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Concentration dependent antioxidative and apoptotic effects of sulforaphane on bovine granulosa cells *in vitro*

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ABSTRACT

Sulforaphane (SFN) has received a great deal of research attention because of its ability to induce the production of a battery of antioxidant enzymes in certain concentrations through the activation of the Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) signaling pathway, which may effectively neutralize reactive oxygen species (ROS) induced oxidative stress. This study was conducted to investigate the potential of different concentrations of SFN in inducing antioxidative and apoptotic effects in granulosa cells (GCs). For this purpose, bovine GCs were collected from preovulatory antral follicles and cultured with different concentrations of SFN (0–80 μM) and based on phenotypic evaluation three concentrations were selected: 2 μM (low), 10 μM (medium), and 20 μM (high) for further investigations. The results showed that there was a dramatic loss of cell viability and higher cytotoxic effects of SFN on GCs at higher concentrations (>15 μM). The expression of *NRF2* increased significantly ($p < 0.05$) with fold change ranged 3–8 in SFN treated GCs, whereas Kelch Like ECH Associated Protein 1 (*KEAP1*) expression was either downregulated or similar as control group under the same conditions. Moreover, the relative expression of the genes (*PRDX1*, *CAT*, *TXN1*and *SOD1*) downstream to *NRF2* activation was found to be highly expressed (fold change ranged from 2 to 5, $p < 0.05$) in SFN treated GCs compared to the untreated control. In addition, ROS accumulation was higher in GCs treated with 20 μM SFN which in turn results in a higher accumulation of lipid droplets. Compared to control, no changes in the mitochondrial activity was observed at 2 and 10 μM SFN concentrations; however, significantly lower mitochondrial activity was found at high concentration (20 μM). The results of this study clearly showed that 10 μM SFN concentration played a crucial role in activating Nrf2 pathway without inducing apoptotic characteristics and this concentration may have beneficial effects in boosting the production of phase II antioxidant enzymes in GCs. However, at high concentration (20 μM), SFN may generate excessive ROS that causes mitochondrial dysfunction and induces cellular stress and eventually leads to apoptosis. These data strongly suggest a concentration dependent antioxidative and apoptotic effects of SFN on GCs.

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1. Introduction

GCs are the major cellular component in an ovarian follicular microenvironment and serve diverse indispensable functions during follicular growth and ovulation [1]. Shortly before ovulation, a massive luteinizing hormone (LH) surge provokes GCs to differentiate into luteal cells. Progesterone synthesis in GCs is orchestrated by steroidogenic acute regulatory (StAR) protein, whose expression is known to be modulated by LH surge [2,3]. Furthermore, progesterone synthesized by luteinizing GCs is essential for the formation of the corpus luteum and maintenance of pregnancy. After the LH surge, inflammatory cells (e.g. vascular endothelial cells, macrophages and neutrophils) are massively recruited at the site of follicular rupture and produce excessive amount of ROS which cause acute inflammation, facilitate follicular rupture, and ovulation [4,5]. It also suggests that GCs are exposed to

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elevated level of ROS during ovulation. On the other hand, in follicular atresia, apoptosis in GCs commenced by oxidative stress partly due to the overproduction of ROS which is supported by the fact that macrophages are found in follicular fluid of the atretic follicle [6].

Imbalance between the production of ROS and the ability of antioxidants to scavenge them potentially creates a condition known as oxidative stress [7,8]. Despite their significant role in living organisms, excessive generation of ROS is potentially responsible for a range of disorders and diseases including heart diseases, muscle degeneration, and cancer [9,10]. Immediately after production, ROS can interact with all biological macromolecules near or at the site of their production and are generally involved in the peroxidative degradation of proteins and lipids which potentially leads to the destruction of cell membrane, loss of mitochondrial activity, degradation of cellular structure and finally cells and tissue damage [11]. Importantly, the endogenous defense system against ROS, i.e. a team of enzymatic antioxidants, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) [12], and non-enzymatic antioxidants including vitamin C, vitamin E, melatonin, albumin, and uric acid are found in the follicles which can scavenge excessive ROS and restore homeostasis.

Enzymatic antioxidant defense mechanism is a very complex process in which the Nrf2-ARE antioxidant pathway plays a central role in inducing the transcription of phase II antioxidant enzyme genes [13,14]. Under homeostatic condition, NRF2 remains inactive by binding with KEAP1 and Cullin-3 and has a very short half-life (around 20 min) in the cytoplasm. However, under oxidative stress condition, electrophiles signal the disruption of the KEAP1-NRF2 bond and NRF2 is translocated to the nucleus. In the nucleus, NRF2 first interacts with small Maf proteins to form a heterodimer [15], which later binds with antioxidant response element (ARE) and initiate the transcription of an array of antioxidative genes including peroxiredoxin-1 (*PRDX1*), catalase (*CAT*), superoxide dismutases (*SODs*) and thioredoxin-1 (*TXN*). NRF2-ARE pathway has been shown to be played an important role against atrazine-induced oxidative stress mediated damages in mouse ovarian tissues [16]. In addition, deletion of NRF2 transcription factor results reduced number of ovarian follicles and accelerates ovarian ageing in mice [17]. However, the role of NRF2-ARE pathway in granulosa cell apoptosis and proliferation is not completely understood. Many compounds including oltipraz, curcumin, SFN, and 3H-1, 2-dithiole-3-thione are well known inducers of the Nrf2-ARE pathway [13]. Among these, SFN clearly stands out as one of the most potent inducers of Nrf2-ARE signaling pathway [18].

SFN is a dietary isothiocyanate produced by the vegetables of the genus *Brassica* such as cabbage, brussels sprouts and broccoli [19]. SFN induces Nrf2 signaling pathway through thiocarbamylation at Cys151 resulting the disruption of the NRF2-KEAP1 bonding and inhibition of NRF2 ubiquitination, followed by translocation to the nucleus and promote the transcription of phase II antioxidative genes [18]. In addition, SFN also enhances the induction of the survival signaling proteins such as B-cell lymphoma 2 (BCL2) and downregulates the proteins of death signaling pathway [20]. In addition, growing number of evidences have indicated that SFN not only protects cells against oxidative stress associated diseases [19], but is also capable of regulating the growth of mammalian cells and causes apoptosis at higher concentrations [21,22]. However, its concentration dependent effects on GCs, whether it activates Nrf2-ARE surviving pathway or induces apoptosis pathways, have not been investigated yet. Thus, the current study aimed to investigate the concentration dependent effects of SFN in inducing antioxidative and/or apoptotic effects on GCs.

