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Original Article

A study on in vitro cytotoxicity of cortisol on madin-darby kidney cells

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Abstract: Stress hormones play a vital role in cancer development and tumor formation. However, this mechanism is still obscure. Cortisol plays at several important biochemical roles in disease progress and development. Evaluating the dose-dependent effect of cortisol on MDCK cells is contemplated as the primary task of this experiment. Cell viability did not change significantly following cortisol exposure. Morphologically, no changes were observed in the MDCK cells. However, few cells showed an oval profile. Fluorescent and confocal analysis showed the occurrence of apoptosis significantly at 0.1 mg/L of cortisol. However, the intensity of apoptosis was significantly reduced at higher concentration (0.2 mg/L) of cortisol. The method of qPCR quantitated the mRNA expression of caspase 3, matrix metalloproteinase-9 (MMP-9) and matrix metalloproteinase-2 (MMP-2). Caspase 3 mRNA expression was significantly altered at low concentration (0.1 mg/L) while it was tremendously reduced at the higher level of cortisol (0.2 mg/L). A remarkable increase in the MRNA expression of MMP-2 and MMP-9 was executed in a dose-dependent manner. Our experimental outcome implies that cortisol may induce the apoptosis in MDCK cells.

Keywords: Cortisol, MDCK cells, CLSM, SRB, caspase 3

Introduction

Stress and behavioral factors are known to play in disease progress and development [1]. Chronic stress and depression are believed to be associated with altered cellular immunity [2]. The psychological stress affects both immune system and the pathway of apoptosis. Immune system expresses receptors for catecholamine secreted by the sympathetic system. Hormone-receptor interaction leads to cellular action, cell trafficking and cytokine production [3]. However, the direct effects of cortisol on normal cells have not been investigated.

The adrenal cortex secretes cortisol. It is usually increased in the acute and chronic stress condition [4]. High levels of pro-angiogenic factors suggest that cortisol may probably expedite tumor metastasis. However, metastasis is contemplated to be an extremely complex process involving several sequential and interrelated processes. Metastatic cells must complete all the necessary steps to produce clinical lesions [5]. In the pathogenesis of metastasis, the process of invasion is contemplated to be a pivotal step [6]. However, there is no analysis of the effect of cortisol on the invasion potential of tumor cells. Norepinephrine may enhance the invasive potential because it affects tumor cell motility [7]. Circulating catecholamine has been associated the expression of matrix metalloproteinase (MMP) [8].

The present scrutiny was aimed to assess the effect of cortisol on the proliferation, viability, and morphology of MDCK cells. SRB assay determined cytotoxicity of cortisol on MDCK cells. Morphology of MDCK cells was observed by an inverted microscope, a fluorescence microscope, and confocal laser scanning microscope (CLSM). Quantitative PCR (qPCR) was applied to determine caspase 3, MMP-9 and MMP-2 mRNA expression.

Materials and methods

Materials

Cortisol, dimethyl sulphoxide (DMSO), sulforhodamine B (SRB) was purchased from Sigma.

