203 overexpression restores energy production and prevents DNA damage in endothelial cells after
204 ischemia/reperfusion injury [41], and so it is possible that GADPH protein levels could increase after

205 rt-PA administration as a protective response. Further studies are, however, needed to confirm this
206 hypothesis.

207 CONCLUSION

208 In summary, in our study we have demonstrated that levels of β-Actin, α-Tubulin, GAPDH and HPRT
209 proteins significantly decrease in murine brain endothelial bEnd.3 cells subjected to 2.5 hours of
210 OGD at 3, 6, 24 and 72 hours of reperfusion, and so these proteins would not be suitable as loading
211 controls for Western Blot analysis in these experimental conditions. Therefore, except GAPDH, β-
212 Actin, α-Tubulin and HPRT could be used as loading control proteins in experimental conditions
213 including rt-PA administration (without OGD conditions) at least until 72 hours post-administration.
214 So, given the lack of a specific housekeeping protein for the experimental conditions analyzed, we
215 consider the total protein normalization using Stain-Free technology to be a reliable option and,
216 therefore, we recommend the use of Stain-Free gels as an alternative to traditional housekeeping
217 normalization in Western Blot.

218

219

220 Author contributions

221 PC performed in vitro procedures and Western Blot analysis with EK and CG help. PC, EK and CG
222 analyzed and interpreted data obtained. PC, EK and CG were the major contributors in writing the
223 manuscript. All authors read and approved the final manuscript.

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229 Notes

230 All authors declare no conflict of interests.

231

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233

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371 ## Figure legends

372 **Figure 1 |** Stain-Free gel image with schematic representation of quantification method. The fluorescent signal
373 allows to detect total protein loaded in each lane and use it to normalize the results. The intensity average of
374 each lane was quantified taking the space between lanes as a background as it is showed (L1: lane 1; B1:
375 background 1; and the same for 2, 3 and 4).

376 **Figure 2 |** Cellular survival rate tested in the fourth conditions analyzed: CTR, CTR + rt-PA, OGD and OGD + rt-
377 PA after 2.5 hours of OGD and the subsequent reperfusion periods. *$p < 0.05$ vs CTR, # < 0.05 vs CTR + rt-PA,
378 **$p < 0.05$ vs OGD. Data represent the mean of n = 3-4 independent experiments and error bars indicate the
379 standard error of the mean (SEM).

380 **Figure 3 |**Stain-Free gel image of a representative experiment. bEnd.3 cells were subjected to 2.5 hours of
381 OGD and/or rt-PA administration and 10 ug of total cell lysates were loaded in each lane. Total protein bands
382 were visible due to trihalo compounds that allowed its rapid fluorescent detection.

383 **Figure 4 |** Protein levels of β-Actin, α-Tubulin, GAPDH and HPRT in bEnd.3 cells after 2.5 hours of OGD and/or
384 rt-PA administration. 10 ug of total cell lysates from the four experimental groups were prepared and
385 subjected to Western blot analysis (A). Protein levels of β-Actin, α-Tubulin, GAPDH and HPRT at 3, 6, 24 and 72
386 hours of reperfusion (B-E) were obtained using total protein a loading control. *$p < 0.05$ vs CTR, # < 0.05 vs CTR
387 + rt-PA. Data represent the mean of n = 3-4 independent experiments and error bars indicate the standard
388 error of the mean (SEM).

