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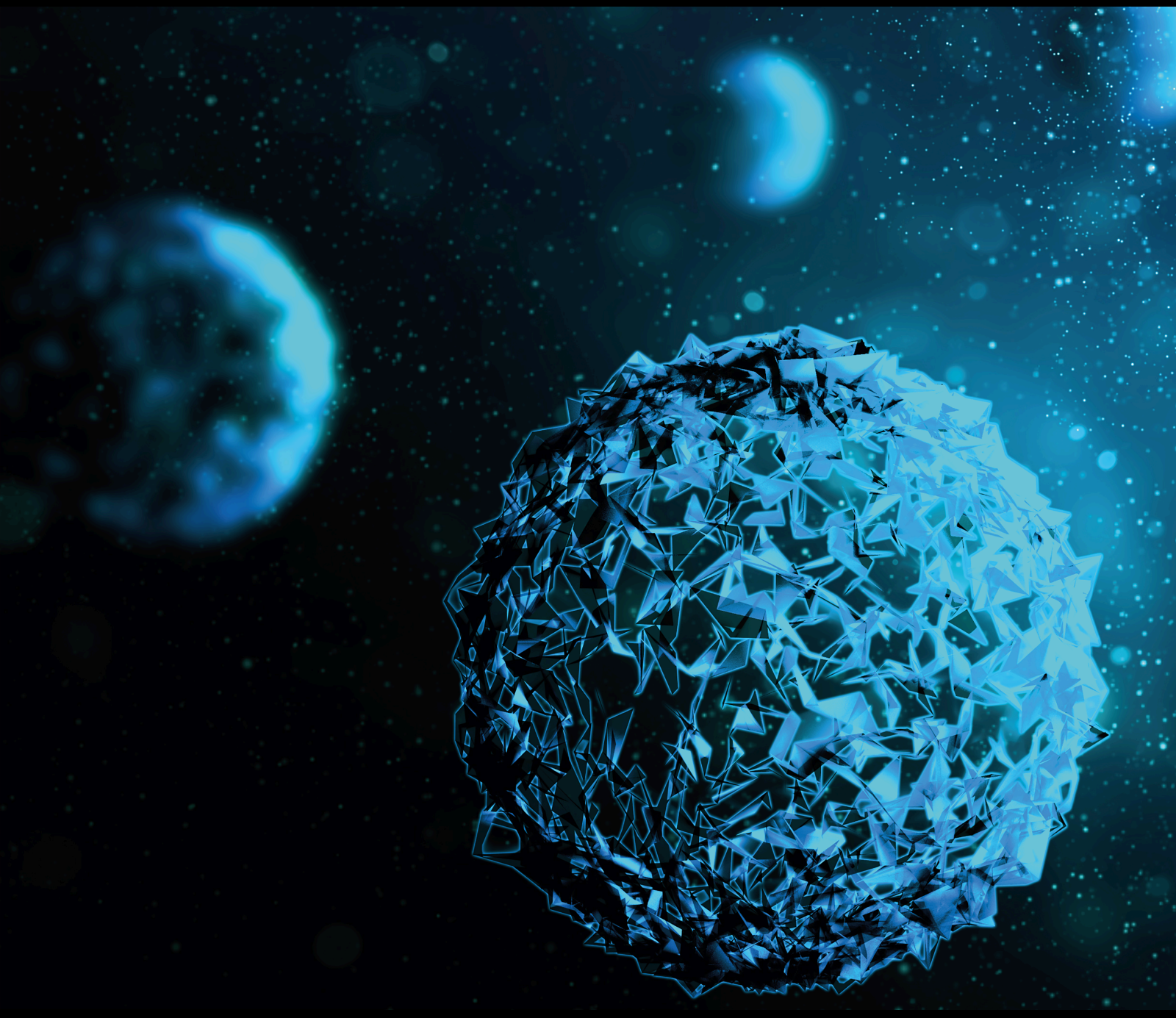

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Research Article

The Neurokinin-1 Receptor Is Essential for the Viability of Human Glioma Cells: A Possible Target for Treating Glioblastoma

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Background. Glioblastoma or glioma is the most common malignant brain tumor. Patients have a prognosis of approximately 15 months, despite the current aggressive treatment. Neurokinin-1 receptor (NK-1R) occurs naturally in human glioma, and it is necessary for the tumor development. *Objective.* The purpose of the study was to increase the knowledge about the involvement of the substance P (SP)/NK-1R system in human glioma. *Methods.* Cellular localization of NK-1R and SP was studied in GAMG and U-87 MG glioma cell lines by immunofluorescence. The contribution of both SP and NK-1R to the viability of these cells was also assessed after applying the tachykinin 1 receptor (*TAC1R*) or the tachykinin 1 (*TAC1*) small interfering RNA gene silencing method, respectively. *Results.* Both SP and the NK-1R (full-length and truncated isoforms) were localized in the nucleus and cytoplasm of GAMG and U-87 MG glioma cells. The presence of full-length NK-1R isoform was mainly observed in the nucleus, while the level of truncated isoform was higher in the cytoplasm. Cell proliferation was decreased when glioma cells were transfected with *TAC1R* siRNA, but not with *TAC1*. U-87 MG cells were more sensitive to the effect of the *TAC1R* inhibition than GAMG cells. The decrease in the number of glioma cells after silencing of the *TAC1R* siRNA gene was due to apoptotic and necrotic mechanisms. In human primary fibroblast cultured cells, *TAC1R* silencing by siRNA did not produce any change in cell viability. *Conclusions.* Our results show for the first time that the expression of the *TAC1R gene* (NK-1R) is essential for the viability of GAMG and U-87 MG glioma cells. On the contrary, the *TAC1R gene* is not essential for the viability of normal cells, confirming that NK-1R could be a promising and specific therapeutic target for the treatment of glioma.

1. Introduction

Glioblastoma (GB) or glioma (WHO grade IV) is the most common and lethal brain tumor [1], occurring in 90% of patients [2]. Several therapeutic strategies have been developed for the treatment of glioma (e.g., Stupp protocol, treatment with bevacizumab, microRNA, immunotherapy, gene therapy, and intranasal drug delivery) [1, 3], but unfortunately, the prognosis is very poor with approximately 15 months of median survival time [4]. Therefore, it is necessary to investigate the mechanisms underlying the development of glioma and the seeking of new therapeutic strategies to target this tumor.

One of these plausible mechanisms could be related to the substance P (SP)/neurokinin-1 receptor (NK-1R) system. Many studies have shown that SP and NK-1R are involved in the growth and development of different tumors (including malignant hematopoietic cells) [5–7]. SP, via the NK-1R, plays a key role in all stages of tumor development such as cell proliferation/migration, invasion, and metastasis

and also exerts an antiapoptotic effect in cancer cells [7–10]. Tumor cells also express NK-1R and SP, suggesting that SP exerts an autocrine action, promoting mitogenesis in cancer cells [6, 11–15]. In melanoma, acute lymphoblastic leukemia, lung cancer, and breast cancer, NK-1R is involved in the viability of human tumor cells [15–17]. NK-1R antagonists have been shown to promote apoptotic mechanisms in tumor cells after binding its receptor [3, 8, 9, 18].

Many studies have demonstrated that the SP/NK-1R system is involved in glioma. Human glioma cell lines [8, 19–22] and glioma samples are extensively described to overexpress NK-1R with respect to normal cells [23]. In these studies, the cellular location of the NK-1R was not reported. SP promotes the proliferation of human glioma cell lines through NK-1R activation [24, 25]. NK-1R antagonists (e.g., L-733,060, aprepitant) inhibit the proliferation in a dose-dependent manner and promote the apoptosis of glioma cells [9, 18, 26, 27]. In GB, the inhibition of NK-1R by L-733,060 decreased the basal kinase activity of Akt, increased the expression of cell cycle regulatory proteins (e.g., p21 and p27), and induced G1/S cell cycle arrest and programmed cell death [28]. A remarkable antitumor synergy between ritonavir and aprepitant was also reported against human glioma cells [3, 9, 26]. Taken together, the data show that NK-1R could be a new therapeutic target against glioma. In this sense, SP/NK-1R system has been previously targeted in radionuclide tumor therapy [29, 30], and the efficacy (e.g., inducing late apoptosis pathways) of targeted alpha therapy with ^{213}Bi-DOTA-SP against low-grade glioma and secondary/recurrent GB has been confirmed in patients [31–33].

To increase the knowledge about the involvement of the SP/NK-1R system in glioma and to develop new antiglioma therapeutic strategies, we have studied the cellular localization and expression of NK-1R and SP in human GAMG and U-87 MG glioma cell lines and the involvement of NK-1R in the viability of these cell lines.

2. Materials and Methods

2.1. Cell Cultures. Three different cell lines were used for this study, two different human tumoral glioma cell lines (GAMG and U87) and a normal human somatic cell (fibroblasts). The human GAMG glioma cell line was obtained from Deutsche Sammlungvon Mikroorganismen und Zellkulturen (DSMZ) (Braunschweig, Germany). The human U-87 MG glioma cell line was generously supplied by Dr. Nelofer Syed (John Fulcher Neuro-Oncology Laboratory, Imperial College London, UK). Skin fibroblasts were obtained from anonymous donors. Both glioma cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine (Glut), and 1% of penicillin/streptomycin (P/S) maintained in standard culture conditions (37°C in a 5% CO_2 atmosphere, refreshing the medium every two days). Similar conditions were applied to human fibroblasts except the culture medium that was RPMI/F10 Ham medium (mixture at equal parts 1 : 1), supplemented with 10% FBS, 2 mM glutamine, and 10 mM HEPES, 2% of Ultroser G (PALL Life Science, NY, USA) and 1% of P/S.

2.2. Small Interfering (Si)RNA Gene Silencing Method. This method was carried out according to the manufacturer's instructions (Life Technologies, Madrid, Spain). 3×10^5 GAMG and 2.5×10^5 U-87 MG cells were seeded per well in 6-well plates containing 1,750 μl of DMEM supplemented with 1% fetal bovine serum and 2 mM glutamine. 250 μl of transfection reagent medium (20 nM, 10 nM or 5 nM of *TAC1R* siRNA or *TAC1* siRNA) (Invitrogen, Madrid, Spain) and 8 μl of Hiperfect reagent (Invitrogen, Madrid, Spain) were added to each well and incubated for 30 min. After 8 h of incubation, cells were refreshed with 2 ml of normal growth medium. Sham group was also performed, but *TAC1R* siRNA or *TAC1* siRNA were omitted.

2.3. Image Acquisition and Analysis. The bright-field images were randomly acquired at different locations using a Nikon Digital Sight camera attached to a Nikon Eclipse TS100 microscope with 20× objective. Three independent experiments were performed. At least two bright-field images were taken for each well. Cells were manually counted and converted to cells/mm^2.

2.4. Western Blot and Subcellular Fractionation. Western blot and subcellular fractionation methods are based on previously optimized protocols by our group [34, 35]. Briefly, cell lines were lysed in RIPA buffer, and protein samples were separated by SDS-PAGE (10% acrylamide) and transferred to a nitrocellulose membrane (Bio-Rad, USA) at 60 V for 2 h. Membranes were incubated with anti-NK-1R antibody (S-8,305, Sigma-Aldrich, Madrid, Spain), diluted 1/1,000, at 4°C. The secondary antibody was a peroxidase-conjugated antirabbit immunoglobulin. Bands were analyzed by densitometry using the ImageJ analysis software (NIH).

For subcellular fractionation, cells were treated as previously described [35]. The cytoplasm and nuclear fractions were processed as previously described for Western blot analysis. Immunoblot was against anti-NK-1R antibody (1/1000), nuclear marker hnRNP (1: 2000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and cytoplasmic marker α-tubulin (1/1000) (Abcam, Cambridge, MA, USA).

2.5. Immunofluorescence. Cells were cultured on coverslips in a 12-well plate. At 80% confluence, the culture medium was removed and washed twice with PBS. Cells were fixed in 4% paraformaldehyde at 4° C for 20 min. The coverslips were washed with PBS and then blocked with 1% fetal bovine serum in PBS for 1 h at room temperature. The coverslips were incubated with rabbit-derived anti-NK-1R antibody (Sigma-Aldrich, Madrid, Spain; 1 : 1,000) or rabbit-derived anti-SP antibody (Sigma-Aldrich, Madrid, Spain; 1 : 1,000) diluted in blocking solution containing 10% serum and 0.25% Triton X-100 in a humid chamber overnight (4°C). Antirabbit Alexa Fluor 488 or Alexa Fluor 647 conjugated (Thermo Fisher, Madrid Spain; 1/500) and Hoechst (Sigma-Aldrich, Madrid, Spain; 1/2000) antibodies were used as secondary incubation. The coverslips were finally

washed 3 times and mounted with fluorescent mounting medium (Dako Colorado Inc., Fort Collins, USA). The images were acquired in a confocal microscope (Zeiss LSM7 DUO).

2.6. Analysis of Cell Viability and Apoptosis by Flow Cytometer. After silencing of *TAC1R* with *TAC1R*-targeted siRNA in human GAMG and U-87 MG glioma cell lines, viability and apoptosis were determined by flow cytometer (FC500, Beckman Coulter, Pasadena, CA, USA), using Annexin V conjugated with fluorescein isothiocyanate (FITC) and propidium iodide (PI) kit according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Viable cells (Annexin V^-, PI^-) were distinguish from cells in early apoptosis (Annexin V^+, PI^-), late apoptosis (Annexin V^+, PI^+), and necrosis (Annexin V^-, PI^+).

2.7. Statistical Analyses. Data are presented as the mean ± SEM. For statistical analysis, the GraphPadPrism Version 5.03 (Graph Pad Software) was used. Statistical evaluation was performed by one-way ANOVA, followed by Tukey's test. A p value of ≤ 0.05 was considered statistically significant.

3. Results

3.1. Localization of SP/NK-1R in Human GAMG and U-87 Glioma Cell Lines. The presence and localization of SP and NK-1R were studied in human glioma cell lines by immunofluorescence (GAMG and U-87 MG cell lines) and Western blot (GAMG cell line) (Figure 1). Both cell lines expressed SP (Figures 1(a) and 1(c)) and NK-1R (Figures 1(b) and 1(d)). Confocal images confirmed that SP and NK-1R were located in both the nucleus and the cytoplasm. Negative control staining (omitting primary antibodies) did not show evidence of unspecified staining (Supplementary Figure 1). Immunoblot analysis confirmed the subcellular location of two isoforms (full and truncated) of NK-1R in the nucleus and cytoplasm of GAMG glioma cells. The full-length 58 kDa NK-1R isoform (fl-NK-1R) was observed mainly in the nucleus, while the level of the 33 kDa truncated NK-1R isoform (tr-NK-1R) was higher in the cytoplasm (Figure 1(e)).

3.2. Effect of TAC1R/TAC1 Gene Silencing by siRNA on the Proliferation of Human GAMG and U-87 MG Glioma Cell Lines. To study the role of the NK-1R and SP in human GAMG and U-87 MG glioma cells, *TAC1R* and *TAC1* were depleted in these cells using siRNA. *TAC1R* siRNA transfection did not produce any abnormality in the phenotype of GAMG glioma cells (Figure 2(a)). However, a significant decrease in NK-1R expression was observed by Western blot (Figure 2(b)) at 24 h and 48 h, showing significant suppression effects compared to control and sham groups. This decline was time-dependent (Figure 2(c)). NK-1R depletion produced a significant decrease in cell proliferation at 48 h after transfection of GAMG glioma cells with si*TAC1R*. The cell number in *TAC1R* siRNA group was significantly lower (446.8 ± 43.0 cell/mm^2) than control (702.1 ± 52.5 cell/mm^2) and sham (673.8 ± 43.1 cell/mm^2) groups (Figure 2(d)). By contrast, the silencing of *TAC1* expression by *TAC1* siRNA did not produce any change in cell proliferation in GAMG glioma cells (data not shown). Thus, the inhibition of SP expression did not affect the survival of these cells.

Inhibition of the *TAC1R* expression in U-87 MG human glioma cell also leads to time-dependent reduction in the expression levels of the NK-1R (Figures 3(a)–3(c)). *TAC1R* siRNA concentration and treatment duration was optimized to 10 nM during 6 h. Importantly, depletion of *TAC1R* resulted in a significantly cell proliferation decrease (48.18%) as showed the cell count number (cell/mm^2) at 6 h compared with control cells (Figure 3(d)). 5 nM of *TAC1R* siRNA was also tested, but no significant difference between the control and treated groups was observed (Supplementary Figure 2). These results showed that the U-87 MG cell line was more sensitive to the effect of the *TAC1R* inhibition than GAMG cell line. Unlike, no detrimental effect of silencing of *TAC1R* gene by siRNA was observed in the proliferation of human fibroblast cell culture (Figure 4).

3.3. Depletion of the NK-1R Reduces Cell Viability in Human Glioma Cell Lines. To investigate the role of the NK-1R on cell survival, the effect of its silencing in GAMG and U-87 MG glioma cells was analyzed. NK-1R-depleted cells showed a significantly decreased cell viability compared to control sham. There was observed a decrease of 48.4% and 51% at 24 h and 48 h, respectively, in GAMG (Figure 5(a)), and 22.6% at 6 h in U-87 MG cells (Figure 5(a)).

3.4. Depletion of the NK-1R Induces Both Apoptosis and Necrosis in Human Glioma Cells. To study whether the decrease in the number of human glioma cell lines after *TAC1R* siRNA gene silencing was due to an inhibition of the cell proliferation or to an induction of apoptotic or necrotic cell death mechanisms, the number of apoptotic and necrotic cells after transfection by flow cytometer using an Annexin V- conjugated with fluorescein isothiocyanate (FITC) kit was assessed. Viable cells (Annexin V-, PI-) were distinguished from cells in early apoptosis (Annexin V+, PI-), late apoptosis (Annexin V+, PI+), and necrosis (Annexin V-, PI+) stage. Depletion of the NK-1R in GAMG glioma cells led to an increase in both early apoptotic and necrosis population (Figure 6(a)). At 24 h, the early apoptotic positive cells were up to 8.6-fold higher. Necrotic positive cells were up to 5.1-fold higher in NK-1R-depleted cells compared to control sham (Figure 6(b)). In the case of 48 h, while the increase of necrotic positive cells was similar to 24 h (5.1-fold), the early apoptotic positive cells increased only 3.7-fold (Figure 6(b)). In NK-1R-depleted U-87 MG glioma cells (10 nM of TAC1R siRNA), the population of early apoptotic positive cells was up to 1.8-fold in control sham compared to NK-1R-depleted cells, and the necrotic positive cells were up to 11.9-fold higher in NK-1R-depleted cells compared to control sham. When 5 nM of TAC1R siRNA was used, necrotic positive cells were only 2.25-fold higher in NK-1R-depleted cells compared to control sham.

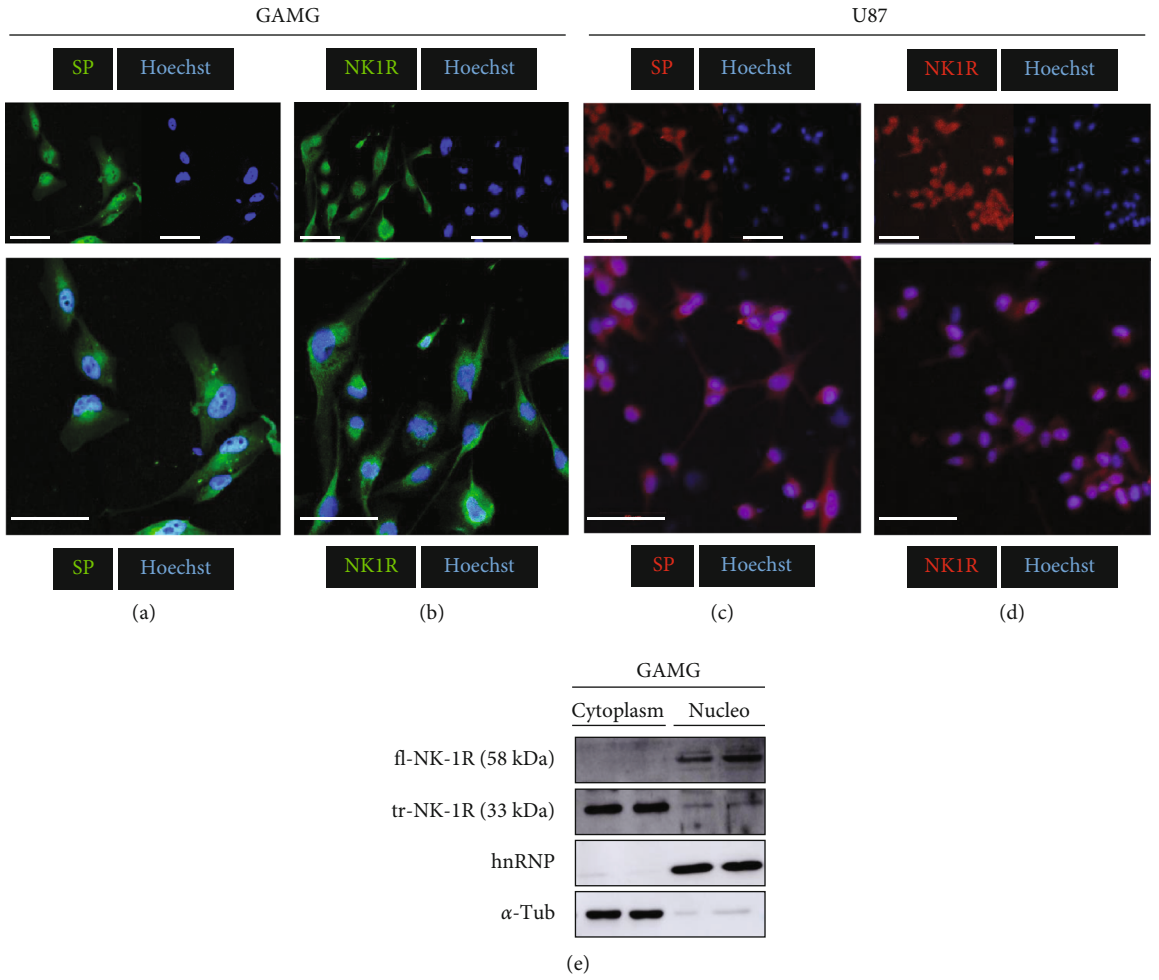

Figure 1: Subcellular localization of SP and NK-1R in human GAMG and U-87 glioma cell lines. Immunofluorescence of SP (a) and NK-1R (b) in GAMG glioma cells and U-87 glioma cells ((c) and (d)). Localization of SP and NK-1R (red) and nuclei (blue). Scale bar =50 μm. (e) Subcellular localization of the NK-1R in GAMG glioma cells by immunoblot. Subcellular fractionation and immunoblot analysis of NK-1R, α-tubulin (cytoplasmic marker), and hnRNP (nuclear marker) were performed as described in Materials and Methods.

4. Discussion

The present findings provide evidence about the antitumor activity of NK-1R gene silencing in human GAMG and U-87 MG glioma cells. We also show for the first time the immunolocalization of SP and NK-1R and the involvement of the NK-1R in the viability of these cells. The fl-NK-1R was mainly observed in the nucleus and the tr-NK-1R isoform in the cytoplasm. U-87 MG cells were more sensitive to the effect of the *TAC1R* inhibition than the GAMG cells. It has been also shown that the *TAC1* gene expression is not relevant for the viability of GAMG glioma cells. Moreover, another interesting finding is that in human nontumor normal fibroblast cells, *TAC1R* silencing by siRNA did not produce any change in cell viability.

The presence of SP and NK-1R in the nucleus and cytoplasm of GAMG and U-87 MG glioma cells is in agreement with previous data reporting the localization of the peptide in the cytoplasm/nucleus of other human cancer cells [7, 14, 15, 23]. These findings confirm that, in general, SP is mainly located in the nucleus and the NK-1R in the cytoplasm of human tumor cells. It is important to mention that here, and in most of the previous studies, the NK-1R was observed in the cytoplasm of tumor cells [7, 14]. The reason is currently unknown, since these receptors are G-protein coupled transmembrane receptors located in the cytoplasmic membrane [36–38]. However, in human primary melanoma, the presence of NK-1R has been found in the cytoplasm and cell membrane of tumor cells [39, 40]. Also, in human primary tumor cells, the NK-1R has been located by immunohistochemistry in the cytoplasm (in all patients studied), cell membrane (80% of patients), and nucleus (46.6%) [15]. Moreover, the number of NK-1Rs in primary tumors of glioma has been reported to be higher than in glioma cultures [3, 41]. It is possible that the immunohistochemical technique applied here in cultured cells was not sensitive enough to detect a clear labeling in the cell membrane and/or the labeling of the cell membrane could be masked by the high labeling for the NK-1R observed in the cytoplasm.

