Effect of 7-methylxanthine on human retinal pigment epithelium cells cultured in vitro

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Purpose: To evaluate the effects of 7-methylxanthine (7-MX) on the growth of human retinal pigment epithelium (RPE) cells and to observe the changes in the expression of adenosine receptors (ADORs) in RPE cells upon 7-MX treatment.
Methods: Human RPE cells (monolayer at about 80% confluence) were cultured in vitro in the presence or absence of 7-MX. Cell proliferation was evaluated with 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The cell cycle distribution and apoptosis level were analyzed with flow cytometry. Quantitative PCR and immunofluorescence assay were used to examine the mRNA and protein expression of ADORs.
Results: 7-MX at low concentrations had no effect on the proliferation of RPE cells, whereas 100 µmol/l 7-MX slightly decreased cell proliferation at 48 h but without a statistically significant difference. The 7-MX treatment was performed at the low concentration of 10 µmol/l in the following experiments. The proportion of RPE cells in the G1 stage was slightly increased at 24 h (p=0.035) but decreased at 48 h (p=0.0045) upon 7-MX treatment; and the proportion was restored to normal at 72 h. No statistically significant change in apoptosis levels was found in RPE cells cultured with 7-MX. The expression of ADORA1, ADORA2A, and ADORA2B in RPE cells was inhibited by 7-MX treatment at 48 h, while the expression levels appeared to rebound at 72 h.
Conclusions: 7-MX has little effect on the proliferation or apoptosis level of human RPE cells; however, in short-term treatment, 7-MX disturbs the proportion of cells in the G1 stage and inhibits the expression of ADORA1, ADORA2A, and ADORA2B.

Adenosine receptors (ADORs) belong to the superfamily of guanine nucleotide-binding G-protein-coupled receptors; this superfamily includes four subtypes (ADORA1, ADORA2A, ADORA2B, and ADORA3) [1]. Animal studies have shown that myopia is associated with changes in retinal dopamine and acetylcholine neurotransmission, which are both modulated by ADORs [2-4]. High ametropia in children is associated with retinal electrophysiological abnormalities associated with abnormal ADOR activity [4,5].

7-methylxanthine (7-MX) is a metabolite of caffeine and theobromine, and has been shown to have low toxicity [6] and no carcinogenic effects [7]. 7-MX is known as a nonselective adenosine antagonist and has been shown to work against myopia [4,8,9]. 7-MX has been confirmed to reduce the severity of myopia and eye elongation induced by forming deprivation in guinea pigs and to counteract the thinning of the posterior sclera and of collagen fibrils induced by form deprivation [10]. A clinical trial showed that 7-MX reduced

eye elongation and myopia progression in childhood myopia [4].

A previous study by our group showed that all four subtypes of ADORs were expressed in human retinal pigment epithelial (RPE) cells [11]. RPE cells play an important role in regulating the chemical composition and balancing the extracellular environment of the retina [12,13]. Adenosine from the retina activates ADORA on the RPE [14]. Adenosine is involved in the regulation of fluid input and output in RPE cells through the ADORs [15,16]. The fluid exchange transits signaling molecules coming from the choroid and the retina [17]. These signaling molecules modulate the ocular growth–related functions [18] and may regulate the growth of the eye [19], therefore playing a role in the progression of myopia [20]. Furthermore, lesions and/or dysfunction of the RPE cells are involved in the pathological changes of myopia [20].

Therefore, it can be hypothesized that the regulation of adenosine signaling in RPE cells may be involved in a mechanism by which 7-MX affects myopia progression. Thus, this study aimed to examine the effect of 7-MX on RPE cells and whether ADORs in RPE cells are modulated by 7-MX. The results may provide evidence of how adenosine and ADORs work in regulation of eye growth and myopia progression.

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METHODS

Tissue source: This study was approved by the Ethics Committee of Sun Yat-sen University (China) and complied with the Declaration of China for Research Involving Human Tissue and with the Declaration of Helsinki. This study was approved by the Ethics Committee of Sun Yat-sen University (China) and complied with the Declaration of China for Research Involving Human Tissue, the Declaration of Helsinki, as well as the ARVO statement on human subjects. Three myopic adult human eyes (from 27-year-old men) were obtained from the eye bank of Zhongshan Ophthalmic Center (Sun Yat-sen University).

