Research Article

Poly(ADP-Ribose)Polymerase 1 Stimulation in Liver and Thymocyte Nuclei of Rats Treated with Benzamide

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Received: 19-07-2018; **Revised:** 22-08-2018; **Accepted:** 05-09-2018.

ABSTRACT

Poly (ADP-ribose)polymerase 1 (PARP 1) inhibitors are entering clinical trials improving efficacy of chemotherapeutic treatment in cancer therapy and their employment tend to increase in time. It is considered that therapeutic endpoint of PARP 1 inhibitors is suppression of PARP 1 activity in cells. However, the data regarding PARP 1 activity in the cells of intact organisms, which underwent treatment with PARP 1 inhibitors are poor and controversial. In present study we evaluated the possible link between the treatment of hall organism with BA, intranuclear NAD+ content and condensation of chromatin in rat liver nuclei. Benzamide is bona fide PARP 1 inhibitor in the in vitro systems. In contrast to this well recognized role, we observed that BA elicited stimulation of PARP 1 activity in liver cell and thymocyte nuclei of rats when administrated to hall animals and this effect tend to increase in time. The data presented here come to show that stimulation of PARP 1 in liver cell and thymocyte nuclei isolated from BA treated rats can be linked to elevation of NAD+ content. Here we determined that increased availability of NAD+ induced by administration of BA to rats could modulate chromatin condensation in liver cell nuclei.

Keywords: poly(ADP-ribose)polymerase 1, benzamide, DNA internucleosomal fragmentation, chromatin condensation.

INTRODUCTION

Poly (ADP-ribose) polymerase 1 (PARP 1) is a chromatin-associated enzyme which has fundamental functions in many physiological and pathological processes. PARP 1 is defined as a main player in recognition and DNA damage repair in eukaryotic cells[1]. Activation of the enzyme is stimulated by allosteric influence of regulatory domains which form contacts with damaged DNA. PARP 1 protein binds to DNA through Zn1 and Zn2 fingers and regulatory domain interfaces (Zn3, WRG), which are critical for the enzyme activity. The binding to damaged DNA supports PARP 1 activity through unfolding of helical domains localized to catalytic domain (CAT) of the enzyme.

PARP 1 inhibitors are recognized as potential therapeutic agents, which can be clinically used not only for monotherapy in treatment of BRCA-deficient malignancies but also as adjuvant therapy to enhance curative potential of antineoplastic drugs[2]. Pharmacologic endpoint of PARP inhibitors that enter into clinical trials is PARP 1 inhibition in cells. The vast majority of reported inhibitors belong to the competitive inhibitors containing nicotinamide as pharmacophore. From the other hand, PARP 1 can be inhibited by the mechanism ensuring the trapping of the enzyme to DNA [3, 4]. This mechanism involves auto-poly(ADP-ribos)ylation (auto-PARylation) of the enzyme molecule and occurs at PARP 1 regulatory domain. Extensive auto-PARylation of PARP 1 facilitates its dissociation from DNA due to high negative charge of poly(ADP-ribose). This event is necessary for stimulating of PARP 1 activity and the completion of DNA repair [5, 6]. DNA binding properties of PARP 1 greatly influence chromatin structure and modulate the character of DNA fragmentation in nuclei [7]. Benzamide (BA) is well known first generation PARP 1competitive inhibitor and is widely exploited pharmacophore in wide range of drugs. In present study we attend to examine whether BA can affect chromatin structure in liver cell nuclei, when BA is administrated to hall animals. Chromatin condensation was assessed according to Hughes and Cidlowski and by DNase1 accessibility assay [8].

MATERIALS AND METHODS

Animals

Animals were handled in accordance with protocols approved by Committee for Bioethics of Yerevan State University. Animals were obtained from the stock of animal house of faculty of Biology, Yerevan State University. Rats were housed in laboratory conditions in polypropelene cages at standard conditions (22±2°C) with a 12 light/dark cycle. Animals were fed with commercial rat feed ad libitum and were given access to water. The procedures were approved by the National Centre of Bioethics (Yereavan, Armenia) and performed according to the International Recommendations (CIOMS, 1985) guidelines.

Albino inbred male rats (6 week old) were used throughout all experiments. The animals were standardized by weight (to 100g). BA and other reagents were purchased from Sigma-Auldrich. Vehicle (saline) and BA (25 mg/kg weight), were injected intraperitoneal. Animals were sacrificed under light ether anesthesia by decapitation after 24 and 48 h treatment with BA.