NK-1R exists in two isoforms, as a full-length form (fl-NK-1R) and in the truncated isoform (tr-NK-1R). There

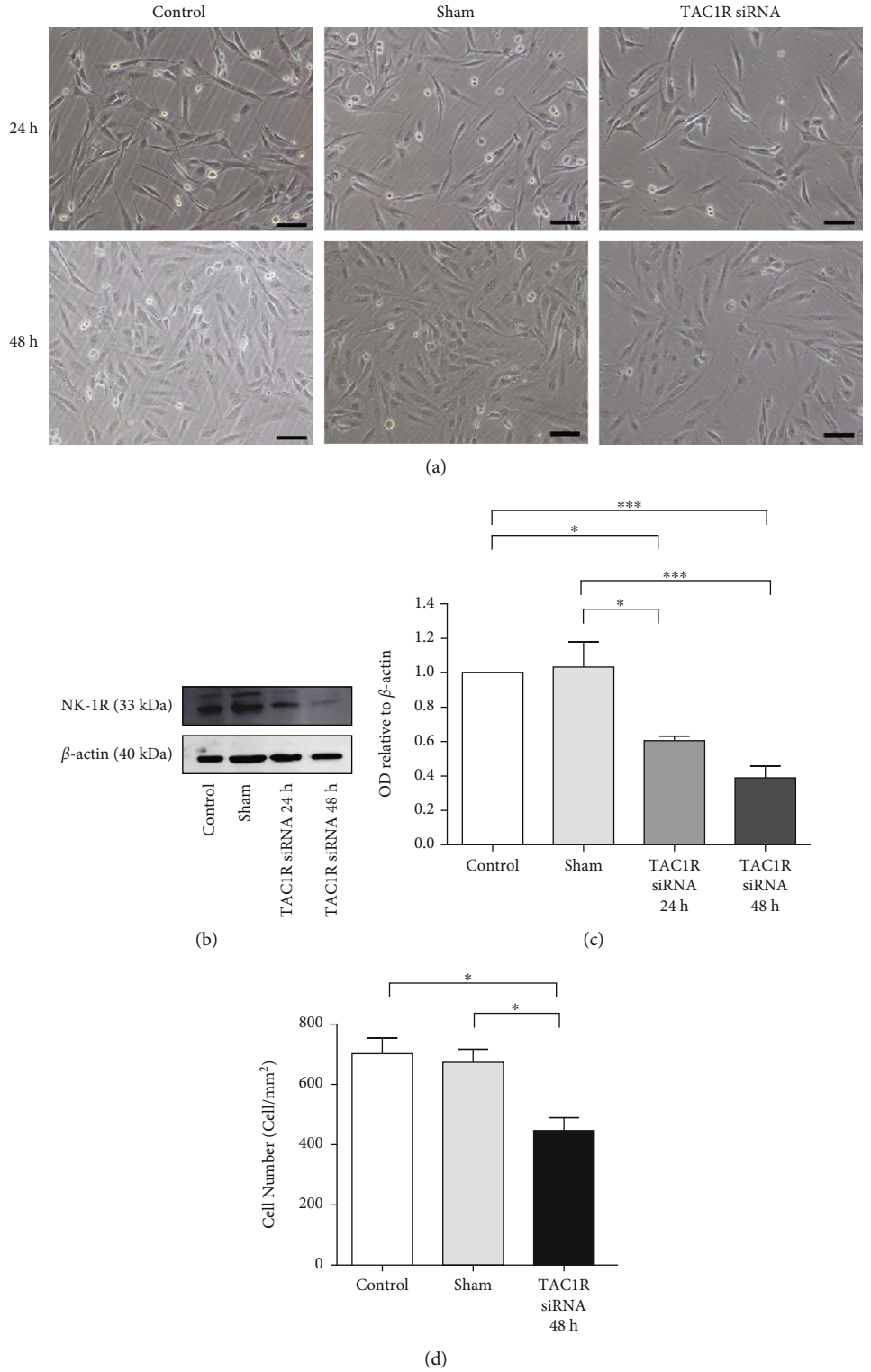

FIGURE 2: NK-1R expression in *TAC1R* silenced by siRNA in GAMG glioma cells. (a) Images from glioma cultures at 24 h and 48 h. (b) NK-1R and β-actin immunoblot. (c) Immunoblot analysis after measuring the OD bands relative to β-actin. Data are shown as the mean ± SEM of three independent experiments ($n = 3$ per group). (d) Cell counting after 48 h of treatment were performed as described in Materials and Methods. $*p < 0.05$; $**p < 0.01$.

are several functional differences between the 2 isoforms depending on the truncation of the 96 C-terminal tail of NK-1R. In our study, the most important finding regarding the localization of the NK-1R is that both the fl-NK-1R and tr-NK-1R isoforms were observed in the cytoplasm (mainly the tr-NK-1R isoform) and nucleus (mainly the fl-

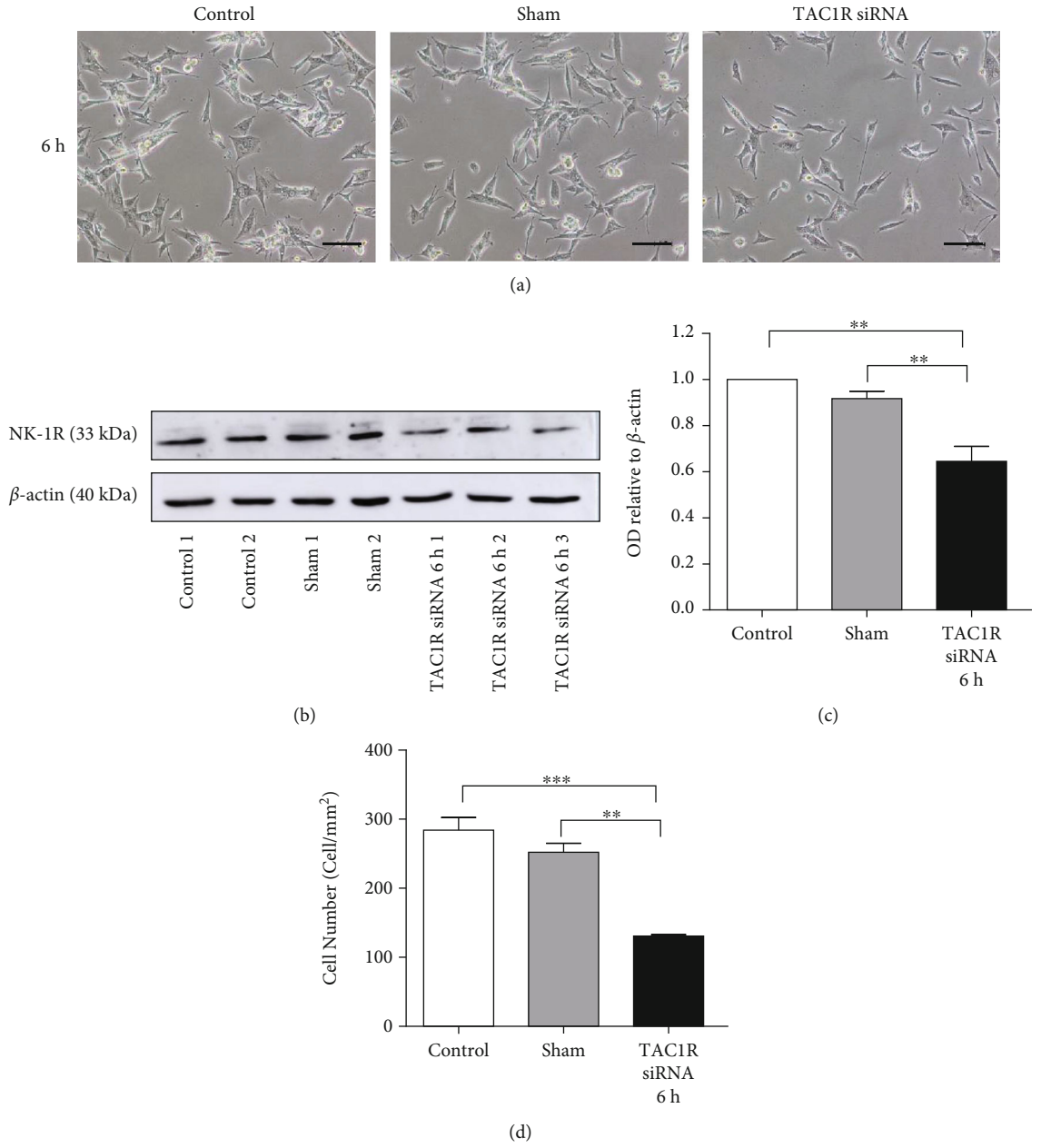

Figure 3: NK-1R expression in s *TAC1R* silenced by siRNA in U-87 glioma cells. (a) Images from glioma cultures at 6 h with 10 nM of siTAC1R. (b) NK-1R and β-actin immunoblot. (c) Immunoblot analysis after measuring the OD bands relative to β-actin. Data are shown as the mean ± SEM of three independent experiments ($n = 3$ per group). (d) Cell counting after 6 h of treatment was performed as described in Materials and Methods. $**p < 0.01$; $***p < 0.001$.

NK-1R) of glioma cells. Our results are also in agreement with those observed in adipose stem cells [35].

Internalization of NK-1R on the cell surface is a mechanism that occurs after ligand binding. This could reduce the number of cell surface receptors and thus participate in the desensitization of the response to SP. The absence of the C-terminal tail inhibits the receptor internalization and recycling processes, resulting in both a receptor resistant to desensitization and a longer response after SP binding [27, 42, 43]. Our study shows how the truncated isoform is also located inside the tumor cell like in the fl-NK-1R.

In cancer cells, the expression of the fl-NK-1R isoform is less expressed, meanwhile tr-NK-1R form is overexpressed. Therefore, the expression of the tr-NK-1R isoform, but not the fl-NK-1R, has been related to an enhanced malignant potential (e.g., Ki67 expression and tumor stage) [44]. The fl-NK-1R isoform promoted a slow growth of cancer cells, whereas the tr-NK-1R form increased the growth of tumor cells, mediated malignancy in cancer cells, and stimulated the synthesis of cytokines which exert growth-promoting actions [21]. These cytokines activated the transcription factor NF-κB that upregulated the truncated isoform and

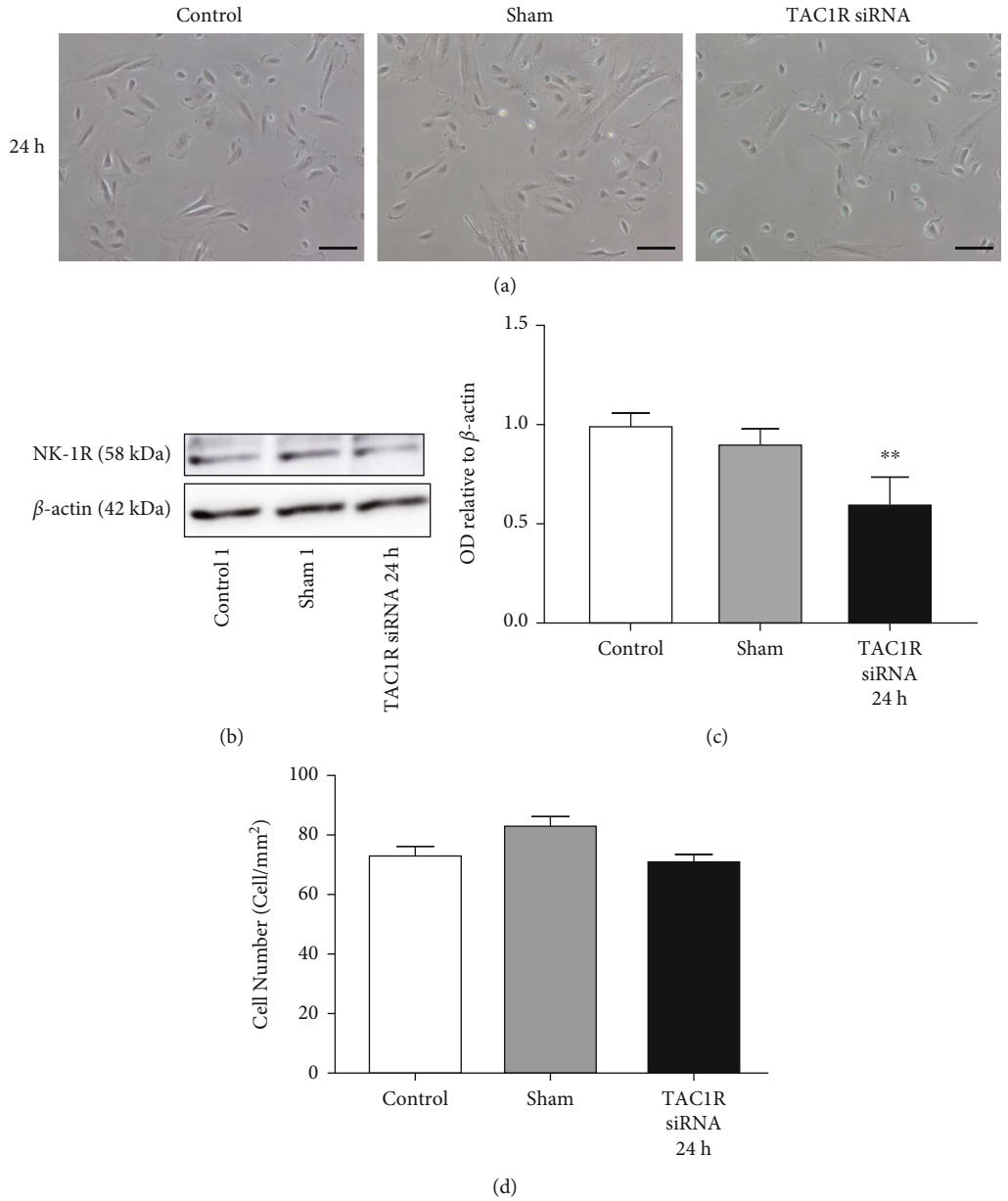

FIGURE 4: NK-1R expression in *TAC1R* silenced by siRNA in human fibroblast cells. (a) Images from human fibroblast cultures at 24 h with 10 nM of siTAC1R. (b) NK-1R and β-actin immunoblot. (c) Immunoblot analysis after measuring the OD bands relative to β-actin. Data are shown as the mean ± SEM of three independent experiments ($n = 3$ per group). (d) Cell counting after 24 h of treatment was performed as described in Materials and Methods. $**p < 0.01$.

slightly increased the full-length isoform [45, 46]. It has been described that tumor cells overexpress miR-206 which regulates the expression of the fl-NK-1R isoform by directly binding the 3′-unstraslated regions of the fl-NK-1R messenger RNA. Overexpression of miR-206 contributes to the malignant phenotype of tumor cells by maintaining a low level of the fl-NK-1R isoform [47]. We describe here that glioma cell proliferation was decreased when glioma cells were transfected by *TAC1R* siRNA. By contrast, in human nontumor normal fibroblast cells, *TAC1R* silencing by siRNA did not produce any change in cell viability. This means that overexpression and continued activation of tr-NK-1R are pivotal for viability of glioma cells. However, less expression and discontinued activation of fl-NK-1R are not pivotal for viability of nontumor cells. This is in agreement with previous results indicating that the expression of NK-1R in human fibroblast cell was much lower than in tumor cells [48]. Moreover, *TAC1R* knockout mice are completely viable and healthy, showing only some cognitive alterations as hyperactivity [49].

An important question is why NK-1R and SP are located in the nucleus of cancer cells. According to previous studies, it seems that SP, via the NK-1R, could regulate the nuclear function and could act as an epigenetic factor in human

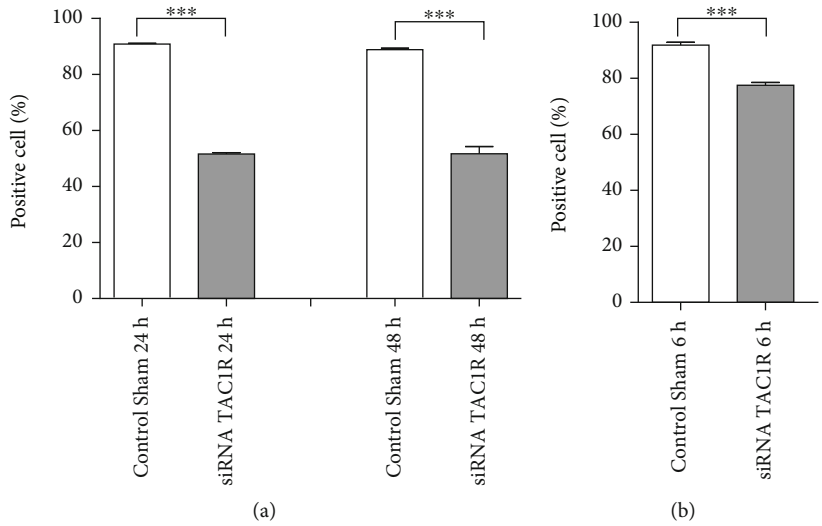

Figure 5: Depletion of NK-1R by TAC1R-targeted siRNA decreases cell viability in human glioma cells. GAMG cells were treated with 20 nM of siRNA for 24 h and 48 h (a), and U-87 glioma cells were treated with 10 nM of siRNA for 6 h (b). Then, cell viability was measured by flow cytometry using Annexin V–FITC and propidium iodide (PI) as described in Materials and Methods. Values are the means ± SEM of three experiments. $***\ p < 0.001$.

cancer cells. Thus, in GAMG and U-87 MG glioma cells, the SP/NK-1R system (e.g., via a SP-NK-1R complex) could exert an important role in the regulation of DNA expression (intracrine or nucleocrine action) through the modulation of proto-oncogenes and transcription factors (e.g., AP-1, c-myc, c-jun, c-fos, and hypoxia-inducible factor) involved in apoptosis, in cellular differentiation/transformation, and in cell cycle progression [41, 50].

The presence of SP in the cytoplasm of GAMG and U-87 MG glioma cells is also important since the secretion of SP, through the NK-1R, could display (1) an autocrine action, in which SP exerts a mitogenic action on the own glioma cell from which the peptide was released [51]; (2) a paracrine action, in which SP promotes the proliferation of glioma and endothelial cells located in the vicinity of the glioma cell from which the peptide was secreted; and/or (3) an endocrine action, in which the release of SP from the tumor mass into the blood vessels increases the plasma level of the peptide, reaching the entire body through the bloodstream [25, 52, 53]. In fact, it is important to note that in recent studies carried out in patients with cancer, both the serum SP level and the number of NK-1Rs were higher when compared with healthy controls [15, 53]. Thus, an increased level of SP in serum could be a predictive factor indicating a tumor development and/or a high risk to develop cancer.

It has been reported that SP, after binding to the NK-1R, activates the mammalian target of rapamycin (mTOR) signaling axis in cancer cells and enhances tumor cell growth and metastasis [25]. NK-1R antagonists (e.g., aprepitant) attenuates mTOR activation by reducing the phosphorylation of its downstream effectors (e.g., p70 S6 kinase) [54]. Moreover, SP released from tumor cells or from other sources (e.g., nerve terminals, immune cells) could also promote the migration of tumor cells [55]. SP also promotes the expression of degradative enzymes (matrix metalloproteinases) favoring tumor cell migration, invasion, and metastasis [43, 56], whereas NK-1R antagonists block tumor cell proliferation, migration, and invasion [55]. The activation of NF-κB by SP increased the NK-1R transcription by binding to the *TAC1R* gene [46]. Moreover, it is known that SP, via the NK-1R located in the endothelial cells (placed within the tumor and in the peritumor region), promoted angiogenesis which is crucial for the development of tumors by increasing the tumoral blood supply [23, 57]. Thus, glioma cells by releasing SP could promote angiogenesis, favoring the development of the tumor. Altogether, these findings show the essential role that the NK-1R plays in cancer, confirming that this receptor is an excellent target for the treatment of tumors.

Regarding the potential development of antiglioma strategies, the main finding of this work is that the NK-1R is necessary for the survival of human GAMG and U-87 MG glioma cells. The suppression of the NK-1R expression in these cells, by a knockdown gene silencing method, induced a decrease (mainly due to a dual apoptotic and necrotic mechanism) in the number of GAMG glioma cells. Similar results were observed in U-87 MG glioma cells, although these cells were more sensitive to the effect of the *TAC1R* inhibition than GAMG cells. The reason is currently unknown, but it could be related to the number of the NK-1Rs expressed by glioma cells and, in particular, to the number of full-length/truncated expressed isoforms. Therefore, further studies are required to elucidate the different *TAC1R* inhibition responses between glioma cell lines. Our findings agree with previous studies showing the essential involvement of the NK-1R in the viability of other human tumor cells (melanoma, acute lymphoblastic leukemia, breast cancer, and lung cancer) [15, 47]. This means that NK-1R is crucial for tumor cell survival, and a common antitumor strategy could be applied to treat any tumor. In these previous studies and after applying the knockdown gene silencing method, the technique to demonstrate necrotic mechanisms

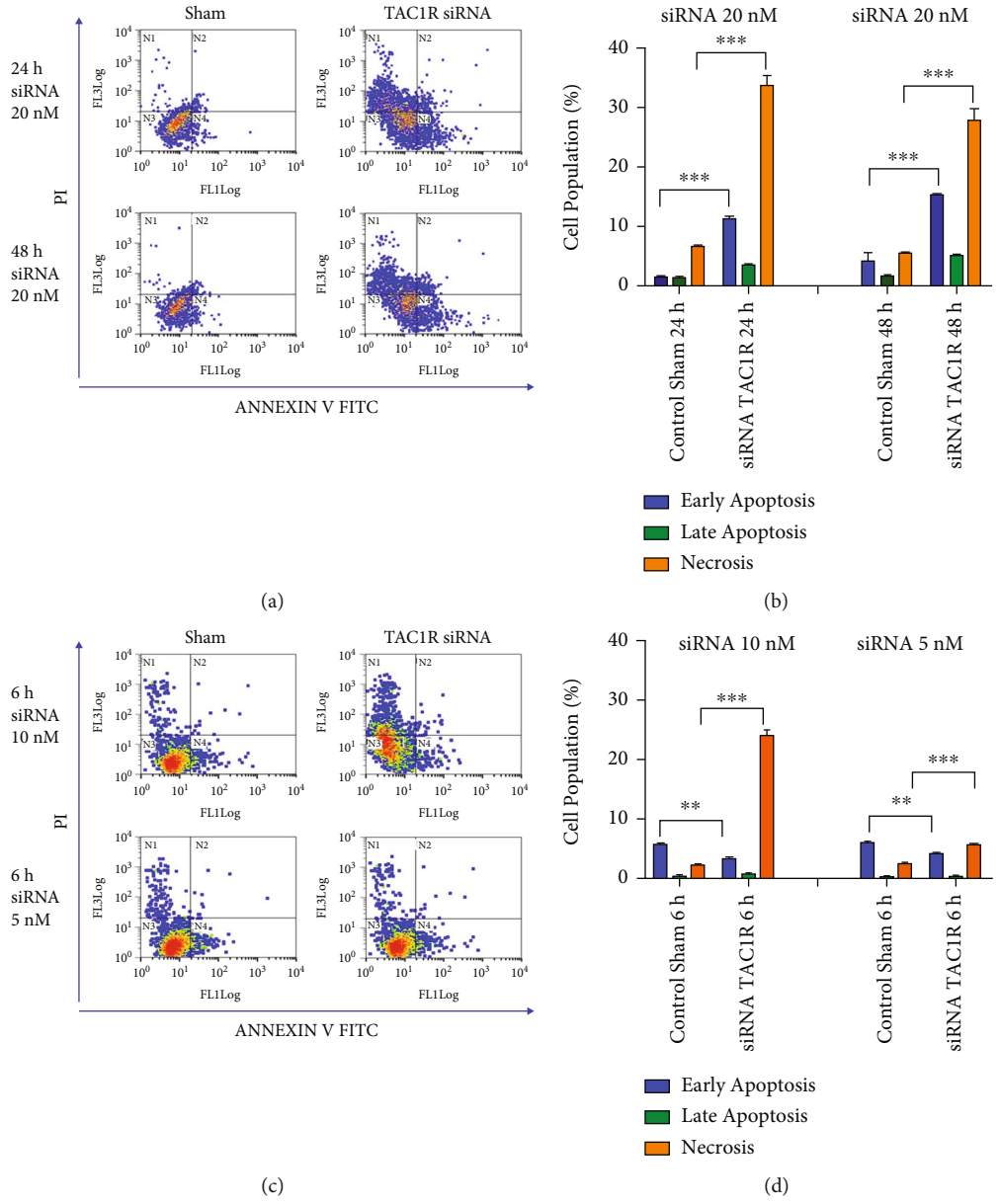

Figure 6: Depletion of NK-1R by TAC1R-targeted siRNA induced both apoptotic and necrotic cell death in human glioma cells. (a) Flow cytometry analysis using Annexin V–FITC and propidium iodide (PI) as described in Materials and Methods was performed to detect cell death in GAMG glioma cells after siRNA transfection at 24 h and 48 h. (b). Cell death rate in GAMG glioma cells. (c). Flow cytometry analysis was used to detect cell death in U87 glioma cells after siRNA transfection at 6 h. (d). Cell death rate in U-87 glioma cells treated with 10 nM and 5 nM of siRNA. Values are the means ± SEM of three experiments. $**p < 0.01$; $***p < 0.001$.

was not performed, and in all cases, the death of tumor cells was reported to be due to apoptotic mechanisms [3, 15, 18, 26, 58]. In cancer cells, NK-1R silencing promoted G2/M phase arrest/apoptosis and suppressed the proliferation of these cells; similar results were found when the NK-1R antagonist aprepitant was administered, but SP rescued the effects of the NK-1R silencing regarding apoptosis and cell proliferation [44]. Here, our study shows that glioma cells died by necrotic and apoptotic mechanisms. It seems that the absence of the NK-1R in glioma cells induces an acute and irreversible lesion, derived from a nonphysiological situation which produces the breakage of the cell membrane causing the death of glioma cells by necrotic mechanisms.

It is known that SP exerts a mitogenic action in tumor cells [22, 36, 59], and hence, it seems that glioma cells need the stimulus mediated by the neuropeptide, and for this reason cancer cells, including glioma, overexpress the NK-1R [24, 43, 60]. Blocking the stimulus derived from ligand-receptor (SP/NK-1R) activation (by antagonists or silencing NK-1R expression) can trigger apoptotic mechanism in glioma cells.

We have also demonstrated that the *TAC1* silencing by siRNA did not produce any change in GAMG glioma cells

and, hence, the absence of SP synthesis in these cells did not affect their survival. SP is not crucial for glioma cells, since the peptide can be synthesized by immune cells (tumor microenvironment) and/or by nerve cells. SP can also reach glioma cells from the blood, and/or the activation of the NK-1R located in glioma cells can be carried out by other peptides belonging to the tachykinin family (e.g., hemokinin-1 (HK-1)). In fact, it is known that HK-1 facilitates the proliferation of glioma cells [19]. This finding highlights the importance of the NK-1R in glioma cells because there are at least two peptides, SP and HK-1, that can activate this receptor. Thus, the crucial point is the expression of the NK-1R (overexpressed in cancer cells), because after the binding of SP to this receptor, all the produced effects are beneficial for human tumor cells: antiapoptotic effect, mitogenesis, facilitation of cell migration, and increased transcription of NK-1R and Warburg effect [5, 38].

As cancer cells overexpress the NK-1R, two different therapeutic strategies could be used not only against glioma but against any tumor type: pharmacological, using NK-1R antagonists [26, 47, 48, 61, 62], and/or genetic by applying the *TAC1R* siRNA method [3, 15]. Both strategies could improve the prognosis and survival of patients suffering from cancer, including glioma, because, in absence of the NK-1R, glioma cells can die by apoptosis as a consequence of starvation.

5. Conclusions

To summarize, the presence of both SP and the NK-1R has been shown in the nucleus (full-length isoform) and cytoplasm (truncated isoform) of human glioma cells. These data suggest that the peptide could exert intracrine (nucleocrine), autocrine, paracrine and/or endocrine actions. It has also been demonstrated that the NK-1R is necessary for the survival of human GAMG and U87 MG glioma cells, but the expression of the *TAC1* gene is not needed for the viability of these cells. In contrast, the NK-1R is not essential for the survival of the human nontumor normal fibroblast cells. Our study increases knowledge about involvement of the SP/NK-1R system in glioma and also confirms that the NK1-R is a promising and specific therapeutic target (acting like an Achilles heel) for the treatment of glioma. In addition, SP mediates a common mechanism for the proliferation of cancer cells, and the overexpression of the NK-1R in these cells opens up the possibility for a specific therapeutic treatment against any type of tumor.

Data Availability

The data that support the findings of this study are available from corresponding authors upon reasonable request.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

MM, MR, RC, and AA carried out the conceptualization; MFM, SA, and RM carried out the methodology; MM, MR, AA, MFM, and SA carried out the validation; MFM, SA, and RM carried out the formal analysis; MM, MR, RC, AA, MFM, and SA carried out the investigation; MM, RC, AA, and SA carried out the resources; MM, AA, MFM, and SA carried out data curation; MM, AA, MFM, and SA carried out the writing–original draft preparation; AA, MR, RC, MM, MFM, and SA carried out the writing–review and editing; MM, MR, RC, and AA carried out the supervision; MM, MR, and AA carried out the project administration; and RC, MM, and AA carried out the funding acquisition. All authors have read and agreed to the published version of the manuscript. Mario F. Munoz and Sandro Argüelles contributed equally to this work.

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Supplementary Materials

Supplementary 1. Negative control to analyze the level of nonspecific background signal produced by rabbit primary antibodies. Primary antibodies were omitted and replaced by nonimmune serum. Antirabbit secondary antibody conjugated to Alexa Fluor 658 (red) (A and B) or Alexa Fluor 488 (green) (C and D) was used and nuclei (Blue) were stained by Hoechst. Scale bar = 50 μm.

Supplementary 2. NK-1R expression in siTAC1R U-87 glioma cells. (A) Images from glioma cultures at 6 h with 5 nM of siTAC1R. (B) NK-1R and β-actin immunoblot. (C) Immunoblot analysis after measuring the OD bands relative to β-actin. Data are shown as the mean ± SEM of three independent experiments ($n = 3$ per group). (D) Cell counting after 6 h of treatment was performed as described in Materials and Methods.

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Research Article

The Therapeutic Potential of Aprepitant in Glioblastoma Cancer Cells through Redox Modification

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Although there is no doubt regarding the involvement of oxidative stress in the development of glioblastoma, many questions remained unanswered about signaling cascades that regulate the redox status. Given the importance of the substance P (SP)/neurokinin 1 receptor (NK1R) system in different cancers, it was of particular interest to evaluate whether the stimulation of this cascade in glioblastoma-derived U87 cells is associated with the induction of oxidative stress. Our results showed that SP-mediated activation of NK1R not only increased the intracellular levels of malondialdehyde (MDA) and reactive oxygen species (ROS) but also reduced the concentration of thiol in U87 cells. We also found that upon SP addition, there was a significant reduction in the cells' total antioxidant capacity (TAC), revealing that the SP/NK1R axis may be involved in the regulation of oxidative stress in glioblastoma cells. The significant role of SP/NK1R in triggering oxidative stress in glioblastoma has become more evident when we found that the abrogation of the axis using aprepitant reduced cell survival, probably through exerting antioxidant effects. The results showed that both MDA and ROS concentrations were significantly reduced in the presence of aprepitant, and the number of antioxidant components of the redox system increased. Overall, these findings suggest that aprepitant might exert its anticancer effect on U87 cells through shifting the balance of oxidant and antioxidant components of the redox system.

1. Introduction

As one of the most challenging malignancies to treat, glioblastoma has an adverse prognosis and poor quality of life [1]. Numerous therapeutic interventions have been developed for managing this cancer; however, these were not successful enough to induce a complete remission [2]. Recurrence of the tumor is also inevitable in glioblastoma, which makes the development of more accurate and less toxic treatment strategies more crucial [3].

Numerous molecular investigations have introduced oxidative stress as a hallmark of the progression of many cancers, including glioblastoma [4–6]. Despite the importance of oxidative stress in the pathogenesis of glioblastoma, the precise molecular mechanism responsible for the regulation of this event has not yet been identified. Recently, attention has been attracted to tachykinins, as they have a critical movement in the pathogenesis of glioblastoma [7] and control both oxidative stress and antioxidant systems [8]. Substance P (SP) is a small neuropeptide that binds to the tachykinins receptor 1 (TACR1), also known as neurokinin 1 receptor (NK1R), which is one of the most important G-protein-coupled tachykinin receptors initially found in central and peripheral nervous systems [9]. Within a short time, NK1R expression was found in other tissues, and the discovery of noncanonical activities of SP/NK1R added a new

perspective to this signaling axis as a regulator of tumorigenesis [7, 10].

In addition to its numerous biological functions, the SP/NK1R signaling pathway plays a significant role in tumor formation due to its ability to regulate cell proliferation and sustains cancer cell survival [11, 12]. The ability to regulate oxidative stress has brought attention to the SP/NK1R axis, especially in glioblastoma, as it can provide an opportunity for cancer cells to increase their metabolic activity. Baek et al. indicated that SP induced cell damage in retinal pigmented cells through PI3K-mediated induction of reactive oxygen species (ROS) production [13]. This finding has opened a new window into the role of the SP/NK1R axis in glioblastoma pathogenesis and provided an opportunity to use the NK1R antagonist for the treatment of this malignancy. Thus far, the anticancer property of several NK1R antagonists has been tested in different glioblastoma-derived cell lines and xenograft models; however, efforts are still underway to find a drug that has maximal tumor suppressor activity and minimal side effects.

