EFFECTS OF THE POLY(ADP-RIBOSE) POLYMERASE INHIBITOR OLAPARIB IN CERULEIN-INDUCED PANCREATITIS

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ABSTRACT—**Objective:** Activation of the constitutive nuclear and mitochondrial enzyme poly (ADP-ribose) polymerase (PARP) has been implicated in the pathogenesis of cell dysfunction, inflammation, and organ failure in various forms of critical illness. The objective of our study was to evaluate the efficacy and safety of the clinically approved PARP inhibitor olaparib in an experimental model of pancreatitis *in vivo* and in a pancreatic cell line subjected to oxidative stress *in vitro*. The preclinical studies were complemented with analysis of clinical samples to detect PARP activation in pancreatitis. **Methods:** Mice were subjected to cerulein-induced pancreatitis; circulating mediators and circulating organ injury markers; pancreatic myeloperoxidase and malondialdehyde levels were measured and histology of the pancreas was assessed. In human pancreatic duct epithelial cells (HPDE) subjected to oxidative stress, PARP activation was measured by PAR Western blotting and cell viability and DNA integrity were quantified. In clinical samples, PARP activation was assessed by PAR (the enzymatic product of PARP) immunohistochemistry. **Results:** In male mice subjected to pancreatitis, olaparib (3 mg/kg i.p.) improved pancreatic function: it reduced pancreatic myeloperoxidase and malondialdehyde levels, attenuated the plasma amylase levels, and improved the histological picture of the pancreas. It also attenuated the plasma levels of pro-inflammatory mediators (TNF-α, IL-1β, IL-2, IL-4, IL-6, IL-12, IP-10, KC) but not MCP-1, RANTES, or the anti-inflammatory cytokine IL-10. Finally, it prevented the slight, but significant increase in plasma blood urea nitrogen level, suggesting improved renal function. The protective effect of olaparib was also confirmed in female mice. In HPDE cells subjected to oxidative stress olaparib (1 μM) inhibited PARP activity, protected against the loss of cell viability, and prevented the loss of cellular NAD^+ levels. Olaparib, at 1μM to 30 μM did not have any adverse effects on DNA integrity. In human pancreatic samples from patients who died of pancreatitis, increased accumulation of PAR was demonstrated. **Conclusion:** Olaparib improves organ function and tempers the hyperinflammatory response in pancreatitis. It also protects against pancreatic cell injury *in vitro* without adversely affecting DNA integrity. Repurposing and eventual clinical introduction of this clinically approved PARP inhibitor may be warranted for the experimental therapy of pancreatitis.

KEYWORDS—Cell death, DNA, mitochondria, multiorgan dysfunction, pancreatitis, shock

ABBREVIATIONS—ALB—albumin; ALT—alanine aminotransferase; AMY—amylase; BUN—blood urea nitrogen; Ca^{2+}—total calcium; CRE—creatinine; GLU—glucose; K^+—potassium; MDA—malondialdehyde; MPO—myeloperoxidase; Na^+—sodium; PAR—poly(ADP-ribose); PARP—poly(ADP-ribose) polymerase; PHOS—phosphorus

INTRODUCTION

Acute pancreatitis remains a life-threatening disease affecting several million people annually worldwide. In the United States, the rate of primary discharge diagnosis for acute pancreatitis is more than 80 per 100,000 US adults per year. In-hospital case fatality is approximately 1%, but in subjects that are older than 65 years, it is several times higher. In the USA, in 2009, over 270,000 acute pancreatitis patients were diagnosed: thus, acute pancreatitis is one of the most common

gastrointestinal diseases that requires acute hospitalization. Treatment costs of the disease are estimated over $2.5 billion/yr (1, 2). Despite extensive research and despite a large number of pivotal clinical trials targeting various mechanisms of the disease, no specific pharmacological or immunological therapies are available for the treatment of this condition; the mainstays of therapy remain supportive care such as fluid, nutrition, and the clinical management of complications (1–3).

Poly(ADP-ribose) polymerase (PARP) is a constitutive nuclear and mitochondrial enzyme with multiple regulatory functions in health and disease (4–10). Pathological overactivation of PARP has been implicated in many forms of critical illness (4–10), including pancreatitis (11–17). Pharmacological PARP inhibitors of various structural classes as well as PARP-1-deficient mice were shown to exert beneficial effects in various animal models of pancreatitis (11–17). Although these data suggest that PARP may be a therapeutic target in pancreatitis, translational advancement of this concept was not possible, because—until recently—there were no clinically approved drugs to inhibit PARP. This situation, however, has changed, due to an independent line of work in the field of oncology, which demonstrated that in certain forms of cancer, PARP inhibitors can provide therapeutic benefit. This work revitalized the pharmaceutical industry's interest in PARP inhibitors, and as a consequence, in recent years, several classes of PARP inhibitors have been approved by various regulatory agencies worldwide and have entered routine clinical practice in the field of oncology (18, 19). One of these clinically approved PARP inhibitors is olaparib (Lynparza). It is a competitive PARP inhibitor that inhibits the binding of the substrate of PARP (NAD^+) into the active site of the enzyme.

