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Research Article

Epigenetic Modifiers Oxamflatin and Ascorbic Acid Modulate the Proliferation, Viability, and Gene Expression in Buffalo Foetal Fibroblast Cells

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Abstract

Epigenetic regulation plays a crucial role in controlling gene expression without altering the underlying DNA sequence. This study investigates the dose-dependent effects of oxamflatin, a histone deacetylase inhibitor, and ascorbic acid, a known antioxidant and epigenetic modulator, on the proliferation, metabolic activity, and gene expression in buffalo fetal fibroblast cells. Cells were treated with varying concentrations of oxamflatin (0.5 µM, 1 µM, 2 µM, 5 µM, and 10 µM) and ascorbic acid (25 µM, 50 µM, 100 µM, and 200 µM). MTT assay results indicated that 1.0 µM oxamflatin and 50 µM ascorbic acid significantly enhanced cell proliferation and metabolic activity across 24, 48, and 72-h intervals ($P < 0.05$), while higher doses showed inhibitory or toxic effects. Gene expression analysis revealed that both compounds significantly downregulated epigenetic regulators *(HDAC1, DNMT1, DNMT3A)*, particularly at 1.0 µM oxamflatin and 50 µM ascorbic acid. Antioxidant-related genes *(SOD1, SOD2, SOD3)* were markedly upregulated at these optimal concentrations, with a decline at higher doses. Additionally, pro-apoptotic genes *(BAD, P53)* were significantly downregulated, while anti-apoptotic genes *(BCL2, BCL-XL)* were upregulated in a dose-responsive manner, peaking at 1.0 µM oxamflatin and 50 µM ascorbic acid. These results highlight the concentration-specific benefits of oxamflatin and ascorbic acid in enhancing fibroblast cell viability, antioxidant response, and epigenetic regulation, suggesting potential applications in regenerative medicine and cellular reprogramming.

Keywords: Epigenetic Modifiers; Oxamflatin; Ascorbic Acid; Cell Proliferation; Gene Expression

Introduction

The term 'epigenetics' refers to genetic changes that do not involve alterations in the DNA sequence [1]. The condensed complex of DNA and proteins, known as chromatin, is found in a cell nucleus; chromatin shape is altered by substances or agents called epigenetic modifiers, which control gene expression. These changes may affect how DNA packaging impacts the accessibility of cellular machinery involved in gene expression. Epigenetic changes are crucial for various biological processes, including disease, differentiation, and cell development [1]. The primary types of alterations include DNA methylation, histone modifications, and regulation by non-coding RNAs. Histone modification (acetylation) frequently regulates gene expression and chromatin dynamics alongside other histone modifications, such as methylation and phosphorylation [2]. The interactions among various epigenetic markers can significantly influence chromatin structure and cellular function. Histone deacetylases (HDACs) promote chromatin condensation and transcriptional suppression by removing acetyl groups from lysine

residues on histone tails [3]. The potential therapeutic applications of HDAC1 inhibitors in various diseases, including cancer, neurological disorders, and inflammatory conditions, have garnered considerable research interest [4].

Recent studies have concentrated on oxamflatin, one of the HDAC1 inhibitors, due to its possible epigenetic effects [5]. This synthetic compound is well known for its potent inhibitory effects on several HDAC1 enzymes. Oxamflatin causes acetyl groups to accumulate on histone tails by inhibiting HDAC1 activity, which relaxes the chromatin structure. This modification of the chromatin structure may lead to variations in gene expression patterns. It significantly influences fibroblast proliferation, viability, and the expression of genes related to apoptosis, antioxidant defense, and epigenetic regulation [5]. In fibroblasts, this often impacts proliferation by inducing cell cycle arrest, typically in the G1 or G2/M phase. Prolonged exposure or higher concentrations can decrease cell viability by triggering apoptosis through the upregulation of pro-apoptotic genes, such as *BAX* and *PUMA,* while downregulating anti-apoptotic genes like *BCL-2* [6]. Additionally, oxamflatin can modulate the expression of antioxidant-related genes, including *SOD* (superoxide dismutase) and *CAT* (catalase), potentially enhancing the cell ability to neutralize oxidative stress. At the epigenetic level, oxamflatin alters the expression of key regulatory genes involved in DNA methylation and chromatin remodeling, such as *DNMT1* (DNA methyltransferase 1) and *TET1* (ten-eleven translocation methylcytosine dioxygenase 1), leading to lasting changes in gene expression patterns [7]. Oxamflatin positively affects fibroblasts by modulating their behavior and gene expression. It promotes controlled cell growth, supports programmed cell renewal through apoptosis, enhances antioxidant defenses, and facilitates extensive epigenetic modifications, underscoring its potential as a powerful regulator of fibroblast function.

Ascorbic acid, commonly known as vitamin C, plays a crucial role beyond its well-known antioxidant properties. Recent studies have highlighted its significance as an epigenetic modifier, particularly in regulating DNA and histone modifications [8]. It has also demonstrated its role in promoting TET (ten-eleven translocation) enzyme activity, which is involved in the demethylation of DNA. During this process, 5-methylcytosine (5mC) is converted into 5-hydroxymethylcytosine (5hmC), which triggers active DNA demethylation and, consequently, controls the expression of certain genes [9]. This has been linked to the modulation of histone demethylation through interaction with histone demethylases, which play a critical role in removing methyl groups from histone proteins, thus affecting chromatin structure and gene expression [10]. Understanding the role of ascorbic acid in epigenetic modifications could have significant implications for manipulating the epigenome, potentially leading to advancements in cellular reprogramming, regenerative medicine, and disease modeling [11]. Ascorbic acid also plays a crucial role in modulating fibroblast proliferation, survival, and the expression of genes linked to apoptosis, antioxidant defense, and epigenetic regulation. It stimulates fibroblast growth by enhancing collagen production and promoting extracellular matrix formation, which aids in cell expansion and tissue regeneration [12]. At optimal concentrations, ascorbic acid boosts cell viability by mitigating oxidative stress through its antioxidant effects, neutralizing free radicals, and upregulating antioxidant genes such as *SOD* (superoxide dismutase) and *GPX* (glutathione peroxidase). However, at elevated levels, it can become cytotoxic, reducing cell viability by inducing apoptosis [13]. This occurs through the increased expression of pro-apoptotic genes like *BAX* and *CASPASE-3* while simultaneously downregulating anti-apoptotic genes, including *BCL-2* [14]. Very few reports are available on oxamflatin and ascorbic acid as epigenetic modifiers for buffalo fetal fibroblast cells. Keeping this in mind, this research examines how oxamflatin and ascorbic acid influence fibroblast cell growth and gene expression so that they could applicable in regenerative medicine and cell-based therapies.

