CHANGES IN THE RAGE/NADPH OXIDASE SIGNALING PATHWAY AND OXIDATIVE STRESS LEVELS IN SH-SY5Y CELLS EXPOSED TO HIGH LEVELS OF FLUORIDE

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ABSTRACT: Excessive fluoride is known to induce oxidative stress and cell damage, particularly, in the nervous system. We aimed to investigate changes in the expression of the receptor for advanced glycation end products (RAGE), p47phox, phospho-p47phox, gp91phox, p22phox, and nuclear factor kappa B (NF-κB) in SH-SY5Y cells exposed to different levels of sodium fluoride and explore the molecular mechanisms underlying cell injury caused by excessive fluoride exposure. The cells were divided into the following groups: control, low-dose fluorinated (NaF 0.4 mmol/L), high-dose fluorinated (NaF 4 mmol/L), and FPS-ZM1 (1 µM). The protein levels of RAGE, p47phox, phospho-p47phox, gp91phox, p22phox, and NF-κB were detected using western blotting. Additionally, levels of superoxide dismutase (SOD) and malondialdehyde (MDA) were detected using respective biochemical methods. Compared with those in the control group, the protein levels of RAGE, p47phox, phospho-p47phox, gp91phox, p22phox, and NF-κB were significantly
upregulated in the high-dose fluorinated group (p<0.05). Moreover, compared with those in the high-dose fluorinated group, the protein levels of p47phox, phospho-p47phox, gp91phox, p22phox, and NF-κB were downregulated in the FPS-ZM1 group (p<0.05). Furthermore, compared with that in the control and high-dose fluorinated groups (p<0.05), the expression of SOD decreased in the high-dose fluorinated group and increased in the FPS-ZM1 group, respectively. Compared with that in the control group and high-dose fluorinated groups (p<0.05), MDA expression increased in the high-dose fluorinated group and reduced in the FPS-ZM1 group, respectively. Thus, excessive fluoride can upregulate the RAGE/NADPH oxidase signaling pathway in SH-SY5Y cells, and these changes may be related to the nerve cell injury caused by fluoride.

Keywords: Fluorosis; FPS-ZM1; NADPH oxidase; RAGE; SH-SY5Y cells

INTRODUCTION

Endemic fluorosis is a major health concern globally. It is attributed to the long-term intake of excessive amounts of fluoride and can cause pathological changes in many organs and systems of the body, including damage to the nervous system that mainly manifests as mental retardation and cognitive impairment. This excessive fluoride intake induces excessive production of reactive oxygen species (ROS), which eventually can lead to neurotoxicity.

Receptor for advanced glycation end products (RAGE), a member of the immunoglobulin superfamily of cell membrane surface molecules, is expressed by nerve, tumor, and vascular endothelial cells. Its expression is low under normal physiological conditions but is upregulated when the immune system is activated or in a stressed state. RAGE mediates multiple signal transduction pathways such as the RAGE/reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase signaling pathway that is closely related to oxidative stress levels. By binding to a ligand, RAGE can activate downstream NADPH oxidase to produce excessive ROS, resulting in cell and tissue dysfunction. Accordingly, this investigation was designed to examine whether oxidative stress induced in the central nervous system by high levels of fluoride is related to the RAGE/NADPH oxidase signaling pathway.
MATERIALS AND METHODS

Materials: Following is the list of the reagents, kits, and equipment used in this study and the respective supplier information presented within parentheses: sodium fluoride (NaF, Sigma Aldrich, St. Louis, MO, USA); SH-SY5Y cells, derived from human neuroblastoma cells (German Collection of Microorganisms and Cell Cultures GmbH, Germany); Superoxide Dismutase (SOD) Detection Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China); Malondialdehyde (MDA) Detection Kit (Beyotime Biotechnology, Shanghai, China); rabbit polyclonal antibodies against RAGE (ab37647), gp91 (ab180642), p22 (ab75941), and nuclear factor kappa B (NF-κB) (ab32536), and goat polyclonal antibodies against p47 (ab795) (Abcam Corporation, Cambridge, UK); rabbit polyclonal antibodies against phospho-p47phox (AF3167) (Affinity Biosciences, Cincinnati, OH, USA); and FPS-ZM1 (ApexBio Inc., Houston, TX, USA).