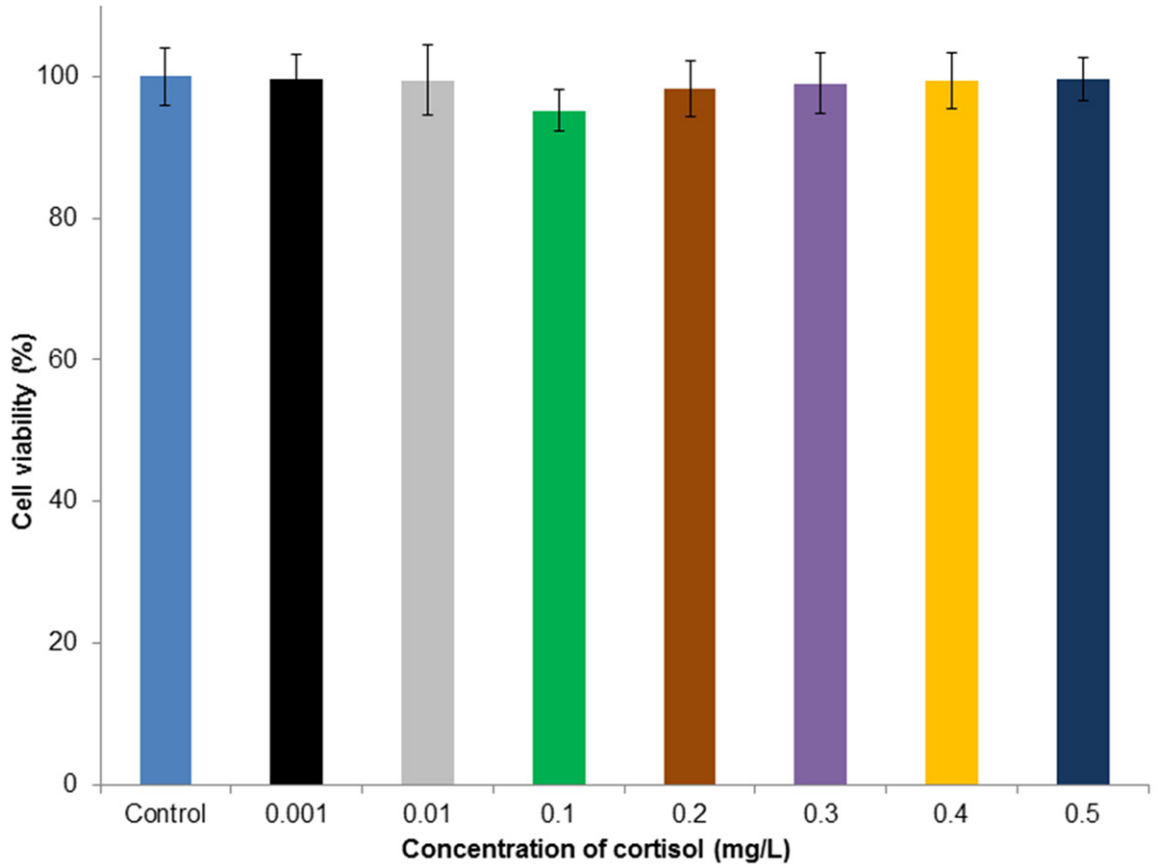

Figure 1. Cytotoxic effect of cortisol on MDCK cells by SRB assay at 48 h. Results are presented as a percentage of growth inhibition compared with the control. Values were expressed as means ± SEM.