Materials and Methods

Establishment and characterization of buffalo fetal fibroblast cells

A buffalo fetus (approximately 45 days old) obtained from a slaughtered animal was separated from the uterus and cleaned twice with antibiotic-fortified saline. The surface of the fetus was made aseptic using 70% ethanol, and the ear pinna was collected and washed 4 to 6 times with DPBS containing 50 µg/ml gentamicin sulfate. These pieces were then cut into small sections (~1 mm^3), washed again 3-4 times with DPBS containing 50 µg/ml gentamicin sulfate, and finally cleaned 2-3 times with cell culture medium (DMEM supplemented with 20% FBS and 50 µg/ml gentamicin sulfate). The tissue pieces were transferred to tissue culture flasks or cell culture dishes and cultured

in the medium in a CO_2 incubator (5% CO_2 in air) at 37°C. The explants were removed after 6-8 days once fibroblast proliferation and establishment were achieved. The fibroblasts were cultured into a monolayer until they reached confluency. The monolayers were then harvested with 0.25% trypsin and subcultured for further cell multiplication. Established fibroblasts were characterized by immunostaining and RT-PCR for fibroblast-specific marker genes.

Effects of oxamflatin and ascorbic acid on fetal fibroblast cells

Cellular metabolic activity (MTT assay)

The MTT substrate [3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl Tetrazolium Bromide] (Sigma, USA) at 10 mg was dissolved in 2 ml of PBS (pH 7.5) to assess the metabolic functionality and proliferation of cells at various concentrations of oxamflatin and ascorbic acid. The fibroblast cells at passage-5 were diluted in DMEM + Ham's F-12 complete medium, and 1,000 fibroblast cells were seeded in each well of 96-well culture plates (3 replicates) for columns 1 to 12 (A to H). This multiwell plate was then incubated in a CO_2 incubator at 37°C with 5% CO_2 and > 95% humidity for 24 h. After 24 h, the cells were treated with oxamflatin and ascorbic acid; columns 1 and 2 (A-H) were kept as controls with no chemical added. Columns 3 to 7 (A-H) received increasing concentrations of oxamflatin (0.5 µM, 1 µM, 2 µM, 5 µM, and 10 µM, respectively), while columns 8 to 12 (A-H) were treated with increasing concentrations of ascorbic acid (25 µM, 50 µM, 100 µM and 200 µM, respectively). Three plates were prepared in the same sequence and cultured for 24 h, 48 h, and 72 h, respectively, in the CO_2 incubator (at 37°C with CO_2 and > 95% humidity). After culturing, 20 µl of stock MTT solution was added to each well and incubated for 4 h in the CO_2 incubator at 37°C. The medium was removed without disturbing the formazan crystals formed in the wells. Finally, 200 µl of DMSO was added, and the optical density was recorded using a multi-well ELISA Reader (TECAN, Germany) at 570 nm.

Cell proliferation

The cells were seeded in 6-well plates for 24 h at 37°C in a 5% CO_2 incubator. After cell attachment, treatments were administered with increasing concentrations of oxamflatin 0.5 µM, 1 µM and 2 µM; and ascorbic acid at 25 µM, 50 µM, 100 µM, and 200 µM, respectively. The plates were incubated for another 24 h at 37°C in a 5% CO_2 incubator. After 48 h, the cell pellet was resuspended in 1 ml of fresh media to obtain a cell suspension. A 0.4% solution of trypan blue (Gibco, Thermo Fisher Scientific, USA) was mixed with 10 µl of the cell suspension from each sample. The mixture was loaded onto TC10 cell counting dual-chamber slides (BIO-RAD, USA) and analyzed using the TC10 Automatic Cell Counter (BIO-RAD, USA).

Quantitative analysis of gene expression

Total RNA was isolated from treated cells using Trizol, followed by the addition of chloroform and isopropanol to the tube for the precipitation of nucleic acids and proteins. The final wash was performed with 70% ethyl alcohol, centrifuging the tube to eliminate all alcohol residues, drying the pellet for 15 to 20 minutes, and then eluting it in 15 to 20 µl of nuclease-free water. For cDNA synthesis, the RNA concentration was measured and adjusted to 100 ng/µl for fetal fibroblast cells. cDNA was synthesized using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Life Sciences, USA) according to the manufacturer's protocol. A reaction mixture containing 1 µl total RNA (100 ng), dNTPs, oligodT, reaction buffer, RNase inhibitor, and RevertAid™ M-MuLV Reverse Transcriptase was prepared with a total volume of 20 µl. Initially, RNA, primer, and nuclease-free water were incubated at 65°C for 5 minutes, then cooled on ice before addition of the remaining components and incubated at 42°C for 60 minutes. The synthesized cDNA was stored at -20°C for subsequent Real-Time PCR. Real-time PCR was performed in a CFX96 system (BIO-RAD, USA) using SYBR GREEN master mix (Thermofisher, USA) in 96-well plates. The PCR conditions included an initial denaturation at 95°C for 3 minutes, followed by 40 cycles of 95°C for 15 seconds, gene-specific primer annealing at X°C for 15 seconds (Table 1), and extension at 72°C for 15 seconds.

Statistical analysis

Statistical analysis was conducted using GraphPad Prism (GraphPad Software Inc., USA), and the ΔΔCt method was employed to compare relative mRNA expression levels. Differences between mean percentages were evaluated with the ANOVA test. $P < 0.05$ was considered statistically significant.