RPE cells isolation and primary culture: The eyes were dissected, and the anterior segment and the retina were removed. The eyecups were rinsed with calcium- and magnesium-free balanced salt solution and incubated with 0.25% trypsin-0.02% EDTA (Gibco, Invitrogen Inc., Carlsbad, CA) for 1 h at 37 °C. The incubation buffer with the released cells was removed, and ten times the volume of Dulbecco's modified Eagle medium (DMEM/F12; Gibco, Invitrogen Inc.) with 20% fetal bovine serum (FBS; Gibco, Invitrogen Inc.), 1.2 g/l sodium bicarbonate (Fisher Scientific, Hampton, NH), and 10 ml/l L-glutamine-penicillin G-streptomycin (2 mM to 100 U/ml to 0.1 mg/ml; Sigma, St. Louis, MO) were added.

Primary RPE cells were cultured in a humidified incubator and kept at 37 °C in 5% CO_2. The medium was changed every 2 to 3 days. At passage 3, cells were photographed, and immunochemistry was performed to detect cytokeratin 18 expression. Then when a monolayer (80% confluence) was achieved, cells were trypsinized in 0.25% trypsin-0.02% EDTA solution in PBS (1X; 155.2 mM NaCl, 1.1 mM KH_2PO_4, 3.0 mM Na_2HPO_4-$7H_2O$, pH 7.4).at room temperature for 2 min and subcultured (i.e., passage 4) for the 7-MX treatment and subsequent examination.

Immunochemistry assay: The RPE cells were cultured on cover glasses in six-well plates until 70–80% confluence. The cells were washed with PBS three times and fixed with 4% paraformaldehyde for 10 min. After washing with PBS, the glasses were immersed in 3% H_2O_2 solution to block endogenous peroxidase and then incubated with 5% normal goat serum (containing 0.5% Triton X-100) at room temperature for 20 min. The slices were incubated at 4 °C overnight with the primary antibody (rabbit anti-human keratin 18 monoclonal antibody; Sigma) diluted at 1:100 in PBS. A negative control was run in which the cells were incubated in PBS without primary antibody. Then the slices were washed with PBS and incubated with goat anti-rabbit immunoglobulin G (IgG) secondary antibody (Chemicon, Temecula, CA) diluted at 1:100 in PBS at room temperature for 30 min. Finally, the slices were colored with 3,3′-diaminobenzidine (DAB) using the DAB developing kit (Maixin Biotech Development, Fuzhou, China) and restained with hematoxylin.

MTT proliferation assay: The RPE cells were seeded in a 96-well plate at a density of 1×10^4/200 µl per well. Incubation took place in the previously described medium until a confluence of 80% was achieved. After a 24-h phase of deficiency in 0.1% FBS-containing DMEM/F12, the primary cultured RPE cells were cultured with different concentrations of 7-MX (from 1 nmol/l to 1 mmol/l; 0 nmol/l was used as control; Sigma) in a FBS-free medium. The control cells were cultured in FBS-free medium without 7-MX treatment.

The proliferation rate was measured dynamically at 24, 48, 72, and 96 h of 7-MX treatment. At the end of the incubation interval, 20 µl of MTT solution (5 mg/ml; Sigma) were added to each well and incubated for 4 h at 37 °C. Thereafter, 150 µl of dimethyl sulfoxide (DMSO; Sigma) were added and incubated at room temperature for 15 min. Optical density (OD) was measured using a microplate reader. Each experiment was repeated three times.

Flow cytometry: Apoptosis and cell cycle distribution were determined with flow cytometry. The RPE cells were cultured in DMEM/F12 with or without 10 µmol/l 7-MX for 24, 48, and 72 h and digested with 0.25% trypsin. Cells without 7-MX treatment were used as control. The medium and the cells were centrifuged at 1,000 ×*g* and 4 °C for 10 min. Precipitates were washed three times with PBS, fixed with 70% alcohol for at least 24 h, and kept at 4 °C until assayed. Then, 1×10^6 cells in 100 µl were washed twice with PBS. Precipitates were dyed with 300 µl of DNA dying solution (PI 100 µg/ml and RNase A 20 U/ml; Sigma) for 30 min. The cells were then read with a FACScalibur system (BD Biosciences, Franklin Lake, NJ). The experiments were performed three times. Cell cycle and apoptosis were analyzed with Multicycle AV analysis software.

RNA isolation and qPCR analysis: The RPE cells with 10 µmol/l 7-MX for 0, 24, 48, and 72 h were harvested and washed with PBS. Total RNA was isolated with TRIzol (Invitrogen Inc.), according to the manufacturer's instructions. The quality and quantity of total RNA were estimated spectrophotometrically. Subsequently, RNA was reverse-transcribed into cDNA using a RevertAid First Strand cDNA synthesis kit (Fermentas, Burlington, Canada). Quantitative PCR (qPCR) was performed as described in a previous study [21]. Reactions were incubated at 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The primers and probes for A1R (Rn00567668_m1), A2aR (Rn00583935_m1), A2bR (Rn00567697_m1), and GAPDH (Rn01775763_g1) were obtained from Applied Biosystems

(Grand Island, NY). The amplification was performed in an ABI 7500 Fast Real-Time PCR system (Applied Biosystems). The $2^{-\Delta\Delta Ct}$ method was used for relative quantification of the gene expression.