Cell culture and treatments: SH-SY5Y cells were cultured in Dulbecco’s modified Eagle medium (DMEM)/F12 supplemented with 10% fetal bovine serum (FBS), 100 mg/mL streptomycin, and 100 U/mL penicillin and maintained in a humidified incubator containing 5% CO₂ at 37 °C. Subsequently the cells were divided into the control (serum-free medium), low-dose fluorinated (NaF 0.4 mmol/L), high-dose fluorinated (NaF 4 mmol/L), and FPS-ZM1 (high-dose fluorinated group [NaF 4 mmol/L] + RAGE blocker [FPS-ZM1]) groups. Briefly, SH-SY5Y cells were treated with different concentrations of NaF (0, 0.4, and 4 mmol/L) for 24, 36, and 48 h (groups were named C24, C36, and C48 for the control; LF24, LF36, and LF48 for the low-dose; and HF24, HF36, and HF48 for the high-dose groups, respectively). The control group was treated with serum-free medium, and the FPS-ZM1 group was treated with 1 µM FPS-ZM1 for 24 h before fluorination (high-dose fluorinated groups [NaF 4 mmol/L] + RAGE blocker [FPS-ZM1]).

Examination of MDA level: The cells inoculated on culture plates were washed thrice with phosphate-buffered saline (PBS) to remove the spent medium, prior to use in the subsequent experiments. MDA levels were measured using the abovementioned MDA Detection Kit (Beyotime Biotechnology) as per the manufacturer’s instructions. Briefly, test samples were mixed with an MDA-working solution and incubated in a water bath at 100 °C for 15 min. Then, the samples were cooled with running water,
centrifuged at 1000 × g for 10 min, and the absorbance of the supernatants was measured at 532 nm. Calculations using the linear regression equation of the standard curve based on the concentration of the standard substance and the corresponding OD value were performed, and the corresponding sample concentration was calculated according to the sample OD value.

**Measurement of SOD activity:** The cells inoculated on culture plates were washed thrice with PBS to remove the spent medium, prior to use in the subsequent experiments. SOD levels were measured using the abovementioned SOD Detection Kit (Nanjing Jiancheng Bioengineering Institute) in strict accordance with the manufacturer’s instructions. Briefly, test samples were mixed with an SOD-working solution and incubated in a water bath at 37 °C for 40 min. Absorbance was measured at 550 nm, and the linear regression equation of the standard curve was calculated using the OD value of the standard substance concentration. The corresponding sample concentrations were calculated using the regression equation and according to the OD values of the samples.

**Western blot analysis of RAGE, gp91, p47, phospho-p47phox, p22, and NF-κB levels in cells:** The inoculated cells were washed thrice with pre-chilled PBS and incubated on ice for 1 h with RIPA buffer (80 µL/well) containing protease and phosphatase inhibitors. The supernatant was collected after centrifugation at 4 °C for 35 min at 27,000 × g. Proteins were separated using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. The membranes were incubated with primary antibodies (1:1000) against RAGE, gp91, p47, phospho-p47phox, p22, NF-κB, and (1:5000) β-tubulin at 4 ℃ overnight. Then, the membranes were incubated with an HRP-conjugated secondary antibody (1:5000 anti-donkey/anti-rabbit) at 20 °C for 1 h. Protein bands were detected with an ECL kit (Solarbio, China). Finally, these membranes were incubated in ECL Plus reagent (Amersham, Sweden) for 3 min and the signals thus produced were visualized by exposure to a Hyper Performance Chemiluminescence film.

**Data analysis and statistics:** Statistical analysis was performed using SPSS v24.0 (SPSS Inc., Chicago, IL, USA). Data are presented as the mean ± standard deviation. Pearson correlation analysis was used to determine correlation; two-tailed Student's t-test and one-way ANOVA (followed by an LSD or T3 test) were used to determine statistical differences between two and among multiple groups, respectively.
P-values < 0.05 were considered statistically significant.

RESULTS

*SOD and MDA levels:* As shown in Table 1, the MDA content and SOD activity were significantly increased and decreased in the HF36 and HF48 groups, respectively, compared to the control group. In contrast, the MDA content in the FPS-ZM1 group was significantly decreased and the activity of SOD was significantly increased in comparison with the HF36 group.