Results

Establishment of primary buffalo fetal fibroblast cells and subculturing

Fibroblast cells emerged from the tissue explants within 3 to 5 days of culture, using DMEM/Ham's F-12 medium supplemented with 20% FBS and 50 µg/ml gentamicin sulfate. The primary culture reached 20-25% confluency within 8 to 10 days. Upon subculturing at a seeding density of 3.2×10^3 cells/cm^2, cells ad-

Table 1: Primers used for cell characterization and qPCR of different genes under study.

Gene Name	Sequence	Annealing temp. (˚C)	Size (bp)	Accession No.
VIMENTIN	F-GGTACAAGTCCAAGTTTGCTG R-TCCAGAGACTCGTTAGTCCC	58	153	XM_006052364.1
B-TUBULIN	F-CAGGTCTTCAGGGCTTCTTG R-GGATGGAGTTGTAGGGCTCA	60	151	NM_005785527.1
DESMIN	F-GCAGCCAACAAGAACAATGAC R-TCCTCTAGCTCCCTCATCTG	60	145	XM_006059028.1
CYTOKERATIN 8	F-CTTCAAGACCAAGTATGAGGA R-TGCCTGTAGAAGTTGATCTC	56	155	NM_001033610.1
CYTOKERATIN 18	F-CAGAGTCAAGTATGAGACAGAG R-TGTAGACCCTTTACTTCCTCC	54	182	NM_001192095.1
HDAC1	F-ATCGGTTAGGTTGCTTCAATCTG R-GTTGTATGGAAGCTCATTAGGGA	60	168	BT030718.1
DNMT1	F-CTCAGAAGGGAGATGTGGAG R-TAGTAGTCACAGTAGCTGAGGA	60	138	NM_182651.2
DNMT3A	F-GTGCTGTCTCTATTCGATGG R-CCATTCCTGGATATGCTTCTG	60	188	NM_001206502.1
BCL-2	F-ACTGCCAGAACAATATTCAACC R-GTAAGAATACCTTCAAAGGCGA	60	103	XM005695059
BCL-XL	F-TTGTGGCCTTTTTCTCCTTC R-GATCCAAGGCTCTAGGTGGT	60	128	ENSBTAT00000008572
BAD	F-CCAGAGCATGTTCCAGATCC R-GTTAGCCAGTGCTTGCTGAG	60	125	XM_005699970
P53	F-GGAAGAATCACAGGCAGAACTC R-ACTTCATTCGGACATTCATCCA	60	176	AB571118.1
SOD1	F-GAGAGGCATGTTGGAGACCT R-TCTGCCCAAGTCATCTGGTT	60	153	NM_001285550.1
SOD2	F-AATCTGAGCCCTAACGGTGG R-CAATCTGTAAGCGTCCCTGC	60	175	XM_018053428.1
SOD3	F-AGGCCTTCTTCCACCTTGAG R-GAAGTTGCCAAAGTCGCCC	60	167	XM_018049136.1
GAPDH	F-GGGTCATCATCTCTGCACT R-GGTCATAAGTCCCTCCACA	60	176	NM_001034034.2

hered rapidly and exhibited a consistent growth pattern of 15-20% confluency within 2 days, 40-50% within 3-4 days, and achieved 80-90% confluency within 4-5 days in early passages (Figure 1). Established fibroblasts were positive for vimentin and negative for cytokeratin-8 and -18 by immunostaining (Figure 2). They were also confirmed by RT-PCR, which showed a sharp band of vimentin, tubulin, and desmin, along with negative for cytokeratin-8 and cytokeratin-18 (Figure 3).

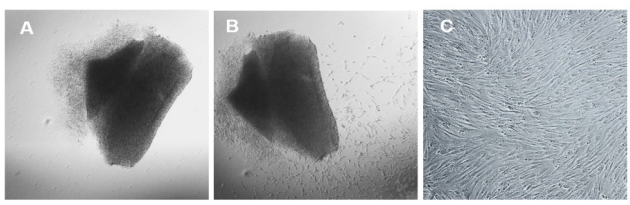

Figure 1: Emerging fibroblast cells from explant tissue (A-B), confluent cells (C).

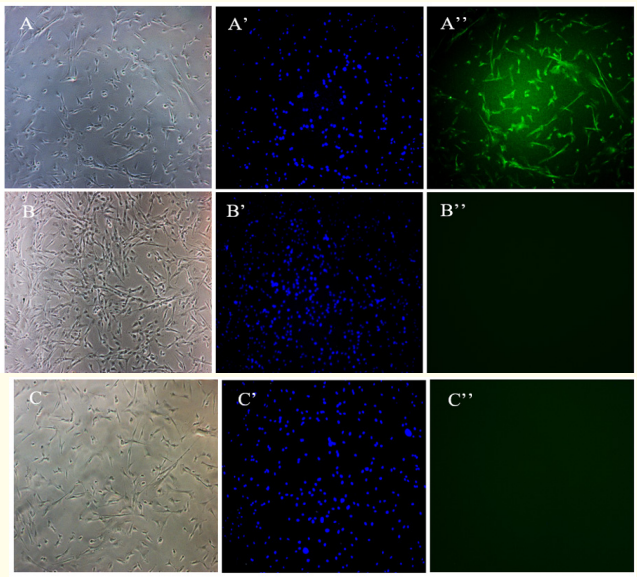

Figure 2: Characterization of fibroblast cells by immuno-staining: Vimentin (A), Cytokeratin-8 (B), Cytokeratin-18 (C). A, B, C are bright-field images; A', B', C' are Hoechst-33342 nuclear staining; A'', B'', C'' are FITC-conjugated Ab in fluorescent light, respectively.