Among different synthesized NK1R antagonists, aprepitant is a competing nonpeptide antagonist of the NK1R, first incorporated into a moderately emetogenic chemotherapy regimen to prevent chemotherapy-induced nausea and vomiting [14, 15]. When it was demonstrated that aprepitant could conveniently pass the blood-brain barrier and block the center of nausea in the brain through halting the attachment of substance P (SP) to NK1R [16], it has been assumed that this agent might have antitumor properties. In xenograft models, aprepitant reversed SP-induced mitogenic stimulation and reduced tumor burden [17]. The abrogation of NK1R using aprepitant in leukemic cell lines was also associated with the induction of G1 cell cycle arrest and caspase-3-dependent apoptotic cell death [18]. Moreover, Berger et al. demonstrated that aprepitant reduced the proliferative capacity of hepatoblastoma *in vivo* and *in vitro* investigations [19]. Apart from monotherapy, there are also several studies suggesting that aprepitant may be a good candidate as an adjunctive drug alongside chemotherapy [20]. The results of a completed phase III trial also shed light on a favorable pharmacokinetic and safety profile for aprepitant [21]. Although multiple studies emphasized the antitumor effect of aprepitant in several cancers, still, there is little evidence on the precise mechanism of action of this NK1R antagonist in human cancers.

Given these, in the present study, we aimed to investigate whether there is a correlation between the activation of the SP/NK1R axis and the induction of oxidative stress in glioblastoma-derived U87 cells. Moreover, it was of particular interest to evaluate whether blockage of this signaling using aprepitant could reduce the viability of the cells via changing the balance of the redox system in favor of antioxidant property in U87 cells.

2. Materials and Methods

2.1. Cell Lines and Reagents. Glioblastoma-derived U87 cells were cultured in DMEM medium supplemented with antibiotics, 10% fetal bovine serum, and 2 mmol/L L-glutamine (Invitrogen) in the presence of 5% CO_2 at 37°C. For evaluating the effect of NK1R stimulation, cells were treated with substance P (SP) at the concentrations of 400 and 800 nM. A stock solution of aprepitant (Santa Cruz Biotechnology Inc., Dallas, USA) was provided by dissolving the agent in sterile dimethyl sulfoxide (DMSO) for the drug treatment. As a negative control, we added an equal volume of DMSO to the drug/SP-untreated cells.

2.2. MTT Assay. To evaluate whether the treatment of U87 cells with aprepitant is coupled with the reduction of cell survival, cells were treated with increasing concentrations of the agent, and the metabolic activity of the cells was examined after 24 h using microculture tetrazolium assay. We plated 8000 U87 cells in each well of 96-well plate, and then, the cells were incubated with indicated concentrations of aprepitant 0 (control), 9.3, 18.7, 37.5, 75, 150, and 200 μM. After 24 h, the media was discarded, and the cells were further incubated with MTT solution (5 mg/mL in PBS) at 37°C for 3 h. Afterward, we dissolved the resulting formazan with DMSO, and the absorption was evaluated at 570 nm in an enzyme-linked immunosorbent assay reader.

2.3. Malondialdehyde (MDA) Assay. To investigate the concentration of MDA upon exposure to SP (400 and 800 nM) in the absence or presence of aprepitant (20 μM), we used malondialdehyde (MDA) assay kit (Kushan Zist, Tehran, Iran). To prepare the cells, 1.2×10^6 U87 cells were lysed in 1X BHT buffer. After centrifugation at 14000 g for 5 min, 250 μL of samples was added to 500 μL TCA, and the mixture was incubated for 5 min at 95°C. For precipitation of the proteins, the mixture was centrifuged at 14000 g for 5 min. Then, 500 μL of the supernatant was added to 250 μL of TBA buffer, and the mixture was incubated at 95°C for 30 min. The absorption of samples was then measured at 532 nm. To evaluate MDA concentration in U87 cells, we plotted a standard curve from the obtained results using GraphPad Prism software.

2.4. ROS Assay. To investigate whether the activation of the SP/NK1R axis in U87 cells is coupled with ROS production, the cells were cultured in a 96-well plate in the presence of SP, aprepitant, or the combination of two agents. After the indicated time interval, the media was discarded from the plate, and 100 μL of Ready assay buffer from cellular reactive oxygen species (ROS) assay kit (Kushan Zist, Tehran, Iran) was added to each well. After removing the Ready buffer, 100 μL of DCF staining buffer was added to each well, except the one considering the blank well. The plate was then incubated at 37°C for 60 min and incubated with 100 μL of R3 stimulator for an additional 20 min in the dark. After discarding the solution and DCF staining buffer, the cells were washed twice with Ready assay buffer and the production of ROS was evaluated by measuring the fluorescence intensity at a wavelength between 480-500 nm. The final concentration of ROS was measured by using a standard curve.

2.5. TAC Assay. U87 cells were treated with SP (400 nM and 800 nM), aprepitant (20 μM), or in the combined modality. After the indicated time, 1×10^6 cells were harvested for

evaluating the total antioxidant capacity (TAC) using the total antioxidant capacity (TAC) assay kit (Kushan Zist, Tehran, Iran). The harvested cells were lysed by repeated cycles of freezing and thawing and then centrifuged at 12000 g for 15 min. The supernatant was collected and transferred into the fresh tube. Afterward, 150 μL, 10 μL, and 30 μL from R2, R3, and %5 reading buffers were added to the samples, respectively. After 5 min of incubation at room temperature, the absorbance of the samples was evaluated at 734 nm using a plate reader. The final concentration of TAC was measured using a standard curve.

2.6. Thiol Assay. The level of total sulfhydryl groups was assessed by the DTNB (2,2′-dinitro-5,5′-dithiodibenzoic acid) reduction method. This reagent reacts with SH groups to produce a yellow color (peak absorbance 412 nm). Briefly, 0.1 mL of Tris–EDTA buffer (pH 8.6) was exposed to the 0.05 mL cell lysate, and after that, the absorbance was measured at 412 nm against Tris–EDTA buffer alone (A1). Next, 20 μL DTNB reagent (10 mM in methanol) was added to the mixture, and after 15 min of incubation at room temperature, the sample absorbance was reread (A2). Additionally, the absorbance of the DTNB reagent alone was measured as a blank group (B). Finally, total SH concentration (μM) was obtained by the following equation: total thiol concentration (μM) = (A2 − A1 − B) × 1.07/0.05 × 13.6.

2.7. Statistical Analysis. The results are presented as the mean ± standard deviation of three independent experiments. All the investigations were performed in triplicate to provide a meaningful result. The significance of the differences between experimental variables, a probability level of $P < 0.05$, was demonstrated using two statistical tests, two-tailed Student's t-test, and one-way variance analysis. The data analysis was performed by using SPSS and GraphPad prism software.

3. Results

3.1. The Antitumor Effect of Aprepitant on the Survival Rate of U87 Cells. Previous studies have declared that exposure of malignant cells to aprepitant is coupled with the reduction in the survival and proliferative capacity of the cells. Given these, it was of particular interest to evaluate the antitumor activity of this NK1R antagonist in glioblastoma-derived U87 cells. We treated the cells with the increasing concentrations of aprepitant, and then, the drug-treated cells were subjected to MTT assay. As presented in Figure 1, we found that upon blockage of the NK1R signaling axis using aprepitant, there was a significant reduction in the viability of the cells. Our results showed that aprepitant at the concentration of 20 μM effectively diminished the number of U87 viable cells. Moreover, the estimated IC50 value for aprepitant in U87 cells was 36.15 μM. These findings were suggestive of the antitumor activity of aprepitant in glioblastoma-derived cells.

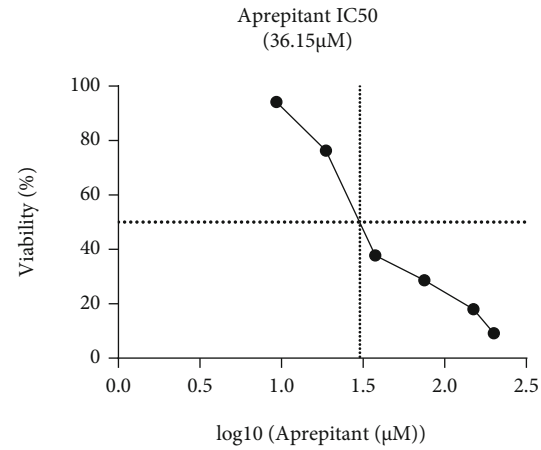

Figure 1: The anticancer effect of aprepitant on glioblastoma cells. The results of the MTT assay showed that aprepitant could remarkably reduce the viability of U87 cells with an estimated IC50 value of 36.15 μM.

3.2. Evaluating the Impact of the SP/NK1R Signaling Axis on MDA Level. The interconnection between oxidative stress and tumorigenesis has been well-established in several reports. It has been claimed that cancer cells exploit the redox system to maintain their survival and proliferative capacity. We aimed to evaluate whether the activation of the NK1R signaling axis in glioblastoma cells could augment the oxidative stress in the cells. We treated U87 cells with SP, a well-known ligand of NK1R, and then, we evaluated the amount of malondialdehyde (MDA), a well-known marker of lipid peroxidation. As depicted in Figure 2, we found that upon SP addition to the culture media of U87 cells, there was a concentration-dependent increase in the amount of MDA. It was demonstrated that SP at the concentration of 800 nM could elevate the concentration of MDA approximately to 25 μM, suggestive of the potent role of the SP/NK1R system in disrupting the balance of the redox system.

Moreover, to confirm that the elevated MDA concentration was due to the stimulation of the SP/NK1R axis in the U87 cells, we treated the cells with the antagonist of NK1R. Of note, our results showed that aprepitant (20 μM), as a single agent, could reduce the concentration of MDA in U87 cells (Figure 2). The favorable activity of aprepitant against oxidative stress became more evident, when we simultaneously treated the cells with aprepitant and sp. As presented in Figure 2, there was a significant reduction in the concentrations of MDA upon exposure of the cells to SP-plus-aperient, suggestive of the antioxidant property of aprepitant in the malignant cells.

3.3. The NK1R Stimulation using SP Increased the Amount of ROS in U87 Cells. To investigate whether NK1R activation in SP-treated U87 cells was associated with the induction of oxidative stress, the intracellular amount of reactive oxygen species (ROS) was evaluated by the ROS assay. In agreement with the results of the MDA assay, treatment of the cells with SP at the concentration of 800 nM significantly elevated the intracellular level of ROS (Figure 3), indicating that SP-mediated stimulation of the NK1R resulted in the induction of oxidative stress probably through increasing the production of ROS in the cells. Interestingly, when this signaling

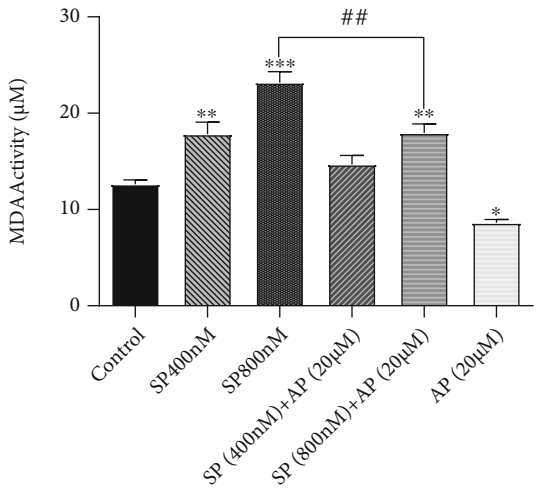

Figure 2: The effect of NK1R stimulation and suppression on the concentration of MDA in U87 cells. While SP increased the concentration of MDA in U87 cells, the treatment of glioblastoma cells using aprepitant (20 μM) diminished the level of this oxidative marker in the cells. Moreover, our results showed that aprepitant could attenuate the effect of SP on the production of MDA. Values are given as the mean ± S.D. of three independent experiments. ## is representative of $P \leq 0.05$, which is statistically significant.

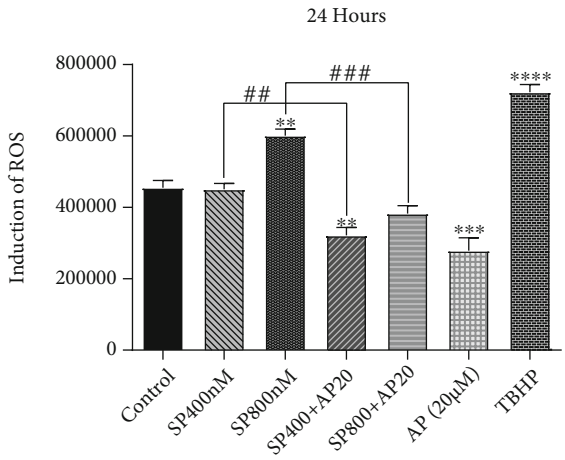

Figure 3: The effect of NK1R on the production of ROS in glioblastoma-derived cells. Using the ROS assay, we evaluated the intracellular level of ROS after the treatment of U87 cells with SP, aprepitant, or the combination of both agents. SP significantly elevated the intracellular level of ROS in U87 cells at the concentration of 800 nM. Aprepitant, either as a single agent or in combination with SP, could halt the generation of free radicals in malignant cells. Values are given as the mean ± S.D. of three independent experiments. ## and ### are representative of $P \leq 0.05$, which is statistically significant.

axis was suppressed by aprepitant, either alone or in the presence of SP, there was a robust reduction in the total production of ROS (Figure 3). As presented, aprepitant, as a single agent, decreased the oxidative stress in U87 cells, but this agent also prevented the SP-induced ROS production in the cells.

3.4. The Oxidative Effect of Activated the SP/NK1R in U87 Cells Was Coupled with the Reduction in Thiol Group Concentration. Given the effect of activated NK1R in the induction of oxidative stress in U87 cells, we aimed to investigate whether SP-mediated stimulation of the NK1R could reduce the activity of the antioxidant system. The thiol group is a well-known component of many antioxidant enzymes used by the redox system to compensate for the harmful effects of free radical spices. The results of the thiol assay revealed that when SP was added to the culture medium of U87 cells, there was a significant reduction in the concentration of the thiol groups. To gain insights into the antioxidant effects of aprepitant in U87 cells, we scrutinized the thiol group concentration upon exposure of the cells to this agent. As evident in Figure 4, we found that culturing the cells with 20 μM concentration of aprepitant resulted in a marked elevation in thiol group concentration. Additionally, the analysis of the antioxidant property of U87 cells treated with aprepitant in combination with SP showed an accumulation in the number of thiol groups. As presented in Figure 4, the addition of aprepitant to SP-treated cells increased thiol group concentrations considerably.

3.5. Activation of the SP/NK1R Signaling Axis Decreased the Total Antioxidant Capacity (TAC) of U87 Cells. Having established that SP-mediated activation of the NK1R signaling axis in U87 cells was coupled with the reduction in the antioxidant property of the cells, it was of particular interest to evaluate the effect of this network on the total antioxidant capacity (TAC) of U87 cells. We found that SP significantly reduced the TAC of U87 cells with the maximal repression observed in the presence of SP at the concentration of 800 nM (Figure 5). Additionally, the ablation of the NK1R using aprepitant (20 μM) was coupled with the remarkable elevation in the TAC capacity, indicating a correlation between the activated NK1R and the reduction of antioxidant capacity of the malignant cells. Previous studies have shown that the induction of the antioxidant process in cancer cells is a promising approach to prevent the rate of tumorigenesis. Accordingly, when we exposed SP-treated cells to aprepitant, we found that this agent could potently compensate the oxidative property of SP, which was in agreement with the results obtained from the thiol group assay. As presented in Figure 5 and compared with SP-treated cells, aprepitant at the concentration of 20 μM was successful in preventing the repressive effect of SP (800 nM) on the TAC capacity U87 cells. While the TAC capacity of U87 cells reached 100 μM in the presence of SP (800 nM), the addition of aprepitant to the culture media increased this capacity up to 200 μM (Figure 3). Taken together, these findings suggested that while SP/NK1R stimulation in U87 cells was associated with the elevation of oxidative stress, blockage of this cascading using aprepitant exerted anticancer effects via altering the balance of the redox system in favor of antioxidant properties.

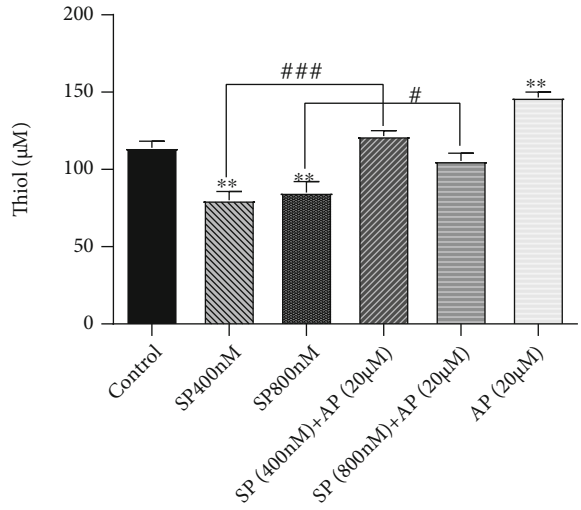

Figure 4: Stimulation of NK1R using SP decreased the concentration of the thiol component in U87 cells. Using the thiol assay, the concentration of proteins containing the thiol group was evaluated in U87 cells after either SP or aprepitant treatment. Unlike SP, which decreased the level of thiol in the cells, aprepitant increased the concentration of thiol-containing proteins, suggestive of the antioxidant property of this agent. Values are given as the mean ± S.D. of three independent experiments. # and ### are representative of $P \leq 0.05$, which is statistically significant.

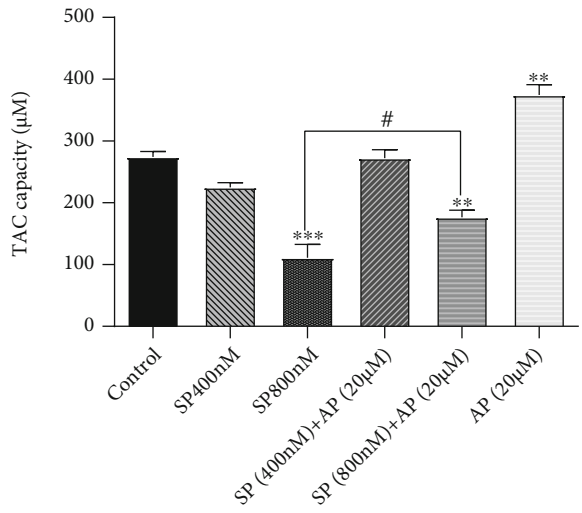

Figure 5: Stimulation of NK1R using SP is coupled with the reduction in the total antioxidant capacity of U87 cells. When U87 cells were treated with SP at the concentration of 800 nM, there was a significant reduction in the concentration of TAC. However, blockage of NK1R using aprepitant (20 μM) resulted in an approximately 2-fold increase in the concentration of TAC in U87 cells. Moreover, in the presence of aprepitant, the ability of SP to reduce the concentration of TAC significantly decreased. Values are given as the mean ± S.D. of three independent experiments. # is representative of $P \leq 0.05$, which is statistically significant.

4. Discussion

Despite striking attempts to ameliorate cancer treatment strategies and improve patient outcome, efforts have not successfully reached the desired results due to the engagement of multiple factors, such as activation of oxidative stress. Recent disclosures showed that changes in redox state in tumorigenesis may correlate with tolerance to chemotherapeutic drugs [22]. A new perspective has been aroused in the treatment strategy that proposes adding antioxidant agents may be befitting for therapeutic protocols. This approach seems to be promising. Nevertheless, since the role of the precise signaling axis in the activation of oxidative stress is not well-established, finding a new agent to change the balance of the redox system in favor of antioxidant property has been postponed.

The importance of the SP/NK1R in the pathogenesis of human cancers coupled with its association with the acquisition of chemoresistance phenotype has raised the question that perhaps the involvement of this axis in these events is mediated through regulation of oxidative stress [7]. Although several studies have examined the effect of aberrantly activated AP/NK1R signaling pathways on the pathogenies of the different human cancers, the connection between this axis and the induction of oxidative stress has not yet been clarified. The results of the present study showed that upon the SP-mediated NK1R activation, there was a remarkable elevation in the concentration of malondialdehyde (MDA) and reactive oxygen species (ROS) in glioblastoma cells; a human malignancy in which the induction of oxidative stress is coupled not only with disease progression but also with poor prognosis [23, 24].

Several lines of evidence have declared that MDA, which is the well-known marker of lipid peroxidation and oxidative stress, could promote the risk of cancer development, including breast cancer [25] and ovarian carcinoma [26]. Lipid peroxidation is also notorious for its key role in inducing chemoresistance against temozolomide (TZM) in glioblastoma cells [27]. It is demonstrated that upon lipid peroxidation and MDA formation, the intracellular amount of ROS increases in neoplastic cells, reducing the cells' sensitivity to chemotherapeutic drugs via activating DNA damage responses [28]. ROS production in neoplastic cells; however, it is a matter of debate. There is a wealth of evidence suggesting that excessive ROS generation is coupled with the induction of apoptotic cell death [29]. It is shown that inhibiting the NK1R in acute myeloid leukemia (AML) cells increases the intracellular levels of ROS and, in turn, induces mitochondria-mediated apoptotic cell death [30]. In acute lymphoblastic leukemia (ALL), it has been claimed that through elevating the intracellular levels of ROS, aprepitant could reduce the expression of antiapoptotic proteins [18]. Many chemotherapeutic drugs such as doxorubicin, cisplatin, and etoposide also eliminate the population of neoplastic cells by requiring ROS [31]. Despite the advantages, the excessive production of ROS within the cancer cells is not always beneficial, as this reactive oxygen species could protect cancer cells from the anticancer agents by increasing the expression of multidrug-resistant proteins (MRDs),

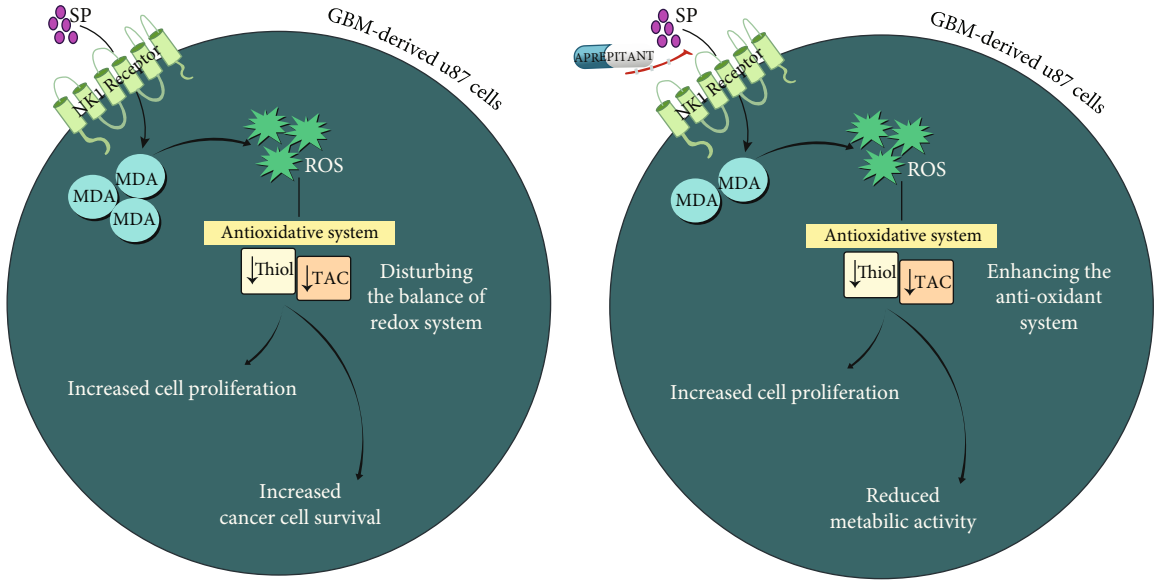

Figure 6: Schematic presentation. Once SP binds to NK1R, the intracellular levels of MDA and ROS increase in glioblastoma-derived U87 cells, which in turn through disturbing the antioxidant system reinforce the survival and the proliferative capacity of neoplastic cells. Aprepitant, an antagonist of NK1R, on the other hand, prevents the oncogenic activity of SP. This agent abrogates the activity of MDA and subsequently the production of ROS within the malignant cells. Moreover, through potentiating the activity of the antioxidant system, as revealed by the elevation in the activity of thiol and TAC, aprepitant decreases the metabolic activity of U98 cells.

overcoming cell cycle arrest, and altering the activity of autophagy flux [32]. The variant of ROS could also facilitate tumor cell migration and metastasis and attenuate the cytotoxicity of the chemotherapeutic drugs [33]. The harmful effect of ROS and its related oxidative stress on the activity of thiol-containing proteins, which have a critical function in maintaining the balance of the redox system and mediating the reversible posttranslational modifications [34], could also be other reasons suggesting that these free radicals might act in favor of tumorigenesis.

In agreement with these findings and the significant elevation of ROS, we found that the activation of the NK1R in U87 cells was coupled with the decrease in the intracellular level of thiol content. This finding suggested that perhaps the results of the constant stimulation of the NK1R using SP in glioblastoma cells are ROS production, which enhances the carcinogenesis process by disturbing the regulation of the redox system. The oxidative property of the SP/NK1R also became more evident when aprepitant, a well-known blocker of the SP receptor, decreased the intracellular level of MDA and ROS in U87 cells and increased the concentration of thiol in the malignant cells. Moreover, we found that the antioxidant property of aprepitant was also coupled with the reduction of the survival of the cells, as revealed by the significant decrease in the metabolic activity of U87 cells. This finding was in accordance with the results of the previous investigations, which reported the cytotoxic property for aprepitant in several cancers ranging from solid tumors [35] to hematologic malignancies [36]. Ghahremanloo et al. also suggested that a single agent of aprepitant diminishes the viability of colon cancer-derived SW480 cells by reducing the intracellular levels of ROS and abrogating the NF-κB signaling axis [37]. In glioblastoma, Korfi et al. suggested that a higher concentration of aprepitant (35 μM) is capable of reducing ROS production while increasing the enzymatic activity of superoxidase dismutase (SOD) and catalase [38]. Aprepitant also showed antioxidant activity in glioblastoma-derived cell lines by reducing the expression of thioredoxin reductase [39]. It has also been reported that this NK1R antagonist suppresses superoxide activity in glioblastoma-induced rats by inhibiting neutrophil activity [40]. To the best of our knowledge, this was the first time that the antioxidant activity of the lower concentrations of this agent (20 μM) has been tied with its anticancer effects, and our study suggested that aprepitant probably reduced the survival of glioblastoma cells via blocking the oxidative stress.

Different factors have been evaluated to determine patients' response rates to both conventional and novel therapeutic approaches in the modern era of cancer management. For a long time, antioxidant components have been claimed to reduce the risk of cancer [22]. However, when epidemiological studies indicated that there is a correlation between the serum level of antioxidant components and the response rate to chemotherapeutic drugs, the common perspective of the antioxidant compound has changed and total antioxidant capacity (TAC) has been introduced as a promising marker for evaluating the prognosis of human cancers. In a study conducted by Santiago-Arteche et al., it was shown that the TAC level was significantly lower in metastatic colorectal cancer patients as compared to patients without metastasis [41]. Likewise, several studies have demonstrated that the serum level of TAC is remarkably lower in breast cancer patients than in healthy counterparts [42, 43]. Although the reduction of the serum level of TAC is reported in patients with glioblastoma [24], its precise molecular mechanism has not been elucidated.

Accordingly, as shown in Figure 6, the present study results showed that the stimulation of the NK1R signaling pathway in glioblastoma-derived U87 cells could be a probable mechanism leading to the reduction of TAC concentration. Moreover, our results showed that aprepitant could effectively bypass the suppressive effect of SP on TAC activity. We found that in the presence of aprepitant, there was a significant elevation in TAC concentrations in U87 cells. The data presented in this study, on the one hand, suggested the aberrant activated SP/NK1R signaling axis as a probable mediator of induction of oxidative stress in glioblastoma cells and, on the other hand, showed that aprepitant could exert anticancer effect on U87 cells by shifting the balance between oxidant and antioxidant components of the redox system. However, since the results presented in this study are obtained based on one cell line and glioblastoma is notorious for its heterogeneous nature [44], further analysis on other glioblastoma cell lines with different genetic characterization is required to more precisely study the role of the SP/NK1R axis in tumorigenesis. Moreover, it should be well-established whether other abnormalities, such as hyperactivation of EGFR, could reinforce the stimulation of NK1R and thereby attenuate the therapeutic value of aprepitant.

Data Availability

Data are available upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Soodabeh Rezaei and Reza Assaran Darban equally contributed as the first author.