2. Materials and methods

2.1. Collection of ovary and GCs

Bovine ovaries (n = 100, from ~60 females) were collected from the local slaughterhouse after the official permission given by the slaughterhouse management to use samples only for research purpose. Ovaries were kept in 0.9% saline solution at 38 °C in a thermo-flask and transported to the laboratory within 2 h of collection. In the laboratory, ovaries were first washed three times with pre-warmed 0.9% saline solution and then dipped into 70% ethanol for 1 min. Following additional three washes with saline solution, ovaries were kept in warm DMEM/F-12 media (Sigma Aldrich, D5546-6X500ML, St. Louis, MO) supplemented with penicillin (100 U/mL) and streptomycin (100 μg/mL) (Sigma-Aldrich, P4333, Steinheim, Germany). The follicular materials (follicular fluid containing GCs) were aspirated by 18-gauge needle attached to a 5-mL syringe form mature preovulatory follicles (13–18 mm in diameter) and placed into a 50-mL sterile falcon tube containing 15 mL DMEM/F-12 media supplemented with penicillin (100 U/mL) and streptomycin (100 μg/mL). Oocyte cumulus complex and cellular debris were allowed to settle down and upper liquid containing GCs was transferred in a 15-mL tube and centrifuged at 500*g* for 7 min.

2.2. GC culture and SFN treatment

GCs were collected and cultured according to the previous protocol [23]. Briefly, GC pellets were resuspended in 1 mL of red blood cell lysing solution (8.26 g/L NH_4Cl) for 1 min to remove erythrocytes. Immediately after incubation 5 mL of DMEM/F-12 media containing 10% FBS was added in order to restore the isotonicity. After two additional washes with DMEM/F-12 media approximately 5×10^4, 1.5×10^5, and 6×10^5 viable cells were seeded in the 8-well chamber slide, 24-well and 6-well culture plates, respectively, containing DMEM/F-12 media supplemented with 10% FBS (Pan-Biotech GmbH, P30-8500, Aidenbach, Germany), penicillin (100 U/mL) and streptomycin (100 μg/mL) at 37 °C in a humidified atmosphere of 5% CO_2 until 50–60% confluence. To investigate the effect of SFN, primary cultures of GCs were exposed to 0.05% DMSO (Miltenyi Biotec GmbH, Teterow, Germany) or different concentration of SFN (1–80 μM) (Sigma Aldrich, S4441-5MG, St. Louis, MO) for 24 h under culture condition. Based on phenotypic evaluation of treated cells, three concentrations (low, medium and high) out of eight concentrations were chosen for further investigations. Following treatment cells were investigated for their phenotypic characteristics or harvested using Trypsin EDTA (Biological Industries, cat no. 03-052-1B, Kibbutz Beit Haemek, Israel) for genetic analysis. The purity of isolated GCs was determined by analyzing the presence of *FSHR* (GC specific marker gene) and *CYP17A1* (theca cell specific marker gene) and the results are presented in Supplementary Material Fig. 1.

2.3. Cell morphology and viability

Untreated control (0.05% DMSO treated) and SFN-exposed GCs were routinely observed under an inverted microscope (Nikon Eclipse TS100, Tokyo, Japan) throughout the experiment for morphological changes and confluence. Viability of cells was determined as previously described [24] with some modifications. Briefly, after the incubation period both adherent and floating cells were collected and resuspended in 1 mL of complete medium by gentle pipetting. Following that, 100 μl of cell suspension and 100 μl

of 0.4% trypan blue were mixed into a micro-centrifuge tube and allowed to incubate for 1–2 min at room temperature. 10 μl of cell mixture/trypan blue was applied to the hemocytometer and placed under a microscope for counting live and dead cells. The percentage of viable cells was calculated as follows: percentage of cell viability = (number of unstained cells/number of total cells) × 100.

2.4. Cytotoxicity assay

Cytotoxic effect of different concentrations of SFN was determined using Cell Proliferation Reagent WST-1 (Roche Diagnostics GmbH, Mannheim, Germany) kit according to the manufacturer's protocol. Briefly, 1×10^4 viable GCs were plated in each well of 96-well microplate (flat bottom, tissue culture grade, Corning Incorporated, New York, USA) containing 100 μl/well culture medium and continue culture for 24 h in a humidified atmosphere (37 °C and 5% CO_2). Old media of each well was replaced by new media containing different concentrations of SFN (0–80 μM) and culture for another 24 h. Following the culture period 10 μl WST-1 reagent was added to each well of the microplate and incubate for 5 h at 37 °C with the presence of 5% CO_2. Following the incubation period, the microplate was shaken for 1 min and absorbance was measured using a microplate (ELISA) reader (Glomax Multi Detection System, Promega Bio Systems Sunnyvale, CA, USA) with the wavelength range of 460 nm. Blank measurements were obtained from wells containing only culture medium and used for normalization.

2.5. Extraction of total RNA and cDNA synthesis

Total RNA was extracted from GCs using miRNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. An on-column DNase digestion step using RNase-Free DNase set (Cat no. 79254, Qiagen, Hilden, Germany) was performed during total RNA isolation in order to remove any additional DNA contamination. Purity and concentration of Total RNA were determined by a BioSpec-nano spectrophotometer (Shimadzu Biotech, Japan). The RNA was then reverse transcribed to cDNA using Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Massachusetts, USA) according to the manufacturer's instruction. The cDNA synthesis reaction consists of 5× RT Buffer (4 μl), 10 mM dNTP mix (1 μl), Maxima H Minus Enzyme mix (1μl), and oligo dT and random hexamer primers (0.25μl each). First, oligo dT, Random primer and dNTP was added to the 200 ng of total RNA and the reaction volume was adjusted to 15 μl by adding nuclease free water and incubated at 65 °C for 5min. After that, other components were added to the mixture and incubated for 10 min at 25 °C followed by 15 min at 50 °C and the reaction was terminated by heating at 85 °C for 5 min.

2.6. Real time quantitative PCR

The resulting cDNA samples were then used to investigate the expression of target genes where GAPDH was considered as reference in Roche Light cycler 480 real-time PCR machine (Roche Life Science, Penzberg, Germany). The stability of GAPDH expression was tested among the samples (data not shown). The qRT-PCR reaction was set up by adding 10 μl 1× Power SYBR Green I (Bio-Rad, Hercules, CA) master mix, 0.3 μM of forward and reverse gene specific primers, 7.4 μl deionized water and 2 μl first-strand cDNA template. The thermal cycling conditions were 3 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Comparative CT (2−ΔΔCT) method was employed to analyze the relative expression of each mRNA. Using a sequence specific primers, a total of 7 genes namely *NRF2*, *KEAP1*, Caspase 3 (*CASP3*), superoxide dismutase-1 (*SOD1*), peroxiredoxin1 (*PRDX1*), thioredoxin-1 (*TXN1*) and catalase (*CAT*) were examined for their expression in GCs. The primers were designed using primer3web version 4.0.4 (http://bioinfo.ut.ee/primer3/) and the sequences are listed in Supplementary Table S1.

