Safa M. Abdulateef et al /J. Pharm. Sci. & Res. Vol. 11(1), 2019, 216-220

ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

Sulforaphane (SFN) And Bardoxolone (CDDO)–Induced Inhibition Of Aflatoxinb1–Mediated Genotoxicity In Human Lymphocytes: Role Of *Gstm1*, *Gst1a1* And *Nrf2* Gene Expression

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Abstract
Real-time PCR used to investigate the ability of sulforaphane (SFN) and Bardoxolone (CDDO) on inhibition aflatoxin B1 genotoxicity in Human lymphocytes *in vitro*. Real time PCR analysis carried out for AFB1 treated lymphocytes with/without SFN and CDDO separately to assess its effects on global transcription through monitoring gene expression variation among genes responsible for AFB1 biotransformation including those involving in AFB1 detoxification like *GSTM1* and *GST1A1* calibrated with *B-actin* housekeeping gene. Lymphocytes incubated with 10 and 100ng/ml of AFB1 separately and simultaneously with (GSTs) inducers. Protective effect of SFN and CDDO required co-treatments with AFB1. Human lymphocytes incubated with 10 and 100ng/ml AFB1 mixed with 10 and 50µM SFN respectively for 2hr., on the other hand lymphocytes incubated with 10 and 100ng/ml AFB1 mixed with 10 and 50µM CDDO respectively for 2hr. Transcriptional induction for genes involved in AFB1 detoxification was showed after treating with SFN and CDDO. SFN able to induce *GSTM1* expression more than CDDO, SFN induce *GSTM1* to (~295.61) fold comparing with separately AFB1 treated cells ($P<0.05$*), in spite of that CDDO was able to induce *Nrf2* more than SFN.

INTRODUCTION

Mycotoxins are secondary metabolites produced by toxigenic strains of different species of fungi. Aflatoxin B1 (AFB1) is one of the most important mycotoxins due to its hepatotoxic and carcinogenic effects on certain animal models and humans [1, 2]. *Aspergillus flavus* and *Aspergillus parasiticus* are the most important fungi responsible for its production [3, 4]. Aflatoxins (AFs) undergo biotransformation, this process aimed to converting the original molecules into more hydrophilic compounds readily excreted in the urine. This process occurs in two phases known as Phase I and Phase II [5].

AFB1 is bioactivated by Phase I [Cytochrome P450 (*CYP450*)], producing reactive metabolite, known as aflatoxin-8, 9-epoxide (AFBO). The AFBO can be detoxified by Phase II [glutathione S transferases (*GSTs*)] through conjugation with glutathione substrate or may be hydrolyzed by an epoxide hydrolase to AFB1-8,9-dihydrodiol, which is able to react strongly with proteins and cause cytotoxicity [6,7]. Depletion of Phase II enzymes activity by oxidative stress through reactive species of oxygen (ROS) and nitrogen (RNS) accumulation leading to damaging cellular DNA, as well as cellular proteins and lipids [8]. Induction of phase II enzymes is an effective mechanism of protection against carcinogenesis, mutagenesis, and other forms of toxicity mediated by carcinogens [9]. Since the discovery of sulforaphane (SFN) in 1992 and the recognition of the bioactivity of this phytochemical, many studies have examined its mode of action in cells, animals and humans. Broccoli, especially as young sprouts, is a rich source of SFN and broccoli-based preparations are now used in clinical studies probing efficacy in health preservation and disease mitigation. On another hand Bardoxolone (CDDO) anti-inflammatory, antioxidant, antiproliferative, anticancer, and anticarcinogeniccompunds belong to triterpenoids, CDDO have been used for medicinal purposes because of their properties. Synthetic triterpenoid analogues of oleanolic acid, bardoxolone [cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO)] are potent inducers of the phase II response as well as inhibitors of inflammation. Triterpenoid, is a highly potent chemopreventive agent that inhibits aflatoxin-induced tumorigenesis [10, 11].