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Research Article

Aprepitant Promotes Caspase-Dependent Apoptotic Cell Death and G2/M Arrest through PI3K/Akt/NF-κB Axis in Cancer Stem-Like Esophageal Squamous Cell Carcinoma Spheres

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The antagonists of the neurokinin-1 receptor (NK1R) are known for their anti-inflammatory, anxiolytic, antiemetic, and anticancer activities. Aprepitant, a nonpeptide NK1R antagonist, is used in nausea and vomiting, the most common side effects of cancer chemotherapy in patients. It has been established that NK1R activation by substance P (SP), which links cancer promotion and progression to a neurokinin-mediated environment, became one mechanism that corresponds to the mitogenesis of tumor cells. Therefore, this study is aimed at explaining and evaluating the anticancer impacts of aprepitant on esophageal squamous cancer cell (ESCC) spheres by using in vitro experiments, such as resazurin, ROS, annexin-V binding, RT-PCR, and Western blot analysis. As a result, we showed that aprepitant had strong antiproliferative and cytotoxic effects on ESCC cell spheres. Also, aprepitant caused significant G2-M cell cycle arrest depending on concentration increase. Further, exposure of cells to this agent resulted in caspase -8/-9-dependent apoptotic pathway activation by modifying the expression of genes involved in apoptosis. Besides, treatment of the cells by aprepitant abrogates of the PI3K/Akt pathway, as shown by reducing the level of Akt, induces apoptotic cell death. In summary, pharmacological inhibition of NK1R with aprepitant seems to have a significant chance of treating ESCC as a single agent or in conjunction with other chemotherapeutic drugs.

1. Introduction

Esophageal squamous cell carcinoma (ESCC) is the dominant histological subtype of esophagus cancer, which constitutes more than 90% of this malignancy [1]. Given the ongoing progress of therapies, like surgery and chemoradiation, the overall survival rate of affected patients remains dismal because of treatment failure and high risk of recurrence. It is, therefore, critically necessary to clarify its pathogenesis and to identify efficient agents as emerging chemotherapeutic potential therapies for its prevention, diagnosis, and treatment [2, 3].

In most cancers, there is a side population of cells characterized by self-renewal ability, differentiation potential, high tumorigenicity, and therapy resistance, known as cancer stem cells (CSCs) [4, 5]. Unfortunately, the presence of CSCs in ESCC can consequently lead to therapeutic failures [6]. There are still no drugs in clinics available to target CSCs specifically; however, chemotherapy for advanced or recurrent ESCC is the primary approach of palliative treatment [7, 8]. Recently, the expression and secretion of peptides by tumors have been shown to influence the growth and development of cancer. As an undecapeptide of the tachykinin

family, substance P (SP) is widely distributed in the central and peripheral nervous systems [9]. Neurokinin-1 receptor (NK1R) shows a preferential affinity for SP, regulating many biological functions, including neurogenic inflammation, pain, and depression [10]. Notably, numerous studies have indicated that the SP/NK1R system may play a significant role in the development of cancer (such as pancreatic cancer, endometrial cancer, colon carcinoma, hepatoblastoma, glioblastoma, and breast cancer), regulating cell proliferation and migration for invasion and metastasis, and controlling cell proliferation for angiogenesis [11–14]. It has been reported that the SP/NK1R system is involved in pancreatic cancer cells via inducing angiogenesis, migration, and tumor proliferation [15]. In contrast, it has been shown that NK1R antagonists could suppress the spread of pancreatic cell cancer, angiogenesis, and pancreatic cancer cell migration [16].

Aprepitant has been shown to suppress cancer cell growth as a selective high-affinity antagonist of the human SP/NK1R system [15]. Moreover, the antitumor action of aprepitant has been previously reported by Javid et al. [17]. In a recent report, Berger et al. observed that NK1R is represented in human hepatoblastoma cells, and its inhibition with aprepitant led to significant suppression of the tumor, both *in vitro* and *in vivo* [15]. In other research, the inhibition of NK1R by aprepitant has been shown to suppress cell proliferation and induce apoptosis in multiple cancer cells by modifying different signal transduction pathways like phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) and nuclear factor-kappa B (NF-κB) [18, 19]. While several types of research highlighted the role of aprepitant in malignant diseases, its antitumor mechanism of action is little understood, and more studies are ongoing to more closely describe the molecular pathways involved in the apoptosis-inducing and cytotoxic effects of aprepitant. Herein, this study is aimed at showing the cytotoxic effects of NK1R antagonist aprepitant and defining its antitumoral action against ESCC.

2. Methods and Materials

2.1. Cell Culture, Chemicals, and Reagents. The KYSE-30 human esophageal squamous cell carcinoma cell line was prepared from the National Cell Bank of Iran (NCBI), Pasteur Institute (Tehran, Iran). It was cultured in medium containing a 1 : 1 mixture of RPMI 1640, and Ham's F-12 (Betacell, Iran) supplemented with 5% fetal bovine serum and 1% penicillin-streptomycin (Grand Island, NY, USA) for 5-6 days until the cells reached the exponential phase of growth ($0.6–1 \times 10^6$ cells/mL). SP and aprepitant were bought from Sigma-Aldrich (St. Louis, MO, USA). A stock solution of SP and aprepitant at the concentration of 70 μM and 74 mM, respectively, was prepared through dissolving the compound in sterile dimethyl sulfoxide (DMSO, Gibco, Scotland). It is then diluted to make a working solution and, cells were treated with relevant amounts of the SP and aprepitant solution to attain a concentration of different and specific doses. So, the final concentrations of DMSO in the culture medium did not exceed 0.1% in all the treatments.

2.2. ESCC Cell Sphere Formation. Single-cell suspensions derived from adherent cells were transferred to the polymer of 2-hydroxyethyl methacrylate- (poly HEMA-) coated petri dishes at the concentrations of 100000 cells/mL for ESCC cells. The cells were maintained at 37°C with 5% CO_2 in serum-free RPMI/F12 medium supplemented with 2% B-27 supplement (Grand Island, NY, USA), 20 ng/mL basic fibroblast growth factor (Grand Island, NY, USA), and 20 ng/mL epidermal growth factor (Sigma-Aldrich Company, USA) to form spheres. To replenish nutrients, the medium was refreshed every two days. After six days, the spheres were separated into the single cells and were cultured subsequently into the new nonadherent petri dishes under the same conditions as before. After three passages, the stem-like properties of sphere cells (tertiary ESCC cell spheres) were analyzed by the following experimental tests.

2.3. Cell Viability Assay. As previously described, a colorimetric resazurin assay is based on the intracellular conversion of resazurin (nonfluorescent) to resorufin and dihydro-resorufin (highly fluorescent) in the presence of mitochondrial enzymes of metabolically active cells [20]. Briefly, 2.5×10^4 ESCC cell spheres were cultured in 96-well plates and administered with various doses of aprepitant 0 (untreated group), 15, 30, 50, 80, and 120 μM for 24 h and 48 hours. Next, the resazurin solution (phosphate buffer saline, 0.01 mg/mL) was added to each well. In the subsequent 3 hours, the absorbance at 600 nm excitation and 570 nm emission was measured on a microplate fluorimeter, and the IC_{50} value was evaluated using the GraphPad Prism® 6 (GraphPad Software, San Diego, CA, USA) software.

2.4. Trypan Blue Assay. The ESCC cell spheres were administered with various doses (0, 15, 30, 50, 80, and 120 μM) of aprepitant for either 24 or 48 hours. Then, the viable cell density was carried out with 0.4% trypan blue staining as described [17].

2.5. RNA Analysis and Quantitative Reverse Transcription-(qRT-) PCR. RNA was extracted from the administrated cells by SP/aprepitant following manufacturer's instructions (Qiagen, Valencia, CA, USA), and qRT-PCR (with specific primers for GAPDH, Bax, Bcl-2, p53, p21, and survivin) was carried out as described [17].

2.6. ELISA. Following exposure of ESCC cell spheres to aprepitant/SP, the protein concentrations of p53 and p21 were determined in cell supernatants according to manufacturer's instructions (Biospeis, China), and ELISA assay was done as described [17].

2.7. Western Blotting. ESCC cell spheres were seeded out, administered with SP (1000 nM)/aprepitant (30 μM), rinsed, and resuspended in ice-cold RIPA lysis buffer. The 30 μg protein samples were measured by a BCA protein assay kit and separated with 10% SDS-PAGE and electrotransferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, HC, USA). The membranes were blocked with 5% nonfat skim milk for two hours at room temperature and then incubated with specific antibodies against PI3K, Akt, NF-κB P65, and β-Actin (1 : 1000; Cell Signaling Technology, MA, USA)

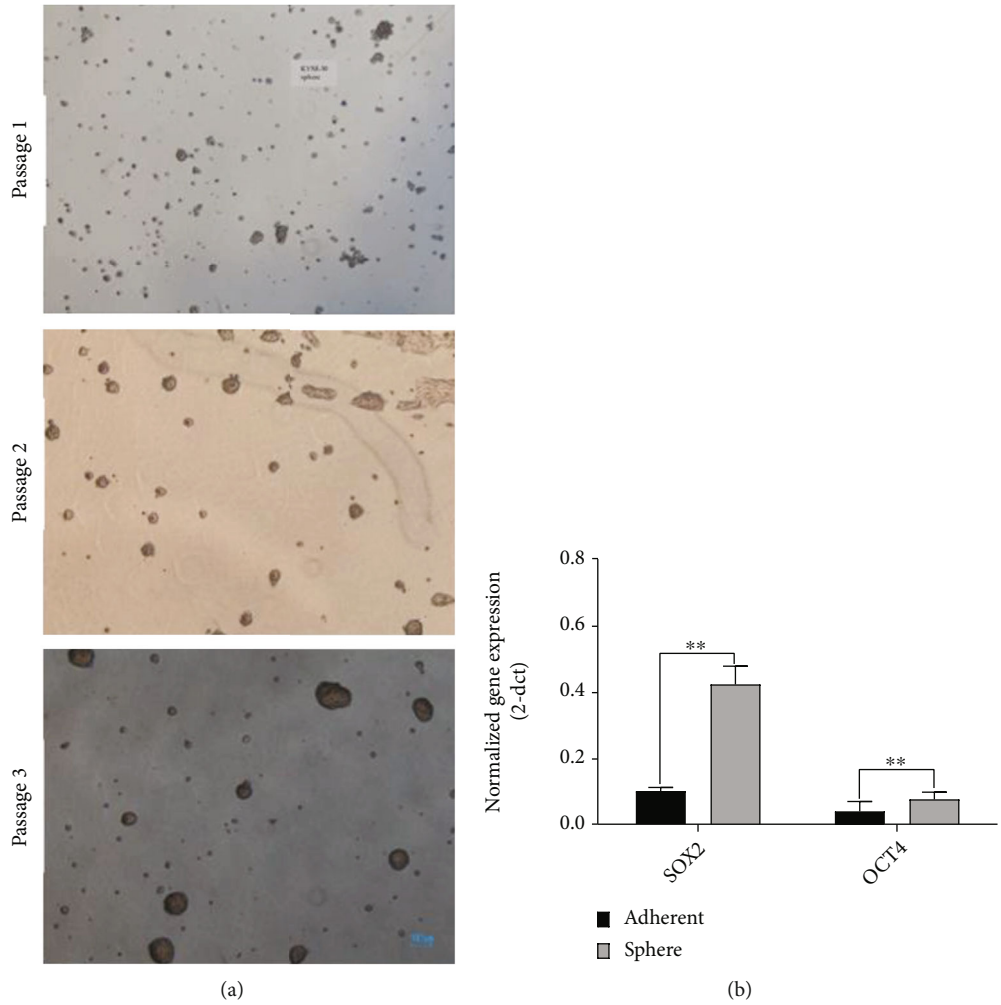

Figure 1: CSC characterization of the KYSE-30 cell spheres. The sphere enrichment process of CSCs is shown (a), the sphere was developed from passage 1 to 3. (b) Upregulation of the pluripotency regulators (SOX2 and OCT4) in spheres of KYSE-30 cells compared to the attached cells. $^{**}p < 0.01$. Gene expression was normalized to the GAPDH gene expression as a reference gene, followed by the $2^{-\Delta ct}$ formula.

at 4°C overnight followed by incubation with HRP-conjugated secondary antibodies (1 : 3000; Cell signaling Technology, MA, USA). After one-hour incubation, immune complexes were visualized using the Chemiluminescence Detection Kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA), as shown by manufacturer's directions. Finally, the intensity of protein bands was performed utilizing the ImageJ 1.52a software (NIH, Bethesda, Rockville, MD, USA) and then compared to the beta-actin protein.

2.8. Caspase Activity. The 1×10^6 ESCC cell spheres were administered with SP (500 and 1000 nM) in the absence or presence of aprepitant (30 μM). Next, the activities of caspase-8 and caspase-9 were measured in cell supernatants according to manufacturer's instructions (R&D System, USA), as described previously [17].

2.9. ROS Assay. The level of reactive oxygen species (ROS) production was examined by the cellular ROS detection kit following manufacturer's instructions. Briefly, 75×10^4 ESCC cell spheres were seeded and incubated overnight. After washing, the cells were exposed to the DCFDA solution (20 μM) for 30 min at 37°C. Next, the cells were rewashed and treated with SP (500 and 1000 nM)/aprepitant (30 μM) for 24 hours. The relative fluorescence intensity was measured (Excitation/Emission: 485/535 nm) with the fluorescence plate reader Perkin-Elmer. Tertbutyl hydrogen peroxide (TBHP, 150 μM) was utilized as a positive control.

2.10. Annexin V-FITC Assay. Apoptosis of ESCC cell spheres administrated 24 hours with SP (500 and 1000 nM)/aprepitant (30 μM) was assessed with the annexin V-FITC kit following manufacturer's directions (Roche Applied Science, Germany). Finally, flow cytometric analysis was performed on a flow cytometer (BD Bioscience, San Diego, CA, USA), and data analysis was assessed by FlowJo software (Treestar, OR, USA). All the treatments were conducted in triplicates.

2.11. Cell Cycle Analysis. The 6×10^5 ESCC cell spheres were treated with SP (500 and 1000 nM)/aprepitant (30 μM) for 24 hours. Next, the cellular DNA content was evaluated with PI staining, as previously described [17].

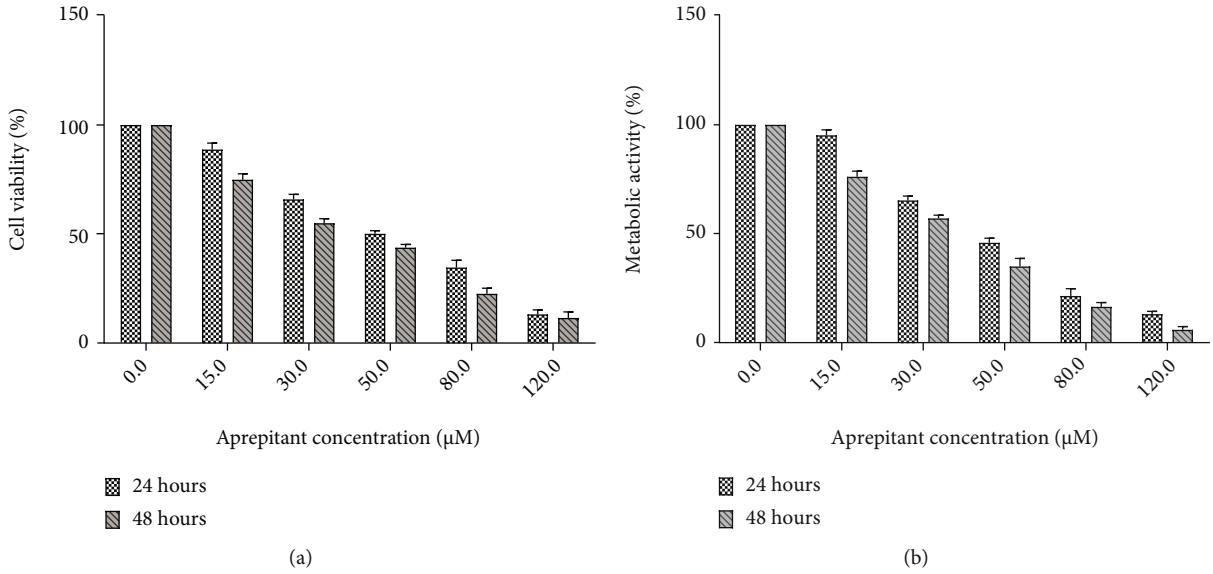

Figure 2: Investigating the effect of aprepitant on cell growth and metabolic activity of ESCC cell spheres. Incubation of the cells with increasing concentrations of aprepitant (0–120 μM) for 24 and 48 h reduced the cell viability and metabolic activity in a dose- and time-dependent manner.

2.12. Statistical Analysis. The experimental data are presented as the mean ± standard error of the mean. The values were analyzed using GraphPad Prism® 6.0 software (San Diego, CA, USA) for Windows. The relative ratios of various groups were compared using the ANOVA followed by Bonferroni's t -test. A statistically significant difference for all data was considered to be a p value < 0.05. All results were assessed in triplicate as compared to the untreated control group.

3. Results

3.1. The KYSE-30-Derived Sphere Represents CSC-Like Characteristics. To confirm the ESCC cell sphere enrichment, the KYSE-30-derived sphere in passage three was evaluated to their original adherent cells. As depicted in Figure 1(a), the cell line showed the most well-shaped sphere appearance in passage 3. Further gene expression analysis indicated that the master regulators of the pluripotency genes (SOX2 and OCT4) are markedly upregulated in ESCC cell spheres as compared to KYSE-30 attached cells (Figure 1(b)). The sphere cells characterized by pluripotency gene overexpression and anchorage-independent proliferation were chosen as cancer stem cell-like cells (CSC-LCs) in the next experiments.

3.2. Aprepitant Effectively Mitigates Cell Viability and Metabolic Activity of ESCC Cell Spheres. Growth inhibition of the ESCC cell spheres by aprepitant was analyzed with the resazurin proliferation assay. ESCC cell spheres were incubated with increasing concentrations of NK1R antagonist aprepitant for 24 and 48 h. As shown in Figure 2, we observed a remarkable dose- and time-dependent growth inhibition and metabolic activity inhibition after treatment by aprepitant. Aprepitant showed robust growth inhibition, with 64.21% inhibition at 30 μM and 47.25% at 60 μM. Thus, the concentration required for a 50% reduction in optical density (IC_{50}) observed in the controls treated with aprepitant was 48.21 μM for 24 h. The 30 μM of aprepitant was selected as the experimental concentration based on the dose-dependent viability change.

3.3. Aprepitant Induces Apoptosis and G2-M Phase Arrest in ESCC Cell Spheres. The cell line has been cultivated with the aprepitant and stained with Annexin-V/FITC, following confirmation of significant growth inhibition of ESCC cell spheres with the NK1R antagonist. After the administration of aprepitant (30 μM), a considerable number of apoptotic cells were found in the ESCC cell spheres after 24 hours (Figure 3). After treatment with agonist SP (as an antiapoptotic agent) at 500 and 1000 nM doses, we observed no significant increases in apoptotic rates compared to the control group. Further, the antiapoptotic effects of SP were reversed after treatment with SP plus aprepitant, significantly.

We also examined the caspase-8/-9 activities as the main executors for apoptotic processes to validate findings obtained from the flow cytometry study. Of particular interest is that after 24 hours of aprepitant treatment (30 μM) compared with the control group, we also observed a dramatically improved enzymatic activity of caspase-8/-9, thus providing additional evidence that NK1R antagonist may contribute to the induction of ESCC cell spheres through caspase-mediated apoptosis.

Recent findings have demonstrated that the treatment of cancer cells with NK1R antagonists, may arrest the progression of the cell cycle, most importantly by down/upregulating the essential proteins that regulate the growth of cells [21]. Notably, DNA content analysis showed a significant increase in the proportion of the G2-M phase of the cell cycle (Figure 4), suggestive of the proapoptotic capability

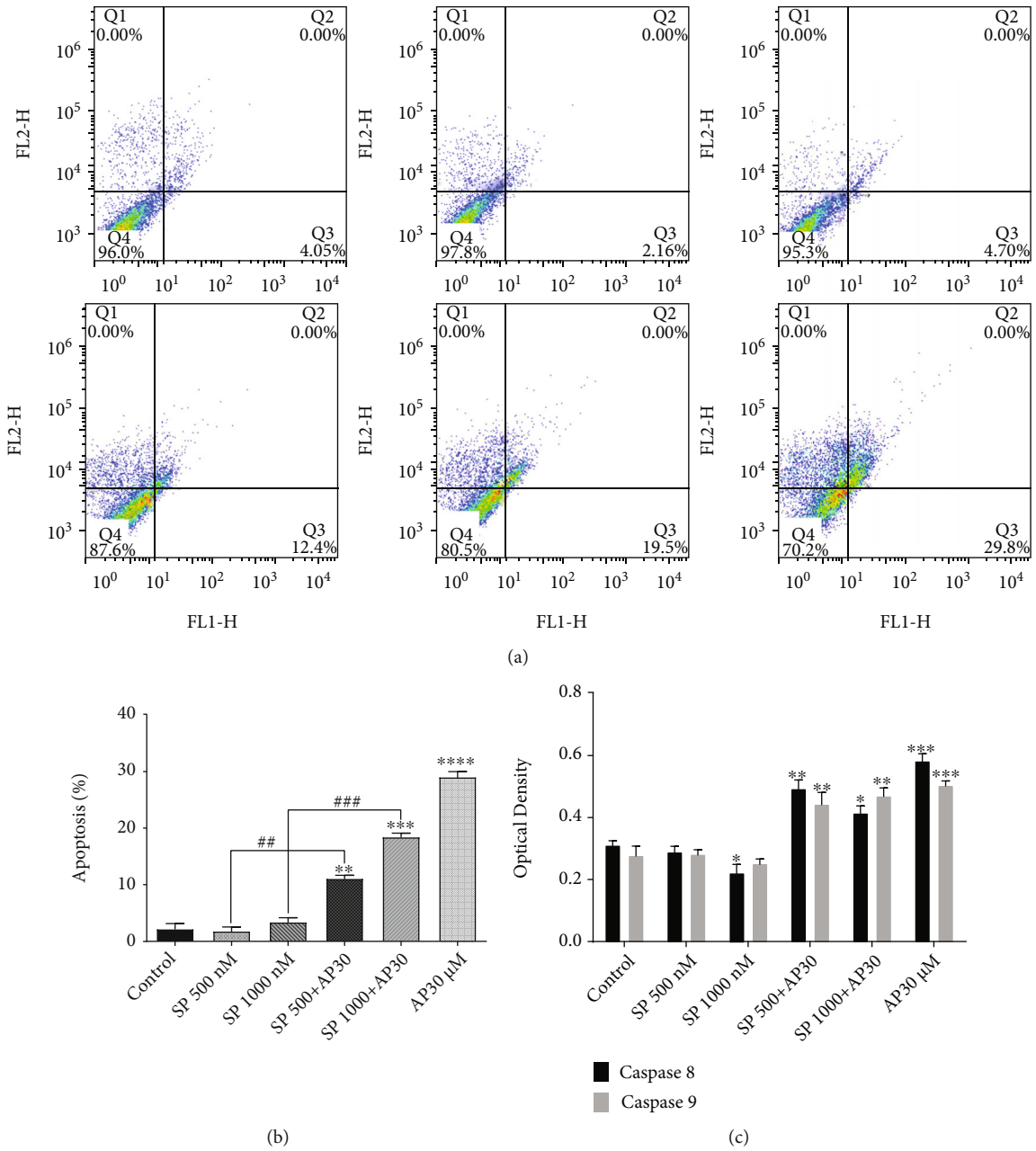

Figure 3: NK1R blockage by aprepitant had a suppressed growth on ESCC cell spheres through a caspase-dependent apoptotic pathway. (a, b) The percentages of annexin-V and annexin-V/PI double-positive inhibitor-treated cells were increased in response to drug treatment after 24 hours, as compared with the untreated group. (b) Also, ESCC cell spheres were subjected to the caspase assay to examine the contribution of caspases-8/-9 to aprepitant-induced apoptosis. Aprepitant exerted a substantial increase in caspase-8/-9 activity, which suggested that the apoptotic effect of aprepitant on the ESCC cell spheres was caspase-dependent. (*) indicates a statistically significant difference compared to controls, while the (#) sign indicates a statistically significant change compared to other treatment groups.

of aprepitant in ESCC cell spheres, which is in accordance with the findings obtained from the annexin-V staining experiment. Moreover, we observed a substantial decrease in the cell proportion in the S phase of the cell cycle with the NK1R antagonist.

3.4. Aprepitant Did Not Alter the Transcriptional Activity of p53 Apoptosis-Related Genes. Regarding cell growth regulation, the p53 gene is often used to facilitate programmed cell death by transcriptionally triggering proapoptotic genes such as Bax, Bad, Bid, and p21 [22]. In this light, we examined whether NK1R antagonist treatment influenced the mRNA level of genes involved in apoptosis, based on the protein concentration and gene expression of p53 and proapoptotic p53 target genes p21 and Bax in ESCC cell spheres utilizing ELISA and qRT-PCR, respectively. As shown in Figure 5, our results demonstrated that aprepitant (30 μM) treatment plus SP (500 and 1000 nM) did not significantly

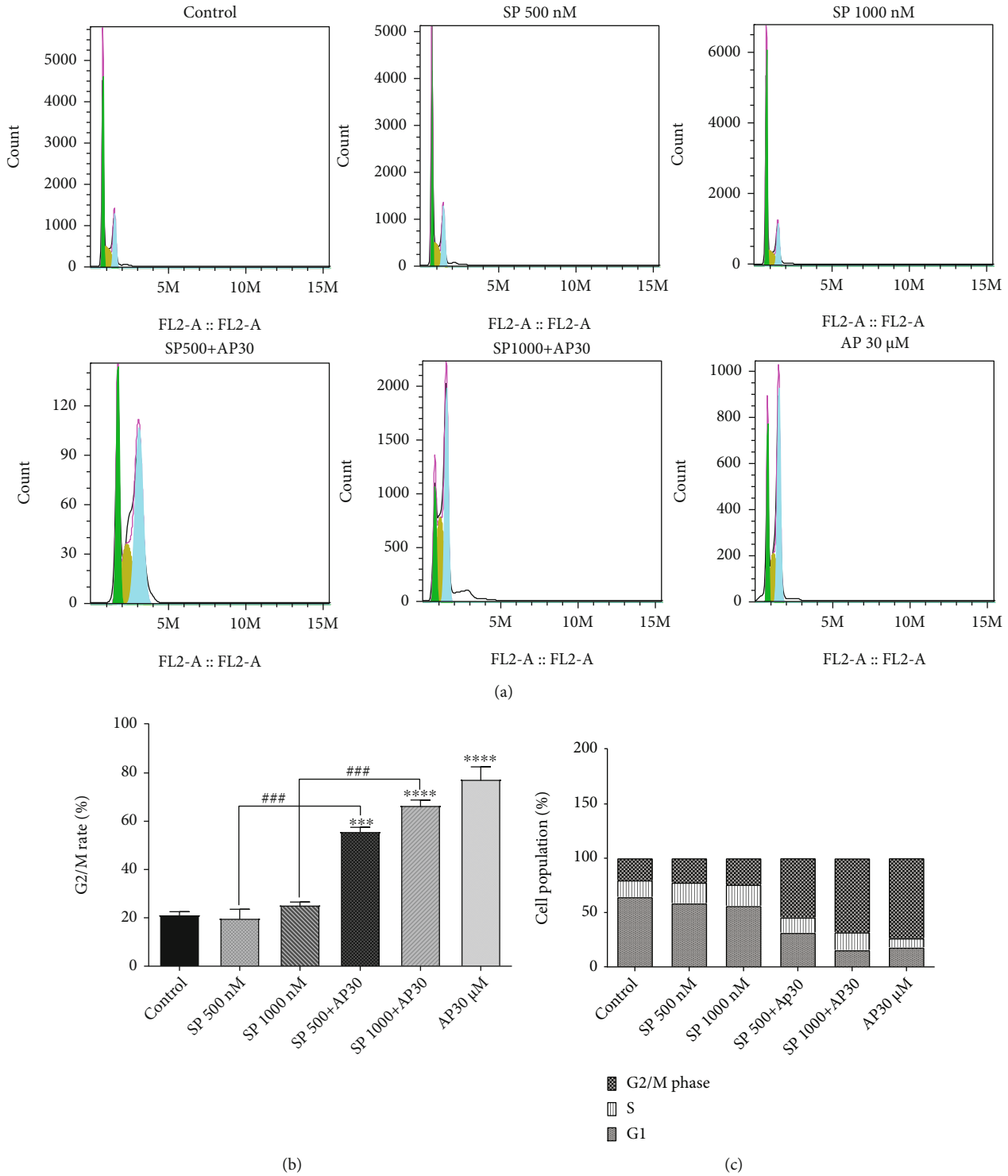

Figure 4: (a) Flow cytometry analysis of cell cycle development of ESCC cell spheres treated with aprepitant. The frequency of cells in G1 (green peak), S (yellow peak), and G2/M (blue peak) was calculated with FlowJo software. (b) Aprepitant raises the percentage of the cell population in G2-M cells (c) and decreases the percentage of cell accumulation in S-phase. (*) indicates a statistically significant difference compared to controls, while the (#) sign indicates a statistically significant change compared to other treatment groups including SP 500 nM and SP 1000 nM.

alter the protein expression level of p53 and p21 as well as the transcriptional activity p53 proapoptotic target genes, such as p53, p21, and Bax.