2.7. Intracellular ROS accumulation

Accumulation of intracellular ROS in untreated control (0.05% DMSO treated) and SFN treated GCs were assessed by the fluorescent dye 2′7′-dichlorodihydrofluorescein diacetate (H_2DCFDA; Molecular Probes, Eugene, OR) according to the manufacture's protocol. Briefly, GCs from each group were first washed two times with DPBS and then 400 μl of 15 μM H_2DCFDA was added to each well of 24-well culture plate and incubated for 30 min in dark at 37 °C. Following the incubation cells were washed twice in PBS, and images were captured immediately under a fluorescence microscope (Nikon Eclipse T*i*-S microscope, Nikon Instruments Inc., Tokyo, Japan) using a green-fluorescence filter. Mean fluorescence intensity of five non-overlapping fields per well was measured using ImageJ software.

2.8. Accumulation of lipid droplets

Accumulation of lipid droplets in SFN treated GCs were assessed using Oil Red O stain (Sigma Aldrich, O1391, St. Louis, MO) using manufacturer's protocol. Briefly, approximately 1.5×10^5 viable GCs were added to each well of 24-well culture plate and grown up to 50–60% confluence. After that various concentration of SFN were added to cells and continue culture for another 24 h. Following the incubation period, media was completely removed and cells were washed two times with PBS and fixed by 10% formalin for 30 min at room temperature. Immediately prior to the staining, Oil red working solution was prepared and then incubated with cells for 40 min at room temperature. Following staining cells were washed three times with distilled water and visualized by Leica DMIL LED inverted microscope (Leica microsystem Ltd., Germany).

2.9. Mitochondrial activity

The activity of mitochondria was examined using MitoTracker1 Red CMXRos (M7512; Life Technologies, Oregon, USA) using manufacturer's protocol. Briefly, GCs from different treatment groups were washed twice with DPBS and then 100 nM Mito Tracker red dye was added and incubated for 45 min. GCs were then washed two times with DPBS and were fixed overnight with 4% formaldehyde. In the next day fixed cells were mounted with Vectashield Mounting Medium with DAPI (H-1200, Vector Laboratories, Inc., CA, USA). Images were acquired by a fluorescence microscope (Nikon Eclipse T*i*-S microscope, Nikon Instruments Inc., Tokyo, Japan) using a red-fluorescence filter. Mean fluorescence intensity of five non-overlapping fields in each well was measured using ImageJ software.

2.10. Detection of apoptosis using TUNEL assay

Apoptosis in GCs was analyzed by Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) assay using Click-iT® Plus TUNEL Assay kit (Life Technologies, Inc., Carlsbad, CA, USA) according to manufacturer's instruction. In brief, at the end of the indicated treatments, 50 μl of TdT reaction mixture was added to each well of 96-well plates and incubated for 1 h at 37 °C, followed by 30 min incubation with the Alexa Fluor® 594 dye (red fluorescence). Then, the cells were counterstained with 5 ng/mL DAPI (ThermoFisher Scientific) in PBS for 10 min and observed under a fluorescence microscope (Nikon Eclipse T*i*-S microscope,

Nikon Instruments Inc., Tokyo, Japan) using a red-fluorescence filter. The TUNEL-positive nuclei of five non-overlapping fields per well were counted, and these counts were converted to percentages by comparing the TUNEL-positive counts to the total number of cell nuclei as determined by DAPI counterstaining.

2.11. Statistical analysis

A minimum of three biological replicates in each experiment were performed. Statistical differences between treatment group means (quantitative variables) were analyzed by one-way ANOVA followed by a multiple pairwise comparison (Tukey test, $\alpha = 0.05$). For statistical analyses and graphs SPSS version 20.0 and Excel 2016 were used. Data are expressed as mean ± SEM of three biological replicates. Differences were considered as significant at $p < 0.05$.

3. Results

3.1. Viability of GCs in different concentrations of SFN

Despite its beneficial role against oxidative stress, higher concentrations of SFN are reported to have detrimental effects on survival and function of different cell types [22]. Therefore, we investigated the effect of a wide range of concentrations of SFN (0, 1, 2, 5, 10, 15, 20, 40, and 80 μM) on GCs to identify the concentration where the cells started to face additional stress and drop their viability significantly and 0.05% DMSO-treated cells were considered as control. The effects of various concentrations of SFN on viability of GCs were determine by trypan blue exclusion test, and results are shown in Fig. 1A. Although no significant difference was found up to 10 μM concentration, consistently reduced viability was observed at higher concentrations. For instance, 98-97% viability was observed at 1–2 μM concentrations, whereas the viability of GCs was ~90% at 10 μM concentration. On the other hand, survival ability of GCs reduced significantly upon a 24-h exposure at ≥15 μM concentrations of SFN and lowest viability was observed at highest concentration (80 μM) of SFN (Fig. 1A).

According to the viability test, it is clear that SFN caused a dose dependent loss of cell viability in GCs which, perhaps, indicating higher level of cytotoxicity on GCs at higher concentrations. Therefore, we sought determine the cytotoxic effects of indicated concentrations of SFN on GCs using WST-1 reagent and the results are presented in Fig. 1B. The results revealed that SFN-induced cytotoxicity was also dose dependent and followed a similar pattern like viability results. For instance, low doses of SFN <5 μM had no significant cytotoxic effect, whereas medium concentrations (~10 μM) had very little cytotoxic effects and higher concentrations >15 μM were found to be extremely cytotoxic to GCs (Fig. 1B). It is important to note that, both viability and cytotoxicity test suggested that lower concentrations of SFN had no cytotoxic effect, while higher concentrations are highly cytotoxic and had detrimental impacts on cell viability.

3.2. SFN enhance the expression of NRF2 and attenuates the expression of KEAP1

Based on phenotypic observations, out of eight concentrations, three different concentrations were chosen for further investigations, i.e. 2, 10, and 20 μM representing low, medium, and high doses of SFN, respectively. We first investigated the expression level of *NRF2* and *KEAP1* mRNAs under different concentration of SFN in order to have an idea whether the 'Nrf2-ARE pathway' is activated or not. Analysis of qRT-PCR data revealed that, no significant changes in the expression of *NRF2* was observed in cells treated with 2 μM SFN, while a significant increase in the expression of *NRF2* was observed in 10 and 20 μM SFN treated groups (Fig. 2A). Although no significant difference in the expression of *NRF2* was observed between 10 and 20 μM concentrations, higher expression trend was found in the cells treated with 20 μM concentration. Expression of *KEAP1* decreased in SFN treated GCs compared to untreated control (Fig. 2B). Compared to control, no significant difference in *KEAP1* expression was observed in cells treated with 2 μM SFN. However, significantly lower *KEAP1* expression was observed in 10 and 20 μM SFN concentrations (Fig. 2B).