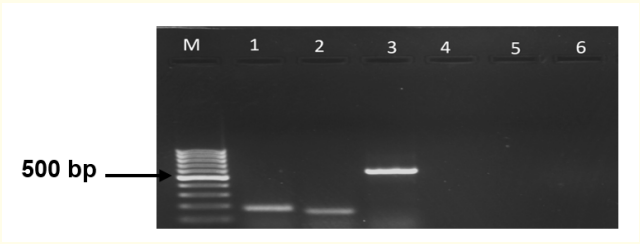

Figure 3: Characterization of cattle fibroblast cells by specific genes *VIMENTIN* (Lane-1: 183bp), *β-TUBULIN* (Lane-2: 151 bp), *DESMIN* (Lane-3: 578 bp), and negative for *CYTOKERATIN*-8 (Lane-4: 155 bp), *CYTOKERATIN*-18 (Lane-5: 182 bp), Lane-6: negative control and Lane-M: 100 bp ladder.

Metabolic activity of cells

The metabolic activity and proliferation rate of cultured fibroblast cells were determined using MTT substrate. The color produced from formazan crystals was observed at 0.5 µM, 1.0 µM, 2.0 µM, 5.0 µM, and 10.0 µM of oxamflatin, as well as at 25 µM, 50 µM, 100 µM, and 200 µM of ascorbic acid at different time intervals, specifically, after 24 h, 48 h, and 72 h of incubation. This was assessed through the relationship between absorbance (at 570 nm) and cell activity, as shown in Table 2 and 3. After 24 h, cell activity significantly increased ($P < 0.05$) at 1.0 µM oxamflatin and 50 µM ascorbic acid compared to the control and other concentrations. However, at 100 µM and 200 µM ascorbic acid, cell activity decreased ($P < 0.05$) relative to 50 µM but remained higher than the control and oxamflatin concentrations. After 48 h, oxamflatin at 1.0 µM significantly increased cell activity ($P < 0.05$) compared to the control, whereas 10 µM led to a significant decrease, suggesting toxicity at higher concentrations. Ascorbic acid at 50 µM also significantly increased cell activity ($P < 0.05$) compared to the control and other concentrations. After 72 h, 1.0 µM oxamflatin significantly increased cell activity ($P < 0.05$) compared to higher concentrations and the control, whereas 10 µM caused a significant decrease, indicating potential toxicity. Other oxamflatin treatments showed no significant change ($P > 0.05$). Ascorbic acid at 25 µM, 50 µM, and 100 µM increased cell activity, with the highest effect observed at 50 µM ($P < 0.05$). These findings suggest that 1.0 µM oxamflatin and 50 µM ascorbic acid are optimal concentrations for cell culture.

Table 2: Effect of oxamflatin and ascorbic acid on mitochondrial dehydrogenase activity in fibroblast cells after 24, 48, and 72 h of treatment.

Oxamflatin Conc.	OD at 570 nm		
	After 24 h	After 48 h	After 72 h
0 µM	0.0817 ± 0.0146[a]	0.1168 ± 0.0063[a]	0.3026 ± 0.0319[a]
0.50 µM	0.0926 ± 0.0054[a]	0.1342 ± 0.0046[a]	0.2872 ± 0.0338[a]
1.00 µM	0.1531 ± 0.0157[b]	0.1579 ± 0.0103[b]	0.4997 ± 0.0205[b]
2.00 µM	0.0846 ± 0.0141[a]	0.1183 ± 0.0055[a]	0.2960 ± 0.0125[a]
5.00 µM	0.0949 ± 0.0066[a]	0.1147 ± 0.0089[a]	0.2810 ± 0.0149[a]
10.00 µM	0.0899 ± 0.0040[a]	0.0410 ± 0.0129[c]	0.0244 ± 0.0101[c]

Different superscripts in columns indicate statistically significant differences (P < 0.05).

Table 3: Effect of ascorbic acid on mitochondrial dehydrogenase activity in fibroblast cells after 24, 48, and 72 h of treatment.

Ascorbic acid Conc.	OD at 570 nm		
	After 24 h	After 48 h	After 72 h
0 µM	0.0817 ± 0.0146[a]	0.1168 ± 0.0063[a]	0.3026 ± 0.0319[a]
25 **µM**	0.1396 ± 0.0289[b]	0.2386 ± 0.0087[b]	0.3868 ± 0.0128[b]
50 **µM**	0.4627 ± 0.0404[c]	0.5619 ± 0.0115[c]	0.5832 ± 0.0373[c]
100 **µM**	0.3187 ± 0.0285[d]	0.3235 ± 0.0033[d]	0.3678 ± 0.0185[b]
200 **µM**	0.2406 ± 0.0300[e]	0.2721 ± 0.0076[e]	0.3110 ± 0.0140[a]

Different superscripts in columns indicate statistically significant differences (P < 0.05).

Effect of oxamflatin and ascorbic acid on cell viability

After 24 h, oxamflatin treatment was administered in each well with concentrations of 0 µM (control), 0.5 µM, 1.0 µM, and 2 µM, respectively. After 48 h of post-treatment incubation, the total cell count is shown in Table 4. Oxamflatin at 1.0 µM significantly increased (P < 0.05) cell numbers compared to 0.5 µM, 2.0 µM, and the control. This indicates that lower concentrations promote fibroblast proliferation more than higher concentrations (Figure 4). Ascorbic acid treatment was administered in each well after 24 h at concentrations of 0 µM (control), 25 µg/ml, 50 µg/ml, 100 µg/ml, and 200 µg/ml, respectively. 48 h post-treatment, the total cell count is shown in Table 5. At 50 µM ascorbic acid, fibroblast cell numbers significantly increased (P < 0.05) compared to 25 µM, 100 µM, 200 µM, and the control. However, 25 µM does not affect cell proliferation and viability (Figure 5).

Gene expression patterns in fibroblast cells treated with oxamflatin

Epigenetic-related gene

The relative mRNA expression levels of acetylation (*HDAC1)* and methylation-associated genes *(DNMT1 and DNMT3A)* in oxamflatin-treated fibroblast cells at concentrations of 0 µM (control), 0.5

Conc. of oxamflatin	Live cell count	Live cell (%)
Control	$4.69 \pm 0.39 \times 10^{5a}$	98.50 ± 0.64^{a}
0.5 µM	$5.07 \pm 0.12 \times 10^{5a}$	95.75 ± 1.26^{a}
1 µM	$6.93 \pm 0.59 \times 10^{5b}$	97.50 ± 0.28^{a}
2 µM	$3.63 \pm 0.17 \times 10^{5c}$	97.25 ± 0.55^{a}

Table 4: Effect of oxamflatin on fibroblast cell proliferation and viability.