*RAGE, gp91, p47, phospho-p47phox, p22, and NF-κB protein levels:* Western blotting results indicated that RAGE (Figure 1A), gp91 (Figure 1B), p22 (Figure 1C), p47 (Figure 1D), phospho-p47phox (Figure 1E), and NF-κB (Figure 1F) expression was significantly higher in the HF24, HF36, and HF48 groups than in the control group. In addition, the protein expression of gp91 (Figure 2A), p47 (Figure 2B), phospho-p47phox (Figure 2C), p22 (Figure 2D), and NF-κB (Figure 2E) was significantly decreased in the FPS-ZM1 group than in the HF36 group.

*Correlation between RAGE expression and SOD or MDA levels:* In SH-SY5Y cells exposed to high levels of fluoride, significant correlations between RAGE and SOD levels (Figure 3A) as well as between RAGE and MDA levels (Figure 3B) were observed.

DISCUSSION

Chronic fluorosis is known to damage the nervous system. Oxidative stress is one of the mechanisms underlying fluoride-induced neurotoxicity. High levels of fluoride can increase the content of free radicals and decrease the activity of antioxidant enzymes in the body, thereby resulting in increased oxidative stress that can induce changes in the structure and function of the brain tissue.

RAGE is a multi-ligand-specific receptor expressed on the surface of several cell types. Its ligands mainly include advanced glycation end products (AGEs), S100, high mobility group protein B 1 (HMGB1), and amyloid β Peptide (Aβ) among others; extensive research has been performed on AGEs, which interact with RAGEs to activate several downstream signal transduction pathways, including the RAGE/NADPH oxidase signaling pathway explored in the present study. Activation of this pathway leads to increased oxidative stress levels in tissues, causes systemic tissue damage, and participates in the initiation and progression of a variety of diseases. NADPH oxidase is a membrane protein widely expressed by several tissues and organs. NADPH oxidase can reduce oxygen molecules into superoxide...
anions via NADPH-dependent single-electron reduction. It is not only the main source of ROS generation in vivo but also the only enzyme that directly produces ROS. The enzyme is composed of multiple subunits, with gp91phox and p47phox being the main functional subunits. Following phosphorylation of p47phox and binding with p22phox, the spatial conformation of gp91phox changes that eventually activates its enzymatic activity, thereby catalyzing ROS production and increasing oxidative stress. A previous study showed that blocking gp91phox transcription and using NADPH oxidase inhibitors can reduce ROS production, suggesting that NADPH oxidase is closely related to increased oxidative stress. Concurrently, another study reported that an increase in oxidative stress level induced the expression of RAGE, which in turn further increased the ROS production, thereby forming a self-amplifying vicious cycle. In this study, we examined the protein expression and oxidative stress levels associated with the RAGE/NADPH oxidase signaling pathway in SH-SY5Y cells exposed to different fluoride concentrations. The results showed that the expression levels of RAGE and the NADPH subunits, phospho-p47phox, p47, gp91, and P22, were significantly upregulated with induction of fluorosis. Simultaneously, oxidative stress levels were also increased. Reportedly, AGE expression levels are increased in the brain tissue of fluorosis-afflicted rats and SH-SY5Y cells exposed to fluoride. It is speculated that fluorosis can lead to increased AGE levels. Based on the outcomes of our study, we hypothesized that fluoride exposure may stimulate the RAGE/NADPH oxidase signaling pathway by increasing the AGE levels, leading to increased oxidative stress and tissue damage. This may be one of the mechanisms by which fluoride induces nerve cell damage. To confirm this hypothesis, we used the RAGE-specific blocker FPS-ZM1 that downregulated the expression of phospho-p47phox, p47, gp91, p22, and NF-κB as well as reduced the oxidative stress levels. We also performed a correlation analysis between the expression levels of RAGE protein and two oxidative stress indicators, SOD and MDA. Our results strongly suggest that fluoride exposure causes oxidative stress-induced injury of the nerve cells via activating RAGE.

NF-κB is a ubiquitous transcription factor that, when activated, regulates several downstream factors, including inflammatory molecules, cytokines, and adhesion factors, and plays an important role in cell apoptosis, inflammatory response, and various immune disorders. According to previous reports, NF-κB levels significantly increase in the liver, kidneys, bone tissue, hippocampi, and
microglial cells\textsuperscript{24} of rat models of chronic fluorosis. Thus, this protein plays an important role in fluoride-induced cell injury. The promoter region of RAGE-encoding genes contains functional binding elements for NF-\(\kappa\)B, suggesting that NF-\(\kappa\)B is a key modulator of RAGE signal transduction.\textsuperscript{25} Reportedly, oxidative stress can induce the expression of NF-\(\kappa\)B.\textsuperscript{26-28} Here, we found that the expression of NF-\(\kappa\)B was significantly upregulated in the cell-based model of fluorosis, and our results are consistent with those in the published scientific literature. The upregulation of RAGE expression due to excessive fluoride activates NF-\(\kappa\)B, thereby upregulating the expression of its target genes and causing nerve tissue damage. In addition, FPS-ZM1 inhibited the expression of NF-\(\kappa\)B, suggesting that blocking RAGE signaling can inhibit the subsequent production of NF-\(\kappa\)B and thus, reduce the cell damage caused by fluorosis.