3.3. Expression of ARE genes downstream to Nrf2 pathway

Next, we investigated the expression level of selected antioxidant genes downstream of *NRF2* activation, namely *PRDX1*, *CAT*, *TXN1*, and *SOD1* using qRT-PCR. Interestingly, almost similar expression patterns were observed for all candidate genes

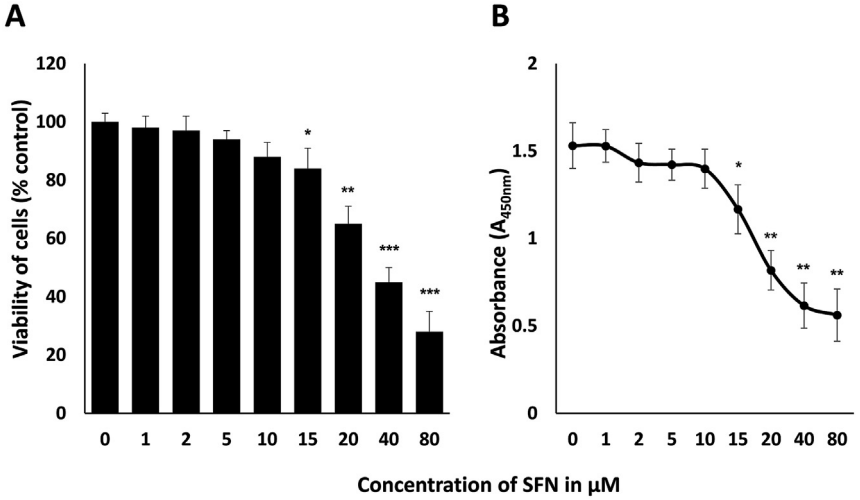


Fig. 1. Viability and cytotoxic effects of different concentration of SFN in GCs. After an exposure with SFN (ranging from 0 to 80 μM) for 24 h, cells were subjected to trypan blue dye exclusion test for cell viability (A) or WST-1 test for cytotoxic effect in different concentrations (B). Data shown are mean ± SEM (n = 3), different asterisks represent statistically significant differences where * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$.

(Fig. 3A–D). For instance, no significant difference was observed in any of the antioxidant genes at 2 μM concentration. However, expression of all genes significantly increased in GCs treated with 10 or 20 μM SFN concentrations compared to control (0.05% DMSO) (Fig. 3A–D). Although expression of *PRDX1* significantly increased at 20 μM compared to 10 μM (Fig. 3A), no significant differences were observed in other genes at these concentrations (Fig. 3B–D).

3.4. High concentration of SFN increases the expression of genes associated with apoptosis in GCs

In order to investigate whether high concentration of SFN induces cell death through apoptotic pathway, we sought to investigate the expression of *BAX* and *CASP3* in GCs treated with different concentrations of SFN. As shown in Fig. 4A, there was no significant difference in the expression level of *BAX* in 2 μM and 10 μM concentration compared to control. A significant increase (3 fold) in the expression level of *BAX* was observed in the cells exposed to 20 μM SFN. Similar to *BAX*, no significant difference was observed in the expression level of *CASP3* in 2 μM SFN and slightly increased expression (~2 fold) was observed in 10 μM concentration; however, significantly higher expression (~5 fold) was found in 20 μM SFN (Fig. 4B).

3.5. High concentration of SFN leads to higher GC apoptosis

To further verify the role of SFN in inducing cellular apoptosis, GCs were grown in 96-well plate and treated according to the experimental plan. The results of in situ detection of fragmented DNA by TUNEL assay in GCs cultured with different concentrations of SFN are presented in Fig. 4. Only 0.5% TUNEL-positive cells were detected in untreated control GCs, while 2.3% and 3.3% TUNEL-positive cells were present in 2 μM and 10 μM SFN treated GCs, respectively (Fig. 5). Significantly higher number of apoptotic cells (32%) were detected after 24 h of incubation with 20 μM SFN (Fig. 5).

3.6. SFN causes higher accumulation of ROS and lipid droplets at higher concentrations

We next investigated the accumulation of ROS in order to understand the SFN induced loss of cell viability is indeed due to excessive production of intracellular ROS. GCs were incubated with different concentrations of SFN and after 24 h incubation cells were subjected to ROS sensitive fluorescent dye H_2DCFDA. As shown in Fig. 6A and B, exposure to SFN generates ROS in GCs in a concentration dependent manner. No differences were observed between 0 and 2 μM SFN treatment and there was a slight increase in ROS accumulation in 10 μM concentration. A sharp increase in ROS accumulation was observed in GCs treated with 20 μM SFN (Fig. 6A and B).

Excessive level of ROS can induce activation of SREBP which eventually enhance lipid synthesis and formation of lipid droplets in cells. Therefore, we next investigated accumulation of lipid droplets in SFN treated cells. Interestingly, compare to control decreased intensity of Oil Red O stain was observed in cells treated with 2 μM SFN indicating lower lipid droplet accumulation (Fig. 6C). While accumulation of lipid droplets slightly increased in 10 μM SFN which is somewhat similar to control, excessive lipid droplet accumulation was observed in cells exposed to 20 μM SFN (Fig. 6C).

3.7. Higher concentration of SFN results in low mitochondrial activity in GCs

Although electron transport chain of mitochondria is the major source of intracellular ROS, mitochondria itself is one of the primary targets of ROS induced damage and dysfunction. In order to assess the effects higher SFN induced excessive ROS on mitochondrial function, mitochondrial activity was determined in cells exposed to different concentration of SFN. Similar mitochondrial activity like untreated control was observed in cells exposed to 2 and 10 μM SFN, as evidenced by similar florescence intensity detected by fluorescence microscopy (Fig. 7B and C). Notably lower activity of mitochondria was observed in cells exposed to 20 μM SFN indicating mitochondrial dysfunction under high concentration of SFN (Fig. 7D).