Immunofluorescence assay: The RPE cells with 10 µmol/l 7-MX for 24 and 48 h were subcultured on cover glasses in six-well plates until 70–80% confluence; cells without 7-MX treatment were used as control. The cells were washed with PBS three times, fixed with cool acetone for 15 min, air-dried, and kept at −20 °C until use. The slices were washed three times with PBS, covered with 10% normal goat serum diluted in PBS, and incubated for 20 min at 37 °C. The slices were incubated at 4 °C overnight with the primary antibody (anti-ADORA1, anti-ADORA2A, and anti-ADORA2B; Chemicon) diluted at 1:500 in PBS. A negative control was run in which the cells were incubated in PBS without primary antibody. The slices of the antibody-treated and the negative control samples were washed with PBS and exposed to fluorescein isothiocyanate (FITC)–conjugated goat anti-rabbit IgG antibody (Zhongshan Golden Bridge Biotechnology, Beijing, China) diluted at 1:50 in PBS at 37 °C for 30 min. The slices were washed with PBS three times, and propidium iodide (PI) was added for 5 min to dye the nuclei in red. Immunofluorescence images were taken using a laser scanning confocal microscope (LSM 510 META, Carl Zeiss GmbH, Oberkochen, Germany). Four cells with clear boundaries in each image were randomly chosen for analysis. The mean fluorescence intensity of cells was measured using the Axio Vision 4.8 software. The experiments were repeated three times.

Statistical analysis: Data analysis was performed using SPSS 16.0 (SPSS Inc., Chicago, IL). The data were expressed as mean ± standard deviation. The ANOVA (ANOVA) was applied to compare variables among multiple groups. P values of less than 0.05 were considered statistically significant.

RESULTS

In vitro culture and identification of human RPE cells: Primary human RPE cells were flat or angular, and rich in brown pigment granules in the cytoplasm. After two to four passages, the pigment granules were gradually reduced, and the cells were spindle-shaped or irregularly shaped (Appendix 1). The cultured cells had cytokeratin expression in the cytoplasm, indicating that they were RPE cells (Appendix 1).

Effects of 7-MX on RPE cell proliferation: To observe the growth of cells treated with 7-MX, primary human RPE cells were incubated with different concentrations (from 1 nmol/l to 1 mmol/l) of 7-MX. No statistically significantly growth alteration was noted at 24, 72, and 96 h (Figure 1A). A slightly decreased cell proliferation rate was seen with 100 µmol/l 7-MX at 48 h, but without statistically significant differences compared to the other concentrations (all $p>0.05$; Figure 1A). We considered 10 µM a safe dose that would not suppress the growth of the cells. Based on previous studies [4,9], the dosages used in animal experiments and clinical trials were around 10 µM. Thus, we chose 10 µmol/l for further experiments.

Effects of 7-MX on cell cycle distribution and apoptosis level of RPE cells: Human RPE cells were cultured in vitro with 10 µmol/l 7-MX for 24, 48, and 72 h. Flow cytometry was used to analyze changes in the cell cycle distribution
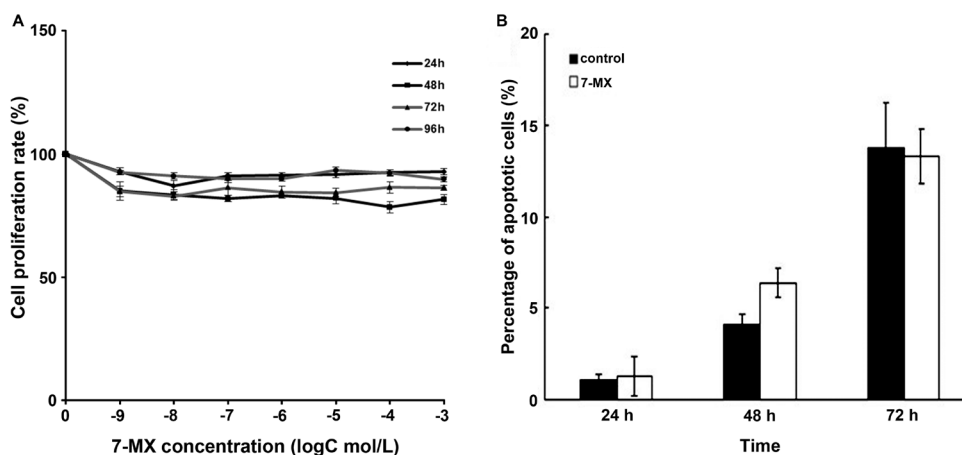