MATERIALS AND METHODS

SFN, CDDO and AFB1: SFN and CDDO were purchased from Cayman chemicals Company while AFB1 was purchased from ENZO life science Company.

Preparation, culturing, and treatment of human Lymphocytes:

Blood samples were taken from several volunteers with no history of using any of known inducer for (GSTs) drugs and almost homogenous (18–21 years old). From those volunteers, heparinized blood samples were applied to lymphocyte isolation[12,13].

Heparinized blood samples separately by syringe take 2ml of blood and diluted with 2ml of Roswell Park Memorial Institute medium(RPMI1640) or phosphate buffered saline (PBS) and then gently layered above 4ml of Ficoll (Lymphprep lymphocyte separation media) and finally centrifuged at 400xg or (2500rpm) for (30min). The cloudy white layer (creamy web like layer) arising between the lymph prep. Layer and plasma layer was transferred to sterile test tube with one ml of RPMI1640. If there is a drop or tiny drop of blood must be lysed by adding 500µl of lysis buffer then set for 5min then centrifuge again at 2500rpm for 10min by adding 1ml of RPMI1640. Discard the supernatant and re suspended the precipitate with 1ml of RPMI and centrifuge at 2500rpm for 10min. Repeat the previous step two times for wannnshing lymphocyte from any debris. After final washing step remove the supernatant and re suspended the precipitate (lymphocyte) with 1ml of RPMI1640. The isolated lymphocyte from each sample were transferred and seeded in polystyrene 24-well tissue culture plates containing RPMI medium with 10% heat-inactivated FBS, final volume in each well 250µl, then incubated for 24 h in a 37 °C incubator containing 95% humidity and 5% CO2.

Cells were treated, in duplicate, with two doses of AFB1 (final concentration of 10 and 100 ng/ml) separately and simultaneously and two doses SFN or CDDO (final concentrations of 50 and 10 µM) with final reaction volume 500µl (250µl cell+250µl treatment). After 2 h incubation with treatments, the lymphocytes in the wells were separately collected by centrifugation (3000_g, 4 min). After elapsing incubation period, the contents of each well were collected and used for molecular analyses.

Molecular analysis of human lymphocyte RNA

RNA isolation accomplished by using TRI-ZOL kit provided from Ambion life Technologies Company. Quantus Florometer was used to detect the concentration of extracted RNA in order to detect the goodness of samples for downstream applications.

Real time PCR was used for measuring *GSTM1*, *GSTA1* and *Nrf2* expression calibrating with *B-Actin* gene for treated and control cells. Total RNA from treated cultures and untreated culture were

extracted and purified using TRIZOL reagent. The expression of genes was quantified using the Syber Green reagent (1-Step RT-qPCR Kit). Primers were designed according to Gross-steinmeyer *et al*., (2005) and checked according to http://www.incbi.com [14]. The real time was performed using MIC System. Primers were obtained from Alpha DNA company .Table (1) showed the primers and their sequences are used in Real time PCR analysis. PCR was performed in optimized conditions as mentioned in tables below. Fluorescence signals were measured over 40 PCR cycles. The cycle number (Ct) at which the signals crossed a threshold set within the logarithmic phase was recorded. Expression levels were quantified using relative quantitation, the difference in cycle threshold (ΔCt) and fold difference evaluated between the treated group and control of each gene. The efficiency of amplification of each pair of primers was determined according to be normalized to *B-actin, GST* and *Nrf2* expression (Bahari *et al*., 2015). Each real time PCR reaction was done in a 10 µl final volume containing 1µl of specific forward and reverses primers, 5 µlGo Taq 1-Step RT-qPCR, 2 µl template and 0.25µl Reverse transcriptase mixture, then completing volume with nuclease free water. Real time PCR conditions for all genes were carried out (in duplicate) using a MIC system (**Mic -4-/Australia**) with a cycling program including holding for 15 min at 37C for Reverse transcriptase and 5 min at 95 _C as Hot start, followed by cycling 45-times at 95, 58, and 72 _C (20 s for each temperature) with melting curve analyses (72°C to 95°C at 0.3°C/s).