Different superscripts in columns indicate statistically significant differences ($P < 0.05$).

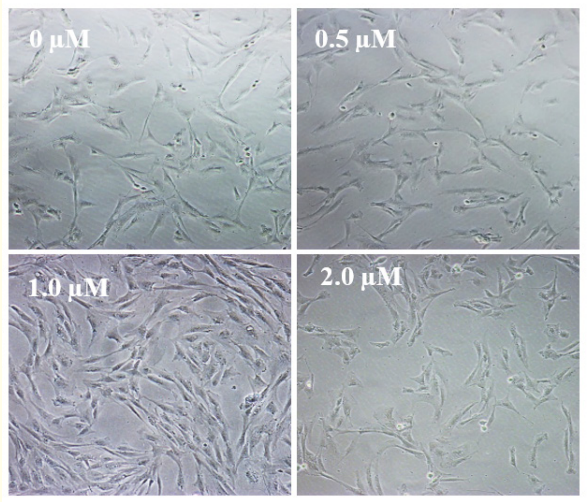

Figure 4: Fibroblast cell confluency and morphology after 48 h of oxamflatin treatment (0 µM, 0.5 µM, 1.0 µM, and 2.0 µM).

Table 5: Effect of ascorbic acid on fibroblast cell proliferation and viability.

Conc. of ascorbic acid	Cell count	Live cell (%)
Control	$5.10 \pm 0.13 \times 10^{5a}$	98.00 ± 0.81^{a}
25 µM	$5.09 \pm 0.41 \times 10^{5a}$	97.50 ± 0.50^{a}
50 µM	$7.53 \pm 0.07 \times 10^{5b}$	97.75 ± 0.47^{a}
100 µM	$6.05 \pm 0.38 \times 10^{5c}$	93.25 ± 0.13^{b}
200 µM	$6.13 \pm 0.36 \times 10^{5c}$	87.50 ± 0.66^{c}

Different superscripts in columns indicate statistically significant differences ($P < 0.05$).

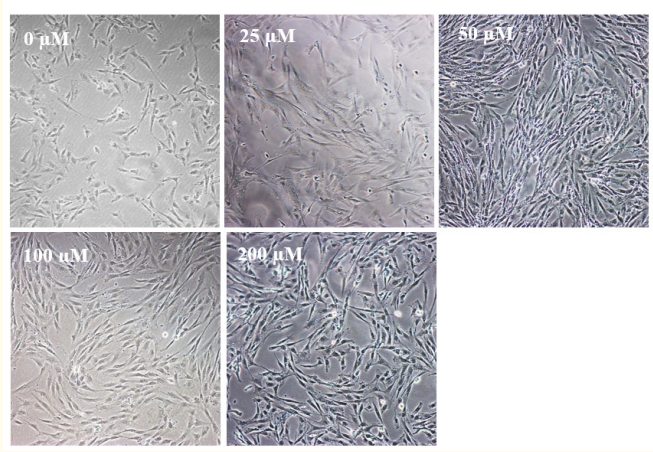

Figure 5: Fibroblast cell confluency and morphology after 48 h of ascorbic acid treatment (0 µM, 25 µM, 50 µM, 100 µM, and 200 µM).

µM, 1.0 µM, and 2.0 µM were as follows: for *HDAC1*, 1.00 ± 0.00, 0.67 ± 0.04, 0.29 ± 0.08, 0.57 ± 0.02; and *DNMT1* and *DNMT3A*, 1.00 ± 0.00, 0.55 ± 0.11, 0.35 ± 0.05, 0.45 ± 0.05; and 1.00 ± 0.00, 0.43 ± 0.10, 0.35 ± 0.05, 0.34 ± 0.09, respectively. The expression levels of the epigenetic genes *HDAC1, DNMT1*, and *DNMT3A* were significantly downregulated at all treatment concentrations (0.5 to 2 µM) compared to the control ($P < 0.05$). *HDAC1* showed a dose-dependent reduction, with 1 µM resulting in the most significant decrease ($P < 0.05$). In contrast, *DNMT1* and *DNMT3A* showed no significant differences ($P > 0.05$) across all treatment doses compared to each other, without exhibiting a dose-dependent trend (Figure 6).

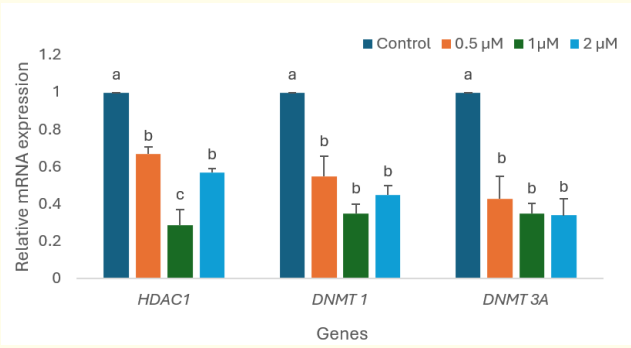

Figure 6: Effect of oxamflatin on epigenetic-related gene expression.

Antioxidant-related gene

The relative fold change expression of antioxidant-related genes *SOD1, SOD2,* and *SOD3* at concentrations of 0 µM (control), 0.5 µM, 1.0 µM, and 2.0 µM were 1.00 ± 0.00, 1.67 ± 0.14, 1.89 ± 0.09, 0.27 ± 0.02; 1.00 ± 0.00, 1.55 ± 0.12, 1.95 ± 0.15, 0.50 ± 0.05; 1.00 ± 0.00, 1.35 ± 0.11, 1.86 ± 0.13, 0.34 ± 0.09, respectively. Treatment with 0.5 µM and 1 µM significantly upregulated ($P < 0.05$) the expression of *SOD1, SOD2*, and *SOD3* compared to control, with 1 µM generally showing the highest expression. In contrast, 2 µM consistently resulted in a significant decrease ($P < 0.05$) in expression across all three genes (Figure 7). These differences are statistically significant ($P < 0.05$), indicating a dose-dependent effect where moderate concentrations enhance and high concentrations suppress antioxidant gene expression.

