Relation between pH alterations in cellular models of amyotrophic lateral sclerosis and TDP-43 protein aggregation

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Abstract

Amyotrophic lateral sclerosis (ALS) is the most common degenerative disease of the motor neuron. TDP-43 aggregates constitute a hallmark of ALS and play a major role in the motoneuron degeneration. Several factors can influence the ability of TDP-43 to form aggregates. It was previously shown that, *in vitro*, mild pH modification can induce a conformational change in TDP-43 structure thus increasing its propensity to aggregate. Therefore, we first analyzed the intracellular pH alterations in a cellular model of ALS as well as fibroblasts derived from ALS patients. HEK293T cells overexpressing wildtype TDP-43 presented an increase in the fluorescence signal of the probe BCECF, which is sensitive to low pH. We also observed an increase in BCECF signal (i.e., low pH) in cultures of fibroblasts obtained from ALS patients as compared to controls. On the other hand, we tested the effect of pH alterations on the localization and aggregation of TDP-43. HEK293T cells overexpressing wildtype TDP-43 were incubated for 1h or 2h with solutions of different pH (6.4, 7.2 and 8.0). Incubation of cells for 1h in solutions of pH 6.4 and 8.0 increased TDP-43 aggregates, an effect lost after a 2h incubation. In these conditions, TDP-43 aggregation was accompanied by its mislocalization to the cytoplasm. Our results suggest that a decrease in cellular pH is observed in a cellular model of TDP-43 proteinopathy and in fibroblasts derived from ALS patients compared to controls. Alterations in cellular pH also seemed to have an effect on the aggregation and cellular localization of TDP-43. Further studies should focus on understanding how cellular pH alterations affect TDP-43, which might offer a new way to mitigate TDP-43 pathology in ALS.

Keywords: amyotrophic lateral sclerosis, TDP-43, aggregates, pH

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a degenerative disease characterized by the progressive death of motor neurons, leading to paralysis of skeletal muscles and eventually respiratory muscles, resulting in the death of patients within 3 to 5 years following the onset of the disease (Hardiman et al., 2017). Despite the progress made in understanding the pathophysiology of ALS, the mechanisms underlying motor neurodegeneration are still poorly understood. An abnormal aggregation of various proteins, particularly the TAR DNA binging protein 43 (TDP-43) has been reported in motor neurons. In fact, TDP-43 aggregates are considered as the hallmark of ALS (Hergesheimer et al., 2019) which has been identified as the major component of intracellular pathogenic aggregates in patients with ALS (Arai et al., 2006; Neumann et al., 2006).

Physiological TDP-43 shuttles between the nucleus and the cytoplasm but in pathological conditions, TDP-43 forms cytosolic aggregates, leading to the loss of protein function and cellular homeostasis. For instance, pathological mechanisms observed in ALS such as ischemia, mitochondrial alterations, and oxidative stress, which can affect intracellular pH have been associated with the cytoplasmic mislocalization and aggregation of TDP-43 (Gao et al., 2019; Wang et al., 2019; Wang et al., 2013). Concomitantly, several studies have shown that alterations in the pH can affect the structure of recombinant TDP-43 (Doke and Jha, 2022; Mompean et al., 2016a). However, no studies have assessed the effect of such alterations on TDP-43 in cells. We therefore hypothesize that there is a bidirectional interplay between pH-altering metabolic alterations in ALS and TDP-43 proteinopathy. The goal of our study was therefore to assess whether the intracellular pH was changed in a cellular model of ALS and patient fibroblasts, and investigate the impact of such changes on the localization and aggregation of cellular TDP-43.

2. Materials and Methods

2.1 Cells

HEK293T cells (ATCC, USA) were cultured in standard conditions (Lanznaster et al., 2019) and plated at a concentration of 0.3×10^6 cells/well the day before transfection.

Dermal fibroblasts from five ALS patients (cultured from the biopsy obtained at diagnosis, information in Supplementary material) and one healthy subject (man, 60 years-old; from Lonza Ltd, Basel, Switzerland) with roughly the same passage number, were cultivated in DMEM supplemented with 10% (v/v) FBS and 1% of Penicillin/Streptomycin.