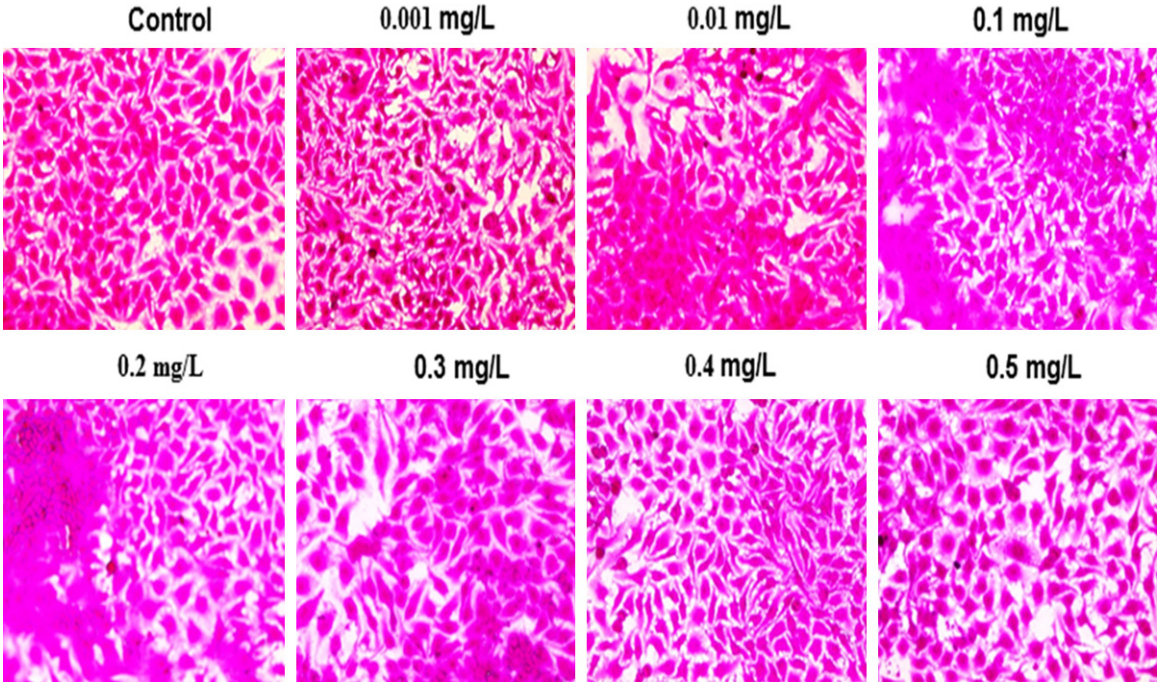

Figure 2. Cytotoxic effect of cortisol on MDCK cells by SRB assay at 48 h. Representative images from three independent experiments.

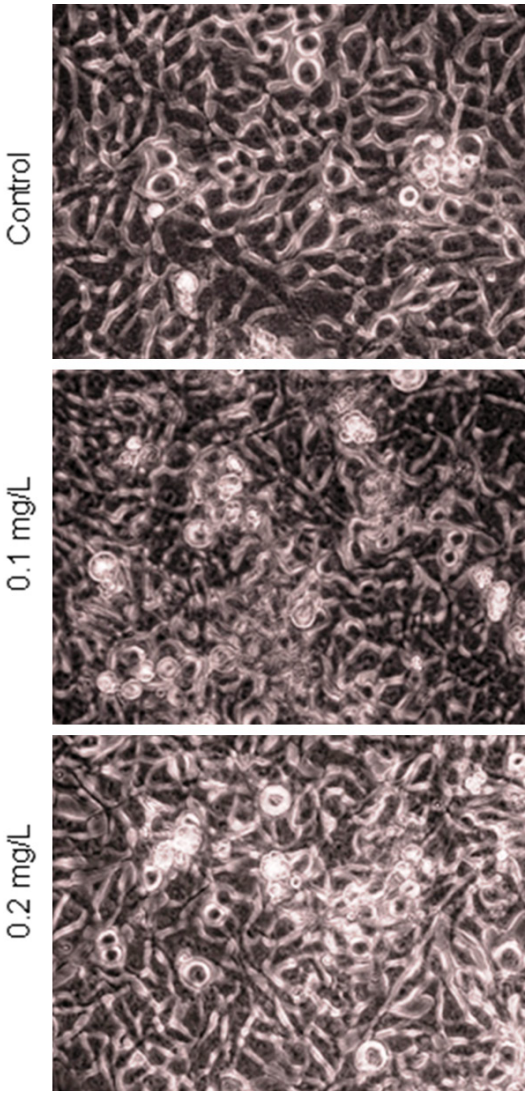

Figure 3. Morphological observation by inverted microscope following cortisol exposure for 48 h. Representative images from three independent experiments.

DMEM, FBS, penicillin-streptomycin, and trypsin-EDTA were acquired from Welgene (China). EB and Acridine Orange (AO) was obtained from Santa Cruz Biotechnology, Inc. (Delaware Avenue, California, USA). Primers were purchased from Macrogen Inc. (China).

Cell culture

MDCK cells were acquired from the China Cell Line Bank (China). Cells were preserved in a growth medium and were supplemented with 10% FBS and 1% antibiotics (penicillin-streptomycin). The cells were developed in a CO_2 incubator at 37°C with 5% CO_2.