Figure 1. Cell proliferation, apoptosis, and cell cycle affected by 7-MX in human RPE cells. **A**: 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay results for cell proliferation. Human RPE cells were treated with 7-methylxanthine (7-MX) at different concentrations (from 1 nmol/l to 1 mmol/l). No statistically significant change was noted at 24, 72, and 96 h. A slightly decreased cell proliferation rate was detected only with 100 µmol/l 7-MX at 48 h but without a statistically significant difference compared to the other concentrations (all p>0.05). **B**: The flow cytometry assay results for cell apoptosis. No statistically significant change in cell apoptosis was found when RPE were cultured with 7-MX for 24, 48, and 72 h. n=3.
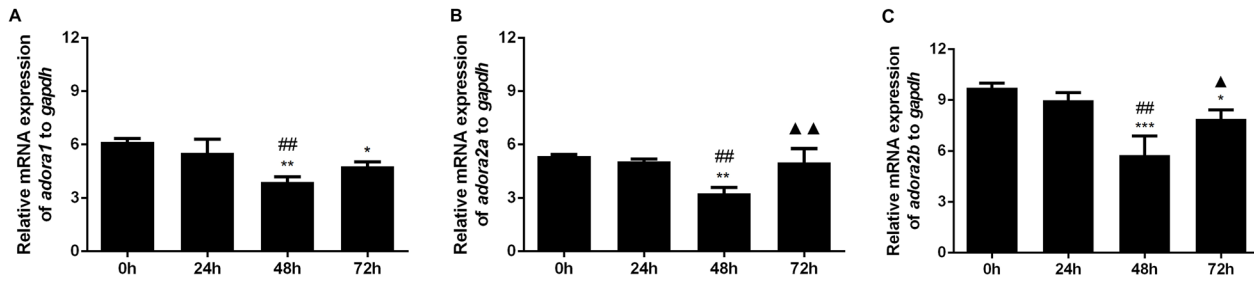
Figure 2. Dynamic changes in ADORs affected by 7-MX at the mRNA level. Human RPE cells were treated with 10 µmol/l 7-methylxanthine (7-MX) for 24, 48, and 72 h. The mRNA levels of ADORA1 (**A**), ADORA2A (**B**), and ADORA2B (**C**) was detected with quantitative PCR (qPCR). GAPDH was used as the internal control. n=3. * $p<0.05$, ** $p<0.01$, and ***$p<0.001$ compared with 0 h; ## $p<0.01$ compared with 24 h; ▲ $p<0.05$ and ▲▲ $p<0.01$ compared with 48 h.

and apoptosis level. The proportion of RPE cells in the G1 stage was slightly increased at 24 h (p=0.035) but decreased at 48 h (p=0.0045) upon 7-MX treatment; and the proportion was restored to normal at 72 h (Table 1). The apoptosis level in RPE cells without 7-MX treatment increased with time (Figure 1B). We suppose that it may be the basal characteristics of in vitro cultured RPE cells even without any treatment, as observed previously [22]. Compared with the non-treated group, no statistically significant change in cell apoptosis was found when RPE cells were cultured with 7-MX for 24, 48, or 72 h (Figure 1B).

Dynamic changes in ADOR mRNA levels with 7-MX treatment: To observe the effects of 7-MX on the mRNA levels of ADORs, RPE cells were cultured with 10 µmol/l 7-MX for 24, 48, and 72 h. The ADOR mRNA levels were analyzed with qPCR (Figure 2). After 24 h treatment with 7-MX, there were no statistically significant changes in the mRNA levels of ADORA1, ADORA2A, or ADORA2B, compared to 0 h. However, the mRNA levels of all three ADORs were statistically significantly decreased at 48 h, compared to 0 h or 24 h (p<0.01 or p<0.001; Figure 2A–C). The mRNA levels of

ADORA2A and ADORA2B appeared to rebound at 72 h, compared to 48 h (p<0.05 or p<0.01; Figure 2B,C).