In our recent position paper (20) we have outlined an approach for the therapeutic repurposing of clinically approved PARP inhibitors for various nononcological indications. Various forms of critical illness are prime candidates for repurposing, because the available therapeutic options are limited, the expected duration of drug therapy is relatively short, and the body of preclinical data—at least conceptually—supports the validity of such efforts. However—as outlined in (20)—additional experiments are needed to expand the groundwork for this concept. Because each class of PARP inhibitors have different pharmacological properties, repurposing efforts require testing the exact same pharmacological inhibitor that would be a candidate for repurposing (as opposed to earlier generation inhibitors) and needs to focus both on efficacy and safety endpoints. The latter point is especially salient, because PARP activity has been implicated in DNA repair (4–10) and it needs to be ensured that the therapeutic benefit of PARP inhibitors in critical illness does not come at the expense of an impairment of DNA integrity.

The current study was set out to begin such a repurposing effort using the clinically approved PARP inhibitor olaparib for acute pancreatitis. From several clinically approved PARP inhibitors, olaparib was selected because several independent experiments have already demonstrated its beneficial effects in various nononcological conditions such as neuroinjury (21, 22), acute lung injury (23), cardiac

reperfusion injury (24), liver injury (25), and various forms of systemic inflammation (26, 27).

Our *in vivo* experiments employed a widely accepted experimental model of pancreatitis (induced by cerulein). We have also conducted an *in vitro* sub-study (human pancreatic epithelial cells subjected to oxidative stress) both focusing on efficacy endpoints (cell viability) as well as safety endpoints (nuclear and mitochondrial DNA integrity). Our *in vivo* results demonstrate the efficacy of olaparib on local (pancreatic injury) and systemic (renal dysfunction) outcome variables in the pancreatitis model, while the *in vitro* results define the concentration range of the PARP inhibitor that exerts cytoprotective effects without adversely affecting DNA integrity. Thus, the data presented in the current report can be considered a potential first step for the future repurposing and clinical introduction of the PARP inhibitor olaparib for the experimental therapy of pancreatitis.

MATERIALS AND METHODS

Materials

Unless stated otherwise, all chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, Missouri).

Animals

C57/BL6 (male and female, 2 mo old) were purchased from Jackson Laboratories. They were housed in a light-controlled room with a 12-h light–dark cycle and were allowed *ad libitum* access to food and water. All investigations adhered to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (Eighth Edition, 2011) and were performed in accordance with the IACUC, University of Texas Medical Branch, Galveston, Texas.

Pancreatitis induced by cerulein

Mice were randomly allocated into the following groups: Sham group: mice were treated hourly (five times) with saline solution, intraperitoneally [IP] instead of cerulein (n = 10); Cerulein group: mice were treated hourly (×5) with cerulein (50 μg/kg, in saline, i.p. plus olaparib vehicle) (n = 10); Cerulein + olaparib group: mice were treated hourly (×5) with cerulein (50 μg/kg, in saline, IP) and received olaparib (3 mg/kg, i.p.) 1 and 6 h after the first dosing of cerulein (n = 10). (The delay in the administration of olaparib relative to cerulein was introduced to mimic the clinical situation where pretreatment with the inhibitor is not realistic.) Mice were killed by exsanguination at 6 h after the first cerulein injection. Blood samples were obtained by intracardiac puncture. The pancreas was removed immediately, frozen in liquid nitrogen, and stored at −80°C until assayed. Portions of these organs were also fixed in formaldehyde for histological examination.

Myeloperoxidase assay

Myeloperoxidase activity was measured in pancreas tissue homogenates using a commercially available myeloperoxidase (MPO) fluorometric detection kit (Enzo Life Sciences, Farmingdale, NY) as described (28). The assay utilizes a non-fluorescent detection reagent, which is oxidized in the presence of hydrogen peroxide and MPO to produce its fluorescent analog. The fluorescence was measured at excitation wavelength of 530 nm to 571 nm and emission wavelength of 590 nm to 600 nm.

Malondialdehyde assay

Tissue malondialdehyde (MDA) levels, an index of cellular injury/oxidative stress, were quantified in pancreas homogenates using a fluorimetric MDA-specific lipid peroxidation assay kit (Enzo Life Sciences, Farmingdale, NY) as described (28). The assay is based on the BML-AK171 method in which two molecules of the chromogenic reagent N-methyl-2-phenylindole react with one molecule of MDA at 45°C to yield a stable carbocyanine dye with a maximum absorption at 586 nm.

Measurement of biochemical parameters of organ dysfunction

Blood samples were collected via cardiac puncture and were analyzed by using a Vetscan analyzer (Abaxis North America, Union City, Calif) as described (26) for various biochemical parameters within 1 h of collection.