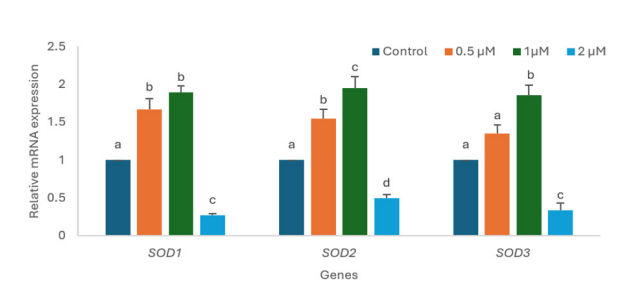

Figure 7: Effect of oxamflatin on antioxidant-related gene expression.

Apoptosis-related gene

The relative mRNA expression levels of the pro-apoptotic genes *BAD* and *P53,* and the anti-apoptotic genes *BCL2* and *BCL-XL* at concentrations of 0 µM (control), 0.5 µM, 1.0 µM, and 2.0 µM were 1.00 ± 0.00, 0.57 ± 0.04, 0.29 ± 0.06, 0.37 ± 0.02; and 1.00 ± 0.00, 0.75 ± 0.15, 0.45 ± 0.17, 0.65 ± 0.11; and 1.00 ± 0.00, 1.39 ± 0.11, 1.75 ± 0.05, 1.2 ± 0.09; and 1.00 ± 0.00, 1.45 ± 0.08, 1.85 ± 0.12, 1.55 ± 0.05, respectively. The pro-apoptotic genes *BAD* and *P53* were significantly downregulated ($P < 0.05$) at all concentrations, with maximum reductions observed at 1 µM and 2 µM ($P < 0.05$) as compared to the control. In contrast, the anti-apoptotic genes *BCL2* and *BCL-XL* were significantly upregulated ($P < 0.05$) at 0.5 µM, peaking at 1 µM. Although expression slightly declined at 2 µM, it remained higher than in the control (Figure 8). These results suggest that moderate concentrations, particularly 1 µM, suppress pro-apoptotic gene expression while enhancing anti-apoptotic gene expression.

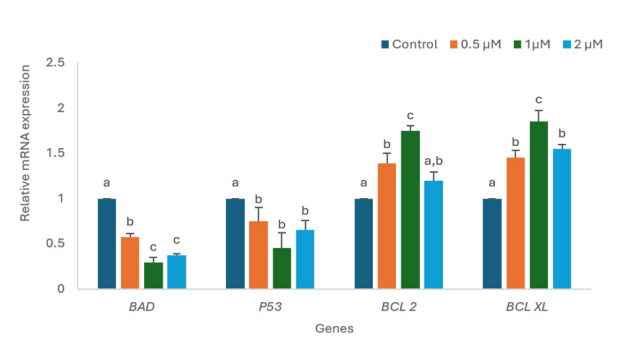

Figure 8: Effect of oxamflatin on apoptosis-related gene expression.

Gene expression patterns in fibroblast cells treated with ascorbic acid

Epigenetic-related genes

The relative fold change of *HDAC1, DNMT1*, and *DNMT3A* at concentrations of 0 µM (control), 25 µM, 50 µM, 100 µM, and 200 µM were 1.00 ± 0.00, 1.13 ± 0.06, 0.67 ± 0.03, 0.60 ± 0.01, 0.24 ± 0.01; and 1.00 ± 0.00, 0.51 ± 0.01, 0.33 ± 0.03, 0.68 ± 0.08, 0.64 ± 0.02; and 1.00 ± 0.00, 0.07 ± 0.01, 0.17 ± 0.05, 0.08 ± 0.01, 0.22 ± 0.04, respectively. *HDAC1* expression remained unchanged at 25 µM but significantly decreased ($P < 0.05$) at higher concentrations, with the lowest expression observed at 200 µM. *DNMT1* was downregulated considerably ($P < 0.05$) at 25 µM and 50 µM, with partial recovery at 100 µM and 200 µM. *DNMT3A* expression was consistently and significantly reduced at all treatment levels. These results indicate that increasing concentrations of ascorbic acid suppress epigenetic gene expression, with significant changes at $P < 0.05$ (Figure 9).

Antioxidant-related gene

The relative fold change of *SOD1, SOD2*, and *SOD3* at concentrations of 0 µM (control), 25 µM, 50 µM, 100 µM, and 200 µM were 1.00 ± 0.00, 1.57 ± 0.20, 1.89 ± 0.05, 1.85 ± 0.06, and 1.65 ± 0.04 for *SOD1*; 1.00 ± 0.00, 1.55 ± 0.05, 1.95 ± 0.03, 1.52 ± 0.06, and 1.45

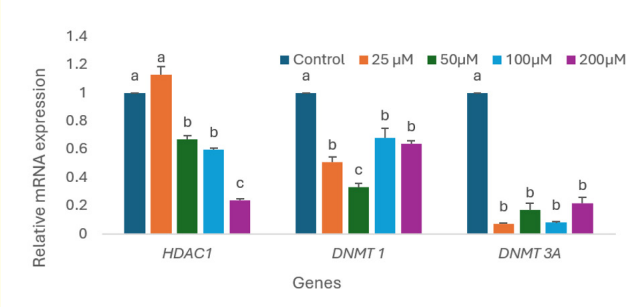

Figure 9: Effect of ascorbic acid on epigenetic-related gene expression.

± 0.03 for *SOD2*; and 1.00 ± 0.00, 1.35 ± 0.09, 1.86 ± 0.05, 1.34 ± 0.02, and 1.25 ± 0.07 for *SOD3*. The expression of *SOD1*, *SOD2* and *SOD3* increased significantly ($P < 0.05$) with treatment at all the concentrations, peaking at 50 µM. These findings (Figure 10) indicate that 50 µM is the most effective concentration for enhancing antioxidant gene expression, with all differences statistically significant at $P < 0.05$.