4. Discussion

One of the major factors in oxidative stress induced cell injury or death is the production of excessive amount of ROS and cell's inability to produce sufficient antioxidant enzymes to scavenge those excessive ROS [25]. On the course of evolution, cell has developed an inherent defense mechanism in response to different stress or stimuli- the activation of Nrf2 signaling pathway to produce a wide range of antioxidant enzymes [26]. Our results clearly demonstrated that medium concentration of SFN is able to activate

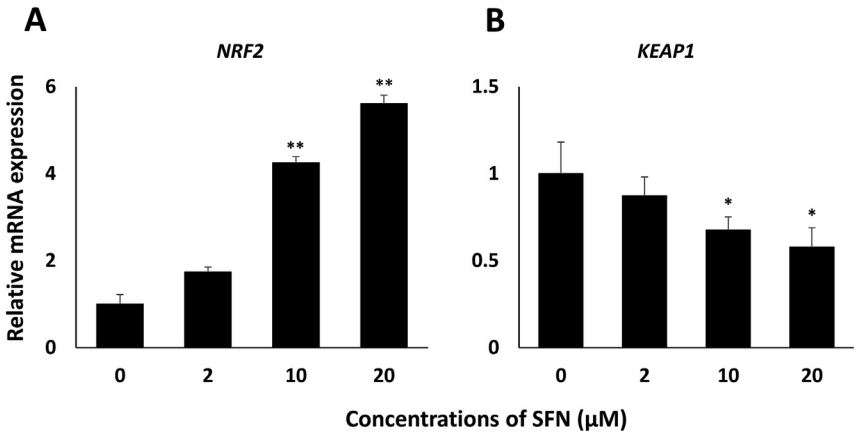


Fig. 2. Relative abundance of *NRF2* and *KEAP1*. To elucidate the Nrf2 pathway activation in response to different concentration of SFN (2, 10, and 20 μM), we employed real time qPCR to investigate the relative expression of (A) *NRF2* and (B) *KEAP1*. The statistically significance of the effect of different treatments on gene expression is marked with asterisks, (where * = $p < 0.05$, ** = $p < 0.01$). Data are presented as means ± SEM of three biological replicates.

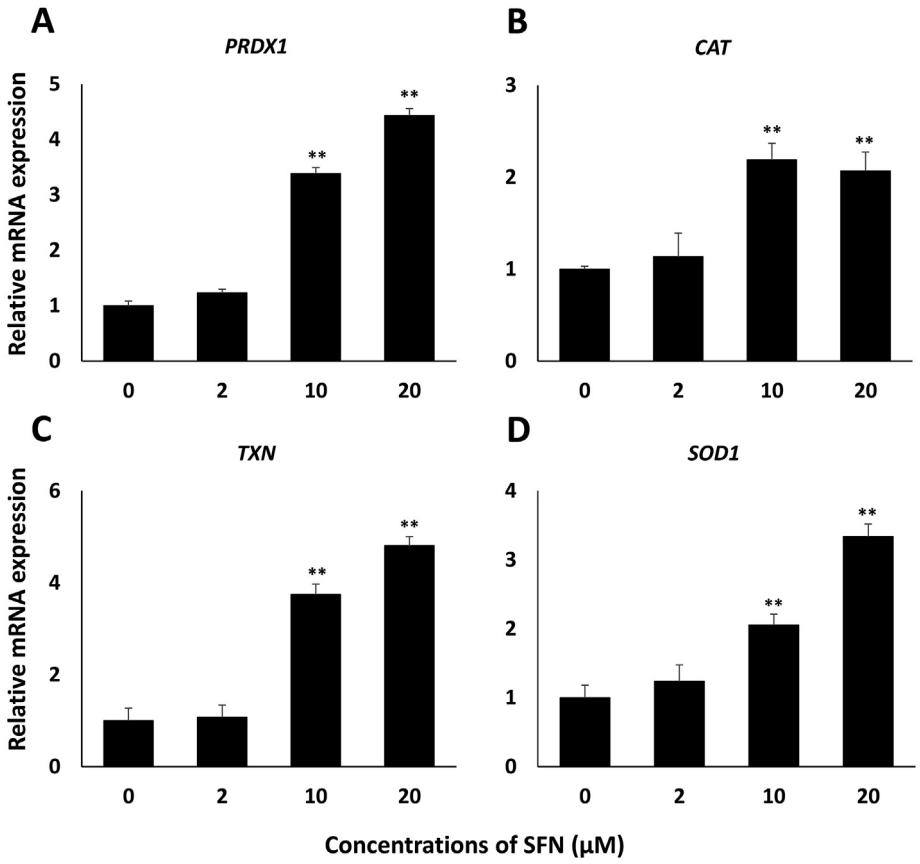


Fig. 3. Expression of ARE genes downstream to *NRF2* activation. Upon exposure to different concentration of SFN (0, 2, 10, and 20 μM), relative abundance of down-stream genes *PRDX1* (A), *CAT* (B), *TXN* (C), and *SOD1* (D) at mRNA level was measured by real time qPCR. Different asterisks represent statistically significant differences where ** = $p < 0.01$. Data are presented as means ± SEM of three biological replicates.

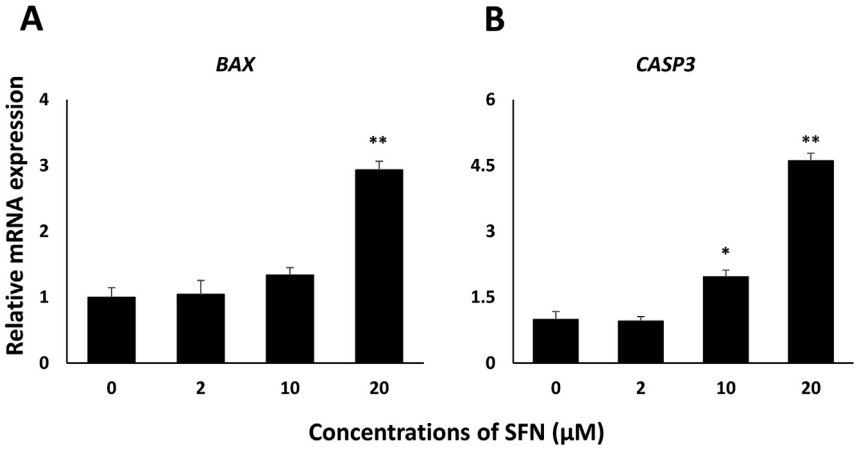


Fig. 4. Expression of *BAX* and *CASP3* genes. In order to identify whether SFN induces the expression of genes related to apoptosis, GCs were exposed to different concentration of SFN (0, 2, 10, and 20 μM) and relative abundance of *BAX* (A) and *CASP3* (B) were measured by real time qPCR at mRNA level. Statistically significant effects of different treatments on gene expression is marked with asterisks where * = $p < 0.05$ and ** = $p < 0.01$. Data are presented as means ± SEM of three biological replicates.