SRB assay

SRB assay [9] was used to measure the cytotoxic effect of cortisol on MDCK cells. MDCK cells were cultured in 96 well plates at a density of 2.5 × 10^4 cells and adhered for 24 h at 37°C. Cortisol was treated to the cells at different concentrations (0.001, 0.01, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/L) for 48 h. Cells were fixed with acetone and air dried at the end of the treatment. After the cells had been fully dried, 100% of SRB solution (0.4% w/v) was added. 1% of acetic acid was used to wash the microplates that are then dried in drying oven. Stained MDCK cells were photographed using an inverted light microscope. Finally, 10 mM of Tris base was added and kept for overnight and measured at 540 nm.

Morphological observation

Inverted microscope: MDCK cells were cultured in 6 well plates at a density of 2 × 10^4 cells/well. The cells were treated with cortisol of different concentrations (0.1 & 0.2 mg/L) for 48 h after a 24 hr adherence. An inverted microscope (Nikon, Eclipse, 80i, Melville, NY 11747-3064, U.S.A) was used to examine the morphology of MDCK cells after 48 h.

Fluorescence microscope: MDCK cells were cultured in six-well plates at a density of 2 × 10^4 cells/well. Cortisol was treated at different concentrations (0.1 & 0.2 mg/L) to the after 24 h adherence. Cells were removed from the wells and centrifuged at 500× g for 3 minutes. Phosphate buffered saline (PBS) was added to the tubes after the supernatant was removed from the tubes. Cell volume was adjusted to 10^5-10^6 cells/ml. 95 µl of cell suspension was added to the Microtube, and then five µl of AO and EB dye mixture was added to the same tube. The tubes were incubated for 15-30 minutes at 37°C with protection from light. A glass slide with a cover glass following the addition of 10 µl of the cell staining solution and viewed under a fluorescence microscope (Axiovert 2000, Carl Zeiss, Germany) [10].

CLSM: MDCK cells were seeded in the confocal dish. Cortisol of different concentrations (0.1 and 0.2 mg/L) was added to the cells after 24 h adherence. After 48 h, PBS was used to wash the cells, and the washed cells were stained with AO (20 µg/ml) for 5 minutes. PBS was