Quantification of plasma cytokine levels

Blood from all groups were collected in heparinized blood collection tubes, and centrifuged at $4°C$ for 10 min at $1,000 \times g$ within 30 min of collection. Plasma was isolated, aliquoted, and stored at $-80°C$ until use. The EMD Millipore's MILLIPLEX MAP Mouse cytokine Magnetic Bead Panel 1 kit was used for the simultaneous quantification of multiple circulating analytes (Merck Millipore, Darmstadt, Germany). Luminex uses a proprietary technique to internally color code microspheres with two fluorescent dyes and to create distinctly colored bead sets of 5005.6 μm polystyrene microspheres or 80 6.45 μm magnetic microspheres, each of which is coated with a specific capture antibody. After an analyte from a test sample is captured by the bead, a biotinylated detection antibody is introduced. The reaction mixture is then incubated with streptavidin-phycoerythrin (PE) conjugate, the reporter molecule, to complete the reaction on the surface of each microsphere. The Luminex instrument acquires and analyzes data using the LuminexxMAP fluorescent detection method and the LuminexxPONENT acquisition software (Thermo Fisher Scientific, Waltham, Mass).

Histological studies and TUNEL assay

Pancreas sections were collected at 6 h after 1st cerulein injection. They were fixed for 1 week in buffered formaldehyde solution (10% in PBS) at room temperature, dehydrated by graded ethanol and embedded in Paraplast (Sherwood Medical, Mahwah, NJ). Tissue sections (thickness: 7 μm) were deparaffinized with xylene stained with hematoxylin/eosin and studied using light microscopy. Histological studies were performed in a blinded fashion. TUNEL assay was conducted using a TUNEL detection kit (ApopTag Peroxidase In Situ Apoptosis Detection Kit; EMD Millipore, Calif) according to the manufacturer's instruction.

Cell culture

Human Pancreatic Duct Epithelial Cells (HPDE) were purchased from the Kerafast, Inc (Catalog # ECA001), and cultured in Keratinocyte Basal Medium (Lonza, Clonetics KBM, Catalog # CC-3111) supplemented with ×1 antibiotic—antimycotic (Gibco Catalog# 15240062).

In vitro oxidative stress induced by hydrogen peroxide

HPDE cells (20×10^3 cells per well) were seeded overnight onto 96-well tissue culture plates with cell culture medium and cultured at $37°C$ at 5% CO_2 atmosphere for 24 h. The cells were then placed in fresh cell culture medium and treated with olaparib (1 μM–100 μM) followed by hydrogen peroxide (H_2O_2) (300 μM–1 mM, 1–24 h) exposure.

MTT assay

The MTT method was performed as described (28). Briefly, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was added to the cells at a final concentration of 0.5 mg/mL and cells were cultured at $37°C$ for 1 h. The cells were washed with PBS and the formazan dye was dissolved in DMSO. The amount of converted formazan dye was measured at 570 nm with a background measurement at 690 nm on a Power wave reader (Biotek).

LDH assay

Measurement of lactate dehydrogenase (LDH) release into the medium (28) was used to assess cytotoxicity. Briefly, 30 μL of supernatant was collected and mixed with 100 μL freshly prepared LDH assay reagent containing 85 mM lactic acid, 1 mM nicotinamide adenine dinucleotide (NAD^+), 0.27 mM N-methyl phenazonium methyl sulfate (PMS), 0.528 mM 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT), and 200 mM Tris (pH 8.2). The changes in absorbance were read kinetically at 492 nm for 15 min (kinetic LDH assay) on a monochromator-based reader (Powerwave HT, Biotek) at $37°C$.

NAD^+ assay

Cellular NAD^+ content was determined using NAD/NADH Quantification Kit (Sigma) according to the manufacturer's protocol. The amount of NAD^+ present in the samples was quantified in a colorimetric assay, measured at 450 nm using a microplate reader. The amount of NAD^+ was normalized to protein content, quantified using the PierceTM BCA Protein Assay.

PARP1 and PAR western blotting

HPDE cells were seeded in 12 well/plates at the confluence of 2×10^5 cells/well and after 24 h they were pretreated with olaparib 1 μM to 100 μM and then treated with H_2O_2 (300 μM) for 1 h. The cells were collected and the total cell extracts were prepared homogenizing the cells in NP-40 lysis buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1% Nonidet P-40) in the presence of protease inhibitor (Roche) followed by clean-up centrifugation at $20,000 \times g$ for 10 min. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane (Bio-Rad, Hercules, Calif). The membrane was blocked in Thermo Scientific Starting Block Blocking Buffer (Thermo Fisher Scientific, Waltham, Mass) followed by exposure to primary antibodies anti-PARP-1(1:1,000; 46D11 Cell Signaling) and poly(ADP-ribose) (PAR, the enzymatic product of the PARP enzyme and a marker of PARP's catalytic activation) (1:1,000; 4336-BPC-100 Trevigen) overnight at $4°C$. The anti-rabbit IgG conjugated to horseradish peroxidase (1:2,000; Cell Signaling) was used as secondary antibody. After incubating with secondary antibody for 1 h at room temperature, the membrane was developed with SuperSignal West Pico Chemiluminescent Substrate (Pierce) in a GeneBox Detection System (Syngene).