Table (1): Primer sequences were ordered for this study form Alpha DNA company:

Primers Name	**Primer sequence (5′ – 3′)**
Beta-actin	AACCCCAAGGCCAACCG
	AGGGATAGCACAGCCTGGA
GSTA1	GACTCCAGTCTTATCTCCAGCTTCC
	TGCTTCTTCTAAAGATTTCTCATCCAT
GSTM1	AAAGTACTTGGAGGAACTCCCTGAAA
	GCTCAAATATACGGTGGAGGTCAA
Nrf2	GCGACGGAAAGAGTATGAC
	GTTGGCAGATCCACTGGTTT

Gene expression was calculated according to lirak method [2^ΔΔCT], the following equation summarized the best way used to find folding for each gene and compared with controlled**:**

Folding =2^-ΔΔCT

ΔΔCT =ΔCT Treated - ΔCT Control

ΔCT =CT gene - CT House Keeping gene

Statistics

Comparisons of the means between the AFB1-treated and control were performed using a student's t-test. All real time assay data were analyzed using a one-way analysis of variance (ANOVA). A p-value≤0.05 was accepted as significant.

RESULTS AND DISCUSSION

To determine whether the oxidative stress of AFB1 could be limited by the affectivity of SFN and CDDO or not, firstly must be Suring if these compound effect on a key regulatory transcriptional factor *Nrf2* of antioxidant reducing genes or not. In this study real time PCR analysis carried out for both AFB1untreated and AFB1 treated lymphocytes with/without inducers (SFN and CDDO) to assess its effect on global transcription (Fig 1). Real time PCR data indicated that *Nrf2* and *GSTM1,* expressed in human lymphocytes while *GST1A1* unexpressed after treated with different doses of AFB1, results revealed that expression levels of *Nrf2,* and *GSTM1* in lymphocytes treated for 2 hr., with AFB1 were higher in those cells that received the lower dose of AFB1 (10 ng/ml) than the higher dose (100 ng/ml) group. *Nrf2* expressed as (~111.27) fold and (~23.82) fold when treated separately with 10 and 100 ng/ml AFB1 respectively, while when lymphocytes co-treated with AFB1 simultaneously with 10 and 50µM CDDO the expression induced to (~239.33) fold and (~530.73) fold respectively. SFN was able to induce *Nrf2* expression but CDDO more efficient in *Nrf2* induction. *GSTM1* expressed as (~80.29) and (~31.61) fold after treated with 10 and 100ng/ml AFB1 respectively, and the expression induced to (~295.60) and (~92.02) fold when co-treated with 10 and 50µM SFN respectively. CDDO was able to induce *GSTM1* expression but SFN more efficient in *GSTM1* induction (Table 2).

Table 2: gene expression values for *B-actin, Nrf2, GSTM1* and *GST1A1* after treating with 10, 100 ng/ml AFB1 with and without inducers (SFN and CDDO) *in vitro*.

TREATMENT	*B-Actin*	*Nrf2*	*GSTM1*	*GST1A1*
Control	19.61	1.00	1	1.00
AFB1 10ng/ml	23.54	111.27	80.29	0.00
AFB1 100ng/ml	22.41	23.82	31.61	0.00
SFN 10µM	22.88	186.85	106.49	0.01
SFN 50µM	16.10	46.39	49.64	0.00
AFB1 10ng/ml SFN 10µM	15.82	83.67	295.60	0.00
AFB1 100ng/ml SFN 50µM	21.65	85.43	92.02	1.60
CDDO 10µM	19.06	414.42	205.70	0.08
CDDO 50µM	18.69	287.39	210.58	10.12
AFB1 10ng/ml CDDO 10µM	18.66	239.33	218.47	2.04
AFB1 100ng/ml CDDO 50µM	19.96	530.73	51.13	0.02

Figure (2) summarized the expression of all studied genes in response to AFB1 separately or simultaneously with SFN or CDDO. *Nrf2*, *GSTM*1 highly expressed than *GST1A*1 in cultured human lymphocytes when treated with AFB1 *in vitro* comparing with control and calibrated with $\boldsymbol{\beta}$ *−Actin* gene.