Nrf2-ARE pathway in bovine GCs as evidenced by higher expression of *NRF2* and lower expression of *KEAP1* genes. In addition, expression of selected antioxidant genes downstream to *NRF2* activation were also found to be highly expressed upon exposure to medium concentration of SFN. It is important to note that the viability and cell proliferation were not affected significantly by medium concentration of SFN. Interestingly, low concentration of SFN had no significant effects on either activating Nrf2 signaling pathway or influencing cell viability. On the other hand, high concentration of SFN exhibited adverse effects on GCs. Although it induced a significant increase in the expression of *NRF2* and its downstream antioxidant genes, it also enhanced the expression of genes related to apoptosis. Furthermore, high concentration of SFN increased the generation of ROS which leads to mitochondrial dysfunction and

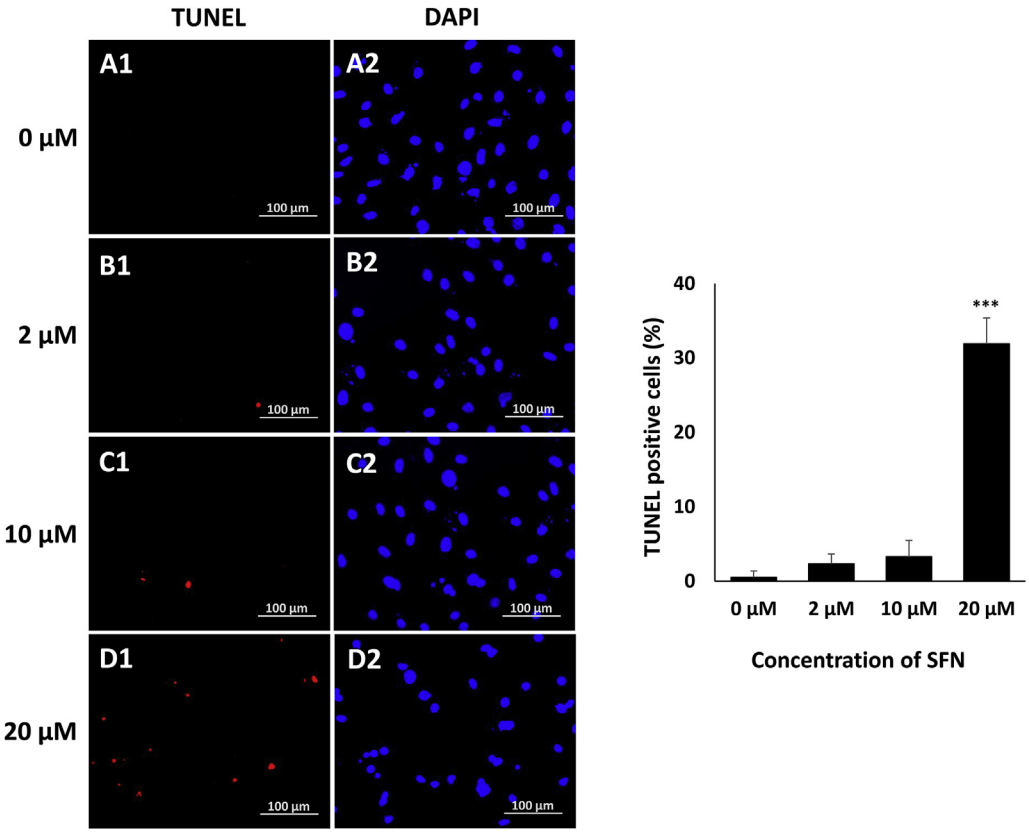


Fig. 5. In situ detection of fragmented DNA by TUNEL assay and simultaneous nuclear staining with DAPI. 1×10^4 viable GCs were seeded in each well of a 96-well plate (flat bottom, tissue culture treated) and grown up to 50–60% confluence and different concentration of SFN was added according to the experimental plan. The results of TUNEL assay showed that 20 μM SFN significantly increased the number of TUNEL-positive apoptotic nuclei after 24 h of incubation, whereas very less TUNEL positive cells were observed in untreated control, 2 μM, and 10 μM SFN concentrations (Fig. 5 A1-D1). Left panel: representative micrographs of double staining of TUNEL (red fluorescent) and DAPI (blue, counter staining), scale bar 100 μm; right panel: quantitative data analysis of TUNEL-positive cells where *** = $p < 0.001$; data are presented as mean ± SEM of three biological replicates. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

apoptosis in GCs.

SFN has been found to be unfavorable for the proliferation and survival of many types of cancer cells [18,27] including ovarian cancer cells [28,29], while in other somatic cells certain concentrations of SFN induce the protection against oxidative and other environmental stresses [30]. Several studies have shown using cell culture, in carcinogen-induced and genetic animal cancer model, and genograft model of cancer that the potential of using SFN as therapeutic agent in cancer treatment [31]. In addition, cell culture and animal studies have provided convincing evidence for the use of SFN to induce the phase II detoxifying and antioxidant enzymes in different kind of somatic cells [32]. It is highly likely that, in near future, SFN will be used as drug to combat against cancer or to maintain cellular homeostasis against oxidative stress in human. However, in either cases the concentration of SFN carries utmost importance as different concentration induces different pathways [32]. Interestingly, the concentration dependent impacts of SFN on GCs have not been investigated yet. By employing a wide range of concentrations (1–80 μM) of SFN, we showed that 2–10 μM SFN had no detrimental effects in terms of survival and proliferation of GCs, whereas high concentrations (≥20 μM) exhibited cytotoxic effects (Fig. 1) characterized by lower number of viable cells. In addition, particular apoptotic morphological characteristics including irregular cell shape, shrinkage of cell wall, and more floating cells in the culture media were detected after the treatment with higher concentrations (data not shown). Similar to our findings, Lee and Lee reported a concentration dependent loss of viability in Human Bronchial Epithelial BEAS-2B Cells that exposed to SFN for 24 h [22]. Perhaps, low concentration of SFN activates the genes related to cell survival pathway, while high concentration induces the expression of genes involved in antiproliferative and apoptotic pathways. Therefore, it is of interest to investigate the effect of low and high concentration of SFN on GCs in activating genes related to both survival and apoptotic pathway.