Nuclei isolation

Nuclei were isolated according to Hewish and Burgoyne [9]. Sucrose solutions utilized throughout the nuclei isolation procedure were buffered with 20 mM Tris containing 15 mM NaCl, 60 mMKCl, 0,15mM spermine and 0.5mM spermidine, pH 7,4.

PARP 1 assay

The enzymatic assay for PARP 1 activity was performed according to the original method based on estimation of residual NAD^+ concentration in PARP assay mix adapted by us to quantify NAD^+ consumed by isolated nuclei [10]. Briefly, nuclei were gently suspended in PARP assay buffer containing 20mM Tris, 6mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.4. Density of nuclear suspension was normalized to 1mg DNA/ml. PARP reaction was initiated by addition of NAD^+ stock solution to 1000 µl aliquot of nuclear suspension (500 µM NAD^+ final concentration). The reaction was carried out for 10 min at 37^{0}C followed by centrifugation at 13 000g, 4^{o}C for 2 min to discard nuclear pellet. 50µl aliquot samples of supernatant were transferred to the Falcon UV-Vis transparent 96-well plate. NAD^+ quantification was performed by sequential addition of 2M KOH, acetophenone (20% in EtOH) and 88% formic acid, in accordance with the original assay. Absorbance of PARP assay mix containing 0,5mM NAD^+ was measured at 378 nm. The amount of NAD^+ was determined by using NAD^+ calibration curve and PARP 1 activity was defined as NAD^+ consumed by nuclei in 10 min per mg of DNA.

NAD^+ assay

The nuclei were pellet from suspension (13000g, 15 min, 1^o C). Extraction of NAD+ from nuclei was performed according to Alano et al [11]. NAD+ was determined by Putt K and Hergenrother [10]. NAD+ standards were used to quantify NAD+ in samples, which were normalized to 1mg/ml DNA.

DNase I assay

Chromatin was isolated by subsequent washing of isolated nuclei in 150 mM NaCl; 75 mM NaCL, 24 mM EDTA in 25 mMTris-HCl pH 8 and several washings in 10 mM and 5mM Tris-HCl pH 7,4. Chromatin was digested for indicated time intervals with 10units/A_{260} DNase I. 1000 µl aliquots were removed from chromatin suspensions and placed in test tubes. The tubes were heated to 37^{o}C and 100 µl DNase I stock solution was added (to final concentrations 10 Kunitz/ml correspondingly). DNase I stock solution was prepared in 25 mM Tris, pH 7,5, 3mM $MgCl_2$). At the end of incubation the tubes were chilled on ice and perchloric acid was added to final concentration 5% and chilled for additional 10 minutes. To determine the amount of acid-soluble nucleotides released from chromatin by DNase I digestion the insoluble material was sediment (9000 g,15 min). Supernatants were removed and absorption in 260 nm was measured.

DNA fragmentation assay

100 µl aliquot samples of nuclear suspension normalized to1000 µg/ml DNA were transferred to the Eppendorf tubes and 60mM $MgCl_2$ and 10mM $CaCl_2$ were added to yield final concentrations of 6mM $MgCl_2$ and 1mM $CaCl_2$ in aliquot probes. The ions were added to activate endogenous Mg^{+2} - and Ca^{+2} /Mg^{+2} -dependent nuclear endonucleases, which initiated internucleosomal DNA cleavage [12]. DNA isolation was performed according to standard protocol [13].

Nuclear DNA was subjected to electrophoresis in 1,8 % agarose gels (8v/cm). DNA was visualized by ethidium bromide staining and DNA fragmentation was assessed after gel densitometry using Fuji Film Image Gauge ver.3.12 program for determination of relative content of DNA fragments.

Statistics

All results were expressed as M ± S.D. Statistical differences in the results between groups were evaluated by the Student's t-test. A probability (P) value of < 0.05 was considered significant.

RESULTS AND DISCUSSION

PARP 1 inhibitors are entering clinical trials improving efficacy of DNA-damaging agents in cancer therapy. Their exploitation tends to increase in time though the knowledge of mechanisms responsible for differences in curative potential are poorly understood [4]. BA represents a broad spectrum of NAD^+-competing inhibitors designed as benzamide derivates and employed in clinical trials [14]. In present study we attempt to describe a link between PARP-1 activation, nuclear NAD+ content and chromatin condensation. Though we realize that fractionation of nuclei take considerable time which could lead to NAD+ degradation, we consider that the standard procedure utilized for nuclei isolation from BA treated and control group animals minimized the risk of uncontrolled fluctuations in estimated NAD^+ content.