Quantification of DNA integrity

Measurement of mitochondrial and nuclear DNA integrity was conducted using gene-specific semiquantitative PCRs as described (29). Briefly, HPDE cells were seeded in 12-well plates (200,000 cell/well). Total DNA was isolated using DNasey Blood and Tissue Kit (QIAGEN). Integrity of the mtDNA was assessed using two set pairs of primers with amplicon of 211 bp and 8.9 kb (short amplicon was used as a normalization factor of mtDNA copies). Integrity of the nuclear DNA was assessed using 10 kb amplicon. Taq DNA Polymerase and LongAmp Taq DNA polymerase (both from NewEngland BioLabs) were used for amplification of short and long DNA targets, respectively. DNA integrity in untreated (control) cells was set as 100%.

Immunohistochemical detection of PARylation in human clinical samples obtained from pancreatitis patients

Pancreas tissue arrays (BBS14011 and PA2081) containing normal pancreatic tissue from autopsy and pancreatic tissues from patients with acute or chronic pancreatic inflammation were purchased from US Biomax Inc (Derwood, MD 20855). Immunohistochemical staining for PAR (the product of PARP) was conducted in deparaffinized tissue sections using a PAR antibody (Tulip mouse monoclonal, anti-human 1:10). n = 3 normal controls, n = 3 acute pancreatitis sections, and n = 6 chronic pancreatitis sections (all from different subjects/patients) were analyzed.

Statistical analysis

All values described in the text and figures are expressed as means ± SEM for n observations. The one-way analysis of variance (ANOVA) is used to determine whether there are any statistically significant differences between the means of the three groups (control, cerulean, and olaparib+cerulean in the *in vivo* experiments or control, oxidative stress, and olaparib+oxidative stress in the *in vitro* experiments). When the one-way ANOVA returned a statistically significant result, we concluded that are at least two group means that are statistically significantly different from each other. At this point, Tukey *post-hoc* test was used to determine whether the insult (cerulein *in vivo* or oxidative stress *in vitro*) has an effect on a given parameter compared with normal control values (animals not subjected to cerulein or cells not subjected to H_2O_2) and to detect if olaparib affected the response in the groups that were subjected to the insult (cerulein *in vivo* or oxidative stress *in vitro*), compared with the groups that were subjected to the same insults in the absence of olaparib. Prism version 5 for Windows (Graph Pad Software) was used. $P < 0.05$ was considered statistically significant.

RESULTS

Olaparib exerts organ protective and anti-inflammatory effects in adult male mice subjected to cerulein-induced pancreatitis

