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Original Article

The pattern of RNA integrity and the expression of housekeeping genes are influenced by sodium hypochlorite and ascorbic acid

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Abstract: Background: Basic biological science research deals with nucleic acid isolation. Post-isolation nucleic acid integrity has a pivotal role in further elucidating gene expression and other molecular mechanisms. RNA (ribonucleic acid), cDNA (complementary deoxyribonucleic acid), and PCR (Polymerase chain reaction) products' integrity and quality are affected by several factors in biochemical and biophysical degradation modes. Inadequate evidence was noted about the direct effects of sodium hypochlorite and L-ascorbic acid. Objectives: This study aims to test the effects of sodium hypochlorite (SHC) and L-ascorbic acid (LAA) in total RNA and PCR products, respectively, in an acellular condition. Methods: The study was categorized into three steps total RNA, cDNA, and PCR product evaluations. mBM-MSCs were used to extract RNA and then treated with SHC. Crude total RNA and, after DNase 1 treatment, the bands of total RNA samples were visualized by agarose gel electrophoresis. cDNAs were synthesized from SHC-treated (0.25%) and untreated RNAs, which were also expressed on the gel. LAA (5 µM, 15 µM, 25 µM, and 50 µM) were added to cDNAs synthesized from SHC- and non-SHC-treated samples. Housekeeping genes, Gapdh (Glyceraldehyde 3-phosphate dehydrogenase), and 18S rRNA (18S Ribosomal ribonucleic acid) were amplified in both groups. Results: SHC-treated samples produced clearer bands on an agarose gel. Its treatment did not affect the integrated densities of agarose bands which revealed non-significant ($P \leq 0.05$) differences in SHC-treated, untreated RNA, and cDNA. However, significant variations were observed at the PCR level. SHC-treated samples expressed decreased housekeeping gene expression in amplified products (Gapdh and 18S rRNA) and slightly but non-significantly high band intensities appeared in the presence of LAA. Significant variable differences ($*P \leq 0.05$) were observed between SHC-treated and non-treated groups after LAA treatment. Conclusions: SHC (0.25%) is favorable in removing RNases and maintaining the integrity of RNA. cDNA synthesis did not affect by SHC treatment, and it follows the same as untreated samples after DNase 1 treatment. LAA drew a positive impact to improve the quality of PCR products in terms of band intensities, which is insignificant in SHC-treated RNA. Interestingly, it was revealed from our study that 5-25 µM LAA has the most beneficial role in the acquisition of PCR products, *i.e.* gene expression. These concentrations can be safely used to improve the quality of gene expression. This phenomenon can be used to achieve other, rarer, desired gene expressions. Further research is needed to explore the effects of SHC on the acquisition of PCR products using other solutions.

Keywords: Bleach, DNases, Electrophoresis, Gapdh, RNA, RNases, Vitamin C

Introduction

Basic biological science research deals with nucleic acids, which include deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) isolation. Post-isolation, nucleic acid integrity has a pivotal role in further elucidating gene expression and other molecular mechanisms. RNA, cDNA, and PCR products' integrity and quality are affected by several factors, including the pH of solutions, temperatures, RNases, and the procedures of storage, and are considered to be biochemical and biophysical degradation modes. Nucleic acids contain genetic information that is used to synthesize RNA and proteins in cells. RNA is the main class of nucleic acids translated into proteins [1]. The removal of genomic DNA (gDNA) contamination in an RNA sample is crucial to attaining perfect results, especially in relation to gene expression. If both cDNA and gDNA sequences are amplified, it may lead to incorrect positive results. Notably,

fragmented DNAs are present in high concentrations with residual gDNA, which is also purified with RNA [2, 3]. The analysis for effective and accurate gene expression required the removal of genomic DNA. The ribonucleases (RNases) are found in the phylogenetic trees of prokaryotes, eukaryotes, and in some viruses as well. The integrity of the total RNA is largely affected by RNases [4, 5]. Since these enzymes are present in microorganisms and are released into the air. The laboratory samples are easily contaminated with these enzymes through the air [6]. Human saliva, tears, sweat, mucus, etc. are also sources of RNases that can quickly degrade RNA [7]. RNases can be active at a wide range of pH (2-10) and temperatures (15-80°C) and are resistant to various chemicals [5, 8-10].

Previous studies have revealed that exposure to hypochlorite breaks down the protein through the mechanism of oxidation [11]. Hypochlorite solution is easily available, inexpensive, and commonly used in molecular laboratories [12]. It was observed that commercial sodium hypochlorite (6% bleach) destroys the secondary structure of the RNA by breaking hydrogen bonds [13]. Another study presented that L-ascorbic acid (LAA; $C_6H_8O_6$) is very useful to neutralize sodium hypochlorite's (SHC; NaClO) effects, which is commonly utilized for disinfection purposes in water storage tanks. Ascorbic acid (AA) can neutralize the total residues of sodium hypochlorite in a few seconds. Usually, $1/4^{th}$ of a teaspoon of AA can successfully remove sodium hypochlorite from a gallon of water. AA is commonly recognized as vitamin C. It is a water-soluble chemical utilized as an additive in food and an antioxidant agent to boost the immune system [14]. It has been recommended as a neutralizer of chlorine toxicity in aquatic life [15]. It has also been suggested that the neutralization of sodium hypochlorite irrigation increases the tubular penetration of resin-based endodontic sealers [15, 16]. AA is also an antioxidant that effectively removes free radicals from reactive oxygen species [17]. It was observed that acellular DNA is damaged by free radical exposure, while low concentrations of Hydrogen peroxide (H_2O_2) are endurable. Enzymatic or non-enzymatic oxidation of LAA follows L-dehydroascorbate (DHA) formation by mono dehydro-L-ascorbate radical [18]. Many intermediates and H_2O_2 are formed by the reaction [19]. The combination of LAA and H_2O_2 generates free radicals, *i.e.,* •OH and DHA, and •OH cause DNA damage [15, 16]. Various chemical-based protocols have been designed to overcome these issues [20, 21]. However, it may have undesired effects on the secondary structure of RNA [2, 5]. Various chemical-based protocols have been designed to overcome these issues [22-24]. The affected RNA can later produce incredible results in research. Thus, laboratory safety measures should be taken to prevent RNA degeneration.