Our data come to show that in the case, when liver and thymocyte nuclei were treated with BA after isolation from animals of control group we observed concentration-dependent PARP 1 inhibition in liver nuclei, whereas enzyme activity in thymocyte nuclei was unaffected (fig.1, A). In contrast, in experimental settings when liver and thymocyte nuclei were isolated after treatment of rats with BA, nuclei displayed elevated PARP 1 activity. Stimulation of PARP 1 activity in liver nuclei of BA treated rats was apparent in 24 hours and increased nearly two-fold during next 24h after BA injection (48h of BA injection to rats) (fig.1, B). Marked PARP 1 activation in thymocyte nuclei was detected only in 48 h of treatment with inhibitor. Coming from this, we consider that PARP 1 activity in thymocyte nuclei was less affected by administration of BA to rats. However, we observed unexpected outcomes of the in vivo treatment of rats with BA. The impact of inhibitor on PARP 1 activity in

nuclei of BA-treated rats was opposite to effect that elicited BA on PARP 1 activity in nuclei isolated from control group animals.

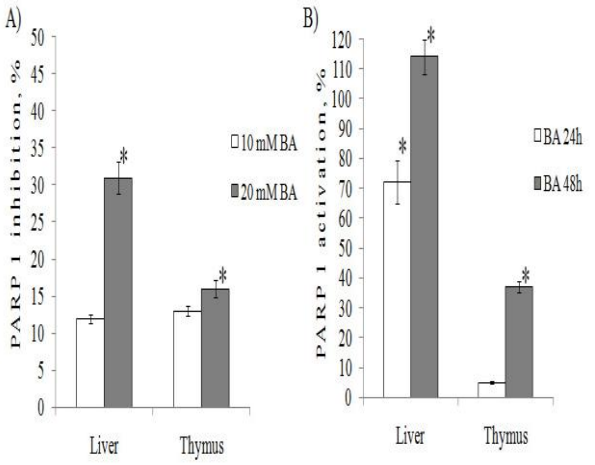

Figure 1: A-inhibition of PARP 1 by BA in liver and thymocyte nuclei isolated from the animals of control group. BA was added into nuclei incubation media 15 min prior to initiation of PARP 1 reaction. B-stimulation of PARP 1 activity in liver and thymocyte nuclei of rats treated in vivo with BA. Constitutive activity of PARP 1 was set as 100%, *P<0.05.

Coming from the knowledge that intracellular NAD^+ content plays prominent role in regulation of PARP 1 activity we hypothesized that PARP 1 stimulation can be determined by elevated NAD^+ content in liver and thymocyte nuclei of BA treated rats [15, 11, 16]. Previously it was shown that BA could significantly increase NAD^+ level in cells [17, 18]. Here we found that liver nuclei contained more NAD^+ that their thymocyte counterparts. In this study we show that BA administration to rats led to dramatic increase in NAD^+ in liver and thymocyte nuclei (nearly two-fold) (fig.2, A). This data supported hypothesis that PARP 1 activation in liver cell and thymocyte nuclei of rats treated with BA could resulted from increased NAD^+ content .

Since poly(ADP-ribos)ylation (PARylation) of chromatin proteins plays prominent role in chromatin structure formation [19], we anticipated that increased availability of NAD+ and PARP 1 stimulation can alter chromatin structure in liver nuclei. We employ chromatin structure-dependent assay, utilizing artificial activation of endogenous nuclear Ca^{+2}/Mg^{+2}- dependent apoptotic endonuclease to perform chromatin cleavage [20].

The data derived from chromatin-structure assay revealed that NAD^+ is capable to suppress DNA internucleosomal fragmentation in isolated nuclei in dose-dependent manner and 500 µM NAD^+ completely suppressed cleavage of DNA (fig.2, B).