NRF2 transcription factor regulates and coordinates more than 200 genes in mammals largely known to be involved in enhancing endogenous antioxidant defense system and survival capability of cells [33]. The endogenous antioxidant defense system consists of a wide range of phase II detoxification enzymes such as glutathione transferase, *SOD*, catalase (*CAT*), and heme oxygenase (*HO-1*). Expression of these antioxidant enzymes is regulated by ARE promoter sequence which is modulated by *NRF2* transcription factor [33,34]. In our study, we found that the expression of *NRF2* increased significantly in GCs exposed to 10 and 20 μM SFN, while the expression of *KEAP1* decreased significantly under similar conditions. SFN has been proven to be an efficient modifier of multiple KEAP1 domains in the NRF2-KEAP1 interactions [35,36]; consequently, NRF2 released from the interactions and be able to translocated into the nucleus where it can modulate the transcription of Phase II antioxidant enzymes by interacting with ARE. Therefore, it is highly likely that the higher expression of *NRF2* and Phase II antioxidant enzymes in our experiment is due to the disruption of the NRF2-KEAP1 interactions caused by SFN treatments. Our findings are consistent with the previous reports

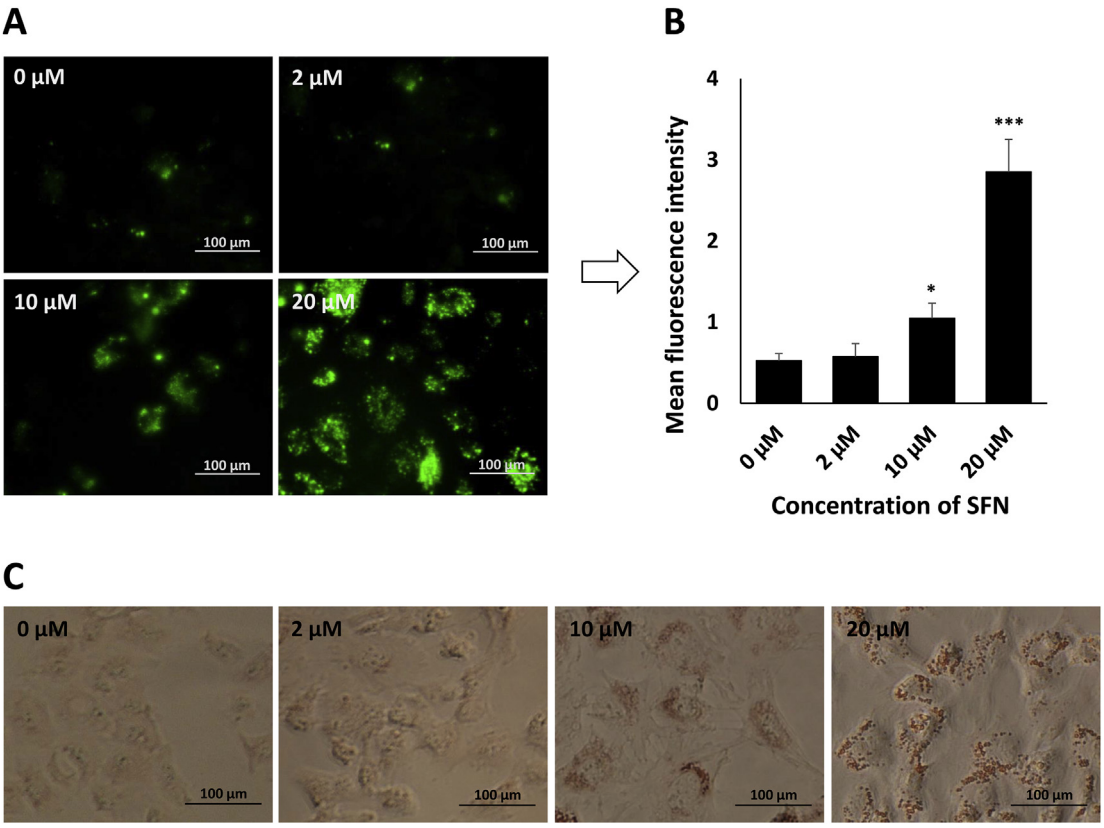


Fig. 6. ROS and lipid droplet accumulation in GCs after treatment with different concentration of SFN. GCs were exposed to SFN (0, 2, 10, and 20 µM) for 24 h, (A) washed and loaded with H_2DCFDA (5 µM for 30 min) and visualized under a fluorescent microscope, scale bar 100 µm (B) Mean fluorescence intensity was quantified by ImageJ software or from five nonoverlapping fields per well, experiments were performed in triplicates; * = $p < 0.05$ and *** = $p < 0.001$; data are presented as mean ± SEM. (C) Following treatment, GCs were loaded with Oil Red O stain working solution (for 40 min), washed several times and visualized with an inverted microscope. Scale bars, 50 µm. A high-resolution version of two images of panel A (10 µM and 20 µM SFN) are available as Virtual Microscope eSlide: VM03999 and eSlide: VM04000.

demonstrated that low concentration of SFN enhances the activity of NRF2 and induces the transcription of phase II antioxidant enzymes in many cell types [37].

One of the crucial elements in the behavior of mammalian cells is the activation of apoptotic pathways under unfavorable environmental conditions [38]. In this process, a series of enzymes participated in a tightly coordinated manner which is regulated by specific genes known as pro apoptotic or apoptotic genes such as *CASP3*, *BAX*, *BAK*, *BID*, *BAD*, *BIM*, *BIK*, and *BLK* [39]. The products of these genes have special significance as they can determine the fate of cells. In our experiment, we found that there was a significant increase in the expression of both *BAX* and *CASP3* in GCs treated with 20 µM SFN which may suggest the activation of apoptotic pathway in the presence of high concentration of SFN which was further supported by results of TUNEL assay. *BAX* can be found predominantly in the cytosol; however, on induction of apoptosis it could partially be translocated to the mitochondria [40,41]. This shift induces the mitochondrial permeability transition pore to open and facilitates the release of cytochrome *c* which eventually activates the caspase cascade [40].

Oxidative stress induced cell death is believed to be associated with higher accumulation of intracellular ROS [42]. Interestingly, it has been shown that SFN induced apoptosis and cell death is also initiated by excessive generation of intracellular ROS [22,43]. Our results revealed that there was higher accumulation of ROS at high concentration as indicated by increased fluorescence activity. On the other hand, slight increase in the accumulation of ROS was observed in GCs exposed 2 and 10 µM SFN. Intracellular ROS generation induced by SFN plays an important role in the induction of apoptosis through cell cycle arrest in many cancer cells. Interestingly, under similar concentrations other somatic cells are not affected as they can scavenge elevated level of ROS through activating antioxidant defense mechanism and show resistance to cell cycle arrest and apoptosis [30,44]. In our study, probably ROS was generated such a high rate, at high concentration, that cells were unable to scavenge by producing sufficient antioxidant enzymes resulting cell cycle arrest and cell death. Elevated ROS level can induce lipogenesis via activating SREBPs in cells under culture condition resulting higher accumulation of lipid droplets [45]. Therefore, it is not surprising that we found elevated accumulation of lipid droplets in GCs exposed to 20 µM SFN. However, in this study we did not check the underlying mechanism of lipid accumulation in GCs exposed to high concentration of SFN which warrant further experimental attention.