In this study human lymphocyte encounter oxidative stress by the effect of AFB1.When lymphocytes subjected to AFB1 undergoes metabolism leading overproduction of ROS producing oxidative stress in cells.

GSTM1 encodes a cytoplasmic glutathione S-transferase that belongs to the mu class. The mu class of enzymes functions in the detoxification of electrophilic compounds, including carcinogens, therapeutic drugs, environmental toxins and products of oxidative stress, by conjugation with glutathione [15].GSH is regarded as the body's master antioxidant compound and maintenance of thiol status. It is found in almost all body cells and plays a critical role in the body's detoxification process. Glutathione is also an essential component of the body's natural defense system. GSH is used as a cofactor to *GST*s that conjugate GSH with exogenous electrophiles like AFs and its metabolites, and other various xenobiotics, once AFBO produced by Phase I metabolism of AFB1is trapped by GSH. Conjugation of AFBO with GSH is catalyzed by glutathione transferases, in which the GSH thiolate anion participates as a nucleophile. These intracellular proteins are protect cells against chemically-induced toxicity and stress by catalyzing the conjugation of the thiol group of GSH and an electrophilic moiety in the substrate, which yield a stable, non-toxic, polar product that is excreted in the bile. The aflatoxin-glutathione product also undergoes sequential metabolism in the liver and kidneys in which it's excreted as a mercapturic acid (aflatoxin-N-acetylcysteine) in urine [16].

Bahari *et al*., (2015) they considered studying *GST* genes in monocytes and lymphocytes in respond to AFB1 exposure and,

also, the effects of AFB1 on the two cell types. They studied the expression of *GSTM1*, *GSTT1* genes in human monocytes and lymphocytes after *in vitro* exposure to 10 or 100 ng/ml AFB1for 2 hr. They improved that both examined genes were present in HepG2 cells, in lymphocytes and monocytes, only *GSTM1* was detected. It was seen that in both the monocytes and lymphocytes, AFB1 caused pronounced over-expression of *GSTM1*, although this effect was diminished with the higher dose 100 ng/ml of AFB1 tested [17].

Nrf2 responsible to regulate the expression of large number of genes including those involved in detoxification of xenobiotic compounds including phase II detoxifying enzymes that metabolize xenobiotics into less-toxic forms by catalyze conjugation reactions to increase the solubility of xenobiotics, thereby facilitating their elimination. When *Nrf2* expression induced due to the effect of oxidative stress, already the expression of genes regulated by this factor increased [18].

Nrf2 activity is regulated by the associated *Keap1* protein, which was initially proposed to act by binding and tethering the *Nrf2* in the cytoplasm. Activation of *Nrf2* in response to stress signals was result from a disruption of this association, releasing *Nrf2* for translocation into the nucleus to affect its transcriptional activity [19].

Nrf2 activation of the antioxidant response element (ARE) is central to cytoprotective gene expression against oxidative and electrophilic stress [20]. Unless activated by inflammatory, environmental or oxidative stress is sequestered in the cytoplasm by its repressor, Keap1 [21] because of its protective capabilities, small molecules that activate *Nrf2* signaling are being examined as potential anti-cancer or anti-inflammatory agents [22]. SFN is an isothiocyanate derived from cruciferous vegetables, including broccoli, that potently induces chemopreventive enzymes via *Keap1-Nrf2* signaling and ARE –driven gene expression [23]. SFN, as a pure chemical, protects against chemical-induced carcinogenesis and other genotoxic models on body organs, this putative effect belong to the chemical biology of SFN that enable it to induce of *Nrf2* signaling and strongly inhibit carcinogenicity. SFN tried for recovering cellular balance by using its electrophilcity. The central carbon of the SFN (–N=C=S) is electrophilic and reacts readily with sulfur-, nitrogen, and oxygen-centered nucleophiles. The most common reaction in mammalian cells is conjugation with sulfhydryl groups, such as the sulfhydryl group of cysteine in proteins. Many putative cellular target affected by SFN, *Keap1-Nrf2* and glutathione considered as validated targets at this time.