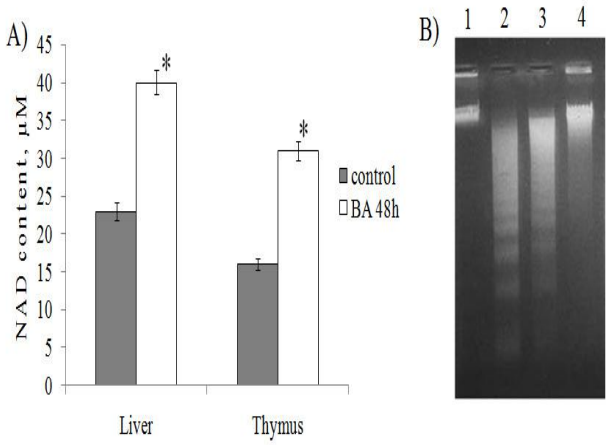

Figure 2: A-NAD^+ content in liver and thymocyte nuclei of rats from control and BA treated groups. B-Effect of exogenously applied NAD^+ on DNA internucleosomal fragmentation in rat liver nuclei. 1-DNA isolated from nuclei incubated 60 min in isolation media, 2- DNA isolated from nuclei incubated 60 min with Ca^{+2} and Mg^{+2} ions, 3- DNA isolated from nuclei incubated in with 100 µM NAD^+ and Ca^{+2}, Mg^{+2} ions, 4- DNA isolated from nuclei incubated with 500 µM NAD^+ and Ca^{+2}, Mg^{+2} ions. NAD^+ was added into nuclei incubation media 15 prior to addition of divalent ions,* P<0.05.

Coming from our results we concluded that increased availability of NAD^+ in liver nuclei stimulated PARP 1 activity and excessive PARylation shielded chromatin areas adjacent to enzyme from nucleolytic attacks.

Next we examined whether BA administration to intact rats could alter chromatin condensation in rat liver nuclei. DNase 1 accessibility of chromatin was examined in nuclei isolated from rats from control and BA treated groups. Our data come to show that BA administration to intact rats modulated the dynamics of DNase 1 digestion in liver nuclei. We revealed that DNase 1 accessibility of chromatin in nuclei isolated from BA treated rats was reduced (fig. 3).

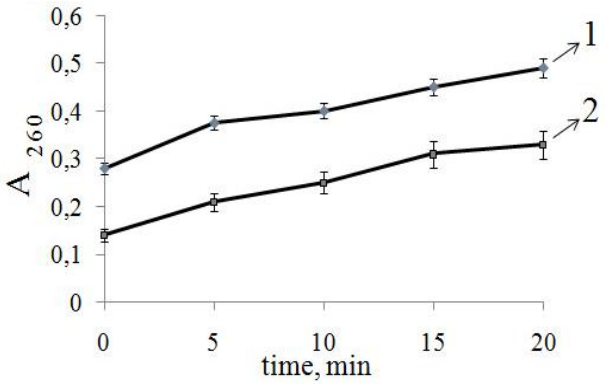

Figure 3: Dynamics of DNase I digestion of rat liver chromatin. 1- chromatin isolated from liver nuclei of control group rats, 2- chromatin isolated from liver nuclei of rats treated with BA (48h).

The results of chromatin-structure assay come to show that in vivo treatment of rats with BA augmented the content of high molecular weight DNA fragments (fig.4, table 1) and reduced the relative content of low-weight fragments.

Chromatin structure-dependent assay and modulation of different length DNA fragments content in concert with DNase digestion assay indicated on chromatin condensation in liver nuclei of BA treated rats.

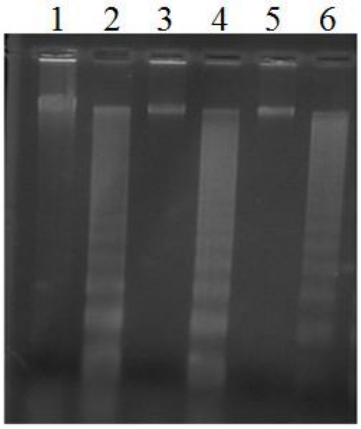

Figure 4: DNA fragmentation in nuclei isolated from liver. 1-nuclei from control group incubated 60 min in isolation media, 2- nuclei from control group incubated with Ca^{+2}, Mg^{+2} ions for 60 min, 3- nuclei isolated from the liver of rats treated with BA for 24h and incubated 60 min in isolation media, 4- nuclei isolated from the liver of rats treated with BA for 24h and incubated 60 min in the presence of Ca^{+2} and Mg^{+2} ions, 5- nuclei isolated from the liver of rats treated with BA for 48h and incubated 60 min in isolation media, 6- nuclei isolated from the liver of rats treated with BA for 48h and incubated 60 min in the presence of Ca^{+2} and Mg^{+2} ions.