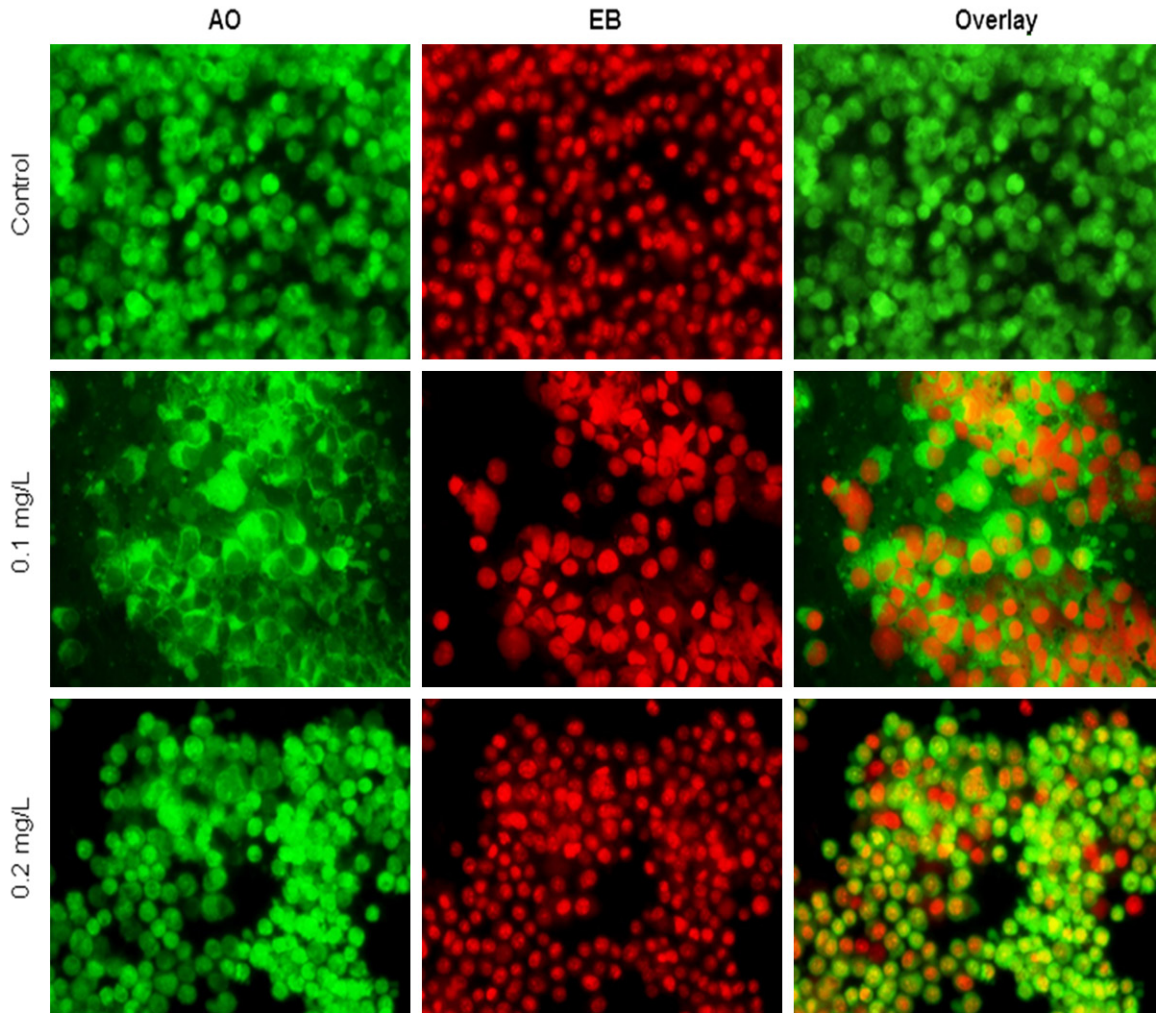

Figure 4. Morphological observation with AO/EB double staining by fluorescence microscope (40×). MDCK cells were seeded in 6-well plates and over 24 of adherence, cells were treated with cortisol (0.1 and 0.2 1 mg/L) for 48 h. At the end of the treatment, the cells were trypsinized, centrifuged and then resuspended in culture medium. 95 µl of cell suspension was then mixed with five µl of the dye mixture, containing 100 mg/l of AO and 100 mg/l of EB in PBS. After staining, cells were visualized immediately under the fluorescence microscope. Representative images from three independent experiments.

used to wash the cell twice, and the stained cells were viewed immediately under CLSM (1 × 81^{R} motorized inverted microscope, Olympus [10].

Gene expression

MDCK cells were seeded T25 flask. Cortisol was treated at different concentrations (0.1 and 0.2 mg/L) to the cells after 24 h adherence. RNA was isolated from the control and cortisol treated cells [11]. qPCR was carried out using primers specific for caspase 3 (forward: 5'-TTAATAAAGGTATCCATGGAGAACACT-3', reverse: 5'-TTAGTGATAAAAATAGAGTTCTTTTGTGAG-3'), MMP-2 (forward: 5'-AGGATCATTGGCTACACACC-3', reverse: 5'-AGCTGTCATAGGATGTGCCC-3', MMP-9 (forward: 5'-CGCAGACATCGTCATCCAGT-3', reverse: 5'-GGATTGGCCTTGGAAGATGA-3' and GAPDH (forward: 5'-GGTCACCAGGGCTGCTTTT-3', reverse: 5-ATCTCGCTCCTGGAAGATGGT-3'). According to the manufacturers instruction, the reaction was carried out in 10 µl using SYBR Green Master Mix (Bioneer). Based on the $2^{-\Delta\Delta CT}$ method [12] relative ratios were calculated. CFX96^{TM} Real-Time System (Bio-Rad) was used to monitor the PCR.