Upon SFN entry into the cell, chemically reacts *Keap1* protein, a protein endowed with a number of reactive cysteine residues which function as sensors for numerous oxidants and electrophiles (termed inducers), including the isothiocyanates [24, 25].

Chemical modification of the sensor cysteines of *Keap1* by inducers, such as SFN, blocks the cycle of *Keap1* -dependent *Nrf2* degradation. This block allows de novo synthesized *Nrf2* to accumulate, translocate to the nucleus, and initiate transcription of its downstream target genes. The depletion of GSH affected by AFB1can be compensate by the activity of SFN, SFN act as inducer and substrates for the *GSTs*, [26, 27].

On other hand, CDDO is a synthetic triterpenoid that blocks the cellular synthesis of inducible nitric oxide synthase by suppressing reactive oxygen and nitrogen species (ROS/RNS) formation. It promotes the cellular control of ROS/RNS levels that would lead to DNA damage associated with tumorgenesis [28]. CDDO has been shown to specifically inhibit proliferation and induce apoptosis, also able to induce genes regulated by *Nrf2* including (*GSTs*) isoforms, that which play a role in ARE signaling activity [29]. The molecular mechanism of action of the triterpenoids is believed to be mediated by the Michael addition with active nucleophilic groups on proteins, such as the thiol groups on cysteine residues. In the development of triterpenoids derivatives, two electrophilic Michael acceptor sites were incorporated in the A and C rings of CDDO. Structure-activity analyses have shown that α,β-unsaturated carbonyl groups at key positions on rings A and C are essential for maintaining the potent anti-inflammatory activity of synthetic triterpenoids [30].