Table 1: Relative content (%) of different length DNA fragments in nuclei isolated from liver of rats treated with BA. DNA content per lane in gel was set as 100%. *P<0,05

The length of DNA fragments	control	BA treated 24h	BA treated 48h
	60 min Ca^{2+}/Mg^{2+}	60 min Ca^{2+}/Mg^{2+}	60 min Ca^{2+}/Mg^{2+}
>1000 b.p.	16.44 ± 0.8	4.8* ± 0.28	23.84* ± 0.95
1000-200 b.p.	74.54 ± 2.9	83.53 ± 5.8	73.59 ± 4.4
<200 b.p.	9.02 ± 0.36	11.67 ± 0.46	2.57* ± 0.07

In purpose to investigate whether chromatin condensation in liver nuclei of rats resulted from direct impact of BA or emerged from BA-induced modulations in biochemical chain of reactions elicited by pharmacologic effect of inhibitor in hall organism, we study the effect of BA on chromatin condensation in naked nuclei isolated from liver of rats from control group. The data obtained in this set of experiments demonstrated, that BA did not affect chromatin condensation in isolated liver nuclei (fig. 5) and did not alter intensity of DNA internucleosomal fragmentation (table 2).

Table 2: Relative content (%) of different length DNA fragments in nuclei incubated with BA. DNA content per lane in gel was set as 100%.

The length of DNA fragments	60 min Ca^{2+}/Mg^{2+}	15 min 20 mM BA + 60 min Ca^{2+}/Mg^{2+}
>1000 b.p.	23.03 ± 0.98	20.5 ± 1.63
1000-200 b.p.	62.3 ± 4.75	56.2 ± 3.93
<200 b.p.	12.4 ± 0.62	23.3 ± 1.16

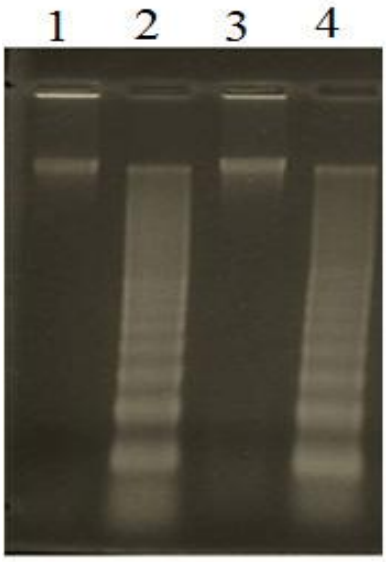

Figure 5: The effect of BA on DNA internucleosomal fragmentation in naked liver nuclei isolated from rats of control group. 1- DNA from nuclei incubated 60 min in isolation media, 2-DNA from nuclei incubated 60 min in the presence of Ca^{+2} and Mg^{+2} ions, 3-DNA from nuclei incubated 60 min in the presence of 20mM BA, 4- DNA from nuclei incubated 60 min in the presence of BA and Ca^{+2},Mg^{+2} ions. BA was added into incubation media 15 minutes prior to addition of Ca^{2+},Mg^{2+} ions.

CONCLUSION

Our results suggest that BA, which is well recognized PARP 1 inhibitor in vitro can display paradoxical PARP 1 stimulating role, when is administrated to intact rats in the in vivo experimental settings. This effect could result from increase of NAD+ content in liver cell and thymocyte nuclei of animals which underwent in vivo treatment with BA. We suppose that capability of BA for paradoxical activation of PARP 1 and modulation of chromatin structure in cell nuclei should be taken into account from the viewpoint of drug superposition in combined therapeutic regimens.