Inadequate evidence was noted about the direct effects of SHC on RNA or cDNA. Here, this study aims to test the effects of SHC and LAA on total RNA integrity and PCR product improvement, respectively, in an acellular condition. LAA was used to check the enhanced intensity of PCR amplified product in SHC-treated and non-SHC-treated groups implicating housekeeping genes, *i.e.,* Gapdh (Glyceraldehyde 3-phosphate dehydrogenase) and 18S rRNA (18S Ribosomal ribonucleic acid).

Materials and methods

Animals

Mice were used in accordance with institutional and international animal care and use guidelines instructions in pathogen-free conditions in the animal facility of Sindh Institute of Urology and Transplantation (SIUT), Karachi-74200, Pakistan, with ethical approval from the institutional ethics committee.

Isolation of mouse bone marrow MSCs

Bone marrow mesenchymal stem cells were isolated from murine BALB/c mice of both sexes of 6-8 weeks old, 25-30 gram weight. ~3-5 mice were used in this study. Mice were euthanized by cervical dislocation. At first, the bone marrow was harvested from the tibia and femur bones of mice in aseptic conditions. A 21 gauge needle syringe containing a culture medium was used to flush bone marrow. The culture medium consists of Dulbecco's Modified Eagle Medium (DMEM, Cat. #. 12440-053, Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Cat. #. 16000-044, Gibco), 1% penicillin/streptomycin (Cat. #. 15140-122, Gibco), and 1% L-glutamax (Cat. #. 35050-061, Gibco). The compact bone

marrow was dispersed in the medium and the cells were maintained in T25 flasks in a humidified, 5% CO_2 incubator at 37°C [25]. The medium was replaced in 3-4 days. The mBM-MSCs were sub-culture or expended using 0.25% trypsin-EDTA (Cat. #. 25200-056, Gibco) after ~70-80% confluence of MSCs. Different passages (P) of MSCs were used in this study. It is stated as P10, P11, P12, P13, and P15.

RNA extraction and sodium hypochlorite (SHC) treatment

Total RNA was extracted from mBM-MSCs using TRIzol reagent (Cat. #. 15596-018, Invitrogen). RNA quality was determined by a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). An aliquot of 1 µg of RNA was mixed with 0.25% and 0.125% in SHC. SHC was previously diluted in distilled water and incubated on ice for ten minutes. Hence, RNA samples were considered treated with 0.25% and 0.125% of SHC.

Treatment of RNA with DNase 1

SHC-treated and non-treated (plane) mBM-MSCs' RNA samples were further treated with DNase 1 (1 µL/µg), Cat. #. M610A Promega). At first, RNA was incubated with DNase 1 for 30 minutes at 37°C. Then, the samples were heated for 10 minutes at 75°C to deactivate the DNase 1. Finally, RNA samples were placed on ice immediately and stored at -80°C.

Gel electrophoresis for RNA

RNA integrity was checked before and after SHC (0.25% and 0.125%) and DNase 1 treatments by agarose gel electrophoresis. 1% agarose gel (Cat. #. A9539 Sigma) was prepared in TBE (0.5%) buffer [54 g of Tris base, 27.5 g of boric acid, and 20 mL of 0.5 µM Ethylenediaminetetraacetic acid (EDTA), pH 8.0]. The agarose was heated in the microwave oven for 45 seconds. 0.4 µg of ethidium bromide (Cat. #. 2218.1, Roth) was added to the agarose solution and left to solidify for gel formation. The wells in agarose gel were loaded with 1 µg of sodium hypochlorite-treated and untreated RNA samples. 1 µL of 100 base pairs (bp) DNA ladder (Cat. #. 10488-058, Invitrogen) was used as a standard. Electrophoresis was carried out at 90 volts for 45 minutes. The ultraviolet (UV) exposure resulted in the detection of RNA bands on Gel Doc (Gel DocTM XR+ and ChemiDoc™ XRS+ Systems with Image Lab™ Software). The expression of each gene was analyzed based on the intensities of bands obtained, via Image J software.

Synthesis and detection of cDNA

The cDNA was synthesized from 1 µg of SHC-treated (0.25%) and untreated RNA using High-Capacity cDNA PCR Master Mix (Applied Bio-Systems, USA). Reverse transcriptase PCR reactions were included in the first step at 25°C for 10 minutes of incubation. The second step was carried out at 37°C for 120 minutes. The third step was 85°C for 5 minutes, and the final Polymerase chain reactions (PCR) products were hold at 4°C. The quality of cDNA bands was detected on 2% TBE agarose gel. The gel images were analyzed as previously described.