In adult male mice subjected to cerulein-induced pancreatitis, olaparib (3 mg/kg i.p.) improved several parameters of pancreatic dysfunction (Figs. 1–3). For instance, the
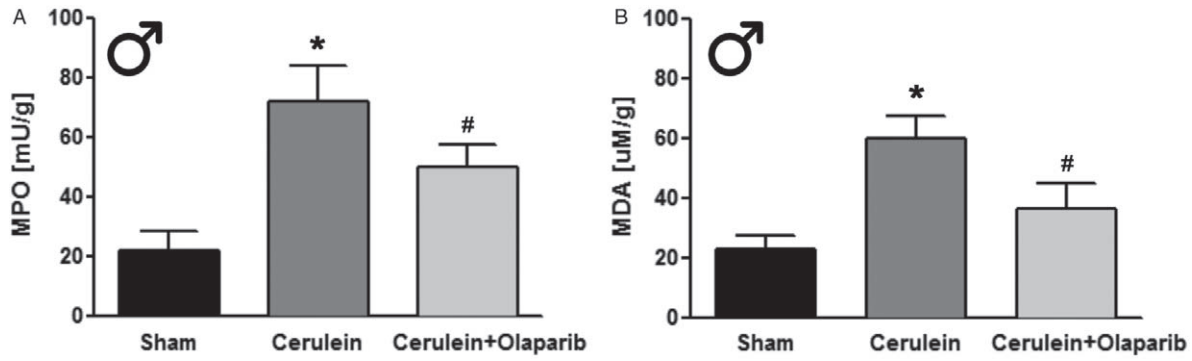
FIG. 1. **Effect of olaparib on pancreatic MPO and MDA levels in male Balb/c mice subjected to cerulein-induced pancreatitis.** (A) Pancreatic MPO levels (expressed as milliunits [mU]/gram tissue) and (B) pancreatic MDA levels (expressed as μM/g tissue) are shown in sham mice (not subjected to cerulein) ("Sham"), in vehicle-treated mice subjected to cerulein at 6 h ("Cerulein") and in mice subjected to cerulein in the presence of olaparib (2 mg/kg $\times$3 mg/kg) (Cerulein+Olaparib). Data are shown as mean $\pm$ SEM of 10 animals for each group; $^*P < 0.05$ shows significant increase in MPO or MDA in response to cerulein, compared with the sham group; #$P < 0.05$ shows significant protective effect of olaparib in cerulein-pancreatitis mice compared with vehicle-treated cerulein-pancreatitis mice.
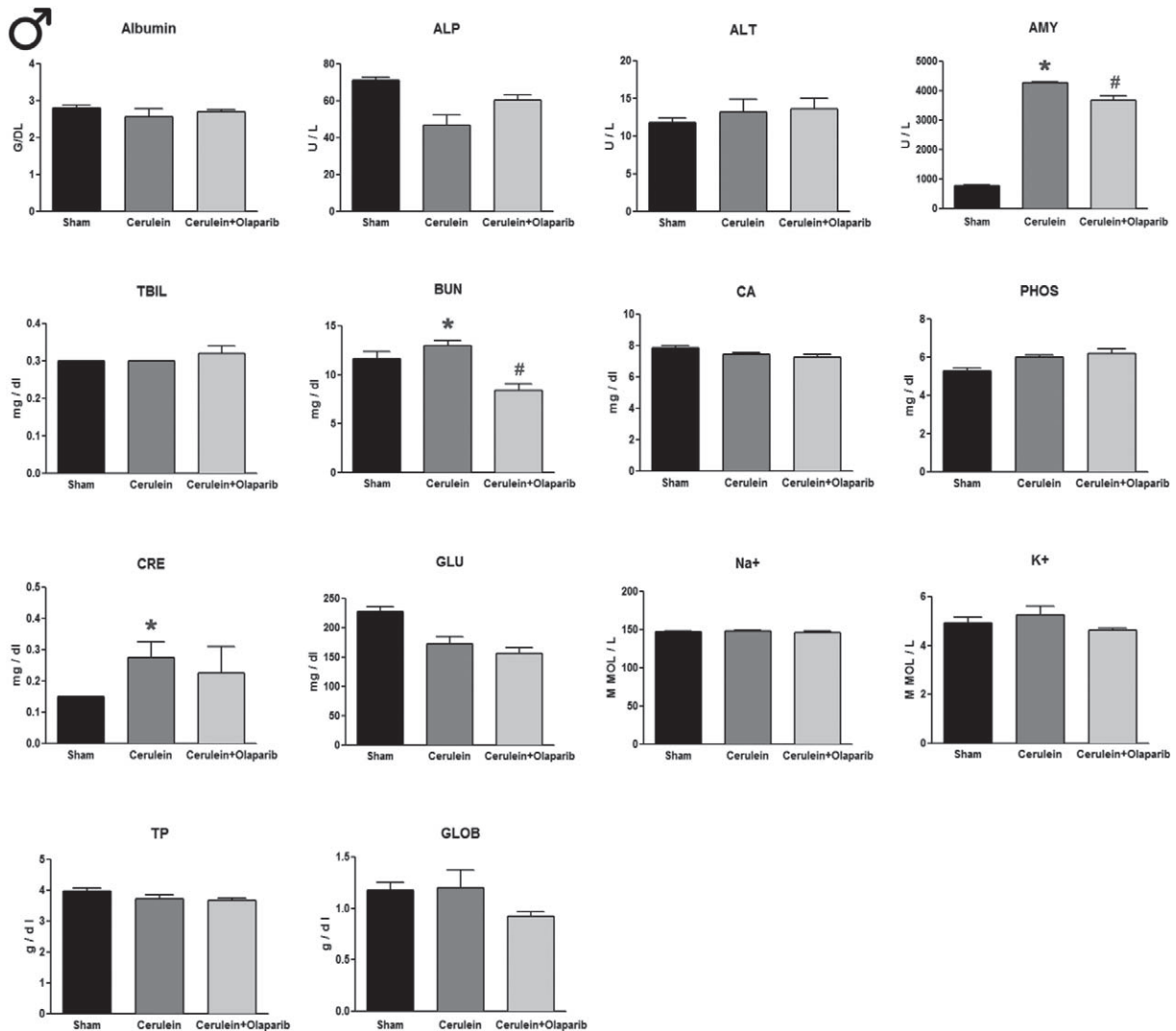
FIG. 2. **Effect of olaparib on selected parameters of organ injury in male Balb/c mice subjected to cerulein-induced pancreatitis.