Dynamic changes in ADOR protein levels with 7-MX treatment: To further verify the effects of 7-MX on ADOR expression, RPE cells were cultured with 10 µmol/l 7-MX for 24 and 48 h. The ADOR protein levels were evaluated with immunofluorescence (Figure 3A, Figure 4A, and Figure 5A). The ADORA1 protein level was decreased after 24 h of 7-MX treatment (p<0.01) and was more obviously decreased at 48 h, compared to 0 h (p<0.001; Figure 3B). The ADORA2A protein was statistically significantly inhibited at 24 and 48 h compared to 0 h (p<0.001; Figure 4B). The ADORA2B protein level declined statistically significantly at 48 h compared to 0 h (p<0.05; Figure 5B).

DISCUSSION

7-MX has an effect on eye development and myopia [4]. The present study showed that 7-MX barely suppresses the growth of human RPE cells cultured in vitro, but 7-MX could statistically significantly inhibit the expression of ADORA1, ADORA2A, and ADORA2B in RPE cells in short-term treatment.

TABLE 1. THE CHANGES IN CELL CYCLE DISTRIBUTION OF RETINAL PIGMENT EPITHE-LIUM CELLS UPON 7-METHYLXANTHINE (7-MX) TREATMENT.

Concentration of 7-MX (µmol/l) and treatment time	Cell percentage (%) in different stages of cell cycle		
	G1	S	G2
0 (24 h)	91.7±1.36	3.7±0.84	4.7±0.60
10 (24 h)	94.1±0.99*	2.5±0.31	3.4±0.69
0 (48 h)	83.1±1.17	9.9±0.42	7.0±0.75
10 (48 h)	81.4±1.02*	11.2±0.96	7.4±0.49
0 (72 h)	78.3±0.25	11.0±0.47	10.7±0.31
10 (72 h)	78.8±0.49	10.3±0.40	10.9±0.26

*p<0.05 and **p<0.01 compared with control (0 µmol/l 7-MX)
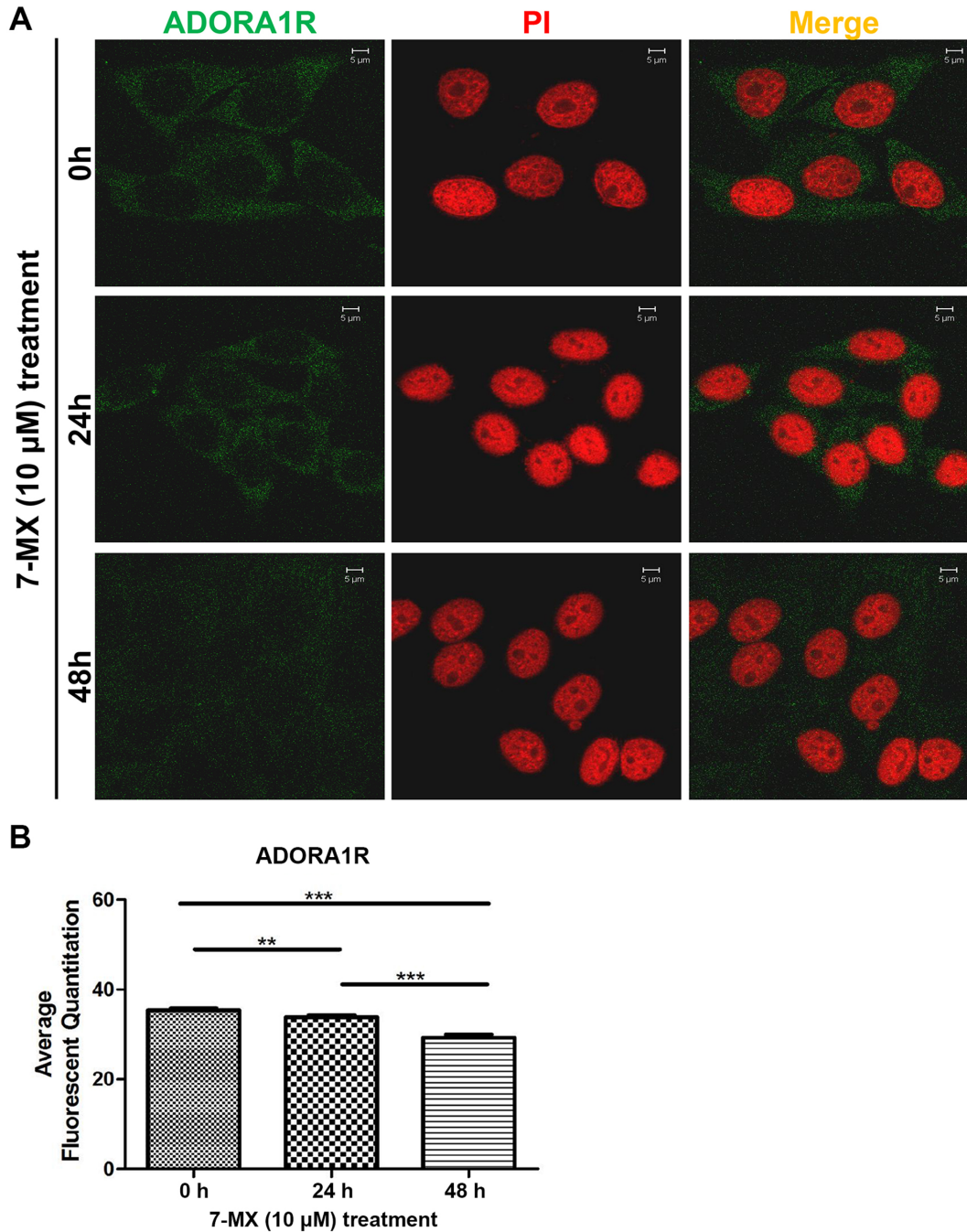
Figure 3. Dynamic changes in ADORA1 protein levels upon 7-MX treatment. **A**: Human RPE cells were treated with 10 µmol/l 7-methyl-xanthine (7-MX) for 24 and 48 h. ADORA1 protein levels were detected with immunofluorescence. **B**: The mean fluorescence intensity of ADORA1R was decreased at 24 h (p<0.01), and even more obviously at 48 h, compared to 0 h (p<0.001). Scale bar: 5 µm. n=3.