2.2 pH solutions

pH solutions were prepared to induce changes in intracellular pH, as previously described (Swietach et al., 2012). The stock solution was prepared with 125 mM NaCl, 4.5 mM KCl, 20 mM Hepes, 20 mM MES, 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (original pH of 4.4). Once this solution was obtained, the pH was adjusted with NaOH or HCl in order to obtain three solutions of pH 6.4, 7.2 and 8.0. Cells were incubated with these solutions for 1h or 2h, which did not modify the viability of the cells (Supplementary material).

2.3 Overexpression of TDP-43

Overexpression of TDP-43 was done as previously described (Lanznaster et al., 2019). Briefly, HEK293T cells were transiently transfected with 2µg or 3µg of TDP-43-expressing plasmid. Control plasmid (pcDNA vector) was transfected at the highest concentration (3µg). Assays were realized 48 h after transfection.

2.4 Assessment of intracellular pH

To analyse the intracellular pH, cells were incubated with 0.5µM BCECF-AM (2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein, Acetoxymethyl Ester, ThermoScientific), a fluorescent probe sensitive to variations in intracellular pH (Supplementary material). After 15 minutes of incubation, the fluorescence was read at 535nm by confocal microscopy (Carre et al., 2015). The fluorescence intensity was analysed using ImageJ software and is given as Relative Fluorescence Unit (RFU).

2.5 Localization and aggregation of TDP-43

To study the localization of TDP-43 and its aggregation in different pH conditions, HEK293T cells were plated on coverslips pre-treated with poly-D-lysine (10 ug/mL) and transfected with the plasmid GFP-wtTDP-43 (3 µg). After 48 hours, cells were incubated for either one or two hours with the different pH solutions (6.4, 7.2, 8.0) previously described. Ten minutes before reading the fluorescence, 1µL of Hoechst 33342 (1:1000) was added. Then, cells were fixed with 4% paraformaldehyde solution (diluted in PBS). Images were obtained by confocal microscopy (Leica SP8) and 100 cells per condition were analysed for TDP-43 localization (nuclear, cytoplasmic, or both) and aggregation.

2.6 Statistics analysis

The statistical analyses were carried out with the GraphPad Prism 8 software. Data is reported as mean ± Standard Error of the Mean (SEM). Differences in pH regarding the fibroblasts of control and ALS patients were analysed with the Mann-Whitney test. The localization of the TDP-43 protein was analysed with the two-way ANOVA test. Differences in pH regarding increasing concentrations of TDP-43 and the influence of pH on TDP-43 aggregation were analysed with the Kruskal-Wallis test. Results were considered statistically significant at $p<0.05$.

3. Results

3.1 pH alteration in cellular models of ALS

3.1.1 pH evaluation in a TDP-43-overexpressing cellular model of ALS

After 48h of TDP-43 overexpression, HEK293T cells were incubated with the fluorescent probe BCECF-AM. The images shown in **Figure 1 (upper panel)** pinpoint to two main observations: 1) for the control cells, a basal level of fluorescence is visible, probably due to ion exchanges present to maintain physiological pH; and 2) cells overexpressing TDP-43 fluoresce more intensely than mock-transfected cells. Control cells (basal fluorescence) show a mean fluorescence of 18.45±3.2 RFU, that is statistically different only from TDP-43-overexpressing cells transfected with 3 µg of plasmid (TDP-43 2 µg: 37.85 ± 5.8 RFU, $p=0.0662$; and TDP-43 3 µg: 43.69 ± 4.5 RFU, $p=0.0127$). These results reflect a tendency for the pH to be altered in cells overexpressing TDP-43, an observation that seems to be dependent on the quantity transfected of TDP-43 plasmid.

3.1.2 pH assessment in ALS patients' fibroblasts

The intracellular pH of dermal fibroblasts obtained from ALS patients is different from that of fibroblasts obtained from a healthy control. The images shown in **Figure 1 (lower panel)** reflect a more pronounced fluorescence of the BCECF probe in the fibroblasts of ALS compared to control (mean ± S.E.M.: 46.2 ± 0.9 RFU and 25.2 ± 1.2 RFU; p=0.007, respectively).