L-ascorbic acid treatment and conventional PCR

To neutralize the bleaching effects of SHC, cDNA samples were treated with various selected concentrations of LAA (Cat. #. A4544, Sigma) comprising 5 µM, 15 µM, 25 µM, and 50 µM. PCR were performed for the amplification of housekeeping genes, including Gapdh (77 bp) and 18S rRNA (138 bp). PCR reactions were amplified using abm 2X PCR Taq Plus Master Mix (Cat. #. G014-dye, Applied Biological Materials Inc.). A PCR program of 35 cycles was set at 56°C Tm. It was performed with a thermal profile at 94°C for 30 seconds, 56°C for 30 seconds, 72°C for 30 seconds, 72°C for 7 minutes, and hold at 4°C.

Expression of reference genes by gel electrophoresis

Electrophoresis was used to detect amplified products of Gapdh (77 bp) and 18S rRNA (138 bp) on an agarose gel (2%). The procedure was the same as mentioned above. The agarose gel (2%) (Cat. #. A9539 Sigma) was prepared in TBE buffer (0.5%) [54 g of Tris base, 27.5 g of boric acid, and 20 mL of 0.5 µM Ethylenediaminetetraacetic acid (EDTA), pH 8.0]. The agarose was heated in the microwave oven for 45 seconds. 0.4 µg of ethidium bromide (Cat. #. 2218.1, Roth) was added to the agarose solution and left to solidify for gel formation. The

Table 1. Oligonucleotide sequences of housekeeping genes

Housekeeping genes	Primer Sequences	Base pairs	ᵀTm
ᵅm-Gapdh-F	5'-TGTGTCCGTCGTGGATCTGA-3'	77	56
ᵅm-Gapdh-R	5'-CCTGCTTCACCACCTTCTTGA-3'		
ᵝm-18S rRNA-F	5'-TAACGAACGAGACTCTGGCAT-3'	138	56
ᵝm-18S rRNA-R	5'-CGGACATCTAAGGGCATCACAG-3'		

ᵅmouse-Glyceraldehyde 3-phosphate dehydrogenase (Forward/Reverse); ᵝmouse-18S Ribosomal ribonucleic acid (Forward /Reverse); ᵀMelting Temperature.

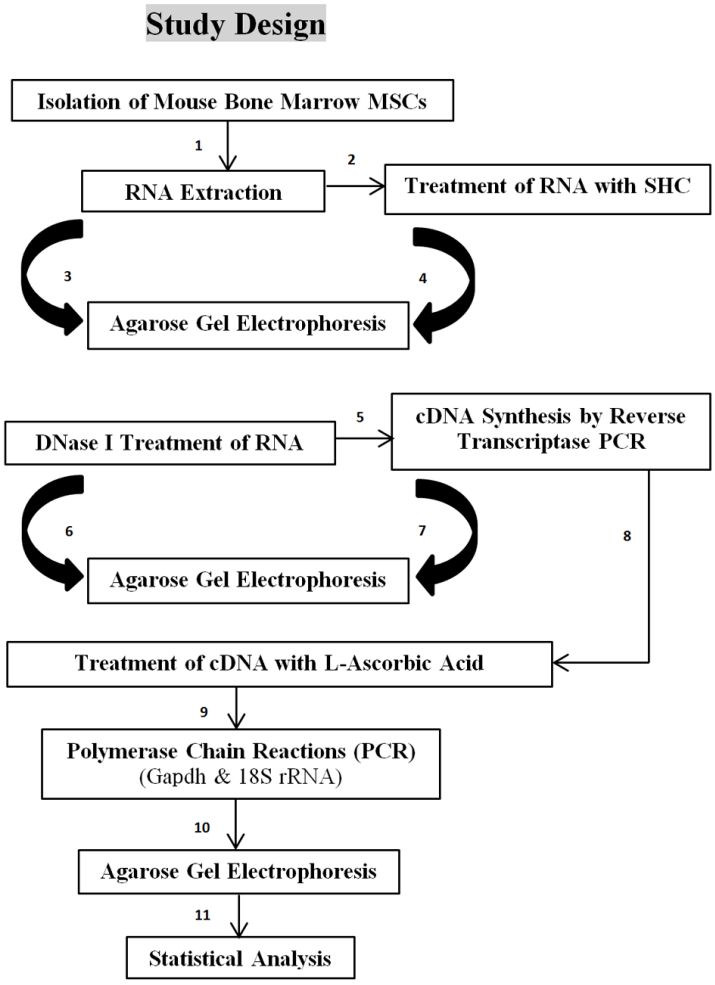

Figure 1. Schematic experimental design of the study.

wells in agarose gel were loaded with 1 µg of sodium hypochlorite-treated and untreated RNA samples. 1 µL of 100 base pairs (bp) DNA ladder (Cat. #. 10488-058, Invitrogen) was used as a standard. Electrophoresis was carried out at 90 volts for 45 minutes. The ultraviolet (UV) exposure resulted in the detection of RNA bands on Gel Doc (Gel DocTM XR+ and ChemiDoc™ XRS+ Systems with Image Lab™ Software). Gel images were recorded. The expression of each gene was analyzed based on the intensities of bands obtained via Image J software. The oligonucleotide sequences of reference genes are mentioned in **Table 1**.

The experimental design of the study is presented in **Figure 1**.

Statistical analysis

Integrated densities were measured by presetting a scale of 100 units/mm in ImageJ software. To evaluate the data and determine the effects of SHC (0.25% and 0.125%) and LAA (5 µM, 15 µM, 25 µM, and 50 µM), data analysis was carried out using IBM SPSS Statistics 20.0 software. The Kruskal-Wallis test and Mann-Whitney tests were used to determine significance at a 95% (alpha = 0.05/P ≤ 0.05) confidence interval.