RPE plays a critical role in relaying retinal growth signals to the choroids and sclera [23]. One of the most important mechanisms of myopia formation is that the visual signal concerned with myopia transfers from the neural retina to the RPE, where fluid exchange transits molecular signals from the retinal and choroid layers to the sclera, followed by scleral remodeling [8]. As confluent monolayers of adult human RPE cultures exhibit characteristics of native RPE [24], it would have been more relevant for our study. 7-MX is a metabolite of caffeine and theobromine shown to work against myopia [4,8,9]. The peak serum concentration after an oral dose of 400 mg 7-MX in adults is around 20 µmol/l with a half-life
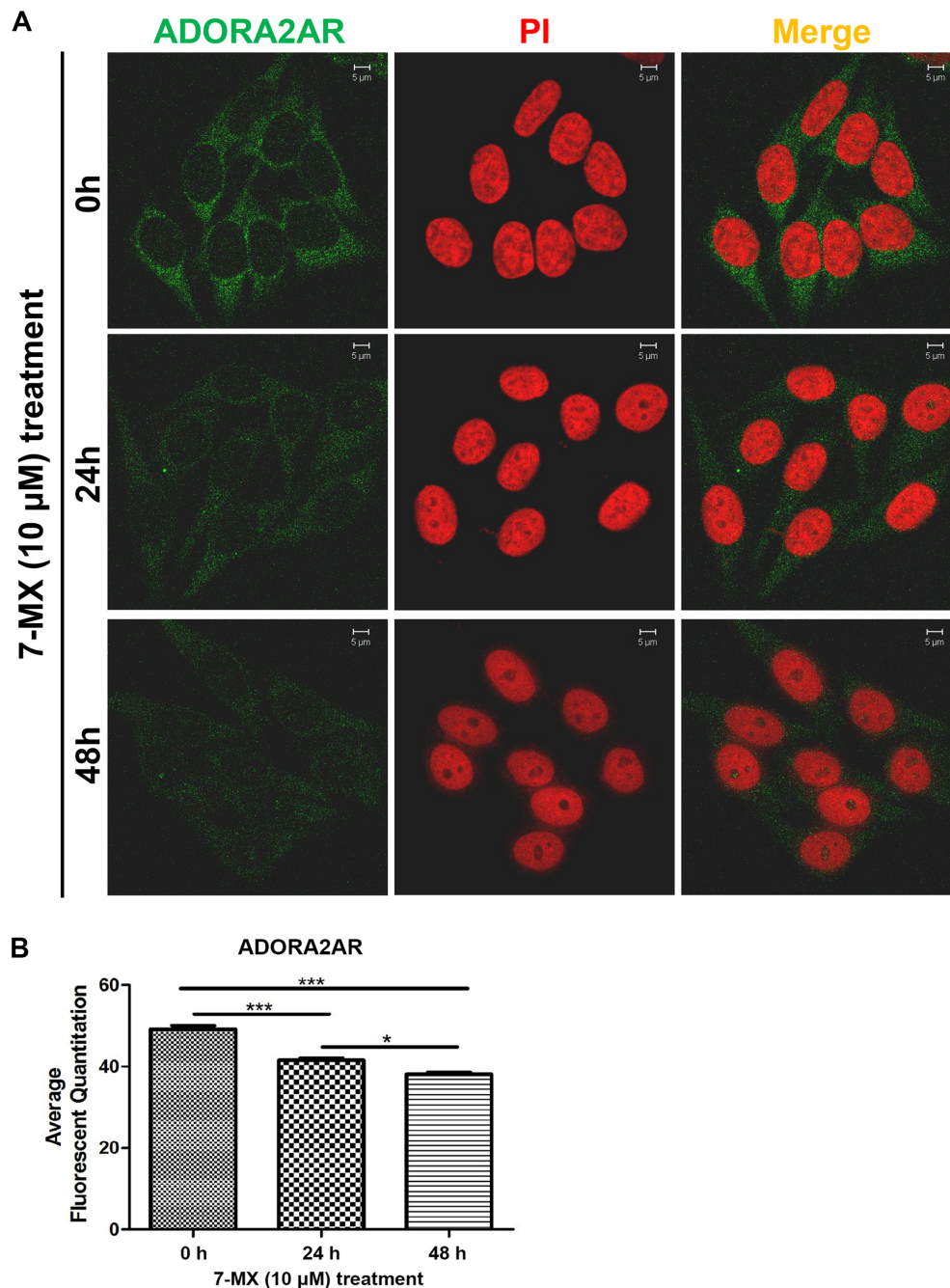
Figure 4. Dynamic changes in ADORA2AR protein levels upon 7-MX treatment. **A**: Human RPE cells were treated with 10 μmol/l 7-methylxanthine (7-MX) for 24 and 48 h. ADORA2A protein levels were detected with immunofluorescence. **B**: The mean fluorescence intensity of ADORA2A was decreased at 24 h (p<0.001) and further decreased at 48 h compared to 24 h (p<0.05). n=3.