Although mitochondria are the major site of ROS generation, it is also highly susceptible to the oxidative damage caused by ROS, particularly mitochondrial DNA and electron transportation chain. In active mitochondria, mitochondrial antioxidant defense enzymes such as glutathione peroxidase and manganese SOD scavenge ROS produced during ATP synthesis [46]. However, under stress conditions, excessive generation of ROS can impair the capacity of this defense system, resulting mitochondrial damage. Importantly, damaged mitochondria can produce even more ROS due to impaired electron transportation chain [47]. Subsequently, excessive ROS can decrease the mitochondrial membrane potential and facilitate the release of cytochrome *c* and apoptosis inducing

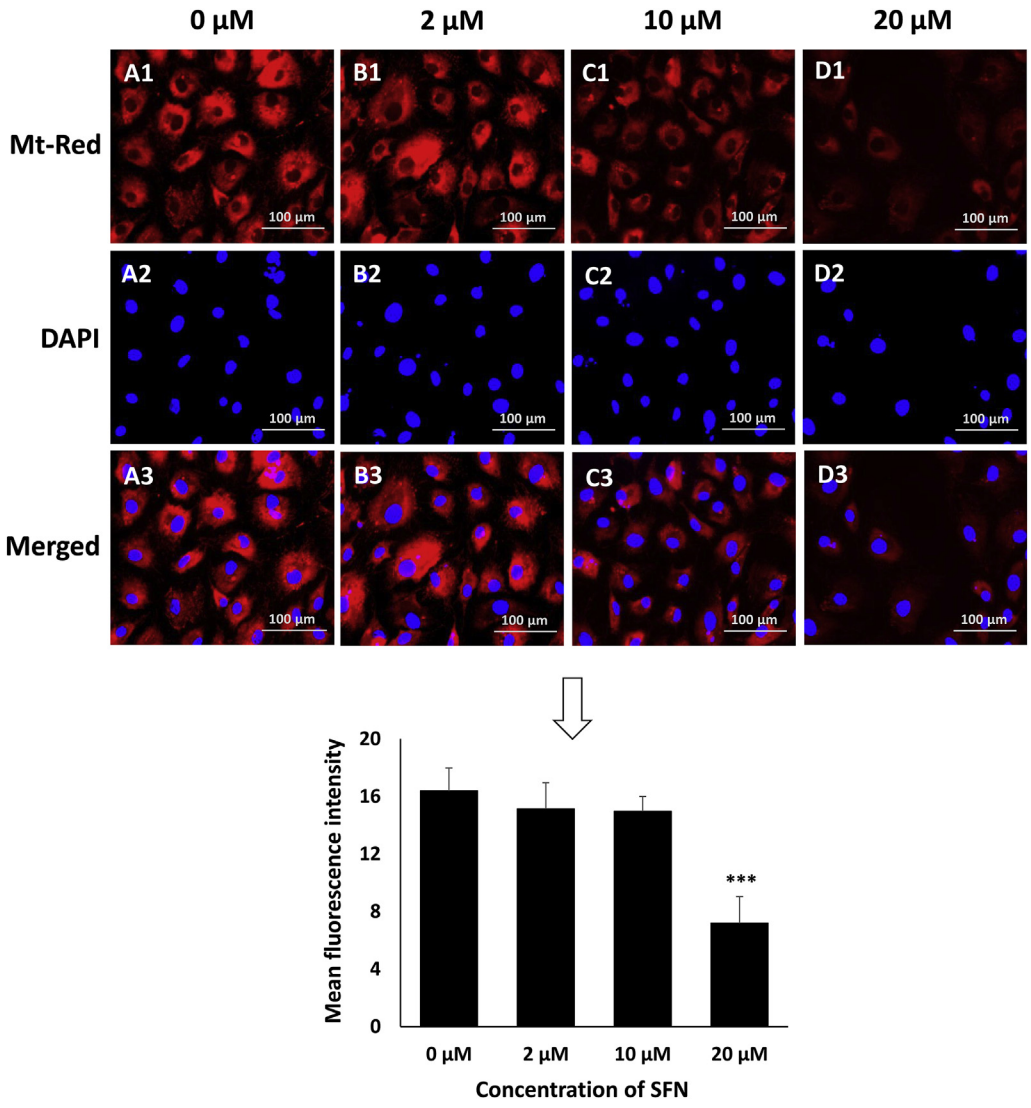


Fig. 7. Mitochondrial activity in GCs at different concentrations of SFN. GCs were treated according to the experimental plan and 100 nM Mito Tracker red dye was added and incubated for 45 min. Images were acquired with fluorescence microscope using a red filter. Higher panel: labels A1-D1 show active mitochondria in red, while A2-D2 show nuclear staining with DAPI in blue color and A3-D3 show merged image of active mitochondria and nucleus staining. Scale bars, 50 µm. Lower panel: mean fluorescence intensity of five nonoverlapping fields per well was quantified using ImageJ software; experiments were performed in triplicates; *** = $p < 0.001$; data are presented as mean ± SEM. A high-resolution version of three panels of this image are available as Virtual Microscope eSlide: VM04001, eSlide: VM04002, and eSlide: VM04003.

factors which eventually activate the pro-apoptotic cascade resulting cell death through apoptosis. Mito Tracker probes are able to differentiate an active mitochondrion from a damaged one, since it can defuse the plasma membrane and accumulate in active mitochondria. In the present study, we found less mitochondrial activity in GCs treated with high concentration of SFN, as evidenced by less fluorescence, perhaps indicating loss of mitochondrial membrane integrity caused by ROS and induction of apoptotic cascade.

5. Conclusion

In summary, our results showed that 10 µM of SFN is able to activate antioxidative response in GCs which is evidenced by higher expression of *NRF2* and its downstream antioxidant enzymes at transcription level. Although we found slight increase of ROS level at this concentration, it did not affect the activity and integrity of mitochondria. Perhaps, low level of ROS was generated in response to 10 µM SFN exposure and instead damaging the cellular macromolecule, it may boost up the antioxidant defense system in GCs. On the other hand, high concentration of SFN are extremely cytotoxic to GCs as we found lower cell viability, high ROS and lipid droplet accumulation and, low mitochondrial activity. These results provoke us to conclude that, depending on concentrations, SFN has both antioxidative and apoptosis effects on GCs.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.theriogenology.2017.04.015.

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