Results

Effect of SHC exposure on RNA integrity

RNA bands on agarose gel were found to be clearer, especially in P13 and P10 SHC-treated (0.25% and 0.125%) mBM-MSC RNAs, as shown in **Figure 2A** and **2B**, but were not observed without SHC or plane RNA. In addition, P10 RNA displayed a light-intensity band, which may be due to a low concentration of total RNA yield. On the other hand, P12 RNA in **Figure 2C** presented visible bands after 0.25% of SHC treatment. However, the 0.125% SHC-treated RNA (P12) sample showed smears in appearance, bands were not detected, and band intensity was comparatively high in untreated RNA. Total untreated RNA showed smear in **Figure 2A** and **2B** bands or disappeared bands in **Figure 2C**. Presently, the analysis of total RNA quality by agarose gel electrophoresis displayed an apparently enhanced quality of total RNA after treatment with SHC as

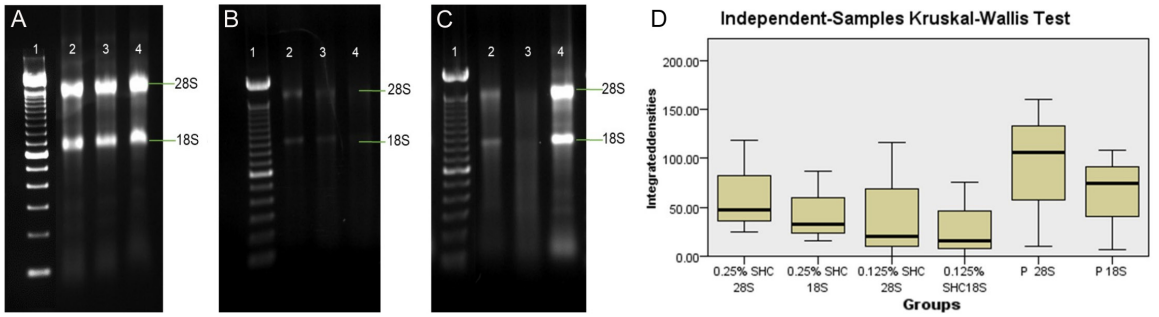

Figure 2. Effects of sodium hypochlorite (SHC) exposure on ribonucleic acid (RNA) integrity. Effects of SHC were checked on RNA integrity at passages P13, P10, and P12 (A-C, respectively). The appearance of RNA bands was enhanced after treatment with 0.25% and 0.125% concentrations of SHC (lanes 2 and 3, respectively) in comparison to SHC-untreated or plane (P, lane 4). The bands on the agarose gel were clearer in SHC-treated lanes. However, the statistical *p*-value was not P ≤ 0.05 (D). Lane 1 exhibited the DNA standard. Confidence level = 95; Criteria *P ≤ 0.05.

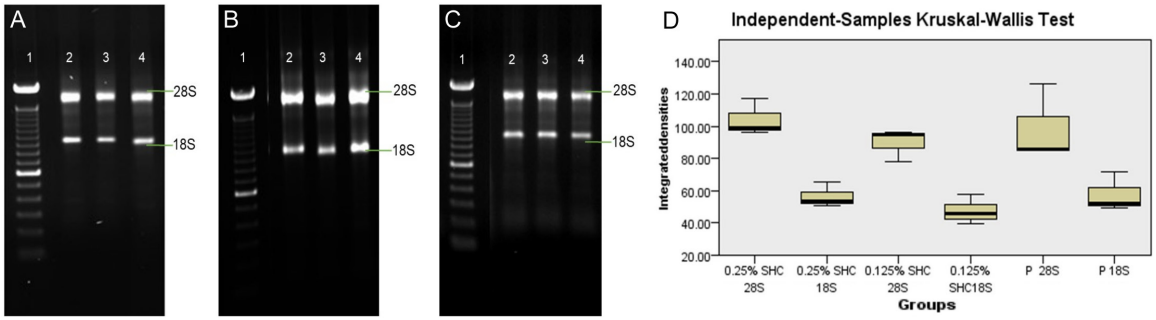

Figure 3. Deoxyribonuclease I (DNase 1) treatment in ribonucleic acid (RNA) samples. The band intensities were distinctly prominent in (A), P13, (B), P15, and (C) P12 samples as a result of DNase 1 treatment of sodium hypochlorite (SCH)-treated (0.25% and 0.125%, lanes 2 and 3, respectively) and SHC-untreated or plane (P, lane 4). However, no significant difference was calculated among both groups (D). Lane 1 exhibited the DNA standard. Confidence level = 95; Criteria *P ≤ 0.05.

compared to untreated RNA, although with an insignificant *p*-value (**Figure 2D**).

Removal of gDNA from RNA samples by DNase 1 treatment

Electrophoresis results of DNase 1-treated RNA (P13, P15, and P12) indicated prominently removed genomic DNA from SHC-treated and untreated RNA, as shown in **Figure 3A-C** individually. Moreover, after the removal of genomic DNA, bands of 28S rRNA and 18S rRNA were found to be clearer among SHC-treated and untreated RNA samples than the RNA without DNase 1 treatment. The higher band intensities were observed apparently in SHC-treated samples, while untreated samples displayed variable results. Furthermore, statistical analysis revealed no statistically significant differences between the untreated and treated groups (**Figure 3D**).