Statistical analysis

Values were expressed as mean ± SEM. ANOVA analysis has been carried out to compare the

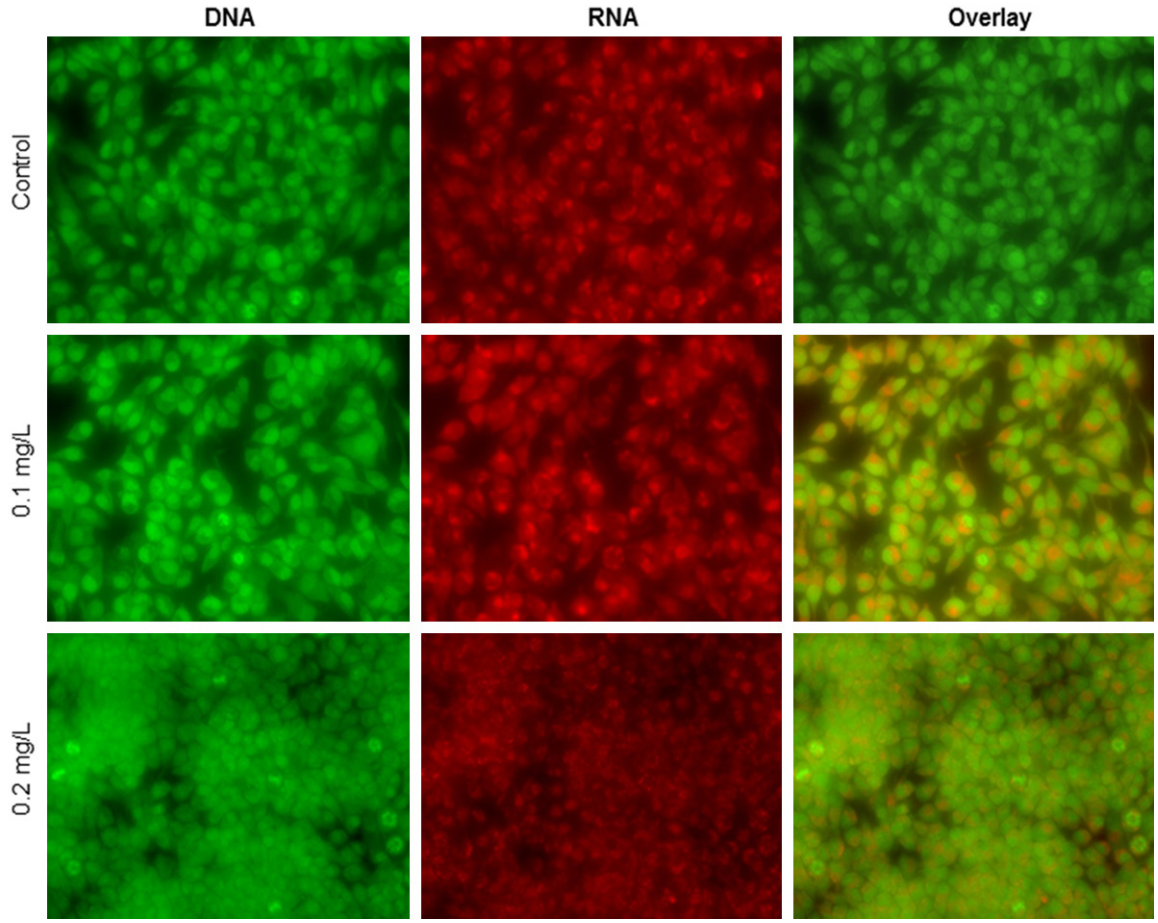

Figure 5. Morphological observation of MDCK cells by CLSM (40×). Cells were grown on the cover glass confocal dish. After 24 h of adherence, cells were treated with cortisol (0.1 and 0.2 mg/L) for 48 h. At the end of 48 h, the cells were stained with 20 μg/ml of AO dye for 5 minutes and washed twice with PBS, and then viewed immediately under CLSM. Green (DNA); Red (RNA); Stack-up merges fluorescence image (DNA and RNA). Representative images from three independent experiments.

groups and control (SPSS 22, Statistical Package) A $P<0.05$ was considered statistically significant.