3.5. Effects of Aprepitant on ROS Levels in ESCC Cell Spheres. To determine whether the cytotoxic effects of aprepitant on the killing of ESCC cell spheres were accompanied by

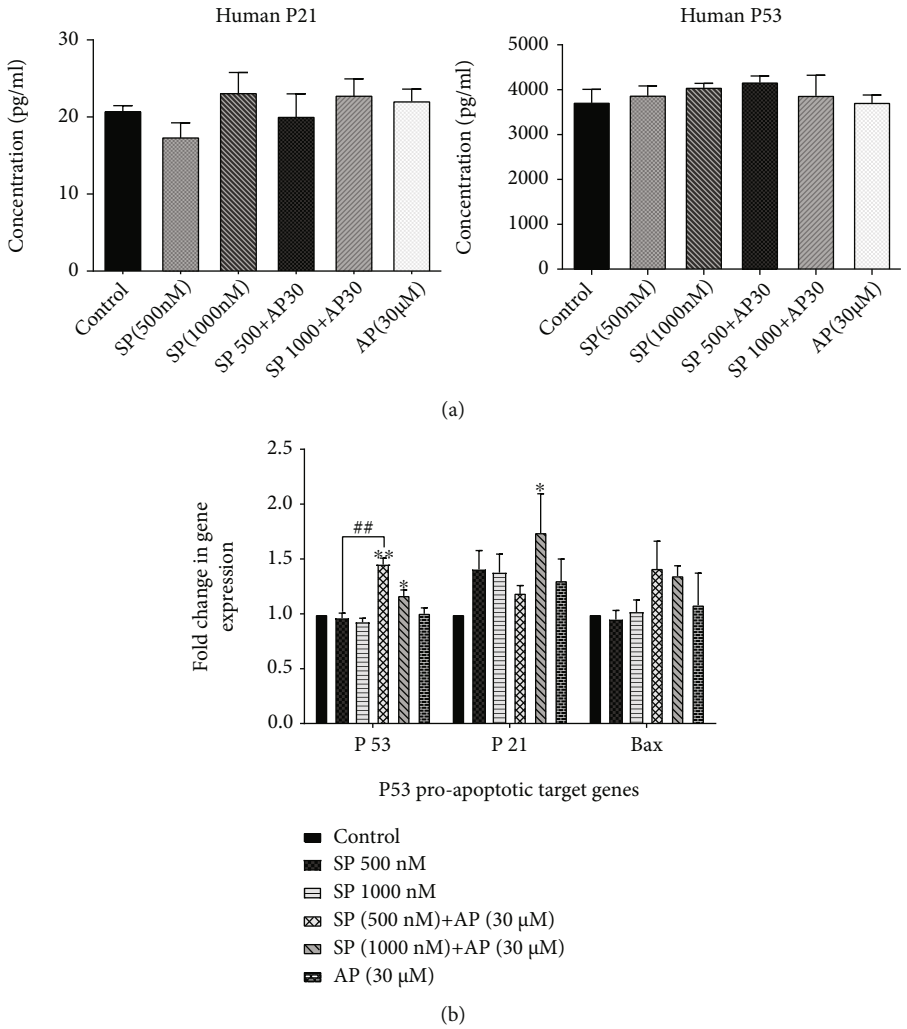

Figure 5: (a) Following exposure to aprepitant (30 μM) for 24 hours, total lysates from ESCC cell spheres were collected and ELISA assay was performed using human p53 and P21 ELISA kit. (b) The mRNA expression levels of three genes (p53, p21, and Bax) were analysed in each group after normalizing the cycle threshold (Ct) values to GAPDH as the internal housekeeping gene. The apoptosis-related genes of p53 were not substantially enhanced in ESCC cell spheres by aprepitant. ELISA and qRT-PCR analyses showed that aprepitant plus SP treatment has no meaningful enhancement impact on proapoptotic target genes of p53 transcription activity. (*) indicates a statistically significant difference compared to controls, while the ($^\#$) sign indicates a statistically significant change compared to SP 500 nM treatment group.

decreased ROS levels, we decided to determine the levels of ROS (Epoch, BioTek® instruments, Inc., USA) in response to aprepitant and SP in treated versus untreated cells. As Figure 6 reveals, the treatment with aprepitant (30 μM, 24 hours) in comparison with the SP alone (500 and 1000 nM) led to a significant reduction in the levels of ROS, demonstrating antioxidative effects of aprepitant in ESCC cell spheres. In comparison to the control group, it was also noteworthy that ROS was elevated by SP at a concentration of 1000 nM. Also, compared to the control group, the combination of SP and aprepitant dramatically reduced the production of ROS caused by SP. Therefore, ROS is not one of the main aprepitant-mediated cytotoxicity pathways in ESCC cell spheres.

3.6. Aprepitant Regulates Cytotoxicity through the PI3K/Akt/NF-κB Signaling Pathway. Numerous molecular experiments have shown that stimulation of NK1R is directly correlated with the NF-κB signaling axis and that SP/NK1R controls apoptosis in malignant cells by targeting this axis [23]. We assessed the quantity of NF-κB p65 protein from drug-treated ESCC cell spheres using Western blot analysis, to evaluate whether blockage of NK1R could increase the cytotoxicity of aprepitant through suppression of NF-κB. Furthermore, the RT-PCR study of antiapoptotic NF-κB target genes has examined the impact of aprepitant on the NF-κB axis. We found that while 1000 nM of SP had a stimulatory effect on NF-κB levels and its downstream target genes (survivin and Bcl-2), aprepitant (30 μM) remarkably reduced the protein level of NF-κB and its antiapoptotic target genes. Besides, these effects were significantly ameliorated by treatment with aprepitant, confirming that aprepitant has antioxidative effects, at least partially, through the modulation of NF-κB activity (Figure 7).

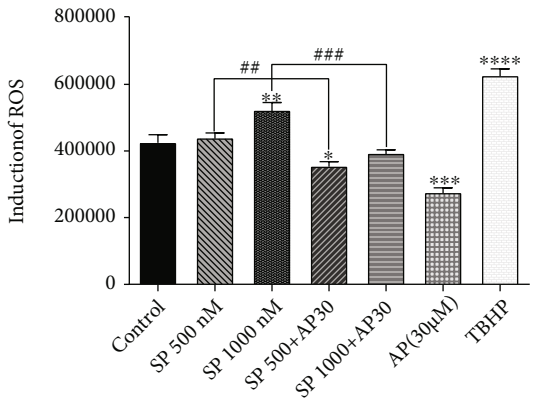

Figure 6: Aprepitant effects on the ROS levels in the ESCC cell spheres. Our results indicate that the aprepitant decreases the level of reactive oxygen (ROS) in 24 hours. The cells were treated by aprepitant (30 μM) for 24 h. ROS levels were measured by a fluorimeter. The intensity of the fluorescence has improved dramatically relative to the control group in the positive control tert-butyl hydroperoxide (TBHP, 150 μM). At 24 hours after treatment, aprepitant significantly reduced the ROS induced by the substance P (SP). (*) indicates a statistically significant difference compared to controls, while the ($^\#$) sign indicates a statistically significant change compared to other treatment groups including SP 500 nM and SP 1000 nM.

Numerous findings show that Akt activation, as the most significant downstream effector of the PI3K signaling pathway, is one of the critical pathways implicated in pathogenesis in the ESCC [17]. On the other hand, recent studies have revealed that stimulation of NK1R in human cancer cells increases the phosphorylation and the activity of Akt. Notwithstanding this, it was challenging to examine whether inhibition of NK1R using aprepitant participates in the inhibition of Akt in ESCC cell spheres. Interestingly, an apparent reduction of the Akt level was found in the inhibitor-treated cells. More notably, aprepitant (30 μM) could decrease the elevation of the Akt level induced by SP (1000 nM). These effects were also observed in PI3K levels, indicating that aprepitant effectively abolishes the proliferation of ESCC cell spheres through PI3K/Akt protein levels inhibition (Figure 7).

4. Discussion

Given that NK1R is considered highly involved in the formation and growth of many carcinomas, intense research is being done to block this receptor with NK1R antagonists for possible anticancer uses [19]. In most of the cancers, there is a side population of cells characterized by self-renewal ability, differentiation potential, high carcinogenicity, and therapy resistance, known as cancer stem cells [4, 5]. ESCC, as world's sixth leading cause of cancer mortality, is histologically the main form of esophageal cancer. In the treatment of ESCC patients, it is possible that the ESCC cell spheres are of elevated NK1R expression as compared to the healthy cells. Aprepitant (Emend®), an NK1R-specific high-affinity antagonist with no toxicity, is widely used for the treatment of chemotherapy-induced nausea and vomiting (CINV) [24]. The safety of aprepitant against human fibroblasts (the IC_{50} for fibroblasts are three times higher than the IC_{50} for tumor cells) has been shown. Further, the IC_{50} for nontumor cells is 90 μM, but the IC_{100} for tumor cells is 60 μM, approximately [25, 26].

Interestingly, several studies are suggesting this medication has an efficient antitumor impact on a wide range of cancers, including melanoma, lung, hepatoblastoma, pancreas, and breast cancer [27]. Specific molecular pathways are still poorly explained by which aprepitant induces antitumor impacts on various cancer cells. Hence, the effects and molecular pathways of aprepitant in the ESCC cell spheres were analyzed throughout this study.

In this context, the concentration-dependent growth inhibition of aprepitant on ESCC cell spheres was discovered. Dysregulation of the cell cycle is a notable feature of cancer, and cell cycle arrest could be an essential guide for anticancer medications [28]. The progression of cell cycles may be regulated by the complexes of cyclin-dependent kinases (CDK). The p21 directly links and prevents kinase activities, which lead to cell cycle arrest, and p53 is known to trigger cell cycle arrest as the tumor suppressor protein [17]. Earlier research has shown that aprepitant prevented cell proliferation in G2/M and significantly downregulated cyclin B1, as well as upregulated p21 in oral squamous cell carcinoma lines (WSU-HN6, UM1, SCC25, and WSU-HN4) [29–31]. Furthermore, the protein behavior can be altered due to posttranslational modifications such as phosphorylation, sulfation, and glycation, which change a local charge of the protein region [32, 33]. Therefore, many changes in gene expression recorded between experiment groups are due to posttranslational modifications, not detected by analyses of RNA. Our studies have shown a similar pattern of no changes in protein expression level in various groups. So, differences in a group of mRNA gene expressions (between SP500 nM and SP500 nM + AP30 μM (can be ignored. Accordingly, our study showed that aprepitant could cause G2/M cell cycle arrest in ESCC cell spheres, in a CDK-independent manner.

Moreover, our study showed that aprepitant could substantially improve the anticancer effects by apoptosis induction. Flow cytometry analysis revealed that apoptosis was markedly induced as compared with the control group by aprepitant treatment in ESCC cell spheres. Two major pathways have currently been established which connect apoptosis: (a) intrinsic or mitochondrial and (b) extrinsic or death-receptor-related [27]. Hence, a caspase activity analysis was conducted to explore caspase activation as a potential mechanism influencing the increased apoptosis level observed in aprepitant-treated ESCC cell spheres. In ESCC cells treated with aprepitant, a substantial increase in caspase-8/-9 activity was reported, which was in line with the results obtained by FACS analysis.

The function of caspases and proapoptotic mitochondrial cascades in control of apoptosis is well established, but the involvement of ROS is elusive for the regulation of apoptosis. A wide range of anticancer agents has been shown to kill cancer cells efficiently and to sensitize different cancer cells by modulating ROS production to other chemotherapeutic

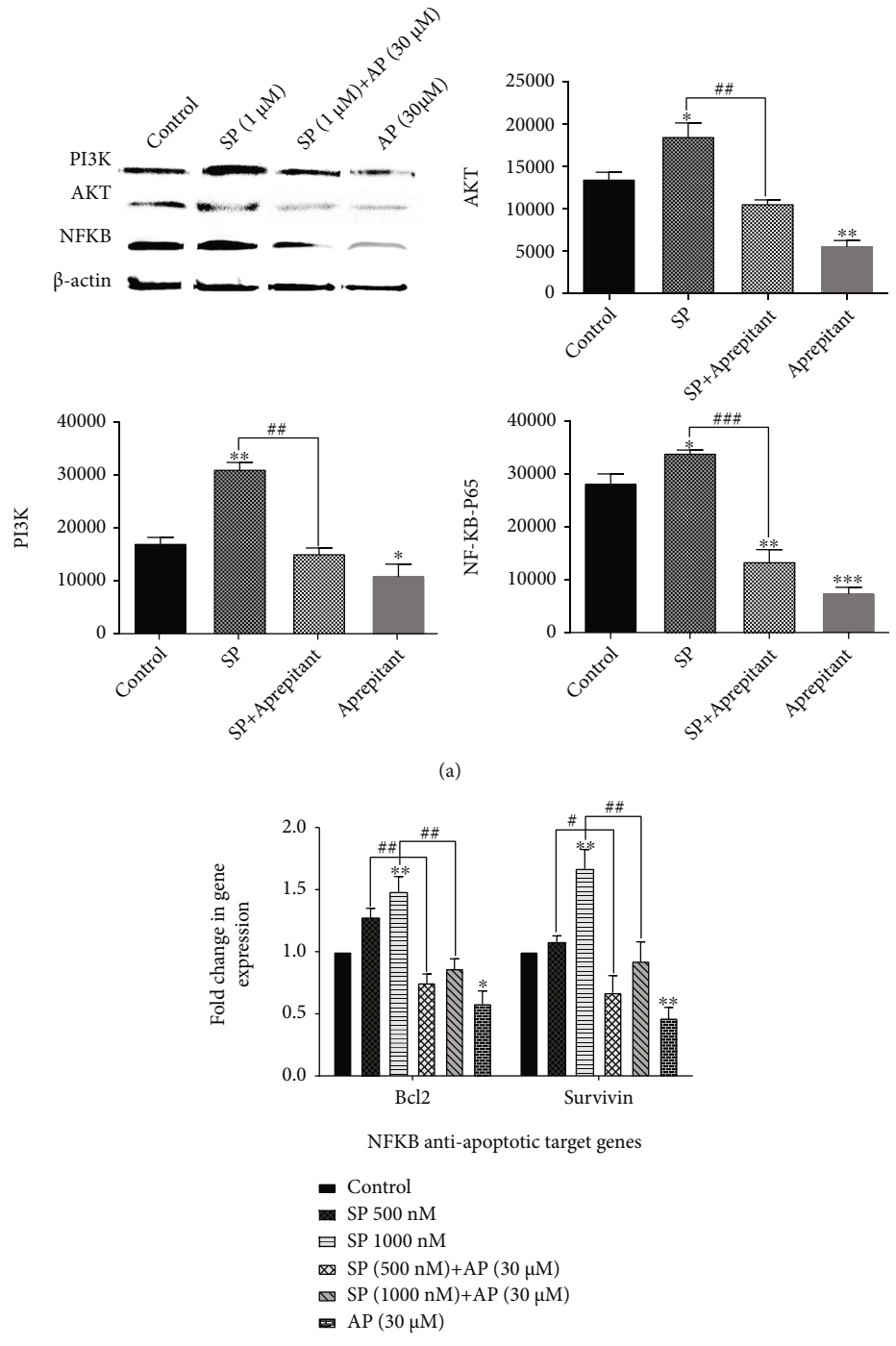

Figure 7: Aprepitant mitigates the PI3K/Akt/NF-κB signaling pathway, as well as the expression of antiapoptotic NF-κB target genes in ESCC cell spheres. (a) Western blotting found that the protein concentration of PI3K, Akt, and NF-κB 65 in the aprepitant (30 μM) group was substantially reduced in comparison with the control group ($p < 0.05$ and $p < 0.01$). (b) Moreover, the administration of aprepitant (30 μM) showed a markedly reduced relative mRNA expression of NF-κB antiapoptotic target genes, including survivin and Bcl-2. The levels of expression of all the target genes were normalized by GAPDH mRNA levels ($p < 0.05$ and $p < 0.01$). (*) indicates a statistically significant difference compared to controls, while the ($^\#$) sign indicates a statistically significant change compared to other treatment groups including SP 500 nM and SP 1000 nM.

agents [34, 35]. Most chemotherapies also fatally influence tumor cells by raising the oxidative stress in these cells, thus stressing them beyond their compensatory capacity, and pharmacological agents, which have increased ROS development in cancer cells, are approved agents for cancer therapy [36]. Based on these findings, our data showed that aprepitant could

significantly decrease the generation of ROS induced by SP in ESCC cells.

In previous decades, a variety of studies have shown that in several forms of malignant tumors, the pathway of PI3K/Akt signaling is aberrant [37–39]. PI3K/Akt pathway activation promotes cell differentiation, development, and angiogenesis and seems to be fundamental for apoptosis, which is considered to be a potential target anticancer therapy [40]. Here, we investigated the role of aprepitant in the PI3K/Akt signaling cascade to understand the mechanisms of aprepitant-inhibited cell growth and induced apoptosis in ESCC. Our findings revealed that apoptotic cell death by inhibition of NK1R was seen, at least partly, through abrogation of the PI3K/Akt pathway, as shown by the significant reduction of the Akt protein level. The PI3K/Akt pathway is of great importance in CSCs, including the maintaining of colony-formation ability and proliferation. Akazawa et al. have consistently demonstrated that NK1R inhibition contributes to decreased Akt activity through the PI3K pathway [18].

Many studies have shown that Akt controls the transcriptional behavior of NF-κB via phosphorylation and subsequent degradation of IκBα, permitting NF-κB to be imported at the nuclear level, followed by the binding and triggering of its anti-apoptotic target genes [41]. In this regard, it was interesting to determine if the Akt/NF-κB axis may be used to mediate aprepitant-induced apoptosis. Our findings revealed that aprepitant decreases the protein expression level of NF-κB and the mRNA level of antiapoptotic NF-κB target genes like survivin and Bcl-2, suggesting that the apoptotic process in ESCC cell spheres is, at least partly, through the NF-κB-dependent pathway induced by aprepitant.

The conclusive proof was documented that a strong dependence on the interaction between p53 and NF-κB signal pathways seems to be because of the susceptibility of cancer cells to apoptosis [42]. Furthermore, two of the most critical regulatory pathways in cells have been identified to be PI3K/Akt and p53, where Akt repression is related to increased p53, subsequent apoptosis, and arrest in growth. In contrast to those as mentioned above, our results reveal that p53 protein expression and mRNA levels of p53 proapoptotic target genes, including p21 and Bax, have not been altered by aprepitant (30 μM). These data proved that aprepitant could exert its apoptotic effects in cell spheres of the ESCC through a p53-independent apoptotic path.

In summary, findings from the present study have demonstrated that aprepitant exerts antitumor activities like proliferation inhibition, cell cycle arrest, and induction of apoptosis against ESCC cell spheres. This study also proposes mechanical pathways, which inhibit NK1R through a possible caspase-dependent mechanism that increases apoptotic cell death. Further, aprepitant showed, at least partly, its anticarcinogenicity by inhibiting the PI3K/Akt signaling pathway. Therefore, more preclinical studies will highlight the mechanisms for the molecular involvement of aprepitant cytotoxicity.

Data Availability

Data are available upon request.

Ethical Approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflicts of Interest

The authors declare there was not any conflict of interest.

Authors' Contributions

Hossein Javid and Amir R. Afshari are co-first authors.

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Research Article

Prognostic Significance of Substance P/Neurokinin 1 Receptor and Its Association with Hormonal Receptors in Breast Carcinoma

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Expression and immunolocalization of Substance P (SP)/Neurokinin-1 Receptor (NK-1R) in breast carcinoma (BC) patients and its association with routine proliferative markers (ER, PR, HER2/neu, and Ki-67) were evaluated. A cross-sectional study was performed on 34 cases of BC. There were 23 cases of group A (grade III), 8 of group B (grade II), and only 3 cases of group C (grade I). All samples were then processed for SP and NK-1R immunohistochemistry for few cases. 14/23 cases (61%) of group A, 7/8 cases (88%) of group B, and 2/3 (67%) cases of group C were SP positive. Overall, strong staining (≥10% tumor cells), labeled as "+3," was observed in 9/14 (64.2%) cases of group A and 1/8 (12.5%) cases of group B. Moderate staining labelled as "+2" (in ≥10% tumor cells) was observed in 3/14 (21.4%) cases of group A and 4/8 (50%) cases of group B. Weak positive staining "+1" was observed in only 2/14 (14.28%) cases of group A, 2/8 (25%) cases of group B, and all 2/2 (100%) cases of group C. SP and NK-1R are overexpressed in breast carcinomas, and there is significant association between the grade of tumor and their overexpression.

1. Introduction

Breast cancer (BC) is the most common cancer in women all over the world with an incidence of approximately 2 million in 2018. The highest rate of BC was observed in Belgium with 113.2/100,000 women [1]. It can occur as a result of cells under the influence of estrogen multiplying and infringing on other tissues spreading to other regions of the body [2]. Worldwide, the occurrence of BC exceeds all female cancers with high mortality rates [3]. Despite the recent advances in BC therapy, the disease is still counted as a major health problem worldwide and remains an elusive disorder. In fact, poor prognosis, late diagnosis, and therapeutic challenges including the evolution of resistant cells and tumor heterogeneity have remained partly unavoidable and are considered as major challenges in the management of this disease.

Table 1: Interpretation of ER, PR, and HER2 by Allred method.

(a)

Allred	Cell stain		Proportion score
	%	Score (3)	
Negative	0	0	0
Weak positive	1	1	1
Moderate positive	1-10	2	2
Strong positive	10-33	3	3
	33-66		4
	66-100		5

(b)

Sum of proportion score and intensity score	
Negative	0-2
Positive	3-8

A number of factors, such as histological grade, type and size of tumor, lymph node metastasis, estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2/neu), influence the prognosis and response to the treatment of cancer [4]. Newer classification methods are also being developed that are based on immunohistochemical, genetic, and molecular findings. Availability of hormone (estrogen and progesterone) receptor markers marked the beginning of molecular classification about 30 years ago [5].

In BC, the malignant cells are enlarged with vacuolated cytoplasm and vesicular nuclei containing prominent nuclei. Most of the time, the stroma was found to be increased and degenerative in nature [6]. The grading of invasive BCs is an important factor in addition to the size and status of the lymph nodes [7]. Benign breast diseases especially fibroadenomas are also important, as some of them (30%) may lead to cancer [8]. The staging of BC is related to the size, location, and number of regional metastases to lymph nodes and sometimes is related to growth [9]. TNM stages IIB, IIIA, and IIIB are tumor stages that help in diagnosis [10].

There are various types of BC; they are classified as in situ and invasive. In situ carcinoma includes lobular carcinoma in situ (LCIS) and ductal carcinoma in situ (DCIS). Invasive carcinoma includes invasive lobular carcinoma (ILC) and invasive ductal carcinoma (IDC) [11]. According to the site, BC is divided into invasive and noninvasive lobular carcinoma. Invasive lobular carcinoma is the second most common type of BC, several histological subtypes exist, most of the tumors are classified as grade II, and the majority of grade III are among the nonclassified subtypes showing a disease-free region as compared to grade II [12]. The number of positive axillary lymph nodes and hormone receptor negative tumors increases among grade III tumors [13].

Further, a new study mandated the molecular classification of human BC by initially dividing BC into four major classes: luminal-like, basal-like, normal-like, and HER2 positive. Subsequently, luminal class was divided into luminal A and luminal B classes, thereby resulting in addition of a fifth class of BC [14]. According to the reported study, molecular subtypes of BC can be classified into luminal A (ER+/PR+/HER2-/low Ki-67), luminal B (ER+/PR+/HER2-/+/high Ki-67), HER2-overexpression (ER-/PR-/HER2+), and triple negative breast cancers/TNBCs (ER-/PR-/HER2-) [15].

Family history of both maternal and paternal relatives is important and has long been known as a risk factor for BC [16]. BC is commonly caused by low-penetrance genes that are involved in the DNA-repairing mechanism. DNA and chromosomal damage may also cause BC. The XRCC3Thr24 Met polymorphism is the most common gene associated to BC [17]. These are repair genes to rectify the DNA damage. These genes are involved in enhancing the cytotoxicity, apoptosis, p53 phosphorylation, and exposure to external factors that cause DNA damage [18]. BRCA1 and BRCA2 are abnormal genes that, when inherited, increase the estimated risk between 40 and 88% of BC. BRCA1 genes tend to develop BC at an early age [19]. In stage 2, about 54% of the women are diagnosed, while in stage 1, only 16% are diagnosed [20]. Sedentary life style, high dietary intake of fat, and obesity particularly in postmenopausal women may cause BC [21]. The use of alcohol is also another cause of BC [22].

Substance P (SP) is a small undecapeptide hormone [23] and the most abundant tachykinin (TK) peptide in the central nervous system of mammals [24]. Many physiological and pathological roles of this peptide have been noticed [25]. Munoz and Covenas [26] suggested a strong role of the SP-Neurokinin-1 receptor (NK-1R) system in the progression of carcinogenesis. SP mediates pain, neurogenic inflammation, and mitogenesis via interaction with its high-affinity receptor NK-1R, which is widely distributed throughout the body. NK-1R is widely distributed throughout the body. BC cells exhibit mRNA for the receptor of SP, NK-1, which is then involved in promoting the cell proliferation and, consequently, metastasis [27]. Additionally, SP is also involved in vasculogenesis, angiogenesis, and neoangiogenesis as observed in both *in vivo* and *in vitro* studies, an essential step towards invasion and metastasis [28, 29].

SP and NK-1R have been detected in tumor cells and in intra- and peritumoral blood vessels [26–28]; furthermore, SP has been shown to protect tumor cells from apoptosis [29]. The relevance of the SP/NK-1 receptor system has been specifically shown in pancreatic cancer, where SP is involved in pancreatic cancer proliferation, neoangiogenesis, and migration of pancreatic cancer cells, and SP receptor antagonism has been shown to reverse these alterations [26, 29–31]. These findings suggest that elevated SP can be detrimental in cancer and suggest that NK-1R antagonism can be beneficial in cancer treatment.

To our knowledge, it is the first study to report the expression and distribution of SP in BC and to suggest a strong association of its expression with the progression of disease and its association to routine proliferative and hormonal markers. Thus, the aim of this study is to evaluate the expression of SP/NK-1R and its relationship with tumor type and clinicopathological parameters of BC patients.

Table 2: Clinicopathological features of studied patients.

$n = 34$	SP+ (23)	SP- (11)	Total (34)	P value
Age(years)				
>60	4 (17.39%)	1 (9.09%)	5 (14.7%)	NS
<60	19 (82.6%)	10 (90.9%)	29 (85.29%)	
Menopause status				
Pre	12 (52.17%)	7 (63.63%)	19 (55.88%)	NS
Post	11 (47.82%)	4 (36.36%)	15 (44.11%)	
Tumor size(cm)				
<2	3 (13.04%)	0	3 (8.82%)	
2-5	15 (65.2%)	8 (72.7%)	23 (67.64%)	NS
>5	5 (21.73%)	3 (27.2%)	8 (23.52%)	
Grade				
I (well diff)	2 (8.69%)	1 (9.09%)	3 (8.82%)	
II (mod)	7 (30.43%)	1 (9.09%)	8 (23.52%)	NS
III (poor)	14 (60.8%)	9 (81.8%)	23 (67.64%)	
TNM				
PT1	4 (17.39%)	1 (9.09%)	5 (14.7%)	
PT2	15 (65.2%)	8 (72.7%)	23 (67.64%)	NS
PT3	2 (8.69%)	2 (18.2%)	4 (11.76%)	
PT4	2 (8.69%)	0	2 (5.88%)	
Tumor type				
IDC	15 (65.2%)	5 (45.5%)	20 (58.82%)	
DCIS	1 (4.34%)	6 (54.5%)	7 (20.58%)	0.005*
ILC	2 (8.69%)	0	2 (5.88%)	
IDC+DCIS	5 (21.7%)	0	5 (14.7%)	
ER status				
+ve	19 (82.6%)	9 (81.81%)	28 (82.35%)	NS
-ve	4 (17.39%)	2 (18.18%)	6 (17.64%)	
PR status				
+ve	19 (82.6%)	9 (81.81%)	28 (82.35%)	NS
-ve	4 (17.39%)	2 (18.18%)	6 (17.64%)	
HER2/neu status				
+ve	18 (78.26%)	4 (36.4%)	22 (64.7%)	0.017*
-ve	5 (21.7%)	7 (63.4%)	12 (35.29%)	
Ki-67 status				
+ve	23 (100%)	7 (63.4%)	30 (88.23%)	
-ve	0	4 (36.4%)	4 (11.76%)	0.002*
Distant metastasis				
Present	3 (13.04%)	1 (9.09%)	4 (11.76%)	
Absent	18 (78.26%)	9 (81.8%)	27 (79.41%)	NS
Unknown	2 (8.69%)	1 (9.09%)	3 (8.82%)	
Lymph node metastasis (axillary)				
1-3 lymph nodes	2 (8.69%)	2 (18.2%)	4 (11.76%)	
>4 lymph nodes	6 (26.08%)	3 (27.3%)	9 (26.47%)	NS
Absent	15 (65.2%)	6 (54.5%)	21 (61.76%)	

*NS: nonsignificant.