3.2 Relationship between pH changes and TDP-43 protein mislocalization and aggregation

3.2.1 Effect of the pH on the localisation of TDP-43

First, we analysed the cellular TDP-43 localisation for each pH condition (**Figure 2A-H**). Our results indicate that the localization of overexpressed TDP-43 varies with the pH. Accordingly, after an incubation of 1h at physiological pH (7.2), TDP-43 was found to be evenly distributed between the cytoplasmic and nuclear compartments. On the other hand, there was an exacerbation of TDP-43 mislocalization following pH alterations. A significant increase of cytoplasmic TDP-43 was observed at pH 6.4 (p=0.04) and pH 8 (p=0.05) compared to pH 7.2. However, after 2 hours of incubation no statistical significance was noted.

3.2.2 Effect of pH on TDP-43 aggregation

The analysis of cells overexpressing TDP-43 aggregates showed that there is a significant increase in protein aggregation following a 1h incubation in solutions of pH 6.4 and 8.0 compared to the physiological pH condition (7.2) (p=0.05; **Figure 2I**). This increase was not observed following 2 hours of incubation (**Figure 2J**), which is therefore in correlation with the results obtained previously.

4. Discussion

Intracellular pH is decreased in cellular models of ALS

We found a decrease in the intracellular pH of fibroblasts from ALS patients, suggesting cellular acidosis. Concomitantly, several studies have shown that TDP-43 is mislocalized and aggregated in fibroblasts from ALS patients (Riancho et al., 2020; Sabatelli et al., 2015). The decrease in intracellular pH was also observed in HEK293T cells overexpressing TDP-43, a cellular model of ALS (Lanznaster et al., 2019). Under prolonged or chronic stressful conditions, the cell's ability to autoregulate its intracellular environment decreases which can explain the eventual acidification of the intracellular environment.

Acidosis is commonly observed in different pathologies of the central nervous system such as Alzheimer's disease, Parkinson's disease, multiple sclerosis, and vascular dementia (Arias et al., 2008; Ding et al., 2000; Friese et al., 2007; Harguindey et al., 2007; Vergo et al., 2011). The metabolic alterations and disease features that have been noted in ALS such as microglial activation (Morihata et al., 2000), impaired oxidative phosphorylation (Erecinska et al., 1993), endoplasmic reticulum stress (Aoyama et al., 2005) can be potentially linked to altered pH regulation. Interestingly, motoneurons were found to be particularly sensitive to acidic environments (Behan et al., 2013). A significant increase in acid-sensing ion channels 2 (ASIC2) was also noted in the motoneurons of G93A-hSOD1 mice and sporadic ALS patients, supporting the involvement of acidosis in ALS (Behan et al., 2013). In one study, increasing the intracellular pH in a mouse model of ALS seemed to increase the lifespan by 13% without affecting the severity of the symptoms (Kuo et al., 2014).

Acute altered intracellular pH exacerbates the mislocalization and aggregation of TDP-43 in a cellular model of ALS

TDP-43 is particularly sensitive to changes in its environment which can shift the equilibrium towards favouring the formation of oligomeric forms (Doke and Jha, 2022). Under stressful cellular conditions, TDP-43 undergoes liquid-liquid phase separation (LLPS) and is recruited to stress granules (Colombrita et al., 2009; Khalfallah et al., 2018). For instance, stress granules can be formed in the context of metabolic dysfunction caused by starvation, hypoxia, or mitochondrial abnormalities (Farny et al., 2009; Jain et al., 2016; Jin et al., 2022; Riback et al., 2017; van der Laan et al., 2012), which can enhance TDP-43 LLPS and aggregation. These stressors are partly linked to intracellular acidosis which affects the protonation state of charged amino acid residues and therefore alters the structure and conformation of proteins (Jin et al., 2022; Yao and Haddad, 2004). Such modifications might allow proteins like TDP-43 to act as biosensors of changes in the intracellular environment (Rabouille and Alberti, 2017).

Changes in the extracellular pH have been shown to induce parallel but less pronounced changes in the intracellular pH (Bouyer et al., 2004; Buckler et al., 1991; Glunde et al., 2002; Putnam, 2001). Here we showed, for the first time, that pH alterations in the cellular environment can increase TDP-43 aggregation and cytoplasmic mislocalization. In cells overexpressing TDP-43, we observed a decrease in the nuclear localization of TDP-43 at pH 6.4 and pH 8 compared to the physiological pH. The extent of TDP-43 mislocalization to the cytoplasm was less pronounced after a 2 h incubation with the different solutions, which might be reflecting the ability of the cell to adjust to acute environmental alterations and restore homeostasis. The same pattern was observed for TDP-43 aggregation which significantly increased following 1h of incubation at pH 6.4 and pH 8 but not after 2 h. For instance, cells were shown to exhibit a quick and sharp fall in their intracellular pH within seconds of being put under acidic stress, but then started to restore their pH after 2 hours (Adam et al., 2021). These results might suggest an implication of TDP-43 in the response of cells to abnormal pH changes.