** Various physiological and organ injury marker levels: alkaline phosphatase (ALP), alanine aminotransferase (ALT), amylase (AMY), total bilirubin (TBIL), plasma blood urea nitrogen (BUN), plasma calcium (CA^{++}), plasma phosphate (PHOS), plasma creatinine (CRE), plasma glucose (GLU), plasma sodium (Na$^+$), plasma potassium (K$^+$), total protein (TP), and plasma globulin (GLOB) measured by Vetscan analysis, are shown in sham mice (not subjected to cerulein) ("Sham"), in vehicle-treated mice subjected to cerulein at 6 h ("Cerulein") and in mice subjected to cerulein in the presence of olaparib (2 mg/kg $\times$ 3 mg/kg) (Cerulein+Olaparib). Data are shown as mean $\pm$ SEM of 10 animals for each group; $^*P < 0.05$ shows significant increase in the respective parameter in response to cerulein, compared with the sham group; #$P < 0.05$ shows significant protective effect of olaparib in cerulein-pancreatitis mice compared with vehicle-treated cerulein-pancreatitis mice.
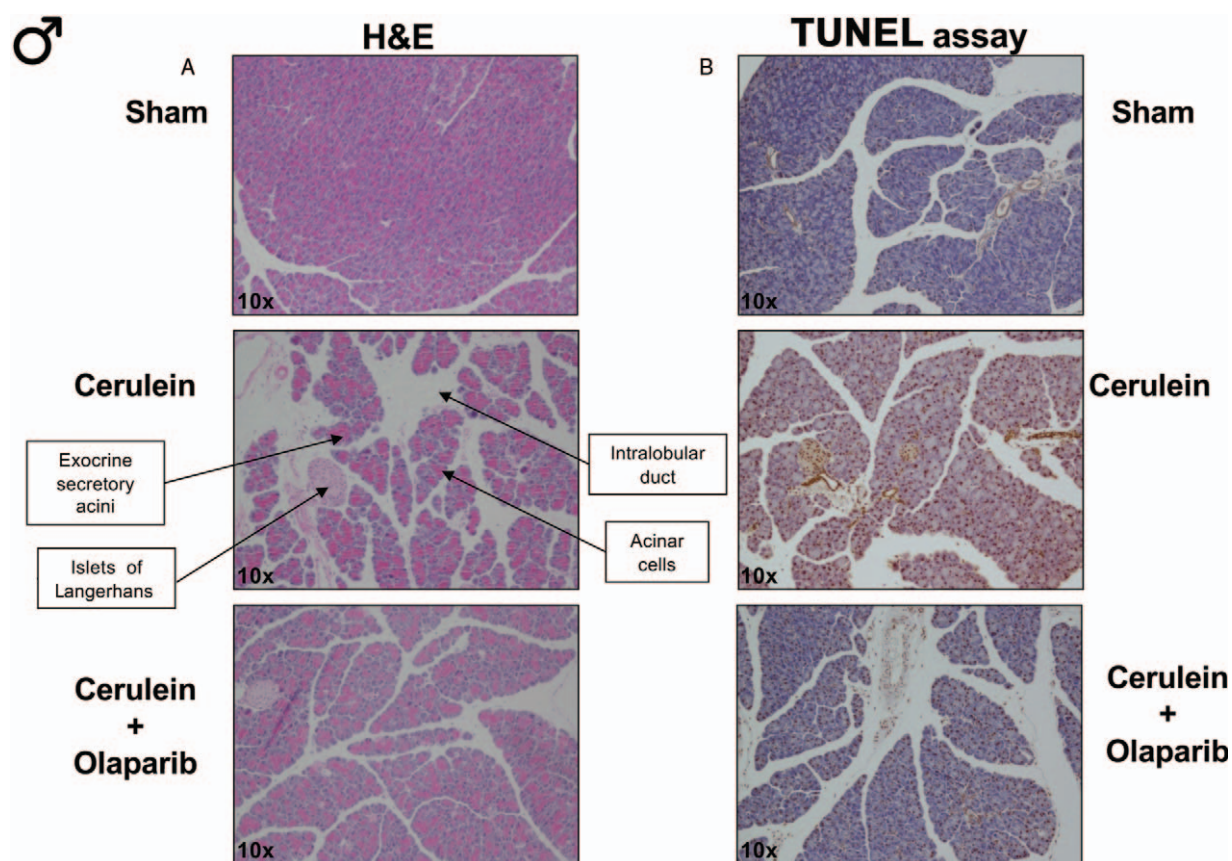
Fig. 3. **Effect of olaparib on histological alterations injury in the pancreas of male Balb/c mice subjected to cerulein-induced pancreatitis.** (A) Representative histological pictures and (B) TUNEL staining are shown in sham mice (not subjected to CLP) ("Sham"), in vehicle-treated mice subjected to cerulein at 6 h ("Cerulein") and in mice subjected to cerulein in the presence of olaparib (2 mg/kg × 3 mg/kg) (Cerulein+Olaparib). Pictures are selected from n = 5 animals for each group. Figure is available in color online.