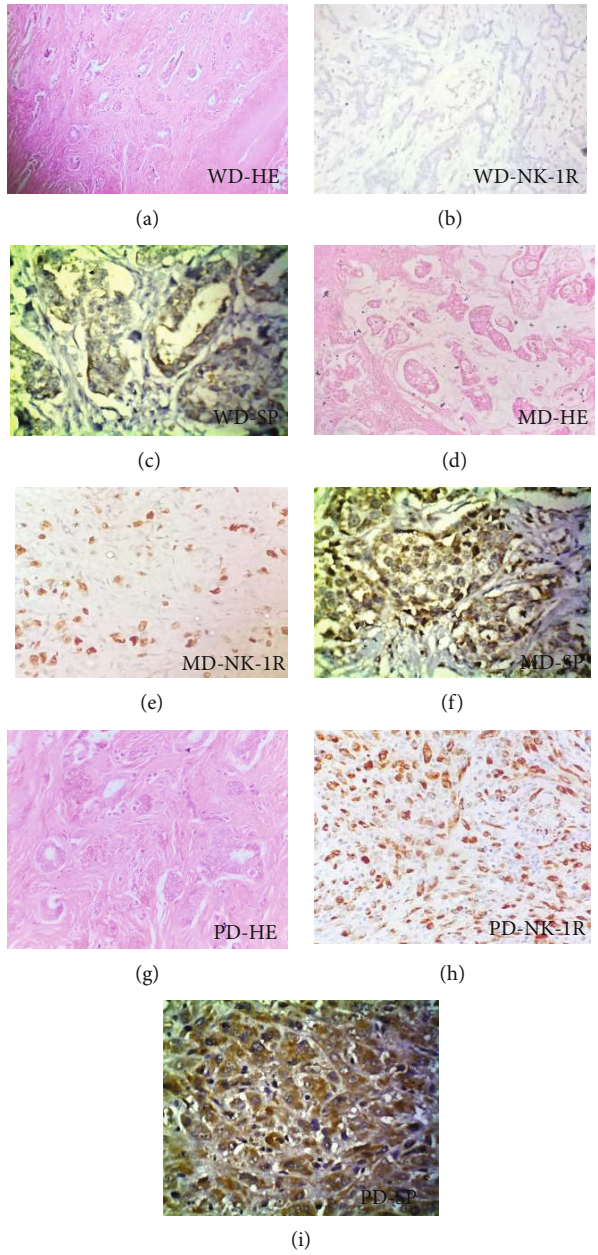

Figure 1: BC at 40x: (a) WD-BC hematoxylin-eosin staining (b) grade 1, NK-1R negative (c) SP weakly positive +1; (d) MD-BC hematoxylin-eosin staining (e) MD, grade 2, NK-1R moderately positive, +2, 40% cells showing positive stain (f) MD, grade 2, SP moderately positive, +2; (g) PD-BC hematoxylin-eosin staining (h) PD, grade 3, strongly SP positive, +3, 90% SP positive cell (i) PD, grade 3, strong positive, +3, 85% cells showing positive stain.

Furthermore, the relationship between the SP/NK-1R and proliferative markers was investigated.

2. Material and Methods

We have followed the same methods for data collection and immunohistochemistry as done in our previous study [30]. The study setting was the Faculty of Allied Health Sciences, the University of Lahore, Lahore, Pakistan. A total of 34 formalin-fixed paraffin-embedded (FFPE) blocks of BC were included. Medical and personal history of patients consisted of age, span of disease, tumor site/size, progression of disease, staging/grading, etc. Age range was 20-80 years. For collection of data, we followed the American Joint Committee for Cancer Staging and End Results Reporting. All the parameters of the Declaration of Helsinki were respected in this study. Classification of the tumor was based on WHO criteria such as well differentiated (WD), moderately differentiated (MD), and poorly differentiated (PD) breast carcinoma for grade I, grade II, and grade III, respectively. All the slides were routinely stained with hematoxylin-eosin to assess the morphology of cells and proper classification of cases. These were interpreted by two histopathologists. Data were entered in SPSS 24.0. A chi square test was

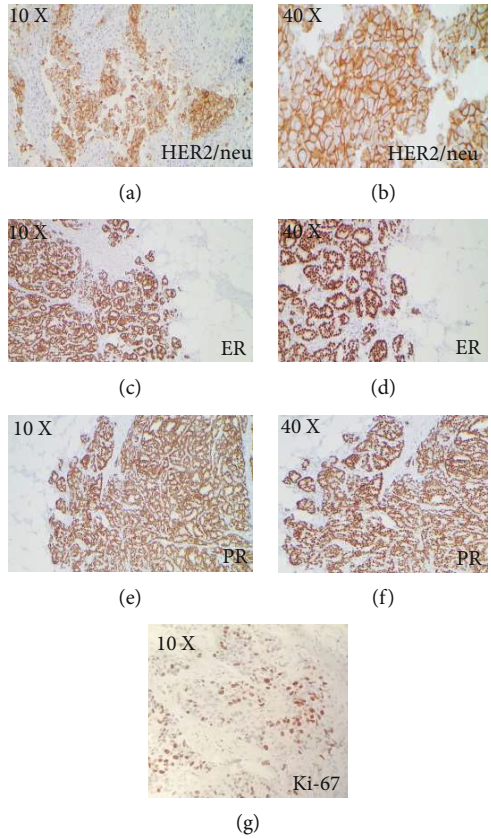

Figure 2: Staining with routine diagnostic markers for BC: (a, b) HER2 strongly positive, complete membranous staining in more than 80% of tumor cells, 10x and 40x; (c, d) ER strongly positive, nuclear staining in 95% of tumor cells, ALLRED score 5 + 3, 10x and 40x; (e, f) PR strongly positive, nuclear staining in 95% of tumor cells, ALLRED score 5 + 3, 10x and 40x; (g) Ki-67 proliferative marker, strongly positive in 30% of tumor cells, 10x and 40x.

Table 3: Clinical classification of breast cancer cases and its association with SP expression.

Types of breast cancer	SP+	SP-
Luminal A (ER/PR+, HER2-)	5	7
Luminal B(ER/PR+, HER2+)	14	2
ER/PR-, HER2+	4	2
Total cases	23	11

applied to check the association between the SP and NK-1R expression (positive and negative stains) and other parameters.

2.1. ER, PR, HER2, and Ki-67staining. Immunohistochemistry (IHC) for ER, PR, HER2/neu, and Ki-67 was accomplished on FFPE tissue segments as part of the routine clinical assessment of these cases. Antibodies against ER, PR, HER2, and Ki-67 were obtained from Dako, Denmark, and used in concentrations as per the manufacturer's protocol. Lobular and ductal normal areas of the breast were used as the control for ER, PR, and HER2 IHC, whereas the appendix tissue was set as the control for Ki-67. Olympus (Model U-DO3) was used for microscopy.

2.2. Substance P/NK-1R Immunohistochemistry (IHC). FFPE sections of 4 μm were deparrafinized with xylene and decreasing grades of alcohol and washed in distilled water and then Phosphate Buffer Saline (PBS). These sections were pretreated with a citrate buffer in microwave and were allowed to cool for at least 20 minutes. Washings in distilled water and PBS were done before 3% H_2O_2 (30 minutes) to block the endogenous peroxidase activity. SP antibody (Bio-Genex) in dilution 1 : 100 and NK-1R antibody (Abcam) in 1 : 100 dilution were applied to the sections for 45-50 minutes in a humid chamber. The washing step in PBS was done for 10-15 min. Slides were then incubated with secondary antibody Horse Radish Peroxidase (HRP) (Abcam ab6789) for 45-50 minutes and washed again with PBS (1x, pH 7.4) (10-15 minutes). 3,3′-Diaminobenzidine (DAB) DAB plus, K3468, Dako, Denmark, was applied for 5-10 minutes and counter stained with Mayer's hematoxylin for 2 minutes. FFPE sections were dipped in increasing grades of alcohol and then xylene for 5 minutes each. DPX mounting medium was used, and slides were cover slipped. Methods are similar to one of our previous studies on oral squamous cell carcinoma [30].

2.3. Grading of IHC. Cell counting at 10x and 40x was done for the evaluation of protein expression, and counts were made as in our previous study (Table 1) [31]. Scoring for

Table 4: Expression and scoring of ER, PR, and HER2 in SP-negative breast cancer cases.

SP-negative cases ($n = 11$)													
Age (years)	Grade	Histoopinion	Expression	% of cell stain	Intensity of stain	Allred score	Expression	% of cell stain	Intensity of stain	Allred score	Expression	TNM	Size
				ER status				PR status			HER2 status		
50	1	DCIS-CB	+++	5	3	8	++	4	3	7	–	PT1	>5
40	3	IDCIS	–	–	–	–	–	–	–	–	+	PT3	>5
40	3	IDCIS	+	–	–	–	+	–	–	–	–	PT2	2-5
37	3	IDCIS	+	2	2	4	++	3	3	6	+++	PT2	2-5
42	3	IDC	+++	5	3	8	+	2	2	4	+++	PT2	2-5
47	3	IDC	++	3	3	6	++	4	3	7	–	PT2	2-5
35	2	DCIS (nipple involved)	–	–	–	–	–	–	–	–	+	PT2	2-5
40	3	IDCIS	+	–	–	–	+	–	–	–	–	PT2	2-5
57	3	IDC	++	2	2	4	+	6	–	–	–	PT2	2-5
72	3	IDC	++	4	2	6	+	–	–	–	–	PT3	>5
57	3	IDC	+++	5	3	8	++	4	3	7	–	PT2	2-5

ER, PR, HER2, and Ki-67 was done by the Allred method proposed by Qureshi and Pervez [32] (Table 2). No protein overexpression or membrane staining in <10% tumor cells was labeled as score "0" and considered negative for SP/NK-1R protein overexpression. Faint/weak staining (in ≥10% of tumor cells) was given the "+1" score, moderate staining as "+2," and strong staining as "+3."

3. Results

3.1. SP/NK-1R Expression. Expression of SP and NK-1R was detected to be cytoplasmic. Expression of SP showed 68% (23) of the BC cases to be positive (Table 2). Cases of well-differentiated (WD) carcinoma had clear cells with cytoplasm and nucleus (Figure 1(a)), and most of them (66.6%) were SP/NK1R positive (Figure 1(c), Table 2). In moderately differentiated (MD) cases, little morphology of cells has been disrupted, but so far, they can be recognized (Figure 1(d)). In poorly differentiated (PD) cases (Figure 1(g), 14 cases, 60.8%), maximum intensity (+3) of SP was observed (Figure 1(i); Table 2), whereas (7 cases, 87.5%) (Figure 1(f), Table 2) MD with +2 intensity of SP expression and low intensity (+1) was seen in WD cases (2 cases) (Figure 1(c), Table 2). In poorly differentiated cases, the cell morphology was extremely distorted, and cells could not be simply distinguished (Figure 2). Immunohistochemical staining for NK-1R was completed in a small number (6) of core biopsies. The expression of NK-1R was similarly found to be related with the progression of BC. Its expression was high in MD and PD cases (Figures 1(e) and 1(h)) while almost negative in WD cases (Figure 1(b)).

3.2. Association of SP and Patient Characteristics with Clinicopathological Features of BC Patients. The maximum number of the SP-positive cases 19/23 (82.6%) belonged to the age group of <60 years. 12/23 (52.17%) SP-positive cases belonged to premenopausal females and 11/23(47.82%) to postmenopausal females. In most cases, 15/23 (65.2%) had tumor sizes ranged between 2 and 5 cm. 14/23 (60.8%) cases of PD or grade III (group A), 7/23 (30.43%) cases of MD or grade II (group B) and 2/23 (8.69%) cases of WD-BC or grade I (group C) were SP positive. According to the TNM staging, 15/23 (65.2%) SP-positive cases had PT2 stage. According to tumor type, 15/23 (65.2%) SP-positive cases were invasive ductal carcinoma. Distant metastasis was absent in the majority (18/23, 78.26%) of the SP-positive cases. Axillary lymph node metastasis was also absent in 15/23 (65.2%) cases (Table 2).

3.2.1. Distribution of Positive Cases of SP according to the BC Classification. Interpreting from the division of BC, 5/23 (21.73%) SP-positive cases belonged to the luminal A group (ER/PR+, HER2-), 14/23 (60.8%) cases belonged to the luminal B (ER/PR+, HER2+) group, and 4/23 (17.39%) cases belonged to the ER/PR- and HER2+ group of BC (Table 3).

3.2.2. SP Association with ER, PR, HER2, and Ki-67. ER was SP positive in 19/23 (82.6%) cases; PR was positive in 17/23 (73.9%) and HER2 was positive in 18/23 (78.2%) SP-positive cases (Figures 2(a)–2(f)). Ki-67 was positive in all the cases (Figure 2(g)) (Tables 2, 4, and 5). *H* scoring; Allred scoring; expressions of SP, ER, PR, and HER2/neu; and intensities of all stains are mentioned in Tables 4 and 5.

4. Discussion

For the first time, it is demonstrated that SP is not only overexpressed but also involved in the progression of BC. It is found to be associated with poor prognosis and advancement of disease as reported by a previous study [27]. BC cells may release SP after binding to its receptor, NK-1R, as a possible mechanism; it may lead to proliferation [27], migration [29], and angiogenesis [33]. SP may also cause inflammation

Table 5: Expression and scoring of ER, PR, and HER2 in SP-positive breast cancer cases.

SP-positive cases ($n = 23$)															
Age (years)	Grade	Histoopinion	SP expression	*H* score	ER status				PR status				HER2 status		Size (cm)
					Expression	% of cell stain	Intensity of stain	Allred score	Expression	% of cell stain	Intensity of stain	Allred score	Expression	TNM	
33	2	IDC	+++	140	−	−	−	−	−	−	−	−	++	PT2	<2
58	3	IDC=DCIS	+++	190	+++	5	3	8	+++	5	3	8	+++	PT4	2-5
57	3	IDC	+++	240	+++	5	3	8	+++	5	3	8	+++	PT2	2-5
34	3	IDC	+++	240	+	2	2	4	++	4	2	6	−	PT2	<2
50	3	IDC+DCIS	+++	150	+++	5	3	8	++	4	2	6	++	PT4b	2-5
62	3	IDC+DCIS	++	120	+	2	3	5	++	−	−	6	++	PT2	2-5
54	3	IDC	+++	210	+++	5	3	8	+++	5	3	8	+++	PT2	2-5
33	2	IDC	++	160	+++	5	3	8	++	4	3	7	++	PT2	2-5
37	3	IDC+DCIS	+++	210	++	4	2	6	+++	5	3	8	+++	PT1c	>5
67	2	IDC+E-DCIS	+	140	+	3	1	4	−	+	−	−	−	PT3	2-5
63	1	DCIS	+	60	+	2	2	4	++	3	3	6	−	PT1	>5
32	3	IDC	+	180	+++	5	3	8	+++	5	3	8	+++	PT2	<2
33	3	IDC	+	70	+	3	2	5	++	3	3	6	−	PT2	2-5
42	2	ILC	+	150	+++	5	3	8	++	4	3	7	++	PT2	>5
57	3	IDC	++	140	−	−	−	−	−	−	−	−	++	PT2	2-5
37	3	IDC	++	150	−	−	−	−	−	−	−	−	+	PT2	2-5
47	2	IDC	++	180	+	3	1	4	−	+	−	3	+	PT2	2-5
34	3	IDC	+++	160	+	2	2	4	+	3	1	4	−	PT1	>5
50	3	IDC	+++	210	+	2	2	4	++	5	2	7	++	PT3	2-5
67	1	IDC	+	160	+++	5	3	8	++	4	3	7	++	PT2	2-5
42	3	IDC	+++	240	+++	5	3	8	++	4	3	7	++	PT1c	>5
32	2	ILC	++	180	+++	5	3	8	+++	5	3	8	++	PT2	2-5
50	2	IDC	++	160	−	−	−	−	−	−	−	−	+	PT2	2-5

by enhancing the permeability of the blood-brain barrier (BBB) [34]. Subsequently, BC cells migrate and metastasize.

Similar findings were observed in our study except that we evaluated SP and NK-1R both in the tissue, but in a previous study [35], only NK-1R was evaluated in the tissue. There is little contradiction in SP evaluation: in our study, we observed an increased expression with an increasing grade of the tumor, while in a previous study, no difference among the grades was observed and it was only performed on the serum. We revealed the SP expression in all grades of BC which was commonly positive, and the intensity increased with advancing grade. It demonstrates that SP expression is associated with the poor prognosis and aggression of this illness. Our outcomes are in concordance with the earlier studies on BC, which showed SP overexpression [27]. SP discharge from BC cells in response to nociceptive stimuli, whose consequences result in proliferation [27], metastasis, and vasculogenesis [29] by functioning of the autocrine role and causes inflammation by the paracrine role. SP raises the absorptivity of the blood-brain barrier (BBB) [33, 34]. An advanced grade of BC showed higher intensity of SP expression; they can be involved in metastasis.

When more SP is released, it can decrease the apoptosis subsequently [36] by modulating the immune markers IL4, IL6, and IL10 [37], resulting in unrestrained cell division, cell progression, and prominent cancer metastasis. All these mechanisms are carried by increased cellularity in human tenocytes [38] resulting in binding of SP to NK-1R. SP has been described to phosphorylate the AKT (antiapoptotic protein kinase) [39]. SP has been studied in bone marrow stem cells showing proliferative effects [40], but it has to be explored extensively in cancer.

Previously, we had demonstrated the immunohistochemical expression of SP in the sudden fetal and infant deaths and neuropathology [41–44]. We also established SP expression in oral squamous cell carcinoma (OSCC), where a strong expression of SP was found to be related with the progression of OSCC and aided as a diagnostic marker [30]. It was directly related to the grade of cancer, i.e., intensity of expression increased with the increasing grade. An *in silico* analysis by us also revealed the possible involvement of the Tachykinin 1 (Tac1) gene, a gene for SP, in cancer [45]. In another study, the SP/NK-1R system is found to be associated with colorectal cancer progression and prognosis [46].

The Tachykinin family is the largest peptide family; its members bind to G-protein coupled receptors at the cells of destination. Hence, a signaling cascade is initiated, leading to mitogen-activated protein kinase activation, mobilization of calcium, and phosphoinositide hydrolysis. The tumor microenvironment plays a crucial role in this regard, and SP carries its role by binding to NK-1R [33]. SP is found to be important for the viability of cancer cells, and NK-1R has been observed to be more expressed in these cells [47]. SP and NK-1R expression has been found to be associated with the progression of several diseases [26, 48]. Our study is in accordance with these studies, and we observed an overexpression of SP in grade III and an intermediate expression in grade II.

Overexpression of SP and NK-1R was also observed in the precancerous epithelium, and it was proposed that it has contribution towards early carcinogenesis by increasing cell growth and cell division [49]; however, in the current study, this trend was found in a later stage of disease. NK-1R antagonists may inhibit cellular growth, proliferation, and metastasis. It may have a therapeutic role for cancer treatment by inhibiting neoangiogenesis and vascularization. It may be explored for potential as antitumor drugs [26]. It may block the signal transduction network in the cancer microenvironment and reduce the proliferation of tumor cells [48]. By contrast, NK-1R antagonists act in a concentration-dependent manner and counteract the pathophysiological functions of SP. So, NK-1R antagonists may inhibit BC cellular growth, proliferation [27], and migration (for invasion and metastasis) [29]. It may have a therapeutic role for cancer treatment by inhibiting neoangiogenesis and vascularization. It may be explored for potential as antitumor drugs [26]. It may block the signal transduction network in the cancer microenvironment and reduce the proliferation of tumor cells [48].

BC cells not showing HER2/neu amplification and not expressing estrogen/progesterone receptors are named triple-negative BC (TNBC) cells. TNBC represents 10-15% of all BC and is associated with an aggressive clinical course. TNBC patient prognosis, survival, and response to current therapies are poor, and for this reason, it is crucial to search for new therapeutic targets in TNBC to develop new therapeutic strategies. One of these targets is the Neurokinin-1 receptor (NK-1R). It is well known that the SP/NK-1R system is involved in cancer progression. TNBC cells overexpress the NK-1R, and after binding to this receptor, SP promotes the proliferation/migration of TNBC cells. Nonpeptide NK-1R antagonists (e.g., aprepitant) are known to exert, via the NK-1R, an antitumor action; TNBC cells die by apoptosis. The review report conducted by Miguel Muñoz updates the data on a promising therapeutic innovation of NK-1R antagonists for the treatment of TNBC patients [50]. The patient remained in good health, with no side effects, and the tumor volume also decreased [51]. Further research and clinical trials must be carried out in order to fully reveal the beneficial effects of NK-1R antagonists in the treatment of patients suffering from BC. NK-1R antagonists can help in inhibition of various cancers by blocking angiogenesis [52]. Recently, we have proposed the NK-1R antagonist, aprepitant, as a therapeutic strategy for inflammation and respiratory symptoms in COVID-19 infection [53–55]. It has also been reported in our recent findings in dental inflammation and pain [56] as well as being associated with miscarriages [57]. We emphasize further research on the SP/NK-1R pathway in breast cancer as well as other cancers.

5. Conclusion

We hereby conclude that increased intensity and overexpression of Substance P and NK-1R is associated with poor prognosis in BC. SP/NK-1R may also be explored further

as a potential diagnostic biomarker for BC to differentiate the grades.

Data Availability

The data will be furnished upon request.

Additional Points

Key Points. Immunohistochemical expression of Substance P and Neurokinin-1 receptor in breast carcinoma tissue was evaluated. It was strongly expressed in grade III, with maximum intensity. It may be investigated further for its role as a prognostic and a diagnostic marker. The therapeutic potential of Neurokinin-1 receptor antagonists must be explored.

Disclosure

This manuscript is available as preprint with the following link: doi:10.1101/2020.06.27.175083v2[58].

Conflicts of Interest

USPTO application no. 20090012086 "Use of Non-Peptidic NK-1 Receptor Antagonists for the Production of Apoptosis in Tumor Cells" was filed by Miguel Muñoz. The other authors declare no conflict of interest.

Authors' Contributions

RM designed and planned the study, wrote the main manuscript, and supervised the project. SAG critically reviewed the manuscript and facilitated this research. AH collected samples and the medical history and did the data analysis. S collected, processed, and stained the samples and contributed to the write-up. IT gave histopathology opinion for the samples and scoring. SJ contributed to the critical review and write-up. SK analysed the work and edited and approved the final manuscript. SH analysed the work and edited and approved the final manuscript. HW contributed to the statistical analysis. AA gave expert opinion and critical review. MM gave expert opinion in conducting the experimental work. All the authors have read the manuscript and finally approved.

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Research Article

Investigation of the Role of Neurokinin-1 Receptor Inhibition Using Aprepitant in the Apoptotic Cell Death through PI3K/Akt/NF-κB Signal Transduction Pathways in Colon Cancer Cells

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Background. Colorectal cancer (CRC) is recognized as one of the most common malignancies with a high mortality rate worldwide, supporting the necessity for an effective novel antitumor drug to improve current therapy's effectiveness. Substance P (SP) is the essential member of the tachykinins (TKs) family, which binds to the specific receptors, known as neurokinin-1 receptor (NK1R), exerting its multiple influences such as tumor cell proliferation, angiogenesis, and metastasis. Aprepitant, as a specific NK1R antagonist, is suggested as a novel antitumor agent, promoting apoptotic processes in tumor cells; however, the exact antitumor mechanism of aprepitant on molecular signaling in CRC is not entirely known. *Method*. The resazurin assay was conducted to assess the cytotoxic effects of aprepitant on the viability of the CRC cell line (SW480). The level of reactive oxygen species (ROS) was measured after 24-hour treatment with SP and aprepitant. PI/annexin V-FITC staining was conducted to assess apoptosis. Also, the expression of NF-κB antiapoptotic target genes and proapoptotic p53 target genes was measured by real-time- (RT-) PCR assay. Western blotting assay was performed to determine the expression of PI3k/AKT/NF-κB proteins. *Results*. We found that aprepitant stimulates apoptotic cell death and attenuates the PI3K/Akt pathway and its downstream proapoptotic target gene, including NF-κB in SW480 cells. Also, the obtained results from the quantitative RT-PCR assay showed that aprepitant could decrease the level of mRNA of NF-κB antiapoptotic target genes. *Conclusion*. Towards this end, this study suggests that SP/NK1R system plays a vital role in the development of CRC, and pharmaceutical targeting of NK1R using aprepitant might be a promising treatment against CRC.

1. Introduction

Colorectal cancer (CRC) constitutes one of the commonly reported malignancies with limited treatment options and a high mortality rate [1]. In the recent years, there is a great deal of evidence that various agents and mechanisms are involved in the onset and progression of CRC [2–4]. In line with this, different molecular mutations have been shown in the advancement of CRC, including modifications in protooncogene KRAS, p53 tumor suppressor, and the transforming growth factor- (TGF-) β pathway [5]. Additionally, several observational researches have failed to indicate any therapeutic efficacy in the survival rate of CRC, despite evidence-based advances in treatment [6]. Toward this end,

attention is focusing on developing new agents with potent antitumor properties to improve the treatment of CRC in the context of targeted therapies [7, 8].

Tachykinins' (TK) family consists of evolutionarily conserved neuropeptides that act as an immunomodulatory agent and regulate a diverse array of tumorigenesis processes in cells [9]. It is found that substance P (SP), hemokinin-1 (HK-1), neurokinin B (NKB), neurokinin A (NKA), and N-terminally extended forms of NKA, including neuropeptide γ (NPγ) and neuropeptide K (NPK), are the most important members of the family of TK neuropeptides in mammalian [10, 11]. The biological functions of these peptides are exerted by binding to specific receptors, entitled neurokinin 1 receptor (NK1R), NK2R, and NK3R [12]. As the leading central member of the TK family, SP has higher binding affinities to NK1R [7, 9]. It is well-known that SP is commonly presented in peripheral and central nervous systems, thereby playing an important role in regulating neurogenic inflammation and immune responses [13, 14].

Recently, an increasing number of cancer studies have been indicated that SP/NK1R system are overexpressed in many types of tumors, such as pancreatic, gastric, larynx, glioblastoma, colon cancer, and acute lymphoblastic leukemia cells [15–20]. Furthermore, recent studies have been demonstrated that SP/NK1R axis could lead to cancer progression, angiogenesis, and metastasis [21, 22]. Taking advantage of these characteristics, the concept of NK1R inhibition recently has received enormous attention as a novel therapeutic strategy in human cancer treatment.

Aprepitant (AP), as a highly specific NK1R antagonist, has already been approved by the Food and Drug Administration (FDA) for the prevention of chemotherapy-induced nausea and vomiting [23–25]. Besides, several pieces of evidence indicate that blocking of NK1R by AP resulted in a considerable reduction of mitogenic activity and inflammation process in some solid malignancies, including colon cancer [26–30]. Despite several studies on AP's antitumor role in different cancer cells, there is relatively little information about the intracellular signal transduction systems responsible for mediating AP's antitumor effects.

Taken together, to distinguish the critical role of the SP/NK1R signaling axis in CRC, our study has aimed to study the anticancer efficacy of AP in an associated CRC cell line (SW480).

2. Methods and Materials

2.1. Cells, Drugs, and Chemicals. SW480 cells were originally purchased from the Pasteur Institute of Iran (Tehran, Iran). RPMI 1640 medium (Gibco-BRL, Life technology, Paisley, Scotland) supplemented with 10% heat-inactivated fetal bovine serum (Gibco-BRL, Life technology, Paisley, Scotland) and 1% penicillin-streptomycin (Gibco-BRL, Life technology, Paisley, Scotland) were used for growing of cells. Cells were seeded in tissue culture flasks (Falcon, Heidelberg, Germany) and retained in a humidified incubator with 37°C and 5% CO2. For the administration of drugs, AP and SP were purchased from Sigma-Aldrich. The stock solution of these drugs was made in sterile dimethyl sulfoxide (DMSO) and culture medium, respectively. After that, they were divided into aliquots and stored at -80°C until use. DMSO concentration was added less than 0.1% of the total volume and adjusted as an applied solution.