How intracellular pH induces TDP-43 mislocalization and aggregation has not been well investigated. Since TDP-43 is known for its ability to spontaneously aggregate, particularly through its intrinsically disordered C-terminal domain (CTD) (Johnson et al., 2009), it is complicated to assess the effect of microenvironmental alterations on the full length protein. Therefore, most studies that investigated the effect of pH on TDP-43 folding have used isolated protein domains rather than the full-length protein (Patni and Jha, 2021; Pillai and Jha, 2019, 2020). In addition, the results obtained seem to vary according to the recombinant TDP-43 forms used and the protocols applied (i.e. protein concentration, temperature, buffer pH, salt concentration).

For instance, under extreme acidic conditions (pH 2.5), the RNA-recognition motifs (RRM1 and RRM2) of TDP-43 were shown to undergo a conformational opening reaction linked to the protonation of buried ionizable residues, thus exposing hydrophobic patches and forming metastable oligomers that act as precursors to solid aggregates (Pillai and Jha, 2020). A subsequent study revealed that the protonation of the side chain of a histidine residue at position 166 is critical for the misfolding of the RRM domains (Patni and Jha, 2021). However, the acidic pH conditions used in these studies are extreme values that are not usually observed in the cytoplasmic environment of cells, even in cases of severe acid-base disturbances (Bouyer et al., 2004; Buckler et al., 1991; Glunde et al., 2002; Tolkovsky and Richards, 1987). Other groups have tried to decipher the biochemical alterations induced by pH changes and affecting the folding and aggregation of TDP-43 under more physiologically relevant conditions. The N-terminal domain (NTD) of TDP-43 was found to be stably folded over a wide range of pH conditions, in part due to the presence of residues with negatively charged carboxylate groups that mutually repel each other at pH values below 10 (Mompean et al., 2016b). Despite it not being itself unfolded, the NTD seems to mediate TDP-43 oligomerization and subsequent CTD misfolding (Tsoi et

al., 2017). While it was first suggested that this oligomerization is mediated by hydrophobic interactions (Li et al., 2018), a more likely explanation is the pH-dependent decrease in electrostatic repulsion (Babinchak et al., 2019; Mompean et al., 2016a). In fact, it seems like the balance between hydrophobic interactions and electrostatic forces, both of which are affected by pH, is a key player in regulating the folding and aggregation of TDP-43, particularly its CTD. Overall, it seems that TDP-43 aggregation is mainly mediated by the CTD at the pH conditions between 6 and 8 that are used in our study.

Although the previously described studies show that TDP-43 domains can be structurally altered upon pH changes, the impact observed on separate protein domains might not be reproducible in the full-length protein given the presence of intramolecular interactions (Wei et al., 2016). In addition, all these studies were performed on recombinant purified proteins, and not in cellular models of TDP-43 proteinopathy. Given the extensive post-translational modifications that TDP-43 can undergo inside cells, such studies do not take into account these modifications that can highly affect the structure and solubility of proteins (Hergesheimer et al., 2019). For these reasons, it is pivotal to study the effect of pH changes on TDP-43 in more physiologically relevant settings, such as cellular models of ALS.

Conclusion

Given the reciprocal effect on one another of TDP-43 and pH alterations, it remains unclear whether pathological TDP-43 drives pH changes or vice versa. This interplay between TDP-43 and pH might take place through intermediary ALS-associated mechanisms (e.g. metabolic alterations, mitochondrial dysfunction, hypoxia) and constitute a feedforward loop whereas TDP-43 proteinopathy decreases the intracellular pH and intracellular pH alterations exacerbate TDP-43 proteinopathy. Our results implicate intracellular pH changes in the pathophysiology of ALS and offer a new potential target for therapeutic interventions. Future studies should look at the effect of pH alterations on TDP-43 in neurons, which are particularly sensitive to changes in their environment.