Post DNase 1 treatment, cDNA quality check by electrophoresis

The RNA bands in SHC samples were found to be absolutely clearer in comparison to those untreated in the cDNA extracted from P13 and P15 mBM-MSCs in **Figure 4A** and **4B** respectively. However, cDNA from the other sample, P12, displayed light bands with a slight difference from mBM-MSCs treated with and without SHC (**Figure 4C**). Although the statistical *p*-value was not significant (P = 0.127, P ≤ 0.05), 10 to 20 units higher band intensity was observed in SHC-treated cDNA as compared to the untreated samples (**Figure 4D**).

Post-LAA exposure effects on Gapdh

Post-LAA treatment revealed that the Gapdh expression was significantly lower (*P ≤ 0.05) in SHC-treated samples compared to SHC-untreated samples presented as SHC and

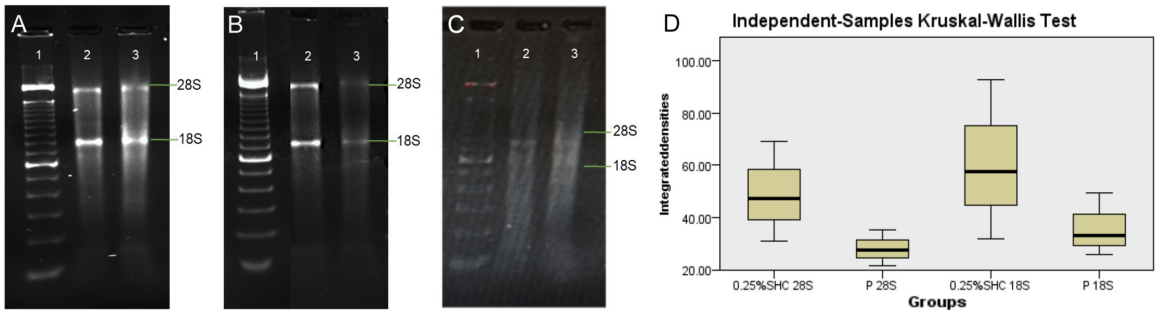

Figure 4. Analyses of complementary deoxyribonucleic acid (cDNA) quality after DNase 1 treatment by electrophoresis. The cDNA of P13, P15, and P12 were synthesized after incubation with sodium hypochlorite (SHC) (0.25%) and without SCH RNA, as shown in (A-C) respectively. The bands of RNA were comparatively clearer in SHC samples (lane 2) than in SHC-untreated or plane (P, lane 3). However, no significant difference was detected (P = 0.128) (D). Lane 1 exhibited the DNA standard. Confidence level = 95; Criteria *P ≤ 0.05.

Plane separately. On the other hand, no significant difference was found in Gapdh expressions in SHC and LAA treated (SHC + 5 μM, SHC +15 μM, Plane + 25 μM, and SHC + 50 μM) and untreated SHC and LAA treated (Plane + 5 μM, Plane + 15 μM, Plane + 25 M, and Plane + 50 μM) samples, as seen in P13, P10, and P12 in the designated **Figure 5A-D**.

Post-LAA exposure effects on 18S rRNA

cDNA quality check showed tightly integrated bands on an agarose gel. Surprisingly, 18S rRNA expression was significantly low (*P ≤ 0.05) in the samples of three different passages samples including P13, P10, and P12 SHC, untreated LAA (SHC), and SHC, LAA, treated samples (SHC + 5 μM, SHC + 15 uM, and SHC + 50 μM) than SHC and LAA untreated samples (Plane) and untreated SHC, LAA treated (Plane + 5 μM, Plane + 15 μM, Plane + 50 μM) indicated in **Figure 6A-C**. Where minor differences were investigated in the expression of 18S rRNA between two groups of samples (SHC + 25 μM) and (Plane + 25 μM), that is statistically not significant. Surprisingly, we noticed significant (*P ≤ 0.05) positive effects of LAA on 18S rRNA expression in untreated SHC samples. Because of the presence of LAA in concentrations of 5-50 μM, the band's intensities of 18S rRNA were relatively high (**Figure 6D**).

Discussions

Initially, the analysis of molecular studies using RNA samples involved a crucial step to check their integrity and quality. RNA integrity can be used to categorize RNA samples for suitability to downstream gene expression analysis, independently of the RNA degradation mechanism. In this study, we used very low concentrations of SHC (0.25% and 0.125%), which are simple to handle and could rapidly improve the total RNA quality. Many procedures for total RNA isolation are available, which take protective measures because of the presence of toxic chemicals in solutions and may include extra washing steps, which make the protocol lengthy [5]. Overall, we inferred that SHC (0.25%) did not affect the RNA integrity as the results were similar to non-treated RNA; further, no truncated band appeared in any sample treated with SHC. Moreover, the appearance of cleared bands indicated the removal of RNases in SHC-treated RNA. While smeared, RNA showed unclear bands, with the possibility of contaminants or RNases' presence in untreated RNA. The effects of different concentrations of SHC on RNA purity were determined in one study by supplementing SHC in an agarose gel. In this work, SHC was utilized for decontamination purposes. They demonstrated that low concentrations of SHC demolished RNases. They revealed that increases in SHC concentration in gel from 0.5-5% further enhanced RNA integrity or purity [5]. Similar results were obtained when RNA was directly treated with SHC in our study; the upper band indicate 28S rRNA (4.8 kb), while the lower band indicate 18S rRNA (2.0 kb). The results pointed to a clearer appearance in all SHC-treated samples. The DNA ladder was completely detected on 1% gel. Currently, in our study, 0.25% SHC was more effective as compared to 0.125% SHC.