2.2. Trypan Blue Exclusion Assay. To assess the inhibitory effects of AP on cell growth, cells were cultured at a density of 450×10^3 cells/mL and treated with increasing concentration of AP (5, 10, 15, 20, 30, and 40 μM) for 24 and 48 h. After that, 0.4% trypan blue and cell suspension were mixed in an equal proportion and incubated for 2 min at room temperature. Finally, the percentage of viable cells was evaluated as follows:

$$\%\text{cell viability} = \left(\frac{\text{viable cell count}}{\text{total cell count}}\right) \times 100. \quad (1)$$

2.3. Growth Inhibition Studies. The cell metabolic activity of AP was assessed by resazurin-based cytotoxicity assay. Resazurin (7-hydroxy-3H-phenoxazin-3-one 10-oxide) is a non-fluorescent dye that can reduce to resorufin and dihydro-resorufin (highly fluorescent), which is directly dependent on the number of viable cells [31]. Briefly, 2.5×10^4 cells were seeded in triplicates into 96-well plates (SPL Lifesciences, Pocheon, Korea). After the administration of cells with various concentrations of AP (5, 10, 15, 20, 30, and 40 μM), the cells were incubated in a humidified incubator for 24 and 48 hours. The blue color of resazurin is altered to pink color resorufin and dihydro-resorufin by living cells. The fluorescent dye production was measured with a microplate fluorimeter under the excitation and emission wavelengths of 600 nm and 570 nm. The cytotoxic effect of AP was calculated by half-maximal inhibitory concentration (IC_{50}) values measured with the dose-response curve in GraphPad Prism.

2.4. Measurement of ROS Activity. The level of intracellular ROS production was measured using 2′, 7′-dichlorodihydro fluorescein diacetate (DCFDA, Sigma, USA) following to the manufacturer's instructions. Briefly, after 24-hour incubation, cells were exposed to the DCFDA solution (20 μM) for 30 min at 37°C. Afterward, cells were treated with SP (100, 400 nM) and AP (15 μM) alone and in combination. Moreover, the tert-butyl hydroperoxide (TBHP) was used as a positive control group. Finally, the fluorescence intensity was measured at 495/529 (Excitation/Emission) in Perkin-Elmer Atomic Absorption Spectrophotometer.

2.5. Flow Cytometry Analysis of Apoptosis. Annexin V-FITC detection assay kit (Roche Applied Science, Germany) was used to detect apoptotic cell death as described previously [30]. Briefly, 35×10^4 cells were seeded into 12 well plates, and the cells were collected after 24 h of treatment. Thereafter, cells were washed with PBS and added a total volume of 100 μL of staining buffer, including annexin V-Flous solution (2 μL/sample). Next, the plate was incubated for 30 min in the dark. Finally, the quantity of necrotic and apoptotic cells was measured by flow cytometer (BD Biosciences, San Jose, CA, USA). FITC-annexin V positive and PI negative were

Table 1: Nucleotide sequences of the primers used for real-time RT-PCR.

Gene	Source	Forward primer (5′-3′)	Reverse primer (5′-3′)
GAPDH	Human	5′-ACAACTTTGGTATCGTGGAAGG-3′	5′-GCCATCACGCCACAGTTTC-3′
Bax	Human	5′-CGAGAGGTCTTTTTCCGAGTG-3′	5′-GTGGGCGTCCCAAAGTAGG-3′
Bcl2	Human	5′-CGGTGGGGTCATGTGTGTG-3′	5′-CGGTTCAGGTACTCAGTCATCC-3′
Survivin	Human	5′-CCAGATGACGACCCCATAGAG-3′	5′-TTGTTGGTTTCCTTTGCAATTTT-3′
P53	Human	5′-GAGGTTGGCTCTGACTGTACC-3′	5′-TCCGTCCCAGTAGATTACCAC-3′

identified as early apoptosis, and annexin V positive, and PI-positive stained cells show late apoptosis.

2.6. RNA Extraction and Real-Time Quantitative PCR (qRT-PCR). Total RNA extraction was realized from 65×10^4 cells after treatment with SP (100 and 400 nM) and AP (15 μM) and combination with each other using total RNA extraction mini kit (Yekta Tajhiz, Tehran, Iran), following the manufacturer's instructions. The RNA concentration was quantified by nanodrop spectrophotometer (NanoDrop 1000™, USA). Afterward, 1 μg of RNA was reverse-transcribed to complementary DNA (cDNA) utilizing cDNA Synthesis Kit (Pars Toos, Tehran, Iran). Quantitative RT-PCR was performed with specific primers such as Bax, Bcl-2, survivin, and p53, purchased from Pishgaman (Pishgaman Co., Tehran, Iran) (Table 1). Also, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene and all amplifications were carried out in Roche real-time thermal cycler (Mannheim, Germany). The relative mRNA expression was calculated by the comparative Ct method ($2^{-\Delta\Delta Ct}$).

2.7. Western Blot Analyses. The CRC cells were lysed with an ice-cold RIPA lysis buffer (250 mM Tris-HCl, pH 7.4, 0.5% sodium dodecyl sulfate (SDS), 5 mM EDTA, 750 mM NaCl, 5% Triton X-100) after exposing of sw480 cells with SP (400 nM) alone or with AP (10 μM) [16]. Protein content was measured using bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Rockford, IL). The 30 μg protein samples were loaded and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and, subsequently, transferred onto nitrocellulose membrane. The membrane was incubated for 24 h at 4°C with specific antibodies against PI3K, Akt, β-Actin (1 : 1000; Cell Signaling, USA) and NF-κB P65 (1 : 1000; Abcam, USA). Following two-hour incubation with anti-rabbit HRP-conjugated secondary antibodies (1 : 3000; Cell signaling, USA). Next, immune complexes on the membrane were detected by adding chemiluminescence detection kit (Thermo Scientific, Rockford, IL) according to the manufacturer's directions. Finally, band intensity was assessed with ImageJ software, and the ratio of proteins to actin expression was normalized.

2.8. Statistical Analysis. Experimental results are presented by mean ± standard deviation. The values were analyzed using ANOVA followed by Bonferroni's *t*-test through GraphPad Prism® 6.0 software (San Diego, CA, USA). Statistical significance was considered lower than 0.05 ($p < 0.05$).

3. Results

3.1. The Antiproliferative and Cytotoxic Effects of AP on Human CRC Cell Line SW480. To investigate the cell growth inhibitory effects of AP, SW480 cells were exposed to increasing concentrations of AP (0-40 μM) for 24 h and 48 h. On the basis of dose- and time-dependent manner, we found that elevating concentrations of AP (0-40 μM) considerably decreased the cell survival and metabolic activity of SW480 cells (Figure 1). As illustrated in Figure 1(b), various concentrations of AP above the IC_{50} (approximately 18 μM after 24 h and 9 μM for 48 h treatment) metabolic activity of the SW480 cells is reduced. Additionally, according to the trypan blue exclusion assay (Figure1(a)), the cell viability is inhibited by various concentrations of AP (0-40 μM) in a dose- and time-dependent manner. With respect to these results, the 10 μM was chosen for experimental concentration. In 10 μM of AP, cell viability and metabolic activity is approximately 65% as compared to untreated control.

3.2. Assessment of the Proapoptotic Effect of AP on Human CRC Cell Line SW480 Using Flow Cytometry. To evaluate the efficacy of AP (10 μM) as a single therapy or in combination with exogenous SP (100 and 400 nM) in programmed cell death, the cells were dual stained with annexin V-FITC and PI. Accordingly, our flow cytometry results (Figure 2) indicated that AP (10 μM) as a single agent or in exposing to exogenous SP (100 and 400 nM) for 24 h could significantly increase the percentage of apoptotic and necrotic SW480 cells as compared to the control group ($p < 0.05$). Quantitatively, apoptotic and necrotic rates in the AP group were 20.7% while in the control groups were 4.01%. Taken together, these findings suggest that SP (100 and 400 nM) could attenuate apoptotic cell death in CRC cells (apoptotic and necrotic rates in the SP 100 nM group were 3.84% and in the SP 400 nM group were 2.06%).

3.3. The Negative Effect of AP on the PI3K/AKT Signaling Pathway in SW480 Cells. Based on emerging evidence, the PI3K/Akt signaling axis is a potentially important treatment target in CRC [32]. Additionally, it has been found the SP/NK1R axis-induced activation of the PI3K and its downstream effectors such as total Akt molecules in various tumors [16, 30, 33, 34]. Towards this end, we assessed SP

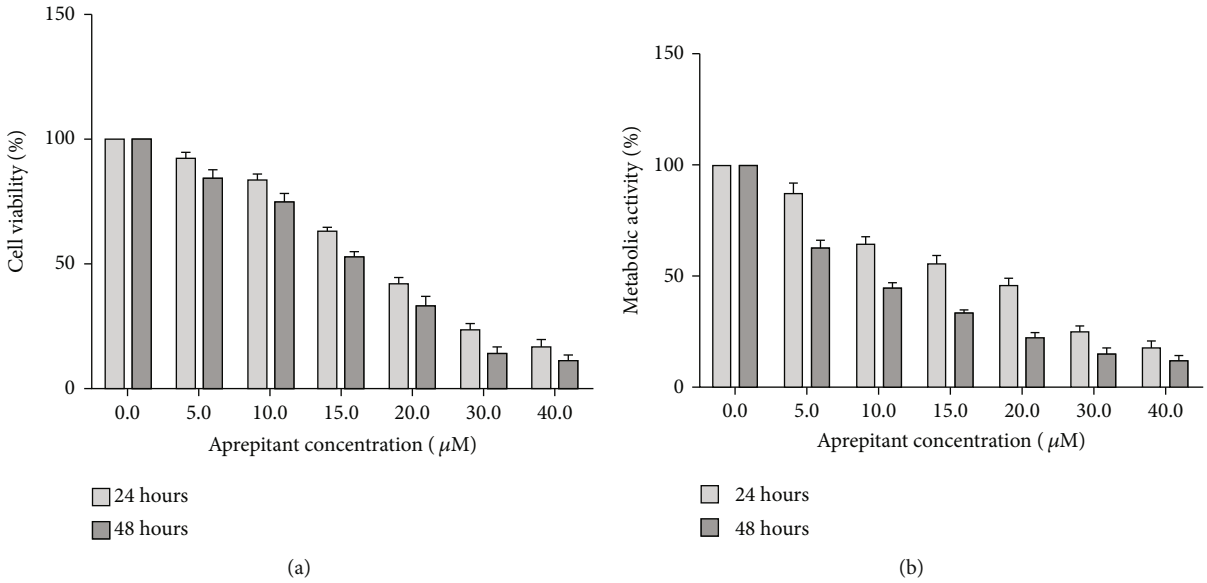


FIGURE 1: The inhibitory effect of AP in the proliferation and viability of SW480 cells. (a) AP has inhibitory activity on the viability of SW480 cells in a concentration and time-dependent based on trypan blue assay. (b) The inhibitory effects of AP on metabolic activity was assessed by resazurin assay after indicated times with doses escalation of AP. The IC50 value was evaluated about approximately 18 μM after 24 h and 9 μM for 48 h treatment. All data were shown as mean ± S.D of three independent experiments.

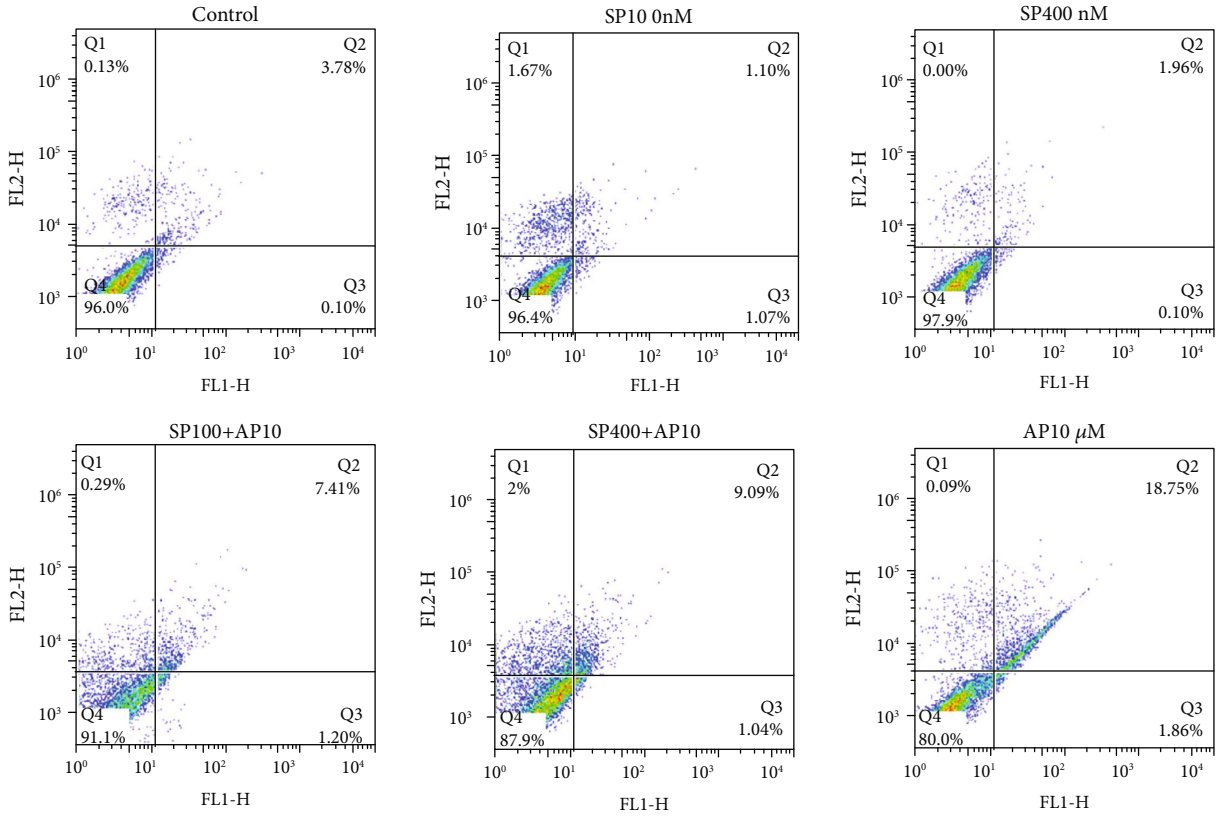
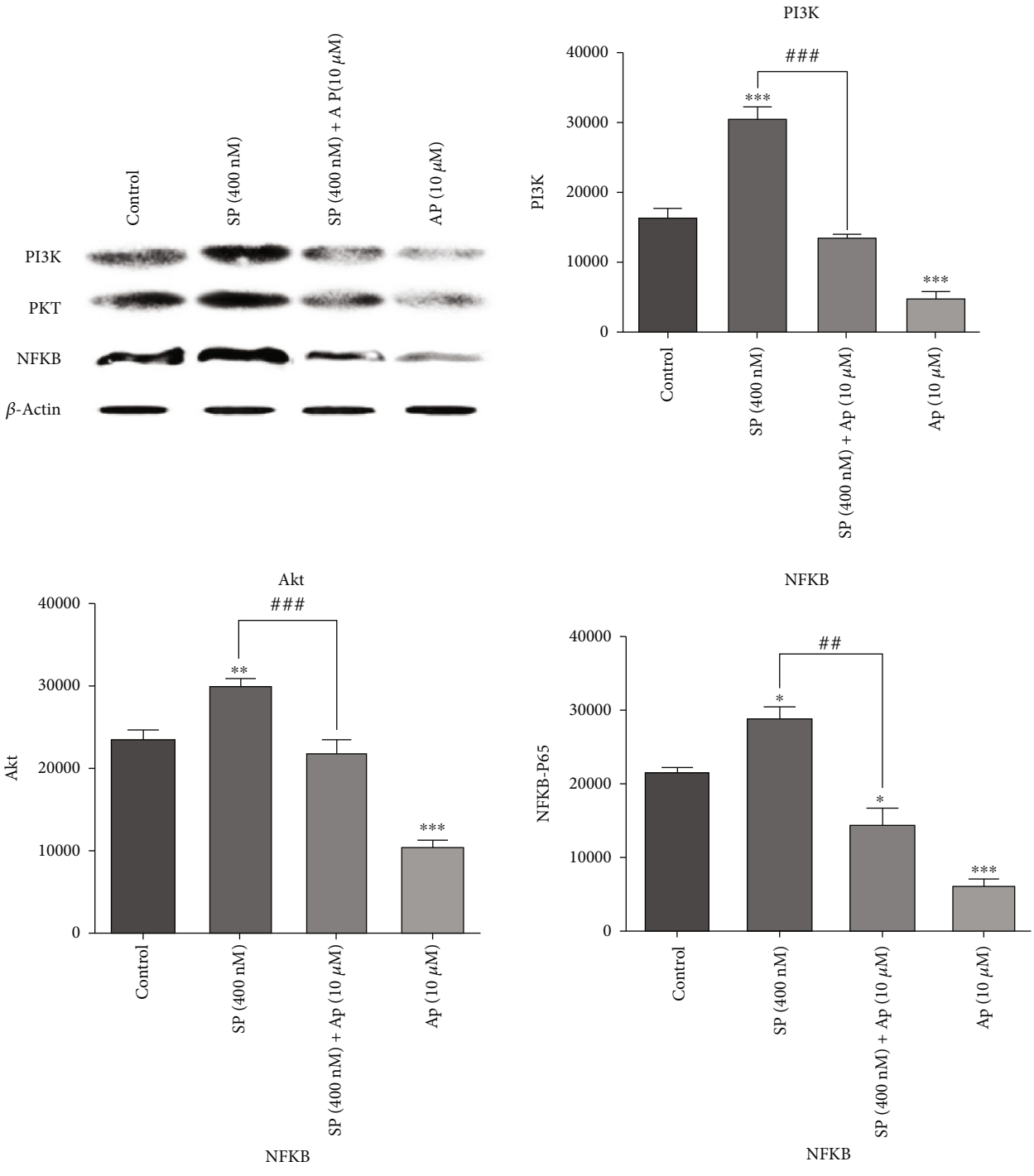


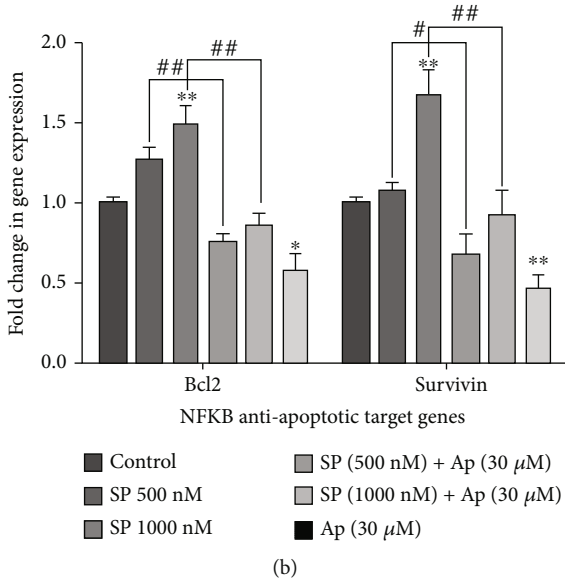
FIGURE 2: Proapoptotic effect of AP in SW480 cell lines. The percentages of annexin-V and annexin-V/PI double-positive in flow cytometry results implicate significantly increased apoptosis in the treated of SW480 cells with AP (10 μM) as compared to untreated control cells.

and AP's impact on the PI3K/Akt ratio in the SW480 cell line. According to the Figure 3(a), we found that the blocking of NK1R with AP alone (10 μM) or in combination with exogenous SP (400 nM) after 24 h could attenuate the PI3K/Akt ratio. Also, these findings were associated with the negative effect of AP (10 μM) on the PI3K/Akt signaling cascade.



(a)

Figure 3: Continued.



(b)

Figure 3: Abrogation of PI3K/Akt/NF-κB signaling axis and downstream of antiapoptotic NF-κB target genes by AP. (a) The cytoplasmic protein was extracted after cell treatment with AP (10 μM) as a single agent or in combination with SP (400 nM), and Western blot assay was conducted using an Ab directed against NF-κB P65, PI3k, Akt, and β-actin. The data were repeated at least two independent experiments. The intensity of bands was analyzed by the ImageJ software. (b) mRNA expression of NF-κB antiapoptotic target genes was decreased by AP. GAPDH mRNA levels were used to normalized the levels of expression of all groups. All results were shown as a mean ± SD ($p < 0.05$).

3.4. Aprepitant Inhibits the NF-κB Signaling Pathway and Attenuates Antiapoptotic Target Genes in CRC Cells. Recently, evidence has been shown that SP via binding to NK1R could enhance the NF-κB signaling pathway activity and subsequently suppress cell apoptosis through targeting NF-κB antiapoptotic target genes [8, 35]. The NF-κB p65 subunit was assessed by Western blotting assay to increase the NF-κB protein phosphorylation in response to SP (400 nM) and AP (10 μM) in SW 480 cell line after 30 min. Furthermore, the mRNA expression of NF-κB antiapoptotic target genes, such as Bcl-2 and survivin, was investigated by quantitative real-time PCR. We found that SP (400 nM) exerted an enhancer effect on the NF-κB p65 subunit based on the obtained results. As shown in Figure 3(a), although the combination of SP (400 nM) with AP (10 μM) decreased the NF-κB protein expression, the AP (10 μM) as a single agent has an additive anti-inflammatory effect compared to the untreated group. Moreover, the results of the mRNA expression level of NF-κB downstream antiapoptotic target genes, such as surviving and Bcl-2, confirmed both the proinflammatory effect of SP and the anti-inflammatory effect of AP (10 μM) in CRC cells (Figure 3(b)). Consistent with these findings, our results indicate that the SP and AP may have a critical regulatory role on the NF-κB signaling pathway and downstream effectors.

3.5. Aprepitant Has No Significant Efficacy in the Enhancement of p53 and Proapoptotic p53 Target Gene Expression. In addition to the NF-κB axis, which is considered a crucial aspect in cancer cell apoptosis, the p53 and its proapoptotic target gene expression could induce apoptotic cell death in several human cancer cells [36–38]. Toward this end, we further evaluated the relative mRNA expression of p53 and downstream proapoptotic target genes such as Bax to investigate the SP/NK1R signaling activity in the SW480 cell line. As presented in Figure 4, our findings demonstrated that the mRNA expression of p53 and Bax was not significantly different among all groups compared to control. Taken together, these data showed that SP/NK1R system could induce a presumptive p53-independent apoptotic cell death in the SW480 cell line.

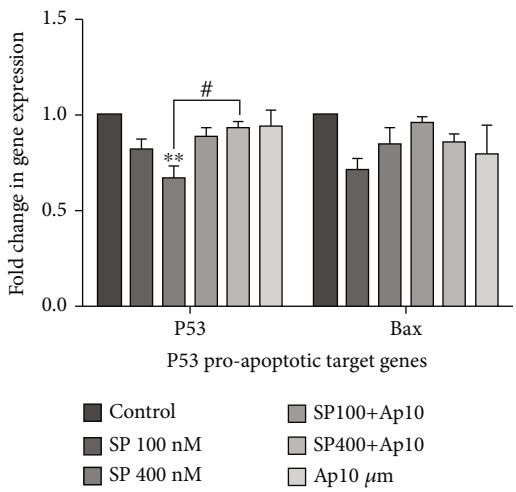


Figure 4: The effect of AP on the p53 and its proapoptotic target genes such as Bax. The expression levels of p53 and Bax were assessed with quantitative RT-PCR and were normalized with the expression level of GAPDH as indicated administration of AP has no significant efficacy on the expression levels of p53 and Bax ($p < 0.05$).

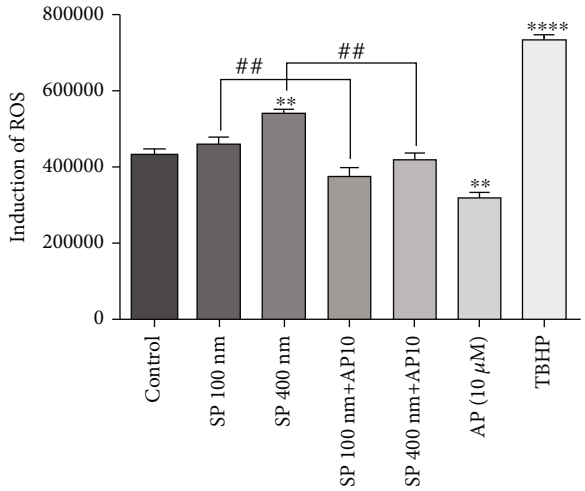


Figure 5: Reactive oxygen species formation is reduced following exposure to aprepitant. ROS formation was determined by DCFH-DA staining after administration of AP (10 μM) alone or in combination with SP (100 and 400 nM). This figure confirms the inhibition efficacy of AP on intracellular ROS formation in the SW480 cell line.

3.6. Aprepitant Decreased the Accumulation of Intracellular ROS in SW480 Cells. Researchers have recently discovered that ROS generation promoting oxidative stress could mediate apoptotic cell death in various tumors [39–42]. Given these notions and the fact that apoptosis relies on the ROS level, we evaluated the influence of the SP/NK1R system on the ROS production level in SW480 cells. As illustrated in Figure 5, although the administration of SP (100 and 400 nM) could lead to increased intracellular ROS production after 24 h, the AP (10 μM) with about 19% change in the AP10 μM + sp100 nM group compared to the Sp100 nM group and about 22% change in the AP10 μM + sp400 nM group compared to the Sp400 nM group or without about 25% change compared to the control group and the exogenous SP (100 and 400 nM) markedly attenuated the intracellular ROS level in CRC cells. Interestingly, these results confirmed the previous observations in NF-κB downstream antiapoptotic target genes. Taken together, these data supported the antiapoptotic effects of AP through decreased ROS production in CRC cells.

4. Discussion

Since SP interaction with NK1R is involved in the development and progression of multiple cancers, an increasing number of investigations focus on blocking human NK1R by various antagonists [6, 27]. Additionally, it is known that the expression of NK1R increases on the surface of tumor cells, such as CRC [17–20]. In the context of cancer treatment, the antitumor influence of AP, as an oral NK1R antagonist, opens up a new avenue to cancer treatment, including melanoma, hepatoblastoma, pancreas, lung, and breast malignancy [21, 28, 43–46]. In line with this, we evaluated the antitumor effect of AP on the molecular signaling in CRC. For this purpose, we found that AP has concentration-dependent antiproliferative and cytotoxic activity on human CRC cell line SW480. As illustrated in Figure1, cell viability and metabolic activity of cells after treatment with 10 μM of AP are approximately 65% as compared to untreated control. We also determined AP-induced apoptotic processes in the SW480 cell line using flow cytometry and found promising results in the treated group with AP. As shown in Figure 2, apoptotic and necrotic cells in the AP group were 20.7% while in the control group were 4.01%. Simply putting analysis of flow cytometry data implied that the blockage of NK1R leads to inhibit the proliferation of CRC cells and stimulate apoptosis in SW480 cells. Similarly, a recent study reported promising results in inducing apoptosis of esophageal squamous cell carcinoma cells after treatment with AP. They found that treatment of esophageal squamous cell carcinoma with AP (15 μM) can impose approximately 83.5% of cells to apoptosis and necrosis, while the apoptotic and necrotic cells were 10.23% in control group [30]. Furthermore, Bayati et al. demonstrated that blockade of NK1R on the surface of acute lymphoblastic leukemia cells with several concentrations of AP (10, 20, and 30 μM) leads to an increase in the percentage of apoptotic and necrotic cells. In this regard, their results show that AP 30 μM induces 59% of cells to apoptosis, while the percentage of apoptotic cells after treatment with AP 20 μM and AP 10 μM was about 25% and 29%, respectively [16].

Additionally, our data demonstrated that the blockage of NK1R with AP (10 μM) could decrease the PI3K/Akt ratio, supporting the findings of apoptosis results. Our results agreed with the data obtained from the recent studies that reported inhibition of NK1R cause to suppress the PI3K/AKT signaling axis and resulted in apoptotic cell death [16, 30, 34]. In line with this, it is known that the PI3K/AKT regulates various transcriptional factors such as NF-κB, leading to imposing several cellular react including apoptosis cell death, invasion, and inflammation [47, 48]. Toward this end, we tried to show that whether AP could stimulate apoptotic cell death by modifying the PI3K/Akt/NF-κB signaling pathway (Figures 3(a) and 3(b)). Our results demonstrated that the treated group with AP (10 μM) decreases both expression of NF-κB p65 protein and mRNA expression of NF-κB antiapoptotic target genes such as survivin and Bcl-2 (Figures 3(a) and 3(b)). To further investigate apoptotic mechanisms by AP, recent evidence has been found that inducing apoptosis by AP is associated with increased p53 in several tumor cells [16, 35, 36]. In support of this idea, it is well found that the activation of PI3K/Akt is correlated with p53 pathways [16]. In contrast to these results, we found that the AP (10 μM) could not increase the mRNA expression level of p53 and, subsequently, the mRNA level of proapoptotic p53 target genes such as Bax (Figure 4). Moreover, in accordance with our data, Javid et al. reported that AP induce program cell death through a p53-independent apoptotic pathway in esophageal squamous cell carcinoma [30]. In harmony with AP-inhibited PI3K/Akt/NF-κB, these data supported that AP might apply its therapeutic effects through a p53-independent apoptotic axis in SW480 cells.