In conclusion, fluoride-induced nerve damage may be related to the activation of the RAGE/NADPH signaling pathway. As we only conducted \textit{in vitro} studies, we intend to validate our findings using \textit{in vivo} models in the future.

ACKNOWLEDGMENTS

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**Table 1.** MDA content and SOD activity levels in SH-SY5Y cells (means±SD). Compared with the control group, *p < 0.05 and **p < 0.01 ; Compared with the high-dose fluorinated group, #p < 0.01 as determined by the analysis of variance (ANOVA), followed by the Student-Newman-Keul’s test.

<table>
<thead>
<tr>
<th>Grouping</th>
<th>MDA content (μmol/L)</th>
<th>SOD activity (U/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24h Control group (Control 24)</td>
<td>0.63±0.07</td>
<td>58.88±2.02</td>
</tr>
<tr>
<td>24h low-dose fluorinated group (LF24)</td>
<td>0.072±0.13</td>
<td>52.76±2.34**</td>
</tr>
<tr>
<td>24h high-dose fluorinated group (HF24)</td>
<td>1.16±0.19**</td>
<td>40.69±1.45**</td>
</tr>
<tr>
<td>36h Control group (Control 36)</td>
<td>0.84±0.15</td>
<td>57.58±0.32</td>
</tr>
<tr>
<td>36h low dose fluorinated group (LF36)</td>
<td>0.99±0.09</td>
<td>54.01±0.48*</td>
</tr>
<tr>
<td>36h high dose fluorinated group (HF36)</td>
<td>1.52±0.19**</td>
<td>40.05±1.33**</td>
</tr>
<tr>
<td>36h high dose fluorinated + FPS - ZM1 group (HFF36)</td>
<td>0.90±0.07***#</td>
<td>47.17±2.61***#</td>
</tr>
<tr>
<td>48h Control group (Control 48)</td>
<td>0.80±0.13</td>
<td>59.00±0.82</td>
</tr>
<tr>
<td>48h low dose fluorinated group (LF48)</td>
<td>1.32±0.16**</td>
<td>51.27±0.06**</td>
</tr>
<tr>
<td>48h high-dose fluorinated group (HF48)</td>
<td>2.13±0.15**</td>
<td>36.94±1.89**</td>
</tr>
</tbody>
</table>
Figure 1. Expression of RAGE (1A), gp91 (1B), P22 (1C), P47 (1D), Phospho-P47phox (1E), and NF-κB (1F) of SH-SY5Y cells in different groups. C24, 36, 48=24h, 36h, 48h Control group; LF24, 36, 48=24h, 36h, 48h low-dose fluorinated group; HF24, 36, 48=24h, 36h, 48h high-dose fluorinated group. The values are shown as the means±SD. *p<0.05 and **p<0.01 in comparison to the control group as determined by the analysis of variance (ANOVA), followed by the Student-Newman-Keul’s test.
**Figure 2.** Expression of gp91 (2A), P47 (2B), Phospho-P47phox (2C), P22 (2D) and NF-κB (2E) of SH-SY5Y cells in different groups. C36=36h Control group; HF36=36h high-dose fluorinated group; HF+FPS-ZM1 36=36h high-dose fluorinated group + RAGE blocker FPS-ZM1(1uM). The values are shown as the means±SD. *p < 0.05 and **p <0.01 in comparison to the control group and #p < 0.05 and ##p < 0.01 in comparison to the high-dose fluorinated group as determined by the analysis of variance (ANOVA), followed by the Student-Newman-Keul’s test.
Figure 3. Correlations between RAGE expression level with SOD and MDA. There were significantly negative-linear correlations between the RAGE and SOD (3A) and positive-linear correlations between the RAGE and MDA (3B) in SH-SY5Y cells. The values were analyzed employing the Pearson correlation test.