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374 **Figures.**

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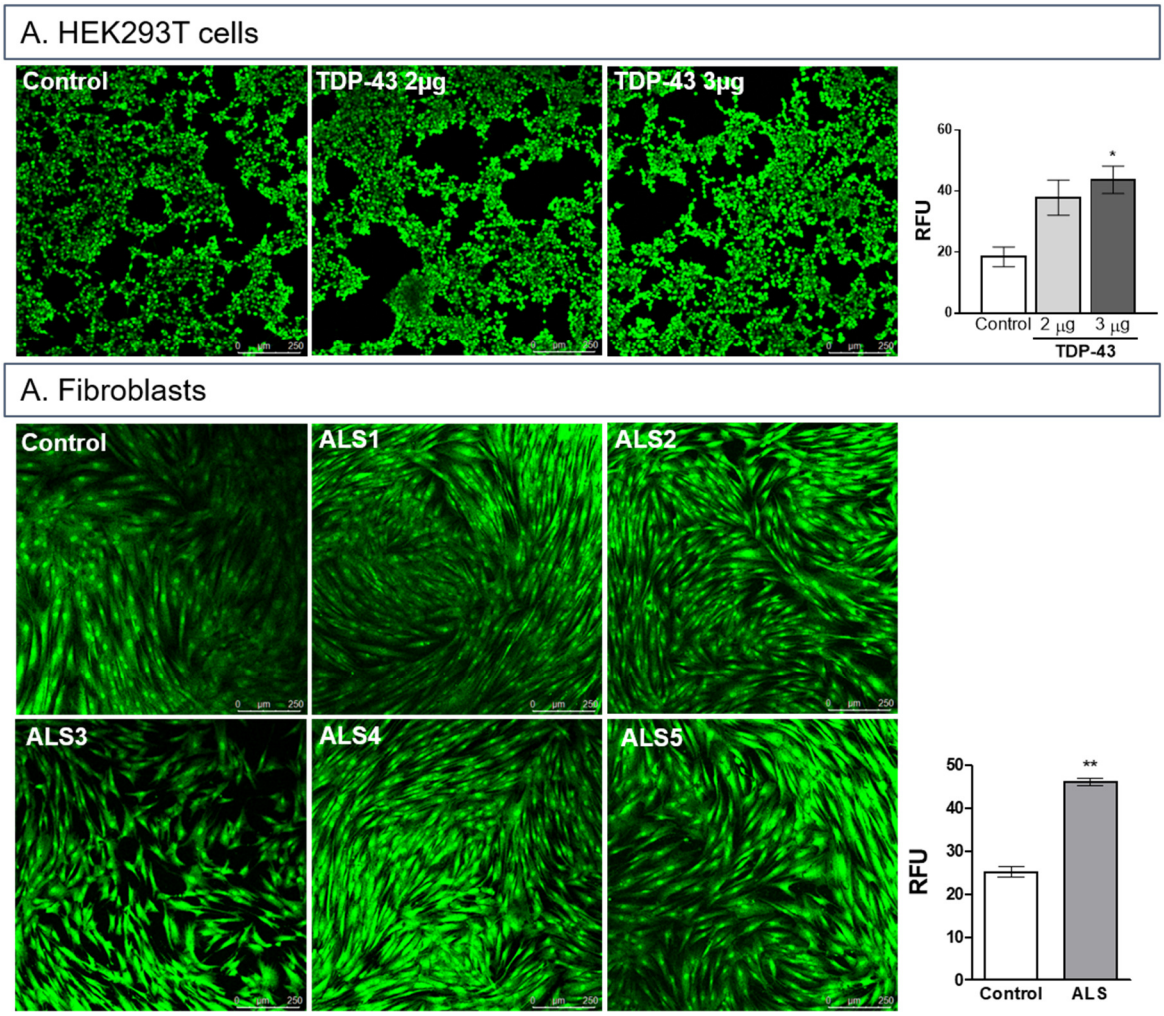

376

377 **Figure 1.** Lower pH in cellular models of ALS. HEK293T cells (upper panel) overexpressing increasing
378 amounts of TDP-43 present more fluorescence due to BCECF than control (mock-transfected) cells.
379 Images represent five independent experiments. N= 5; *p=0,0127; Kruskal-Wallis test. Dermal
380 fibroblasts (lower panel) obtained from ALS patients also present increased BCECF fluorescence
381 compared to fibroblasts from a control subject. Images represent two independent experiments. N= 2;
382 **p=0,0007; Mann Withney test.