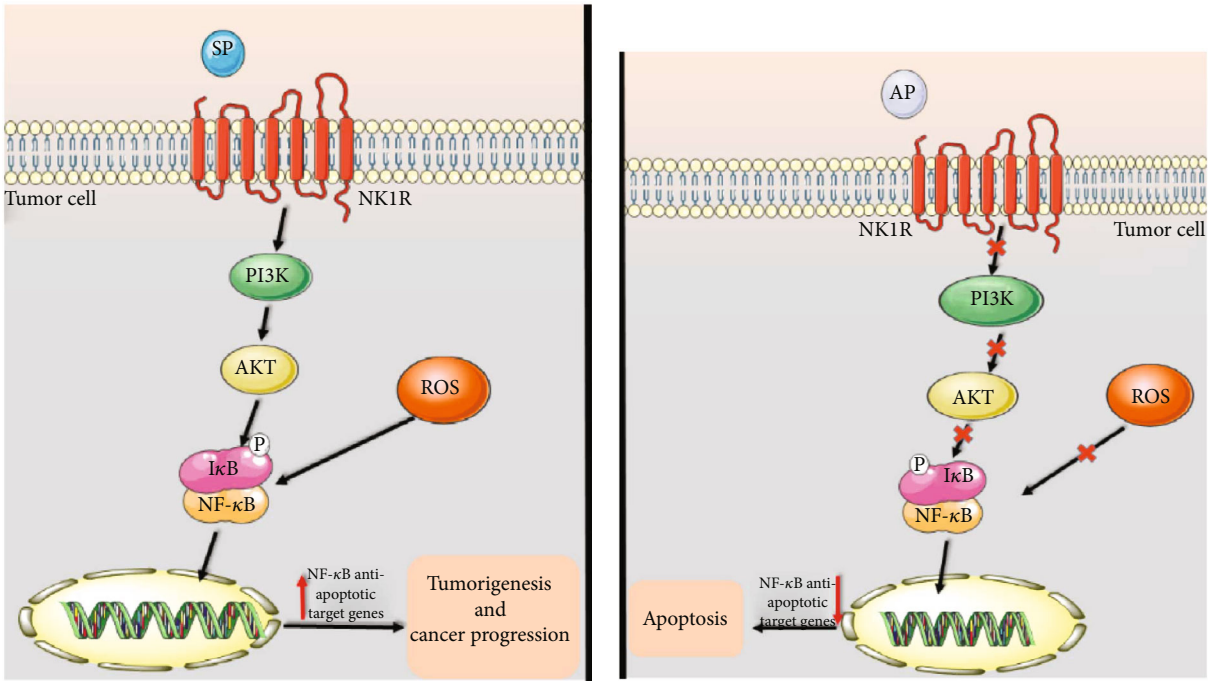


Figure 6: Schematic representation of the possible mechanism of the SP/NK1R system and aprepitant in CRC-derived SW480 cells. (a) A model of signaling of the SP/NK1R system in the absence of aprepitant. (b) A model of signaling of the SP/NK1R system in exposure to aprepitant.

Additionally, it is well established that mitochondrial ROS elevation plays a vital role in apoptosis induction [49, 50]. Furthermore, intracellular production of ROS is associated with activation of the NF-κB signaling pathway and the suppression of apoptotic cells death, survival, and critical role in tumorigenesis of cancer cells [51, 52]. Given this, as illustrated in Figure 5, our data indicated that AP could reduce about 25% change ROS production compared to the control group based on the NF-κB axis regulated in SW480 cells.

5. Conclusions

Overall, our research study revealed that blockage of NK1R with the specific antagonist, AP, has displayed anticancer efficacy against the SW480 human CRC cell line by abrogating PI3K/Akt/NF-κB axis (Figure 6). Additionally, according to the pharmacologic safety of AP against chemotherapy-induced nausea and vomiting as a positive characteristic, our outputs suggest that AP can play an essential therapeutic against CRC as a single therapy or in combination with other typical anticancer therapeutics. However, despite numerous studies on the antitumor activity of AP against various tumor cells, more researches are required to further elucidate the underlying functional mechanisms of AP in multiple cancers.

Data Availability

Data are available upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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Research Article

Enhanced Neurokinin-1 Receptor Expression Is Associated with Human Dental Pulp Inflammation and Pain Severity

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Substance P (SP) is a peptide involved in many biological processes, including nociception and inflammation. SP has a high affinity for its receptor neurokinin-1 (NK-1R). SP/NK-1R complex plays a major role in the interactions going on during the onset of dental pain and inflammation. *Objective*. To identify the expression of NK-1R in healthy and inflamed human dental pulp, as well as to identify any association with severity of dental pain. *Methods*. This case-control study included ten irreversibly inflamed samples of dental pulp, which were extirpated from patients presenting with chief complaint of dental pain due to caries. Ten healthy pulps, extirpated from those teeth which were indicated for extraction due to orthodontic reasons, were used as the control group. Visual analog scale (VAS) and modified McGill Pain Questionnaire were used to assess the characteristic and severity of pain. Immunohistochemical study was performed using monoclonal antibodies against NK-1R. *Results*. The results showed that the NK-1R was expressed intensely in patients with higher pain score. The mean pain score in cases was 7.0 ± 2.0. The healthy dental pulps had negative or mild NK-1R staining of +1 intensity. The NK-1R score in cases was 2.4 ± 0.516 and 0.2 ± 0.4216 in controls. There was significant difference in NK-1R score between both groups (p value <0.05). There was a strong positive correlation between the pain score and NK-1R expression score. As the pain increased, the NK-1R expression score was also increased (0.95**, p value 0.000). *Conclusions*. NK-1R is overexpressed in inflamed dental pulp. SP/NK-1R modulation may provide a novel approach for the treatment of pulpal inflammation and pain.

1. Introduction

Dental pain perception is due to an inflammatory reaction going on inside the tooth pulp [1]. Neuropeptides are now known to be major indicators of the inflammatory process in peripheral tissues. Neuropeptides take part in the process of transmission and modulation of pain and inflammatory process. These neuropeptides include neurokinin A (NK-A), calcitonin gene-related peptide (CGRP), neuropeptide Y (NP-Y), vasoactive intestinal polypeptide (VIP), and substance P (SP), among others [2].

Prior studies have demonstrated SP to be involved in both inflammation and pain [3]. SP is abundantly present in the fibers that innervate the dentin and dental pulp. As the inflammatory processes begin, the amount of SP released by each sensory fiber is further increased, which perpetuates the vicious circle that is underlying inflammation [4]. SP binds to its receptor NK-1R preferentially and exerts all its biological actions by binding to this high-affinity G-protein-coupled receptor. These receptors are located on many inflammatory cells, such as macrophages and mast cells, and also on other connective-tissue cells [5]. SP can also interact with NK-2 and NK-3 receptors, if present in higher concentrations. There are reports suggesting the presence of NK-1R, as G-protein-coupled receptor subunits have been identified in normal human dental pulp [6, 7].

This molecule's production and release is significantly enhanced when the dental pulp is stimulated by multiple factors such as noxious, thermal, mechanical, and chemical triggers [8]. The increase in blood vessel permeability, thus

enabling plasma extravasation and degranulation of mastocytes, is due to induced vasodilatation caused by the interaction between SP and its receptors. Histamine is released from mastocyte granules, which in turn activates and enhances vascular mechanisms.

Lymphocytes, granulocytes, and macrophages have SP receptors, and cytokine production is induced by stimulation of these cells [9]. The interaction of SP with mast cells describes the reason for the enhanced vascular permeability and blood pressure due to induction of release of histamine. Moreover, the macrophages, lymphocytes, and granulocytes contain sites of NK-1R, and these cells are stimulated by SP and induce the production of proinflammatory mediators and cytokines. In addition, SP serves as a powerful chemical agent, which results in further incorporation of inflammatory cells in the pulp tissue.

There is an increased sensitivity towards pain because of dramatic sensitizing of nociceptors due to incorporation of more inflammatory and nociceptive mediators, also resulting in stimulation and abundant release of SP not only in the spinal cord but also within the pulpal tissue [10, 11]. SP affects the dental pulp in many ways. Multiple studies have marked the presence of NK receptors in rodent and human teeth. Animal studies established the pattern of expression of the tachykinin receptors NK-1, NK-2, and NK-3 in different types of epithelial cells, fibroblasts, hard tissue cells, endothelium, and blood vessel walls in teeth and surrounding oral tissues [12].

It has been documented that NK-1R and NK-2R are present both on ameloblasts (enamel-forming cells) and odontoblasts. As predicted, there is an abundance of NK-1Rs on smaller blood vessels, and capillaries and both NK-1R and NK-2R are densely distributed on the capillary plexus underneath dentin [13]. Although, presence of NK-Rs has been reported in human pulp tissue, but the methods used for their evaluation like radio-receptor assay were not precise enough to measure which type of receptor (NK-1, NK-2, or NK-3) was primarily present [9]. However, there is no exact evidence of the sequence and expression of NK-1R in healthy and inflamed human dental pulp while transitioning during different stages of inflammation. This study was thus aimed at comparing expression of particularly NK-1R in human pulp tissue extirpated from teeth clinically diagnosed with irreversible pulpitis to those of healthy teeth in association with severity of pain.

2. Methodology

2.1. Tissue Collection. The study was approved by Ethical Review Committee of The University of Lahore and performed in accordance with the guidelines of the Declaration of Helsinki for Human Research [14]. Complete history was taken from all the patients after taking written informed consent. Twenty pulp samples were obtained from 2 groups of patients of both gender with age ranging between 15 and 35 years [15]. After fulfilling the clinical diagnostic criteria [16], group 1 (cases) included inflamed samples of dental pulp obtained from teeth clinically diagnosed with irreversible pulpitis, and these patients presented in the outdoor patient department (OPD) with chief complaint of dental pain. Group 2 (controls) included pulp samples obtained from healthy teeth with fully developed roots, which needed extraction due to orthodontic reasons, and these patients had no complaint of pain [11]. Both of these groups had no history of antibiotic administration, and they were systemically healthy. Smokers, pregnant, and patients with history of trauma to orofacial region or a TMJ surgery were excluded from this study. Any patient who was suffering from irreversible pulpitis but did not complain of dental pain was excluded from group 1, and patient complaining of dental pain associated with the tooth to be extracted was also excluded from group 2. Group 1 was given the VAS and modified McGill Pain Questionnaire to assess the characteristics and intensity of dental pain [17].

Teeth belonging to group 1 were anesthetized by 1.8 ml 2% lidocaine, injection technique was infiltration for maxillary teeth, and inferior alveolar nerve block was given for mandibular teeth. To obtain pulp sample from group 1, access opening was made and pulp extirpation was done using a no. 15 barbed broach [4]. Immediately after the extirpation, the tissue was placed in 10% neutral buffered formalin, embedded in paraffin, and cut with a microtome to a thickness of 3-4 μm. For each tissue sample, three different depths of cut were made, which were first stained with Hematoxylin-Eosin (H&E). Observation

Table 1: Demographic variables.

Variable	Cases	Control	Total
Gender			
Male	5	2	7
Female	5	8	13
Marital status			
Single	5	10	15
Married	5	0	5
Qualification			
Illiterate	1	2	3
Middle	0	5	5
Intermediate	5	2	7
Bachelor	4	1	5
Use of medication to reduce pain			
No	2	10	12
Pain killer	8	0	8
Oral hygiene			
Poor	2	0	2
Good	7	10	17
Excellent	1	0	1
Diagnosis			
Acute pulpitis	7	0	7
Chronic pulpitis	3	0	3
NK-1 stain			
Negative	0	8	8
Mild	0	2	2
Moderate	6	0	6
Severe	4	0	4

Table 2: Mean difference of pain score and NK-1R between cases and controls.

Independent sample t-test						
	Group	N	Mean	Std. deviation	t-test	p value
Pain score	Case	10	7.00	2.000	11.068	0.000
	Control	10	.00	.000		
NK-1R scoring	Case	10	2.4000	.51640	10.436	0.000
	Control	10	.2000	.42164		

of H&E stained sections involved determination of the form and intensity of inflammation.

For the group of healthy pulps, the teeth were anaesthetized and extracted in the same manner. 5.25% sodium hypochlorite was used to wash the teeth, soon after extraction in order to eliminate remnants of periodontal ligament which can result in contamination of the pulp sample. The sectioning of teeth was done by using a cylindrical diamond bur in a high-speed hand piece with simultaneous irrigation with saline solution. Extirpation of pulp was done by a barbed broach.

2.2. Immunohistochemical (IHC) Staining Protocol. 3-4 μm thick sections of the dental pulp were mounted on 3-aminopropyltriethoxysilane- (APES-) coated slides, deparaffinized in xylene, and rehydrated via graded ethanol solutions. Then, sections were rinsed with distilled water and washed three times with PBS (pH 7.4). Heat-induced pretreatment for antigen retrieval (sections were immersed in a 10 mmol/l citrated buffer, pH 6.0, at 60°C for 5 min) was carried out prior to incubation with the primary antibody. The endogenous peroxidase activity was inhibited by incubation of the samples with 0.3% hydrogen peroxidase in methanol for 30 minutes at the temperature of 4°C. After blocking the nonspecific reactions with 10% normal rabbit serum, the sections were incubated with the primary antibody against NK-1R for two hours.

2.3. Evaluation of Immunohistochemical Staining. The slides were examined by light microscopy using an Olympus BX40 microscope (Artisan Scientific, Champaign, Illinois, USA) at magnification of 400x. The product of the immunohistochemical reaction was detected in the cytoplasm of the endothelial cells, fibroblasts, and inflammatory cells. NK-1R staining was identified by displaying brown color, ranging from light brown to dark brown. The cytoplasmic staining intensity was scored semiquantitatively, according to a previously described method [18], as follows:

0 – 10% = negative
10 – 30% = 1 + (weak staining)
30 – 60% = 2 + (moderate staining)
60 – 100% = 3 + (strong staining)

3. Statistical Analysis

Data were analyzed by using SPSS 25.0. All the quantitative variables were presented by frequencies and quantitative variables in mean ± SD. Independent sample t-test was applied to check the mean difference of pain score and NK-1 between cases and controls. Pearson correlation was applied to check the relationship between pain and NK-1R scoring.

Table 3: Pearson correlation.

Correlations			
		Pain score	NK-1R scoring
Pain score	Pearson correlation	1	.955**
	p value		.000
	N	20	20

**Correlation is significant at the 0.01 level (2-tailed).

4. Results

Levels of NK-1R were much higher in the irreversibly inflamed pulp versus the healthy pulp. Patients with a diagnosis of healthy pulp had a pain rating on the visual analog scale (VAS) of 0. Patients with a diagnosis of irreversible pulpitis had a lowest pain rating score of 4 out of a maximum score of 10. Scores above 5 are associated with moderate to very severe pain. This study included 10 cases of irreversible pulpitis (7 were acutely inflamed cases, and 3 were chronic cases as diagnosed clinically). A positive correlation between expression of NK-1R, level of inflammation, and pain score was observed in 7 cases clinically diagnosed as acute pulpitis. These cases showed higher pain score ranging from 6 to10 and mild to severe levels of inflammation histologically. The NK-1R expression also ranged from moderate to intense staining. Level of inflammation and staining increased with the increasing pain score. On the other hand, 3 cases clinically diagnosed as chronic pulpitis showed moderate levels of inflammation and NK-1R expression. However, the pain score for chronically inflamed cases was moderate (6 as rated on VAS) as compared to acutely inflamed cases where pain score ranged from moderate to severe (above 6 on VAS).

Table 1 shows the demographic variables. The mean age of patients was 21 ± 6.665 years. There were 5 males and 5 females in group 1 (cases) and 2 males and 8 females in group 2 (controls), and majority were single (cases = 5 and control = 10), qualification level of intermediate (cases = 5 and control = 2), using no medication for pain (cases = 2 and control = 10), good oral hygiene (cases = 7 and control = 10), negative NK-1R staining (cases = 0 and control = 8), and (cases = 4 and control = 0) had severe staining.

According to Table 2, the mean pain score in cases was 7.0 ± 2.0. The NK-1R score in cases was 2.4 ± 0.516 and 0.2 ± 0.4216 in controls. There was significant difference in NK-1R score between both groups (p value <0.05). There was a strong positive correlation between the pain score and NK-1R. As pain increased, the NK-1R score also increased (0.95**, p value 0.000) (Table 3).

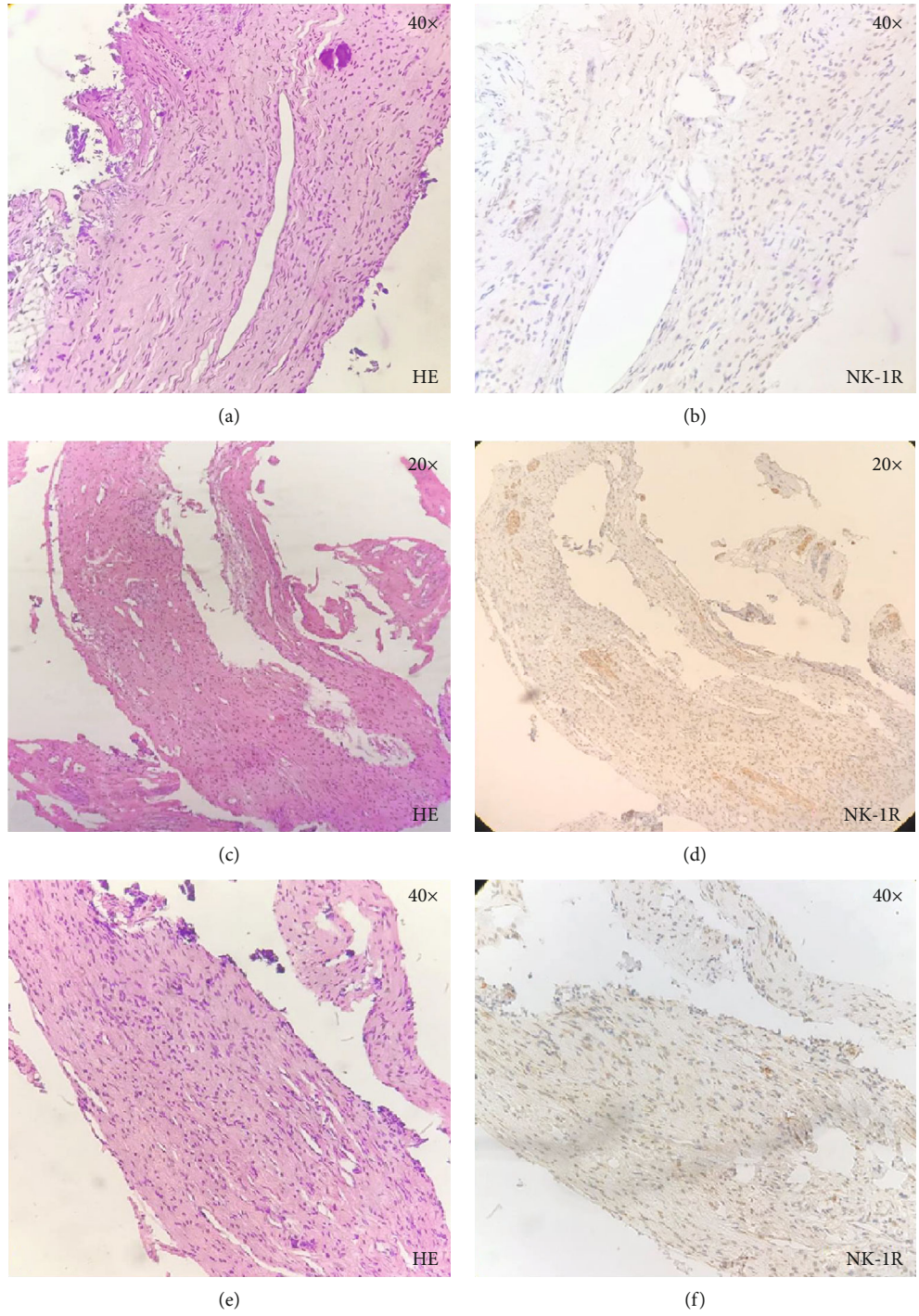


Figure 1: Histological sections of healthy dental pulp.

Figure 1 is representing the preservation of normal morphology of dental pulp. Figures 1(a), 1(c), and 1(e) show H/E staining, consisting primarily of mesenchymal cells, fibroblasts, and some macrophages, exhibiting a stellate morphology. Figures 1(b), 1(d), and 1(f) show NK-1R staining. Figures 1(b) and 1(d) show negative staining, and Figure 1(f) shows mild staining. Figures 2(a), 2(d), and 2(g) show vascular congestion, and there is presence of diffuse inflammatory infiltrate, predominantly formed by lymphocytes and plasma cells. Figures 2(b), 2(e), and 2(h) are showing intense NK-1R staining in inflamed dental pulp at 20x and Figures 2(c), 2(f), and 2(i) at 40x.

5. Discussion

Induction of inflammatory reaction is due to increased amount of SP in peripheral tissues including the dental pulp, but the regulatory effects can only be witnessed in target

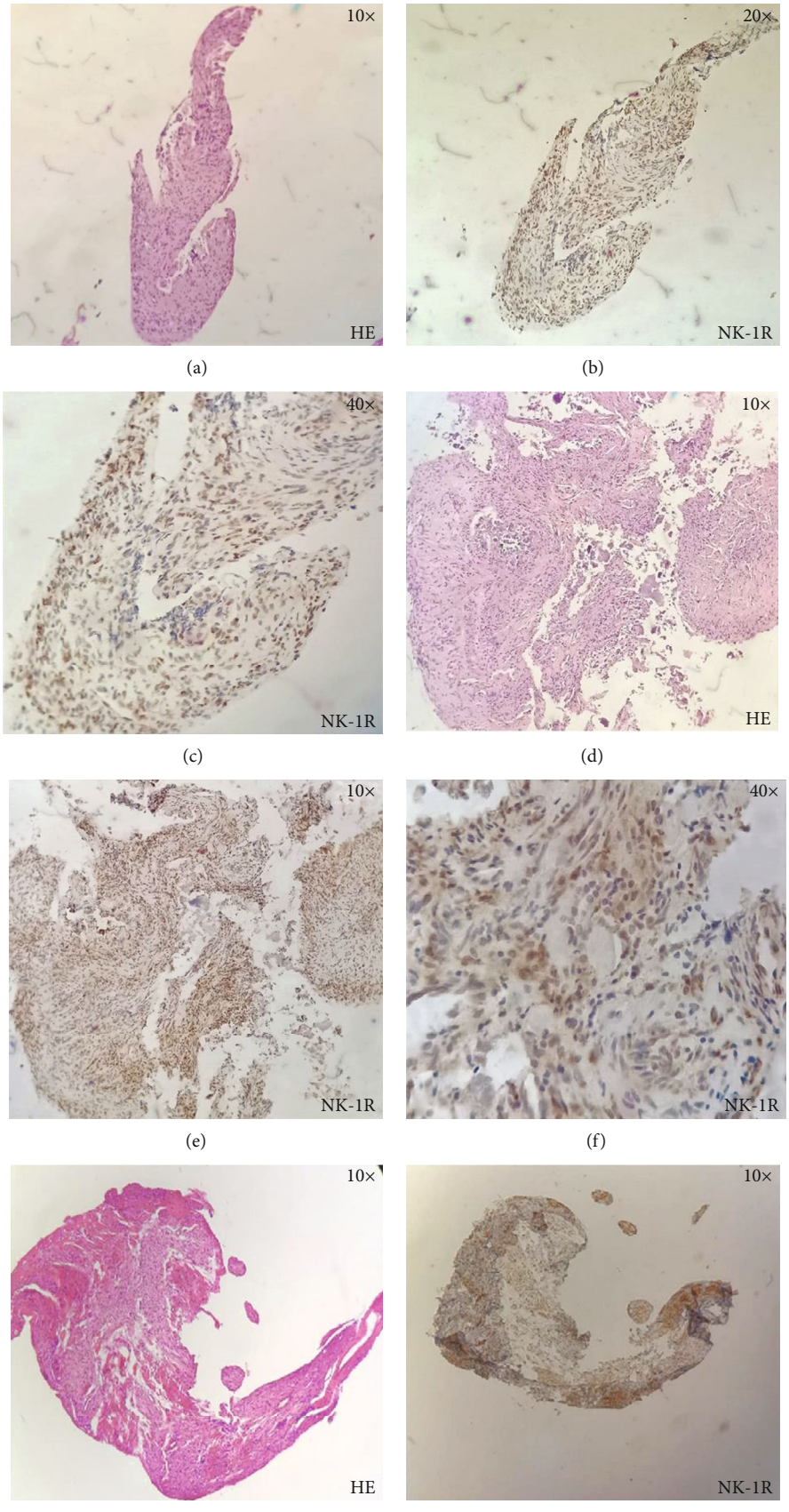
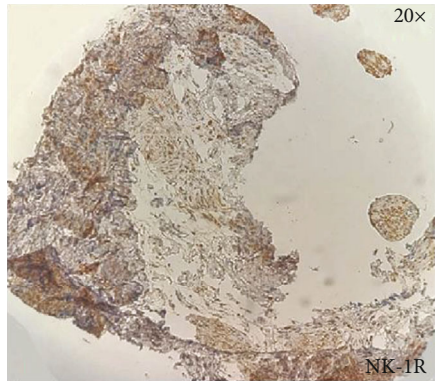


Figure 2: Continued.



(i)

Figure 2: Histological sections of inflamed dental pulp.

tissues if there is enhanced receptor signalling [19]. Therefore, the objective of this study was to compare SP receptor, NK-1R expression in pulpal tissue with clinical diagnosis of irreversible pulpitis, to that of in healthy pulp. The mechanism by which pain arises when an inflammatory reaction is going on within the dental pulp is not completely understood.

Studies reveal that irreversible pulpitis is associated with different expression of various biomarkers, as compared to noninflamed controls. One study reported findings in rat's dental pulp that immunoreactions for NK-1R were detectable in nerve terminals associated with cytoplasmic processes of the odontoblasts [20]. Several other studies suggest that SP/NK-1R has a definitive role in inflammatory processes. This was confirmed due to considerably higher SP/NK-1R expression in the inflamed pulp [21, 22]. One study reported an 8-fold increase in levels of SP, and it was eminent in pulpal tissue clinically diagnosed with irreversible pulpitis versus that of clinically healthy pulp tissue [22]. Consequently, irreversible pulpitis is associated with momentous instigation of this peptidergic system but there is lack of sufficient data related to expression of NK-1R in human dental pulp during inflammation. Another study highlighted that SP, IL-8, and MMP-8 levels were found to be higher in pulpal samples from teeth with irreversible pulpitis, with higher pain scores than those with low pain scores [23].

Another study reported that NK-1R was elevated along with SP in gingival crevicular fluid around painful teeth [23]. Recently, the involvement of SP-NK-1R in oral pain and inflammation has also been reported in a study from Spain [24]. SP has been a major player in the neurogenic inflammation in the afferent neurons and may contribute to the pulpal disease. SP mRNA and protein were expressed by pulpal fibroblasts. N-1R mRNA was also detected in these fibroblasts. SP levels were also observed to be high as compared to the healthy pulps [25]. Only a single study has been performed previously, reporting the expression of SP receptor, NK-1R in human dental pulp. This study proved that SP receptor expression was present in all human pulp tissue samples, and there was an intense expression of NK-1R in the group of pulps from teeth clinically diagnosed with acute irreversible pulpitis [4]. Cytokine interleukin 1 (IL-1) has been observed to be main player in the periodontitis [26].

In a study conducted on 25 human dental pulps, a histological and radioimmunological assay was performed to correlate the concentration of prostaglandin E2 (PGE2) and grade of inflammation. The study confirmed an association, and PGE2 was reported as a marker to distinguish the reversible and irreversible pulpitis [27]. Results from our study correlate with prior statement, as there was an increase in expression of SP receptors during inflammatory processes, and this indicates SP active contribution in the development of pulpitis. Our study is first to demonstrate the comparative expression of NK-1R in healthy and inflamed human dental pulp in association with severity of dental pain. While these results cannot be inferred to address existing endodontic clinical problems, these results have clinical implications, as they have the potential for inspiring future research modalities when proposing alternative methods of biologic pulp therapy. Identifying SP receptor in human pulp, while focusing on its regulatory effects on immune reactions, specially taking into account its role in the induction of fibroblastic proliferation, is of utmost importance in order to broaden the scope of knowledge of biological action of these receptors.

6. Conclusions

The expression of NK-1R in human pulp tissue is remarkable during the process of pulpal damage caused by an inflammatory reaction. Considering the correlation between the symptomatic teeth with increased SP levels and patient VAS scores, SP/NK-1R modulation may provide a novel approach for the treatment of pulpal inflammation and pain. Definitely, familiarity with different processes involved in pulp inflammation is required in order to diagnose, plan, and formulate treatment modalities leading towards better management of the diseased pulpal tissue, thus not only maintaining its vitality but also preventing subsequent loss of resistance and retention of tooth.

Data Availability

The datasets used to support the finding of this study are available from the authors upon reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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