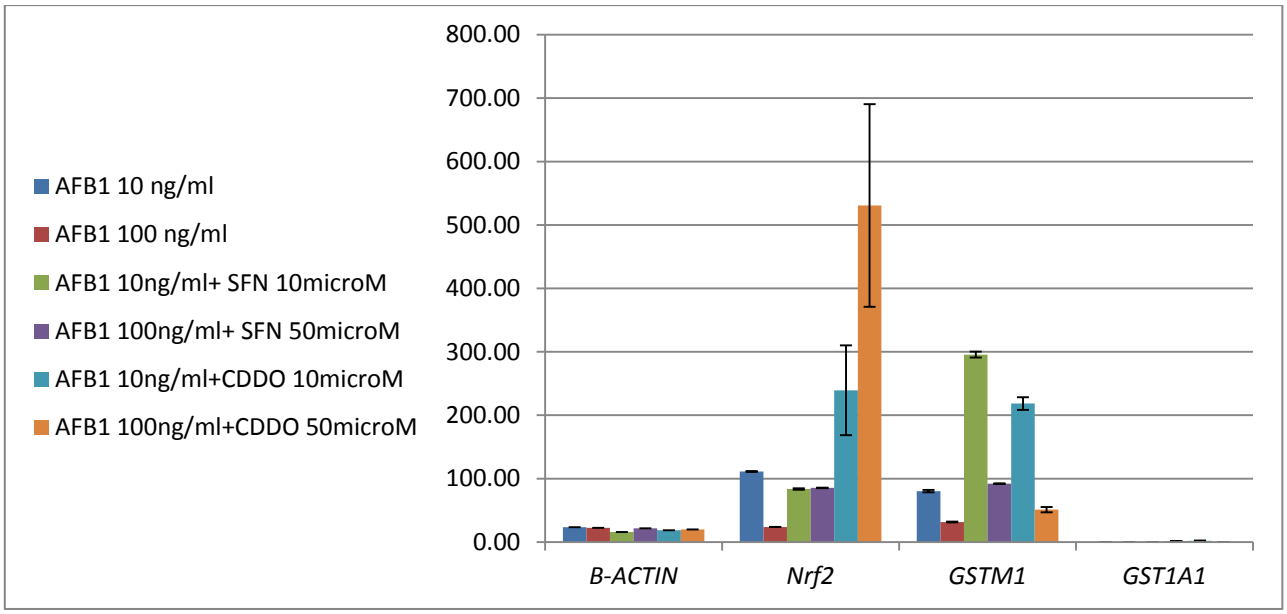

Figure 2. Gene expression quantification of GSTs isoforms including (*GST1A1* and *GSTM1*) and *Nrf2* in human lymphocytes exposed to different doses of AFB1 with/without GSTs inducers (SFN and CDDO) *in vitro*. Lymphocytes were exposed to 10 and 100 ng/ml of AFB1 separately and simultaneously with/without SFN and CDDO for 2 hr. Transcripts of *GSTM1* and *Nrf2* genes were up-regulated in the presence of both doses of AFB1 with/without inducers.