of 200 min (unpublished results, SM Ribel-Madsen and K Trier, 2004). In a rabbit experiment [9], the peak serum level of 7-MX was around 70 μmol/l, and the half-life was around 1 h. As 7-MX inhibit the expression of ADORs in human RPE cells [4,10,16], adenosine and ADORs may be involved in regulation of eye growth, which would be one of the reasons to explain the effect of 7-MX on myopia progression.

Different subtypes of ADORs have different suppression levels by 7-MX, which might indicate that different subtypes might play different roles in visually driven changes in eye growth when treated with 7-MX. Inhibition
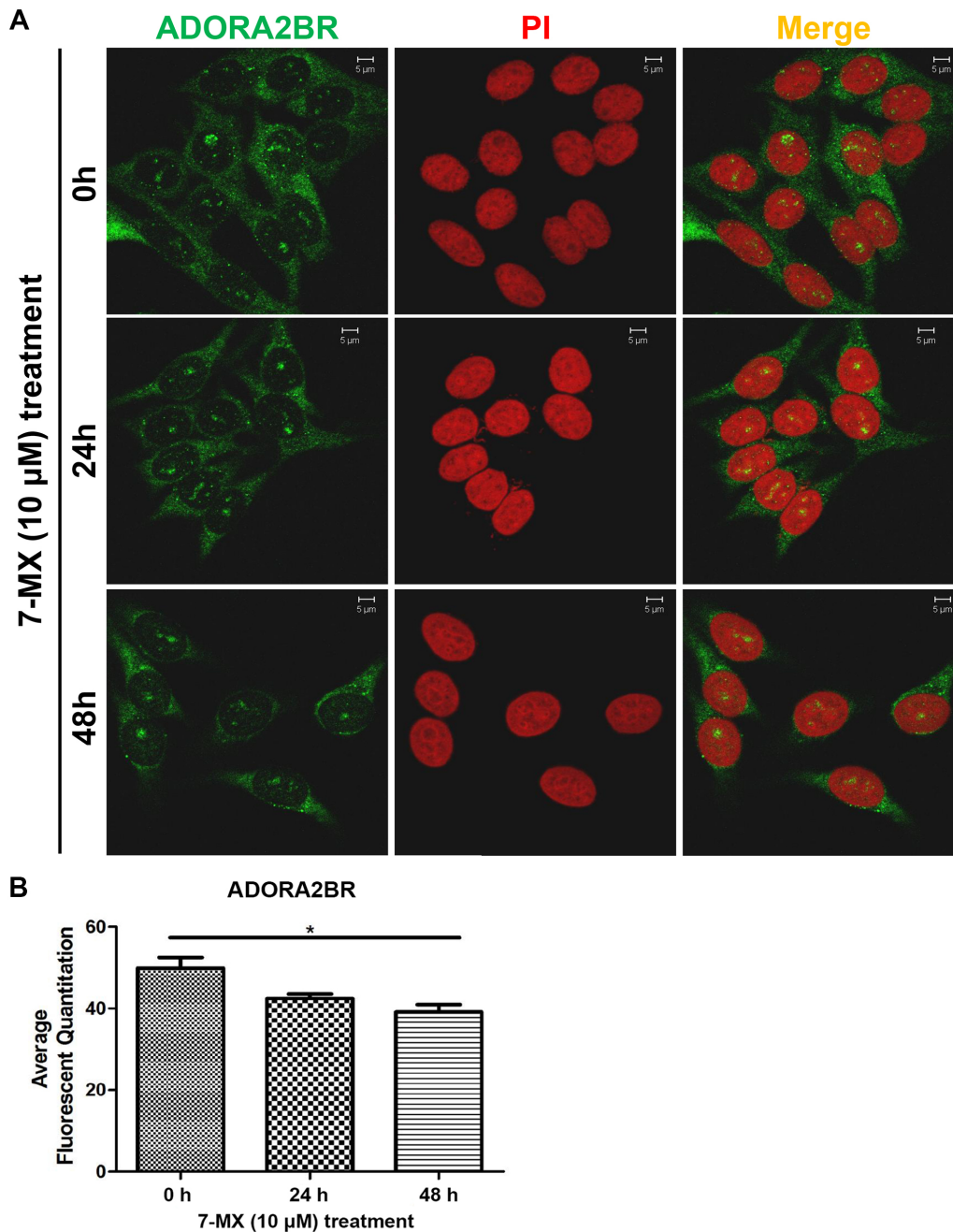
Figure 5. Dynamic changes in ADORA2BR protein levels upon 7-MX treatment. **A**: Human RPE cells were treated with 10 µmol/l 7-methylxanthine (7-MX) for 24 and 48 h. ADORA2B protein levels were detected with immunofluorescence. **B**: The mean fluorescence intensity of ADORA2B was decreased statistically significantly at 48 h compared to 0 h ($p<0.05$).

of neurotransmitter release by ADORA1 activation appears to be mediated by the blockade of Ca^{2+} channels or activation of K^+ channels and hyperpolarization [25], whereas ADORA2A activity mediates neuroprotection and facilitation of neurotransmitter release, such as dopamine, noradrenalin, acetylcholine, glutamate, and serotonin [26].

After the observation of adenosine-dopamine interactions in the central nervous system, initial findings indicated the existence of ADORA2A-D2R heterodimers and ADORA1-D1R heterodimers in the striatum, followed by indications for the existence of striatal ADORA2AR-D3R and ADORA2A-D4R heterodimers [27]. In the chick embryo retina, the long-term