Results

Cortisol on cell viability

The cytotoxic effect of cortisol on MDCK cells was observed (**Figures 1** and **2**). However, 0.3, 0.4 and 0.5 mg/L of cortisol are not related to physiological levels during normal and stress conditions. Therefore, 0.1 and 0.2 mg/L of cortisol was used for the further study. The inverted microscope could be used to observe the cell shape and its morphological changes. Control MDCK cells have a regular shape and morphology. Cortisol treated cells showed the sporadic distribution, loss of adhesion and oval profile at low concentration. However, these effects were reversed marginally at a higher level (**Figure 3**).

Effect of cortisol on morphology

Fluorescence microscopy was carried out to resolve whether the cytotoxic effect of cortisol was related to the growth and proliferation of cancer cells. This method combines the dual uptake of the fluorescent DNA binding dyes EB and AO. The stained nucleus contains chromatin condensation that is used to differentiate viable, apoptotic, and necrotic cells. Control MDCK cells have typical morphological features. Cells treated with cortisol showed fragmented chromatin in the nucleus, and the necrotic cells showed a uniform bright orange core. Significant morphological changes such as chromatin condensation, fragmented nuclei, and apoptotic bodies are observed at a low

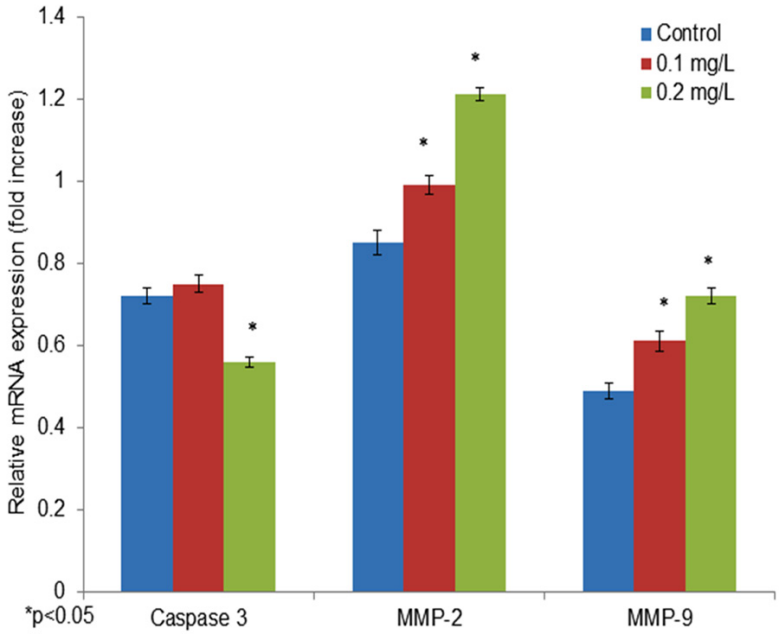

Figure 6. A mRNA expression of caspase 3, MMP-2 and MMP-9 by qPCR. MDCK cells were grown in a T-25 flask and allowed for the adherence for 24 h. Cells were treated with cortisol (0.1 and 0.2 mg/L) for 48 h. At the end of treatments, RNA was isolated from the cells and qPCR was carried. Values were expressed as means ± SEM.

concentration of cortisol (0.1 mg/L). However, the effect of cortisol was reduced at a higher level (**Figure 4**).

CLSM has been widely used to investigate in the morphological studies. AO is mainly used as a nucleic acid-selective fluorescent cationic dye that is applied to the determination of the cell cycle. AO interaction with DNA and RNA can occur by intercalation or electrostatic attractions respectively, and spectrally fluorescein are bound to DNA with an excitation maximum at 502 nm and an emission maximum at 525 nm. Control cells have bigger and smoother nucleus than cortisol treated cells. Morphological changes such as rounding, compact granular masses in the nucleus and reduced nuclear volume at a low concentration of cortisol treatment. However, these effects were significantly reduced at the higher level of cortisol exposure (**Figure 5**).