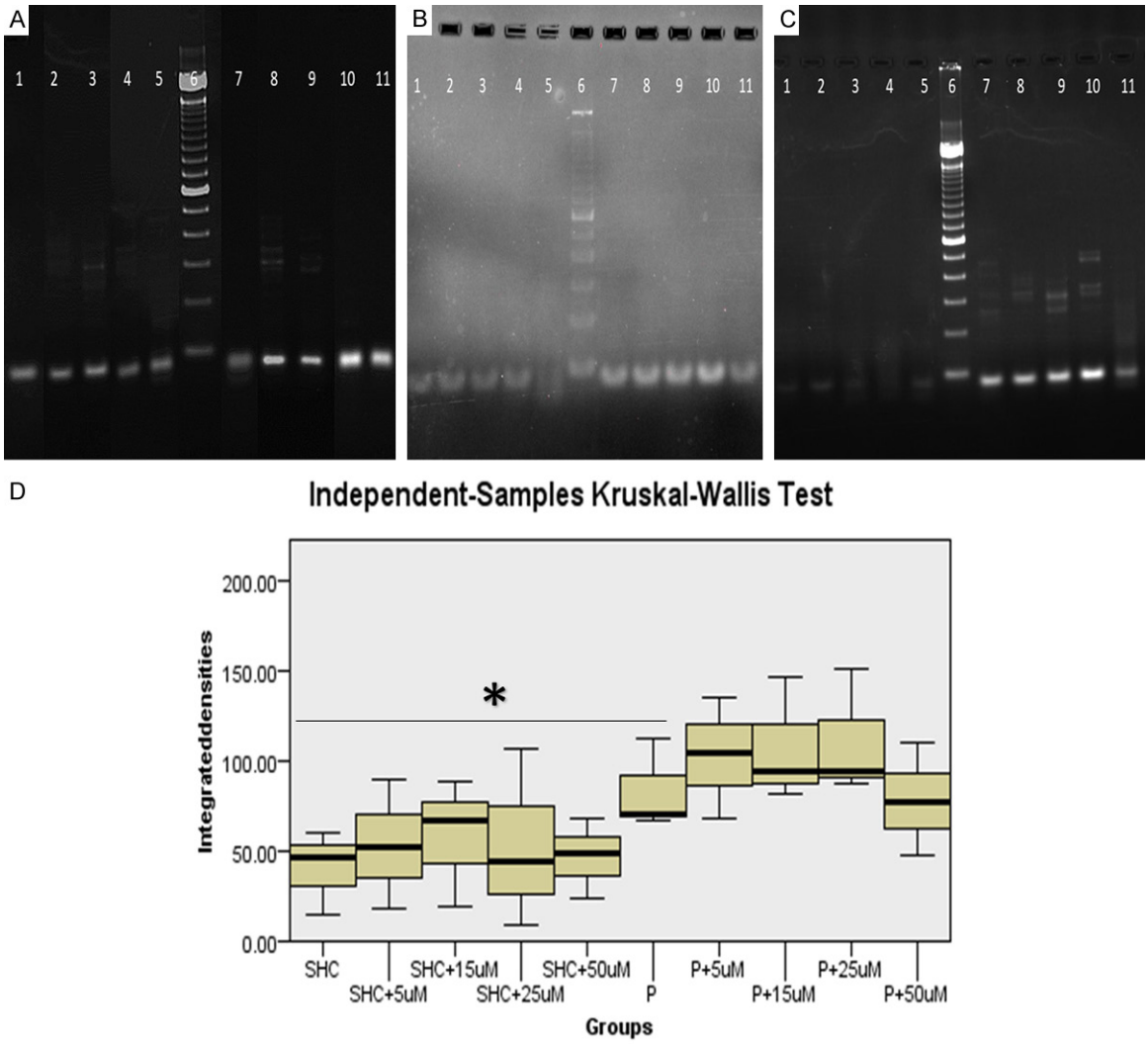

Figure 5. Expression of Glyceraldehyde 3-phosphate dehydrogenase (Gapdh) after sodium hypochlorite (SHC) and L-ascorbic acid (LAA) treatment. The Gapdh expression was meaningfully low (*P ≤ 0.05), in the SHC-treated sample in P13 (A), P10 (B), and P12 (C), respectively. *e.g.,* SHC (lane 1) in comparison to the SHC-untreated plane (P, lane 7). However, no significant differences in Gapdh expression were detected between SHC and LAA-treated samples, such as SHC + 5 μM (lane 2), SHC + 15 μM (lane 3), Plane + 25 μM (lane 4), and SHC + 50 μM (lane 5), as well as SHC untreated and LAA, treated samples labeled as Plane + 5 μM (lane 8), Plane + 15 μM (lane 9), Plane + 25 μM (lane 10) (D). Lane 6 exhibited the DNA standard. Confidence level = 95; Criteria *P ≤ 0.05.

Genomic DNA (gDNA) contamination and amplification lead to incorrect positive results. A research study conducted described that in general, DNase 1 is very useful to remove DNA contamination (up to 10 μg/mL) from RNA, present in traces and at moderate levels [3]. In agarose gel, genomic DNA appears as a band above the 28S band. DNase 1 treatment did not affect SHC-treated samples. Therefore, it proceeded for the PCR amplification, to observe housekeeping genes expression.

As 0.25% of SHC was found to be more effective in preserving RNA integrity, for this reason, cDNA was synthesized only from 0.25% SHC-treated and untreated RNA. Past studies have shown that SHC-based chemicals are extremely effective in removing nucleic acid even after a short period of reaction [12]. All the results were contrary to previous studies that had to use low concentrations of SHC to remove nucleic acid.