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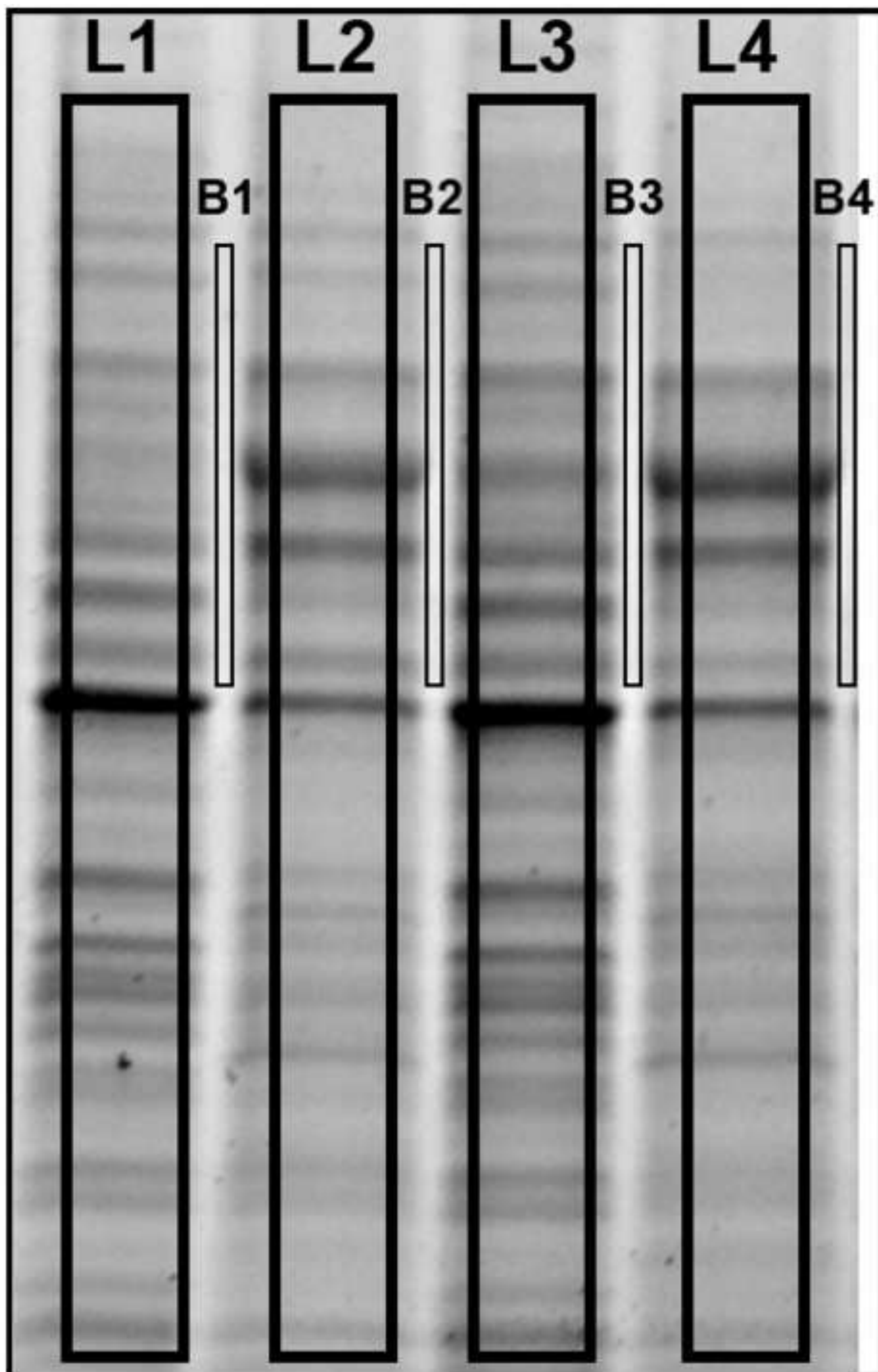

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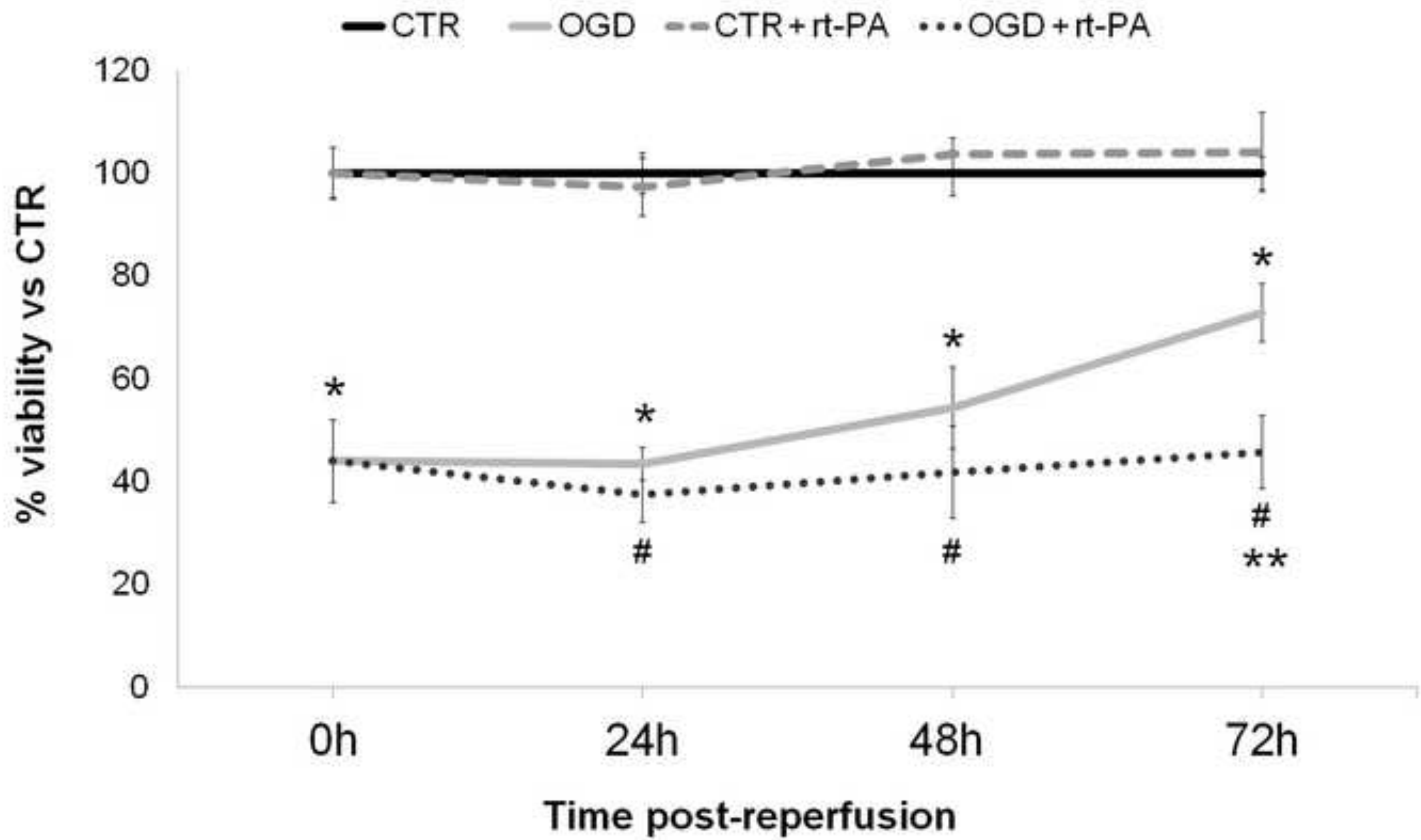