activation of ADORA2A is able to promote a dramatic increase in ADORA1 expression [28]. In addition, inhibition of ADORA2A could result in an increase in dopamine D2 receptors [29]. 7-MX could block ADORA1 and ADORA2s in human RPE cells [4,8,10], which may change the expression of dopamine or other neurotransmitters that are associated with myopia.

The present study suggests that the effect of 7-MX treatment in inhibiting ADOR expression was somehow reversed at 72 h (Figure 2, Figure 3, Figure 4, and Figure 5). Consistently, a mild disturbance in cell cycle distribution was observed only at 24 and 48 h, but not at 72 h (Table 1). It implied that the 7-MX treatment might also activate negative feedback regulation in RPE cells as time went by, which then counteracted the effects of 7-MX in blocking the cell cycle in the G1 stage or inhibiting the expression of the ADORs. Nevertheless, how 7-MX works in different subtypes of ADORs during long-term treatment still needs to be studied further in the future.

In conclusion, the study showed that 7-MX has little effect on the proliferation or apoptosis of human RPE cells; however, 7-MX disturbs the proportion of cells in the G1 stage and inhibits the expression of ADORA1, ADORA2A, and ADORA2B in short-term treatment. The long-term effect of 7-MX treatment and other related signaling requires further studies.

APPENDIX 1. IN VITRO CULTURE AND IDENTIFICATION OF HUMAN RPE CELLS

At passage 3 of in vitro culture, RPE cells were photographed (A) and immunochemistry was performed to detect cytokeratin 18 expression. Magnification: 100 X. (B) Scale bar: 50 μm. n=3. To access the data, click or select the words "Appendix 1."

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