cerulein-induced increases in pancreatic MPO and MDA content (indicative of neutrophil infiltration and intraorgan oxidative stress, respectively) were attenuated by olaparib (Fig. 1). In addition, the cerulein-induced increases in the plasma marker of pancreatic injury (amylase) and renal dysfunction (BUN) were also attenuated by olaparib treatment (Fig. 2). The histopathological pictures of the pancreas showed evidence of cellular damage, as well as DNA damage/apoptosis (assessed by the TUNEL assay); these alterations were also improved by olaparib treatment (Fig. 3). Representative images are shown in Figure 3; histopathological images from five animals per group are shown in Supplemental Fig. 1, Supplemental Digital Content 1, http://links.lww.com/SHK/A909.

Olaparib attenuated the cerulein-induced increases in the levels of several circulating mediators in the plasma (e.g., TNFα, IL-1β, IL-2, IL-4, IL-6, IL-12, IP-10, KC), while plasma levels of other circulating mediators (e.g., KC, IL-10, RANTES, VEGF) were unaffected (Fig. 4).

The beneficial effects of olaparib are maintained in female mice subjected to pancreatitis

As discussed in our position paper (20), in many experimental models, the beneficial effects of pharmacological PARP inhibition or genetic PARP deficiency are gender-dependent, with the effects diminishing or nonexistent in female animals. Importantly, this gender difference is very prominent in endotoxin-induced systemic inflammation models (30) and in cecal ligation and puncture induced sepsis models (27). In the current pancreatitis model, however, the therapeutic effect of olaparib remained significant in female mice (Figs. 5–8). Olaparib attenuated the cerulein-induced increases in pancreatic MPO levels in female mice, although to a lesser degree than in males (Fig. 5); however, it failed to significantly affect pancreatic MDA levels. In addition, the cerulein-induced increases in plasma amylase, BUN, and creatinine were also attenuated by olaparib treatment (Fig. 6). In contrast to the male mice, in female mice we have detected a significant cerulein-induced increase in circulating ALT levels (indicative of hepatic dysfunction); however, this was unaffected by olaparib (Fig. 6). There was also evidence of cerulein-induced hyperglycemia in female mice (perhaps indicative of pancreatic beta cell damage), and this was mitigated by olaparib (Fig. 6). The histopathological pictures of the pancreas showed evidence of cellular damage, which was improved by olaparib treatment (Fig. 7).

Olaparib exerts cytoprotective effects in HPDE cells subjected to oxidative stress in vitro, without adversely affecting DNA integrity

In HPDE cells subjected to oxidative stress, olaparib inhibited PARylation (as measured by the quantification of
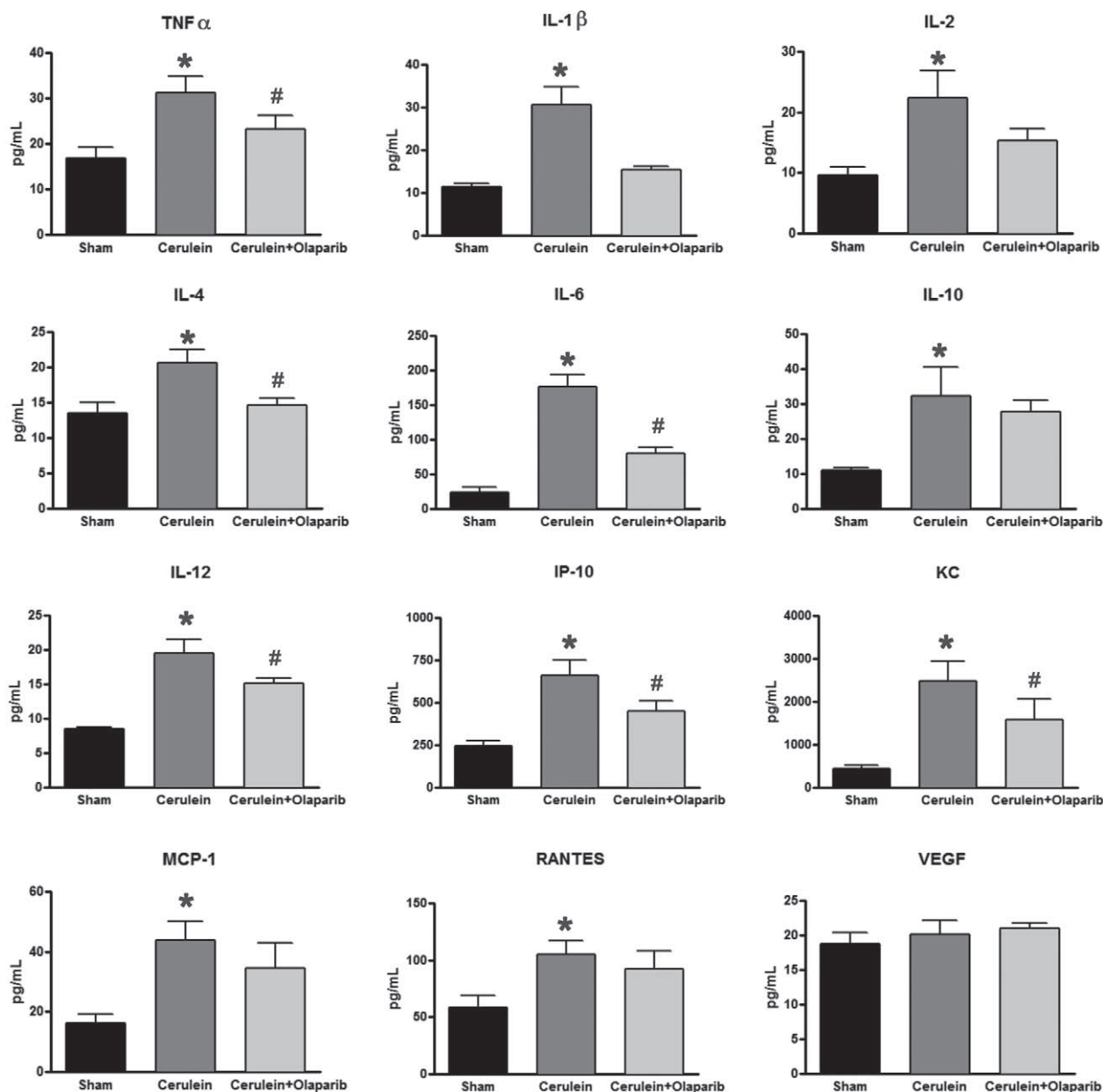
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F$_{IG}$. 4. **Effect of olaparib on the levels of various circulating mediators (cytokines, chemokines, growth factors) in male Balb/c mice subjected to cerulein-induced pancreatitis.** Values are shown in sham mice (not subjected to CLP) ("Sham"), in vehicle-treated mice subjected to cerulein at 6 h ("Cerulein") and in mice subjected to cerulein in the presence of olaparib (2 mg/kg × 3 mg/kg) (Cerulein+Olaparib). $^*P < 0.05$ shows significant increase in the respective parameter in response to cerulein, compared with the sham group; $\#P < 0.05$ shows significant protective effect of olaparib in cerulein-pancreatitis mice compared with vehicle-treated cerulein-pancreatitis mice.