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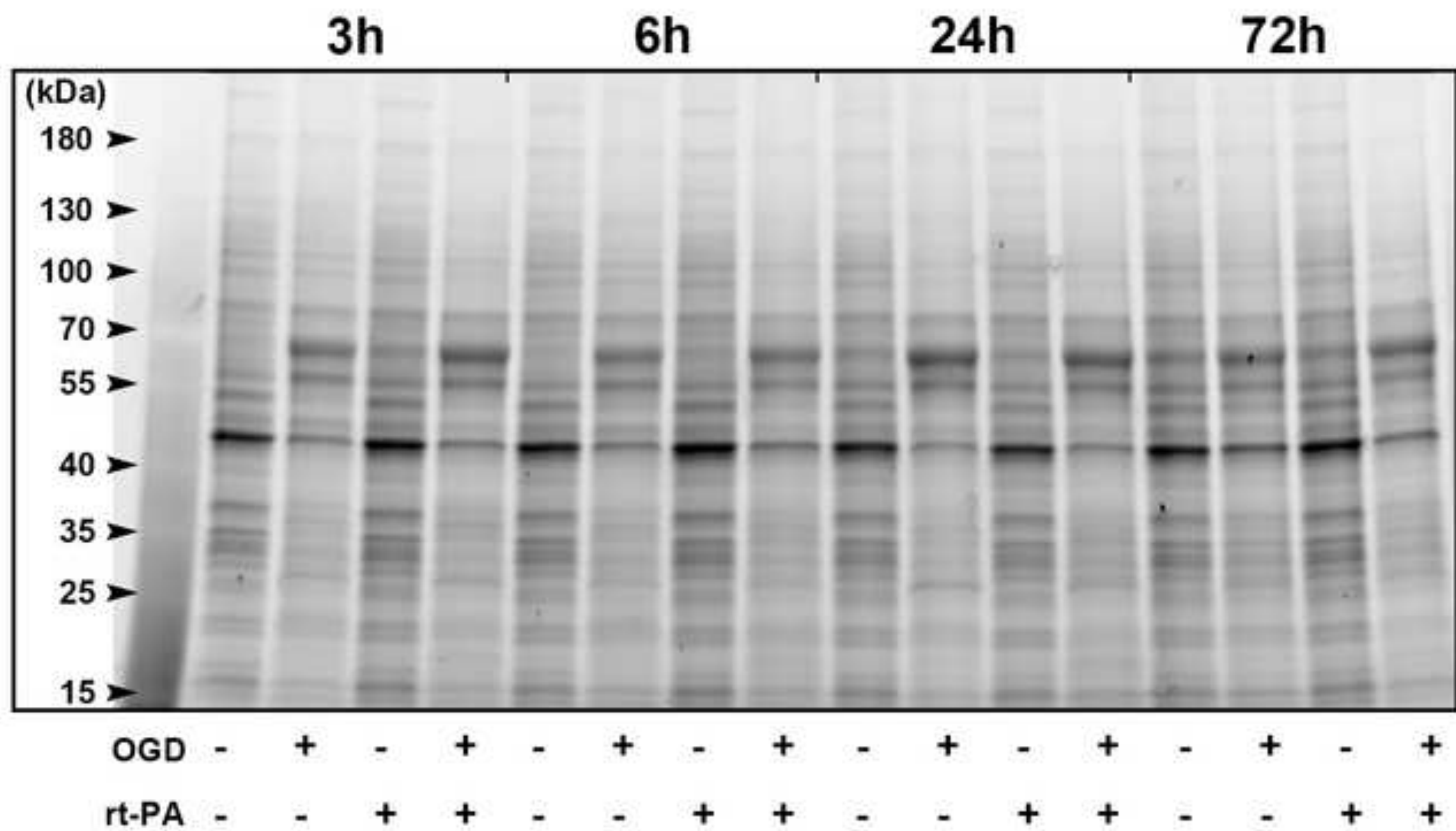