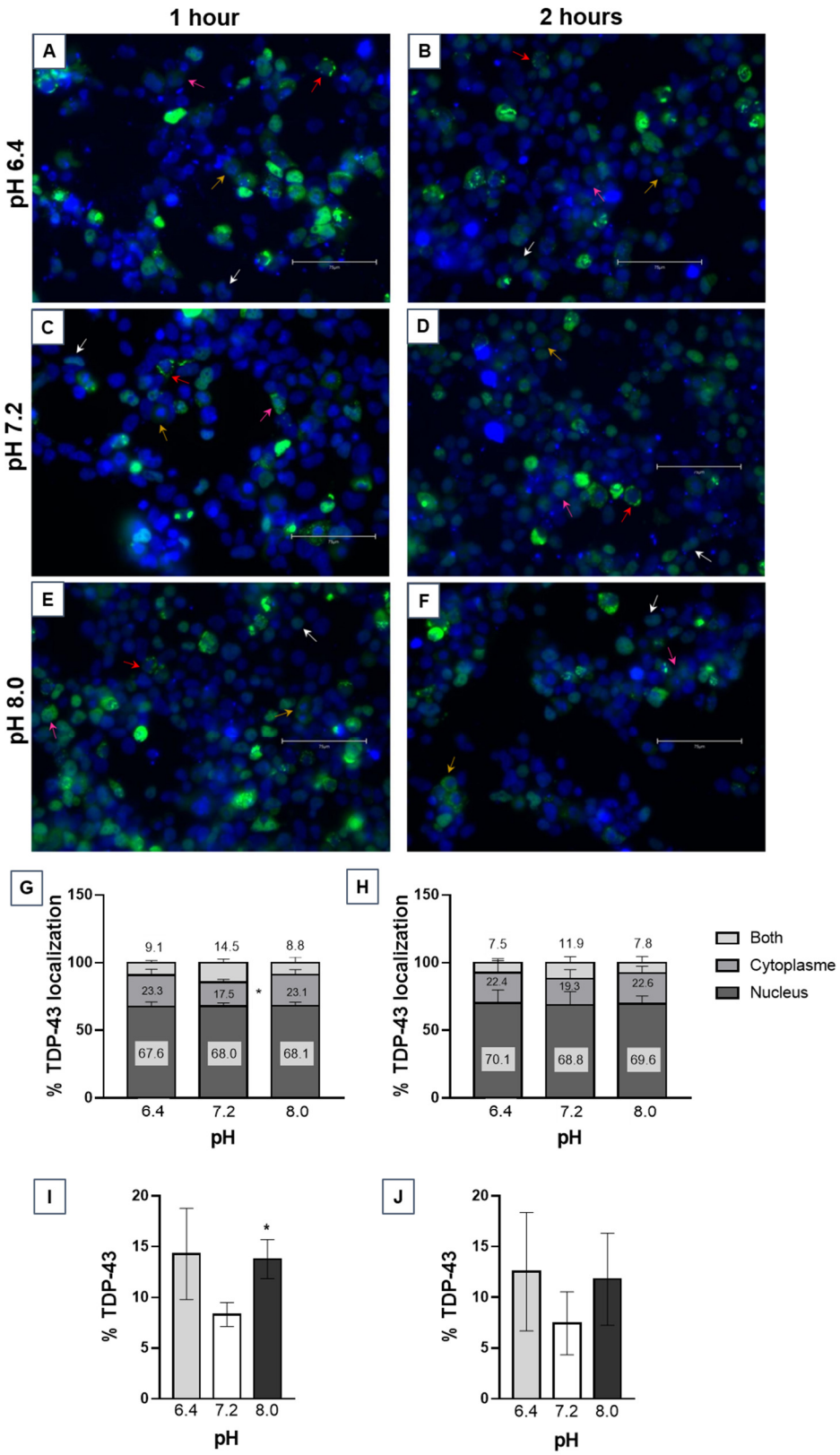

Figure 2. TDP43 localization in function of pH and incubation time : 1h (A,C,E,G,) or 2h (B,D,F,H). n = 4 ; *P = 0,04 (two-way ANOVA); I: percentage of TDP43 aggregation after 1h incubation with different pH solutions or (J) after 2h incubation. n = 4, *P = 0,05 (one-way ANOVA followed by a Kruskal-Wallis test).