Basic cellular functions in all cells of an organism are maintained by certain constitutive gene expression or housekeeping genes, including Gapdh and 18S rRNA. Irrespective of physiological and pathophysiological degradation of

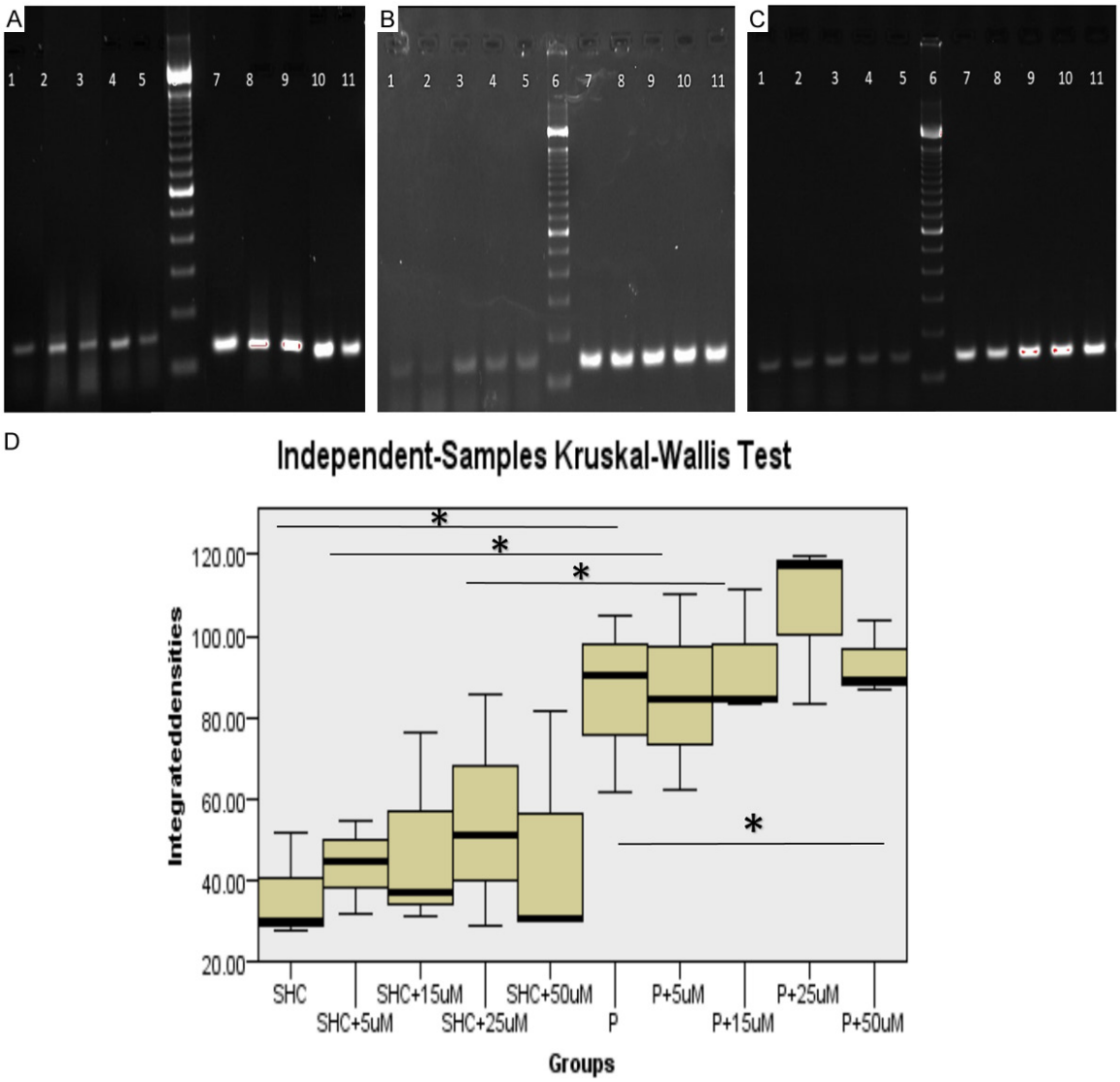

Figure 6. Expression of 18S ribosomal ribonucleic acid (18S rRNA) after sodium hypochlorite (SHC) and L-ascorbic acid (LAA) treatment. Sodium hypochlorite (SHC), L-ascorbic acid (LAA)-treated and untreated SHC samples, and LAA-treated samples in P13 (A), P10 (B), and P12 (C) were tested for 18S rRNA expression. The expression of 18S rRNA was remarkably lower after SCH treatment (lane 1) than in SHC-untreated or plane (P, lane 7) (*P ≤ 0.05). In particular, LAA treatment also results in significantly lower (*P ≤ 0.05) expression of 18S rRNA in SHC + 5 μM (lane 2), SHC + 15 μM (lane 3), and SHC + 50 μM (lane 5) in contrast to the plane + 5 μM (lane 8), plane + 15 μM (lane 9), and plane + 50 μM (lane 11). In addition, no considerable changes in 18S rRNA expression were examined in SHC + 25 μM and Plane + 25 μM samples after LAA exposure (D). Lane 6 exhibited the DNA standard. Confidence level = 95; Criteria *P ≤ 0.05.

RNA; isolated or acellular RNA integrity is required for the correct and meaningful interpretation of comparison and correlation in gene expression data. The quality and intact RNA quantity is required for downstream applications in molecular biological methods like PCR, qRT-PCR, microarray, and RNA sequencing for research and clinical implication. Impaired quality of RNA may present false positive and negative results, for example under or over-expression of oncogenes.