REFERENCES

1. Sistigu A, Manic G, Obristb F, Vitalea I, Trial watch – inhibiting PARP enzymes for anticancer therapy. Molecular & Cellular Oncology, 3(2), 2016, e1053594 , http://dx.doi.org/10.1080/23723556.2015.1053594.
2. Scott CL, Swisher EM, Kaufmann SH, Poly (ADP-Ribose) Polymerase Inhibitors: Recent Advances and Future Developmen,. J Clin Onco , 33(12), 2015, 1397-1406.
3. Murai J, Huang SN, Das BB, Renaud A, Zhang Y, Doroshow1 JH, Ji J, Takeda Sh, Pommier Y, Trapping of PARP 1 and PARP2 by Clinical PARP Inhibitors, Cancer Res, 72(21), 2012, 5588–5599.
4. Shen Y, Aoyagi-Scharber M, Wang B,. Trapping Poly (ADP-Ribose) Polymerase, J Pharmacol Exp Ther, 353, 2015, 446–457.
5. Langelier M, Planck JL, Roy S, Pascal JM, Structural basis for DNA damage-dependent poly(ADP-ribosyl)ation by human PARP-1, Science, 336, 2012, 728-732.
6. Luo X. Kraus L.W, On PAR with PARP: cellular stress signaling through poly(ADP-ribose)and PARP-1, Genes & Development, 26, 2012, 417–432.
7. Kim MY, Mauro S, Gevry N, Lis JT, Kraus WL, NAD+-Dependent Modulation of Cgromatin Structure and Transcription by Nucleosome Binding Properties of PARP-1, Cell, 119(6), 2004, 803-814.
8. Hughes FM Jr, Cidlowski J, Apoptotic nuclease assays, Methods enzymol, 322, 2000, 47-62.
9. Hewish DR, Burgoyne LA, Chromatin Substructure, The Digestion of Chromatin DNA at Regularly Spaced Sites by a Nuclear Deoxyribonuclease, Biochemical and Biophysical Res Com, 52(2), 1973, 504-510.
10. Putt KS, Hergenrother PJ, An enzymatic assay for poly(ADP - ribose)polymerase - 1 (PARP-1) via the chemical quantitation of NAD+: application to the high-throughput screening of small molecules as potential inhibitors, Anal Biochem, 326(1), 2004, 78-86.
11. Alano C, Ying W, Swanson A, Poly(ADP-ribose) Polymerase-1-mediated Cell Death in Astrocytes Requires NAD+ Depletion and Mitochondrial Permeability Transition, The Journal Of Biological Chemistry, 279(18), 2004, 18895–18902.
12. Yakovlev AG, Wang G, Stoica BA, Simbulan 0, Rosenthal CM, Yoshihara K, Smulson ME, Role of DNASIL3 in Ca^{2+}- and Mg^{2+}-dependent cleavage of DNA into oligonucleosomal and high molecular mass fragments, Nucleic Acids Res, 27, 1999, 1999-2005.
13. Sambrook J, Russel D, Molecular Cloning, A laboratory manual, 3rd edition, v.1(6), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York , 2001, 4-6.
14. Davar D, Beumer JH, Hamieh L, Tawbi H, Role of PARP Inhibitors in Cancer Biology and Therapy, Curr Med Chem, 19(23), 2012, 3907–3921.
15. Loetscher P, Alvarez-Gonzalez R, Althaus FR, Poly(ADP-ribose) may signal changing metabolic conditions to the chromatin of mammalian cells, Proc. Natl. Acad. Sci. USA, 84(5), 1987, 1286-1289.
16. Alano C, Tran A, Tao R, Ying W, Karliner JS, Swanson RA, Differences among Cell Types in NAD+Compartmentalization: A Comparison of Neurons, Astrocytes, and Cardiac Myocytes, Journal of Neuroscience Research, 85, 2007, 3378–3385.
17. Nakanishi S, Nishio A, Uyeki EM, Effect of benzamide on cell growth, NAD and ATP levels in cultured chick limb bud cells, Biochem. Biophys. Res. Commun, 121(2), 1984, 710-716.
18. Ghosh U, Bhattacharyya NP, Induction of apoptosis by benzamide in K562 cells was triggered by inhibition of poly(ADP-ribosyl)ation of protein (S). J. Biotechnology and Biomaterials, S 13, 2013, http;//dx.doi.org./104172/2155-952X.S13-008.
19. Kraus WL, Transcriptional Control by PARP-1: Chromatin Modulation, Enhancer-binding, Coregulation and Insulation, Curr Opin Cell Biol, 20(3), 2008, 294–302.
20. Widlak P, Li P, Wang X, Garrard WT, Cleavage Preferences of the Apoptotic Endonuclease DFF40 (Caspase-activated DNase or Nuclease) on Naked DNA and Chromatin Substrates, J Biol Chem, 275(11), 2000, 8226–8232.

Source of Support: Nil, **Conflict of Interest:** None.