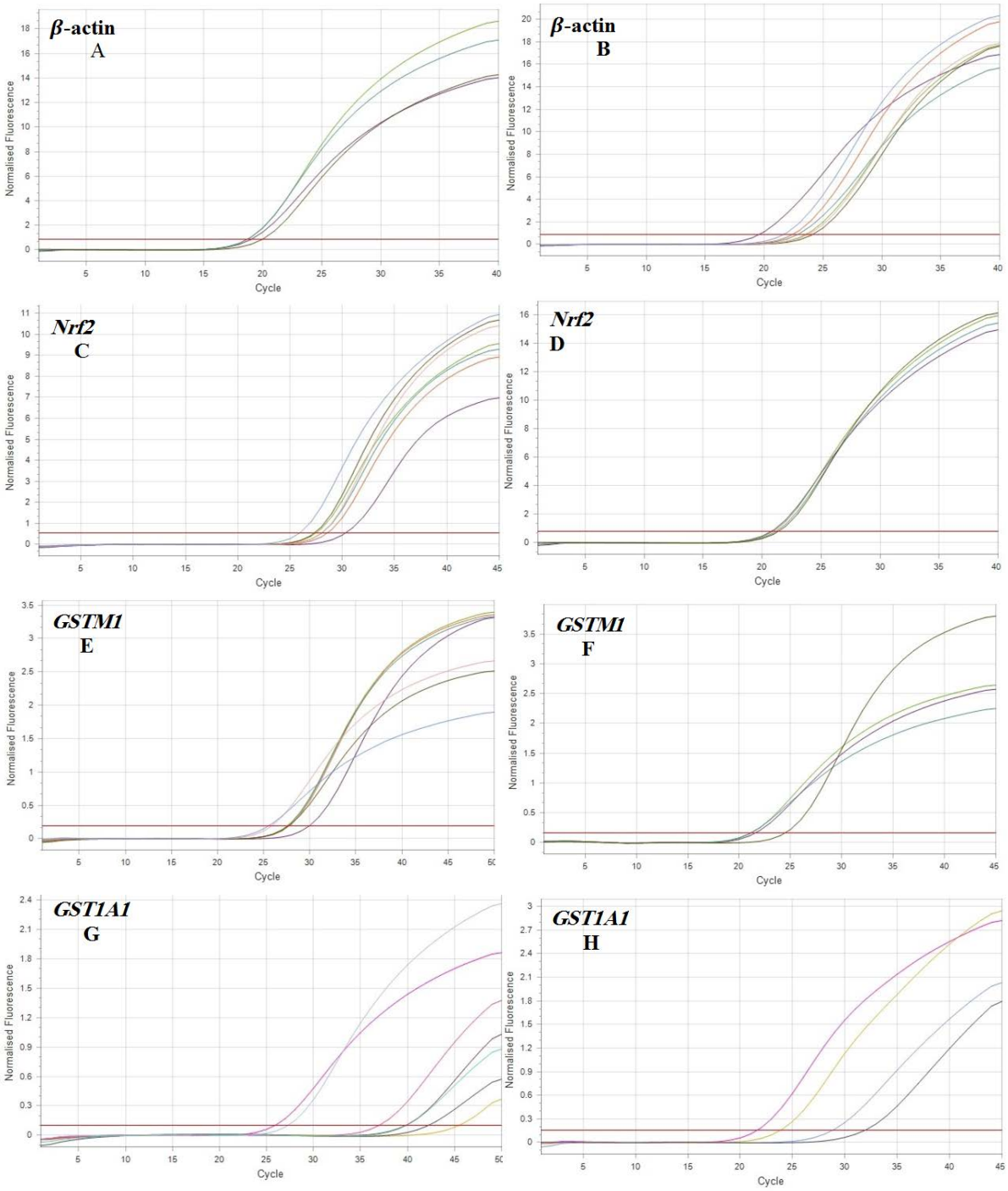

Figure (1): Real time PCR cycling for all genes after 2hour exposure to different concentration of AFB1, SFN and CDDO, (A)*B-actin* cycling of 7 samples including: control, AFB1 10ng/ml, AFB1 100ng/ml, SFN 10µM, SFN 50µM, AFB1 10ng/ml + SFN 10µM and AFB1 100ng/ml + SFN 50µM), (B)*B-actin* cycling of 4 samples including CDDO 10µM, CDDO 50µM, AFB1 10ng/ml + CDDO 10µM and AFB1 100ng/ml + CDDO 50µM. (C)*Nrf2*cycling of 7 samples including: control, AFB1 10ng/ml, AFB1 100ng/ml, SFN 10µM, SFN 50µM, AFB1 10ng/ml + SFN 10µM and AFB1 100ng/ml + SFN 50µM, (D)*Nrf2* cycling of 4 samples including CDDO 10µM, CDDO 50µM, AFB1 10ng/ml + CDDO 10µM and AFB1 100ng/ml + CDDO 50µM. (E) *GSTM1*cycling of 7 samples including: control, AFB1 10ng/ml, AFB1 100ng/ml, SFN 10µM, SFN 50µM, AFB1 10ng/ml + SFN 10µM and AFB1 100ng/ml + SFN 50µM, (F) *GSTM1* cycling of 4 samples including CDDO 10µM, CDDO 50µM, AFB1 10ng/ml + CDDO 10µM and AFB1 100ng/ml + CDDO 50µM. (G) *GST1A1*cycling of 7 samples including: control, AFB1 10ng/ml, AFB1 100ng/ml, SFN 10µM, SFN 50µM, AFB1 10ng/ml + SFN 10µM and AFB1 100ng/ml + SFN 50µM, (H) *GST1A1* cycling of 4 samples including CDDO 10µM, CDDO 50µM, AFB1 10ng/ml + CDDO 10µM and AFB1 100ng/ml + CDDO 50µM.

CONCLUSION

1. Human lymphocytes showed different model of expression after treating with AFB1, SFN and CDDO. The diversity of expression results from the differences mechanism of action for each compound on human cells.
2. Excellent induction was observed for *GSTM1* expression after co- treatment with SFN or CDDO, SFN and CDDO act as potent inducers for *GSTM1* expression. Natural protective SFN take the upper hand for inducing *GSTM1*, whereas when 10ng/ml AFB1 treated cells incubated with 10µM SFN for 2hr., expression up- regulated to **(~295.61)** fold.

3. Substation effect for CDDO was observed on *Nrf2* induction, CDDO able to induce *Nrf2* expression to **(~530)** fold under co-treated with 100ng/mlAFB1 and 50µM CDDO for 2hr.
4. In spite of CDDO capability to induce *Nrf2* more than SFN, SFN get the butter effect in *GSTM1* induction.

Acknowledgements

I wish to express my profound gratitude to my supervisor Dr. Neamat Jameel Abdul-Baqi for her guidance, scientific support and useful suggestions to solve any unsolvable problems throughout the period of this Research.

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