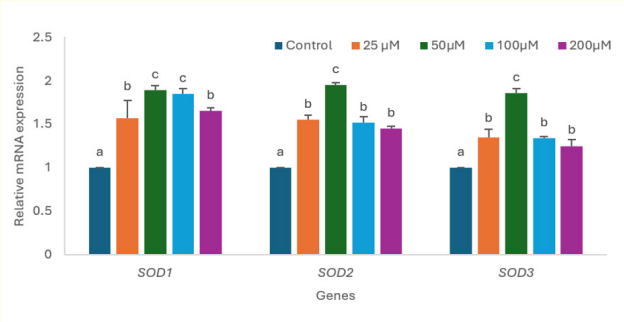

Figure 10: Effect of ascorbic acid on antioxidant-related gene expression.

Apoptosis-related genes

The relative mRNA expression of the pro-apoptotic gene *BAD, P53,* and the anti-apoptotic genes *BCL2* and *BCL-XL* at concentrations of 0 µM (control), 25 µM, 50 µM, 100 µM, and 200 µM were 1.00 ± 0.00, 0.67 ± 0.04, 0.35 ± 0.05, 0.37 ± 0.05, 0.32 ± 0.02, and 1.00 ± 0.00, 0.78 ± 0.05, 0.45 ± 0.06, 0.55 ± 0.05, 0.61 ± 0.01, and 1.00 ± 0.00, 1.39 ± 0.02, 1.85 ± 0.10, 1.72 ± 0.08, 1.75 ± 0.04, and 1.00 ± 0.00, 1.45 ± 0.09, 1.92 ± 0.18, 1.55 ± 0.04, 1.62 ± 0.08, respectively. Gene-wise comparisons across concentrations revealed distinct expression patterns. *BAD* and *P53* were significantly downregulated at higher doses, with the most significant suppression observed at 50-200 µM, respectively ($P < 0.05$). In contrast, *BCL2* and *BCL-XL* were significantly upregulated ($P < 0.05$) from 50 µM onward, with 50 µM showing the highest expression for both. This indicates a concentration-dependent shift from pro-apoptotic to anti-apoptotic gene expression, with 50 µM emerging as the most effective dose for promoting cell survival (Figure 11).

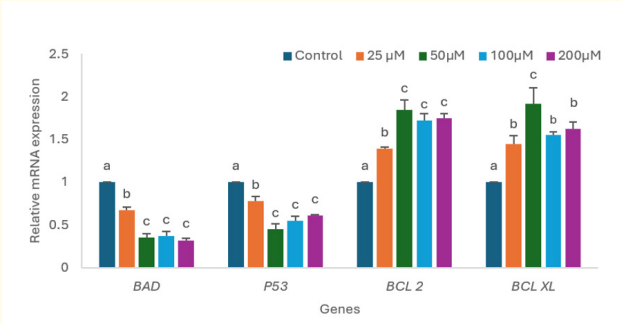

Figure 11: Effect of ascorbic acid on apoptosis-related gene expression.

Discussion

This study investigated the effects of oxamflatin, a histone deacetylase inhibitor (HDAC1i), and ascorbic acid, a well-established antioxidant and recognized epigenetic modulator, on buffalo fetal fibroblast cells. The objective was to evaluate individually the influence of these compounds on cell viability, metabolic activity, and the expression of genes associated with oxidative stress, apoptosis, and epigenetic regulation. Trypan blue exclusion and MTT assays were employed to assess cell viability and proliferation. Simultaneously, quantitative real-time PCR was used to analyze the expression profiles of key genes involved in DNA methylation and histone acetylation, antioxidant enzymes, and apoptotic markers. The results provide valuable insights into how modulation of the cellular redox state and epigenetic landscape can impact the bi-

ological behavior of somatic cells, with potential implications for cell-based therapies, improving cellular reprogramming and somatic cell nuclear transfer (SCNT) efficiency in buffalo.

Treatment with 1 µM oxamflatin and 50 µM ascorbic acid yielded the most favorable outcomes regarding cell viability, metabolic activity, and gene expression among the concentrations evaluated. The choice of 1 µM oxamflatin aligns with previous findings that demonstrate the effectiveness of low concentrations of histone deacetylase inhibitors (HDACi) in inducing epigenetic alterations without causing substantial cytotoxic effects in mammalian cells [15,16]. At this concentration, oxamflatin likely enhances histone acetylation, leading to a more open chromatin structure and improved transcription of genes involved in cellular protection and survival [17]. In contrast, the reduction in cell numbers at 2 µM indicates a threshold beyond which HDAC1i exposure may initiate stress responses or apoptosis, a trend consistent with dose-dependent toxicity commonly reported with other HDAC1 inhibitors [18]. Ascorbic acid at 50 µM appeared to support cellular antioxidant defense mechanisms by increasing the expression of key antioxidant enzymes, including *SOD*, *CAT*, and *GPx*, indicating better regulation of oxidative stress. This concentration aligns with reports from other cell systems where ascorbic acid promoted proliferation, mitigated oxidative damage, and contributed to improved cellular reprogramming [19,20]. Lower concentrations (25 µM) were insufficient to elicit a significant biological effect, while higher concentrations (200 µM) likely induced pro-oxidant behavior or altered redox signaling, negatively impacting cell viability [21]. These results align with, in sheep embryo cultures, supplementing the medium with 50-100 µM of vitamin C under both low (5%) and high (20%) oxygen levels enhanced cleavage rates, enhanced blastocyst development, and increased total cell counts. In contrast, higher doses negatively affected embryonic growth [22]. This biphasic effect of ascorbic acid has been documented in various cell types, where low-to-moderate levels support proliferation. At the same time, excessive concentrations may lead to cytotoxicity due to reactive oxygen species (ROS) imbalance [23].