Usually, LAA is very useful in counteracting the effect of SHC. Although improved procedures may impact the quality of RNA, cDNA, and PCR products, a few studies were found on the direct impact of SHC and LAA. It was planned to examine the effects of SHC-treated cDNA and

LAA on the expression of housekeeping genes in mBM-MSCs. A significant increase in glutathione was reported by the addition of LAA. It plays a role as a cofactor in multiple enzymatic reactions. However, the depth of the molecular mechanism of action is indefinite [26]. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is a highly abundant cellular protein, and an excessively available protein, in stress conditions. It is known to be one of the most prominent targets of H_2O_2 during cellular stress [27], which causes the oxidation of GAPDH catalytic cysteine to a sulfenic acid [28]. Moreover, in the presence of oxidizing agents, GAPDH goes through conformational alterations [29]. The role of AA was described for the treatment of cancer and the synthesis of DNA during mammalian cell development [30]. AA can inhibit GAPDH expression in colorectal cancer cells (CRCs) [31, 32]. Cultured colorectal cancers (CRCs) are specifically targeted when exposed to high levels of vitamin C. Because of the elevated consumption of the oxidized form of vitamin C, dehydroascorbate (DHA). Increased DHA intake via GLUT-1 receptor induces oxidative stress, as cellular DHA has converted to an oxidized form of vitamin C-reducing glutathione. As a result, ROS accumulates and inhibits GAPDH [31]. These findings also support the hypothesis that LAA ultimately affects the DNA at the transcriptional level for gene expression. In this study, the direct effect of LAA exposure was observed in acellular conditions on PCR amplification in terms of housekeeping gene expression. The treatment of cDNA with varying concentrations of LAA (5 μM, 15 μM, 25 μM, and 50 μM) produced diverse effects on the expression of Gapdh as well as on 18S rRNA.

Cumulatively, SHC (0.25%) did not affect RNA integrity or cDNA synthesis, but reduced the level of PCR products. Therefore, it can be suggested that LAA could react with sodium hypochlorite if it was remaining in cDNA-prepared samples, or due to some unknown reason, they produce negative effects that ultimately mark the expression of housekeeping genes, as shown in SHC-treated samples, which had very low expressions of Gapdh as well as 18S rRNA in terms of agarose gel bands appearance. The underlying mechanism of low expression of SHC-treated RNA and post-cDNA formation only at PCR levels is unknown. It is known that the reaction of LAA and H_2O_2 generates OH radicals, which damage the DNA [20, 21]. An apparent negative effect of bleach was not demonstrated on cDNA, but displayed a negative effect on the expression of housekeeping genes in PCR-amplified products. Therefore, we used LAA to maximize the PCR products. In general, about 2.5 parts of ascorbic acid are required to neutralize one part of chlorine. Unexpectedly, we observed that increasing concentrations of LAA reduced gene expression. Therefore, we were unable to check higher concentrations (agarose gel data not shown).

In untreated SHC samples, mostly housekeeping gene expression was elevated after LAA treatment, indicating a positive effect of LAA on PCR products. Plane and SHC-untreated samples produced an improved quality of gene expression. It was inferred from the effect of LAA on the expression of the transcription that 5-25 μM LAA are effective concentrations for both, Gapdh and 18S rRNA band intensities in both SHC and non-SHC-treated PCR products. This phenomenon can be used to achieve other, uncommon or rarer, desired gene expressions. A study demonstrated the effects of different concentrations of L-ascorbic acid-2-phosphate on the proliferation and metabolism of human MSCs through serial gene expression and metabolome analysis. The addition of L-ascorbic acid 2-phosphate (0.1, 0.3, 1, or 3 mM), to the culture medium, clearly encourage and dose-dependently promoted cell proliferation at 24 hours post-seeding, without a significant difference between tested concentrations. Furthermore, LAA was shown to increase Hypoxia-inducible factor 1-alpha (HIF1α) hydroxylase activity, inhibit HIF1α transcription, cause mitochondrial activation, affecting cell metabolism and proliferation [32]. Further studies are required to profoundly understand the effects of LAA on gene expression levels.

Conclusions

SHC (0.25%) is favorable in removing RNases and maintaining the integrity of RNA. cDNA synthesis did not affect by SHC treatment, and it follows the same as untreated samples after DNase 1 treatment. Housekeeping gene amplified products (Gapdh and 18S rRNA) were influenced by LAA. LAA drew a positive impact to improve the quality of PCR products in terms of band intensities, which is insignificant in SHC-

treated RNA. Interestingly, it was revealed from our study that 5-25 μM LAA has the most beneficial role in the acquisition of PCR products, *i.e.* gene expression. These concentrations can be safely used to enhance the quality of gene expression. This phenomenon can be used to achieve other, rarer, desired gene expressions. Further research is needed to explore the effects of SHC on the acquisition of PCR products using other solutions.

Acknowledgements

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Disclosure of conflict of interest

None.

Abbreviations

18S rRNA, 18S Ribosomal Ribonucleic Acid; AA, Ascorbic Acid; ANOVA, Analysis of Variance; bp, base pairs; EDTA, Ethylenediaminetetraacetic acid; gDNA, Genomic DNA; H_2O_2, Hydrogen peroxide; HIF1α, Hypoxia-Inducible Factor 1-Alpha; Gapdh, Glyceraldehyde 3-phosphate dehydrogenase; LAA, L-ascorbic acid; mBM-MSCs, Mouse Bone Marrow Mesenchymal Stem Cells; P, Passage; SHC, Sodium Hypochlorite; TBE, Tris-Borate-EDTA.

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