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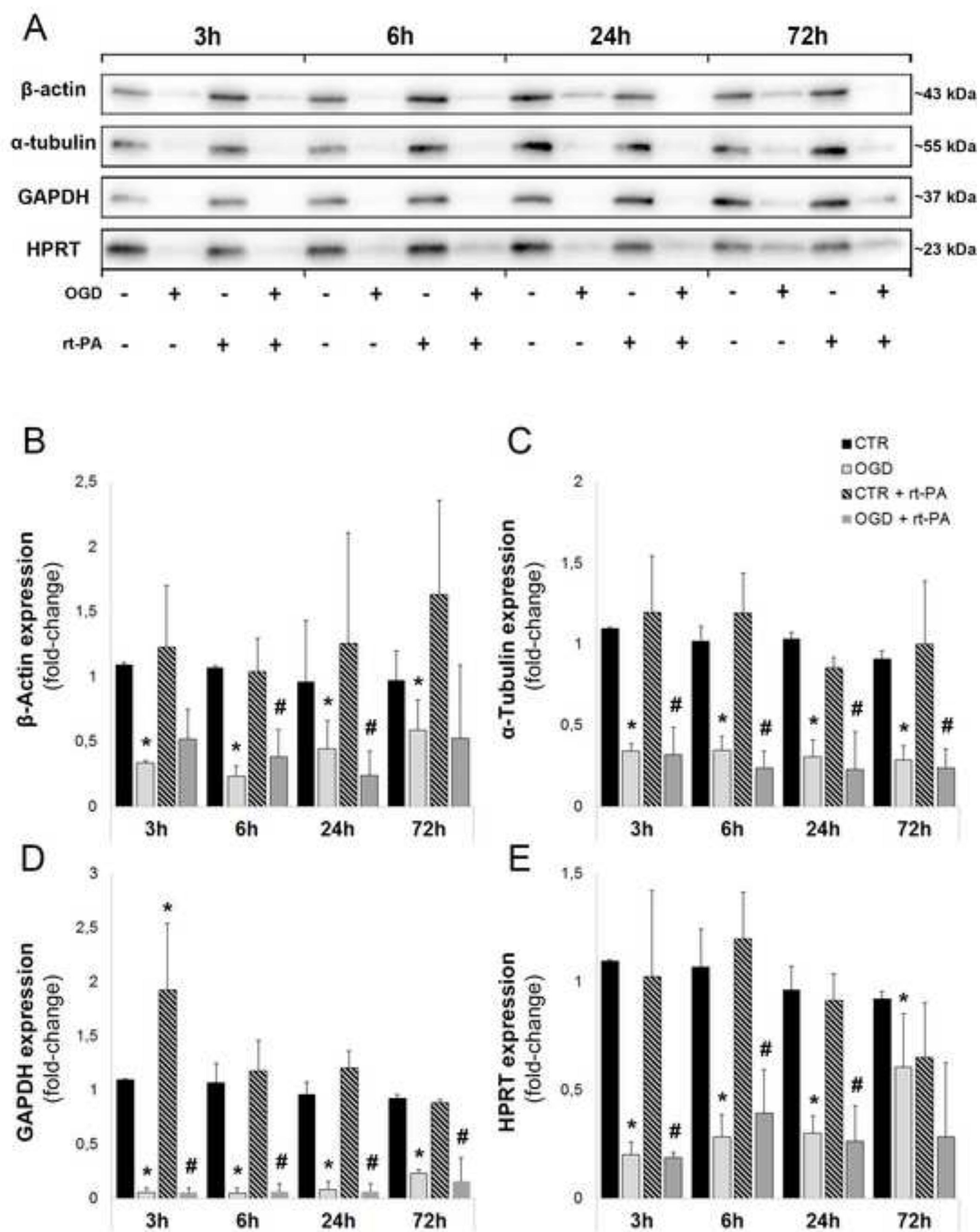

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