Importantly, the combined use of oxamflatin and ascorbic acid at these concentrations will produce more pronounced effects than either compound alone, suggesting a potential additive or synergistic interaction that enhances cell performance and gene regulation. The expression profiles revealed a distinct pattern characterized by increased transcription of antioxidant and anti-apoptotic genes, alongside reduced expression of pro-apoptotic and DNA methylation-related genes. These molecular changes point to a more favorable intracellular environment, which may be especially beneficial for applications such as somatic cell nuclear transfer (SCNT), where donor cells functional and epigenetic state plays a pivotal role in reprogramming success and embryo development. Treatment of fibroblast cells with oxamflatin significantly downregulated *HDAC1, DNMT1,* and *DNMT3A* expression, with *HDAC1* showing a clear dose-dependent response. This agrees with previous findings where 1 µM oxamflatin significantly reduced *HDAC1* expression in porcine fetal fibroblasts [24]. Both *DNMT1* and *DNMT3A* were downregulated, though without a dose-dependent trend, consistent with studies showing that oxamflatin reduces *DNMT1* expression and global DNA methylation in somatic cell nuclear transfer embryos [25]. While direct reports on *DNMT3A* are limited, HDAC1 inhibitors are broadly known to suppress DNA methyltransferases [26]. The lack of a dose-dependent response in *DNMTs* may reflect post-transcriptional regulation, as seen in TGF-β1-treated human fibroblasts [27]. These results highlight oxamflatin potential as an effective epigenetic modulator targeting both histone acetylation and DNA methylation pathways.

Our results demonstrate that moderate concentrations of oxamflatin (0.5 and 1.0 µM) significantly upregulated antioxidant genes *(SOD1, SOD2, SOD3*), while high concentration (2.0 µM) suppressed their expression, indicating a dose-dependent response. This aligns with studies showing that moderate oxidative signals enhance *SOD* expression through pathways like NRF2, whereas excessive stimuli can lead to downregulation or oxidative damage [28]. Similarly, pro-apoptotic genes *BAD* and *P53* were downregulated, and anti-apoptotic genes *BCL2* and *BCL-XL* were upregulated at moderate doses, with 1.0 µM showing the strongest effect. These findings are supported by evidence that GSK3β inhibitors reduce *P53*-mediated apoptosis and promote cell survival [29] and that *BAD* activity is suppressed under prosurvival conditions [30]. Together, this suggests that 1.0 µM of oxamflatin is an optimal dose for enhancing antioxidant defenses and suppressing apoptosis.

Ascorbic acid has been shown to play a significant role in epigenetic regulation, particularly in the context of immune response and cell differentiation [31]. In the case of ascorbic acid treatment, our findings show that increasing concentrations of the treatment led to significant downregulation of epigenetic regulators *HDAC1, DNMT1,* and *DNMT3A,* with the strongest suppression observed at

200 µM. Ascorbic acid is involved in epigenetic regulations through the control of TET enzymes [32]. These results align with previous studies demonstrating that certain small molecules, ascorbic acid and GSK3β inhibitors, reduce *DNMT* and *HDAC1* expression, thereby promoting a more open chromatin state and enhancing transcriptional activity [33,12]. Notably, *DNMT3A* expression was consistently suppressed across all concentrations, suggesting higher sensitivity to the treatment. Ascorbic acid enhances TET enzyme activity in IVF embryos, restoring proper DNA demethylation and increasing 5hmC levels. This leads to improved blastocyst quality, higher cell counts, and better implantation outcomes, resembling in vivo development [34].

In contrast, the antioxidant genes *SOD1, SOD2,* and *SOD3* were significantly upregulated, reaching a peak at 50 µM before gradually declining at higher concentrations. This is consistent with reports that moderate levels of oxidative modulators activate antioxidant responses, primarily through NRF2 signaling, while excessive exposure may lead to a plateau or reduction in expression due to cellular feedback mechanisms [12,28]. In rat testis, ascorbic acid is protective, reducing apoptosis and increasing cell proliferation in response to malathion-induced toxicity [35,36]. Our findings also demonstrate that treatment with ascorbic acid induces a dose-dependent shift in apoptotic gene expression, characterized by significant downregulation of pro-apoptotic genes *BAD* and *P53*, and upregulation of anti-apoptotic genes *BCL2* and *BCL-XL*, with the most pronounced effects observed at 50 µM. This aligns with previous studies, adding ascorbic acid to embryo culture media (around 20 µg/ml) helps lower oxidative stress by reducing reactive oxygen species (ROS). This improves the rate of blastocyst formation and reduces cell death by increasing *BCL-XL* and decreasing *BAX* [37]. It has also been reported that compounds such as CHIR99021, PD0325901, and ascorbic acid can modulate apoptotic pathways to enhance cell survival. For example, CHIR99021, a GSK3β inhibitor, has been shown to suppress *P53*-mediated apoptosis and enhance the expression of survival-related genes via activation of the Wnt/β-catenin pathway [29,38]. Similarly, ascorbic acid has been reported to reduce *P53* expression and oxidative stress-induced apoptosis while promoting *BCL2* upregulation [39]. Our results support these findings, suggesting that 50 µM treatment optimally promotes cell viability by tipping the balance toward anti-apoptotic signaling and represents the optimal balance for enhancing antioxidant defenses while suppressing epigenetic repressors.

Conclusion and Prospects

This study highlights the potential of oxamflatin and ascorbic acid as effective modulators of cellular redox balance and epigenetic regulation in buffalo fetal fibroblast cells. When applied at optimal concentrations, both compounds significantly improved cell viability and metabolic activity while enhancing antioxidant responses and favorably altering apoptotic and gene expression profiles related to epigenetics. Specifically, treatment led to the upregulation of antioxidant and anti-apoptotic genes, while suppressing pro-apoptotic factors and epigenetic repressors, thereby promoting a more survival-supportive and transcriptionally active cellular state. These findings are highly relevant to reproductive biotechnology, as oxamflatin and ascorbic acid enhance donor cell quality, potentially improving SCNT efficiency in buffalo. Moreover, these findings extend beyond SCNT, offering potential applications in iPSC generation, cell therapy, regenerative medicine, and tissue engineering. Further studies on their combined and long-term effects could refine donor cell preparation for advanced applications.

Conflict of Interest

The authors have no conflict of interest.

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