Cortisol on gene expression

The effect of cortisol on selected gene mRNA expression and quantification was carried out by qPCR. MDCK cells were exposed to different concentration of cortisol for 48 h. No significant changes were observed at low concentration (**Figure 6**), while the mRNA expression of caspase 3 was reduced to 0.22 fold at 0.2 mg/L of cortisol. The mRNA expression of MMP-2 was increased 0.16 and 0.42 folds following 0.1 and 0.2 mg/L of cortisol exposure respectively. The mRNA expression of MMP-9 was increased 0.24 and 0.47 folds following 0.1 and 0.2 mg/L of cortisol exposure respectively.

Discussion

Our results showed that under stress condition cortisol enhances the capacity of cervical carcinoma cells to invade the extracellular matrix (ECM). Alpha and beta adrenoceptors are stimulated by cortisol [13]. The membrane-bound enzyme, adenylate cyclase is activated by the beta component, which converts adenosine triphosphate to cyclic adenosine 3', 5'-monophosphate (cyclic AMP). This, in turn, leads to phosphorylation of various proteins due to activation of several protein kinases. One of the proteins Myosin light chain regulates ATPase-actin activity and cell contraction [14].

The fluorescence microscopy study showed that morphological changes, including chromatin condensation, cell shrinkage and formation of apoptotic bodies. However, there was a reverse trend occurring at a higher level of cortisol exposure. CLSM has been widely used to study typical morphological changes [15] in cortisol treated cells, stained with AO dye. Our experimental results showed that the cortisol exerted the less cytotoxicity at a higher concentration compared to the low level. Morphological changes in cells are associated with general cellular functions that include mitosis, locomotion, phagocytosis, cytokinesis and secretion of macromolecules [16]. Actin filaments are used to mediate Cell-to-cell and cell-to-substrate attachments [17]. Enhanced levels of calcium ion, which are essential for the formation of the mitotic spindle, can be related to cortisol-induced cessation of mitotic activity [18]. Prolonged exposure and repeated administration

of cortisol could produce oxidative free radicals. Due to an insufficient amount of protective enzymes, especially at a higher concentration of cortisol is one of the putative explanation for changing the cell shape and morphology.

Increased production of MMP-2 and MMP-9 occur when the invasive potential of cancer cells mediated by β-adrenergic receptors. Cervical carcinoma penetration of extracellular matrix may be facilitated by altering MMPs expression. B-Adrenergic receptors and MMP expression are believed to be key regulators in the pathogenesis of cervical carcinoma cells. The average level of cortisol ranges from 1 to 10 pmol/L and increases up to 10 nmol/L in the circulatory system under stress condition [19]. Stress can also increase the tissue cortisol level through increased sympathetic activity, which has been associated with metastasis [20]. The invasion to ECM is believed to be a critical factor in metastasis and blood flow in the tumor area. Cancer cell penetration in the host basement membrane contains an attachment, matrix dissolution, motility, and penetration [21]. MMPs degrade components of the extracellular matrix and core protein of proteoglycans [22]. Abnormal expression of MMPs leads to tumor cell invasion and metastasis [23]. MMP-9 play crucial role in the angiogenic switch that occurs during carcinogenesis [24]. Our data show that stress hormone can increase expression of MMPs under stress condition. Our data indicate that MMPs might play a role in stress hormone-mediated changes in the cervical carcinoma cell metabolism. Yang et al. [25] have reported that the effect of stress on MMPs expression in a blister chamber wound model. Studies have indicated that at the psychological stress can increase MMP expression in mice. The increased level of MMP-1, MMP-2, and MMP-9 mRNA expression occurred in colon tumors, and liver tissues of stress induced mice [8]. Cortisol reduced caspase 3 mRNA expression. Caspases act as central executioners of apoptosis [6]. The down-regulated caspase-3 reduces auto-catalysis [26].

Conclusion

Our experimental results show that cortisol can induce apoptosis in MDCK cells through up-regulation of MMP-2 and MMP-9. Cell viability, proliferation, and morphology of MDCK cells were significantly altered at a low concentration while a reverse trend occurred at a higher level of cortisol. Stress and behavioral factors could contribute a significant role in cancer pathogenesis and prevention.

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Disclosure of conflict of interest

None.

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