poly(ADP-ribose) [PAR], the product of the enzyme) already at the lowest concentration (1 μM) used, confirming the well-established (31) potent inhibitory effect of olaparib on PARP1 catalytic activity in this cell line (Fig. 8). PARP1 enzyme levels were downregulated by the oxidative stress challenge; this downregulation was also partially attenuated by the lower concentrations (1 μM – 3 μM) of olaparib (Fig. 8). In line with prior findings demonstrating that PARP inhibition prevents the cellular depletion of its substrate, NAD$^+$ (4, 32), olaparib also protected against the H$_2$O$_2$-induced loss of cellular NAD$^+$ levels, with the effects already near-maximal at its lowest concentration tested (1 μM) (Fig. 9).

Consistent with prior findings demonstrating the protective effect of PARP inhibitors in cells exposed to various oxidants (4, 32, 33) olaparib (1 μM) protected against the H$_2$O$_2$-induced loss of cell viability, indicated by continued MTT reduction in the presence of olaparib treatment (Fig. 10). Although the H$_2$O$_2$-induced inhibition of MTT reductive capacity, as well as the marked drop in NAD$^+$ levels predicted the development of significant cell dysfunction, this was not associated with any apparent necrosis, as LDH levels in the cell culture supernatant did not increase after H$_2$O$_2$ challenge (Fig. 10).

In a broad concentration range (1 μM – 100 μM), olaparib did not have any adverse effects on nuclear or mitochondrial DNA
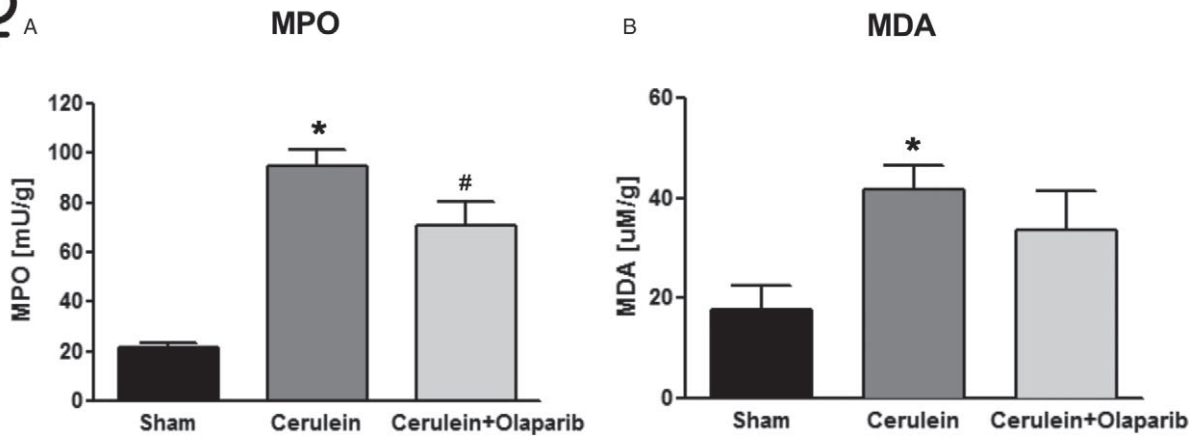
Fɪɢ. 5. **Effect of olaparib on pancreatic MPO and MDA levels in female Balb/c mice subjected to cerulein-induced pancreatitis.** (A) Pancreatic MPO levels (expressed as milliunits [mU]/g tissue) and (B) pancreatic MDA levels (expressed as μM/gram tissue) are shown in sham mice (not subjected to cerulein) ("Sham"), in vehicle-treated mice subjected to cerulein at 6 h ("Cerulein") and in mice subjected to cerulein in the presence of olaparib (2 mg/kg × 3 mg/kg) (Cerulein+Olaparib). Data are shown as mean ± SEM of 10 animals for each group; $^*P < 0.05$ shows significant increase in MPO or MDA in response to cerulein, compared with the sham group; #$P < 0.05$ shows significant protective effect of olaparib against the increase in MPO in cerulein-pancreatitis mice compared with vehicle-treated cerulein-pancreatitis mice.
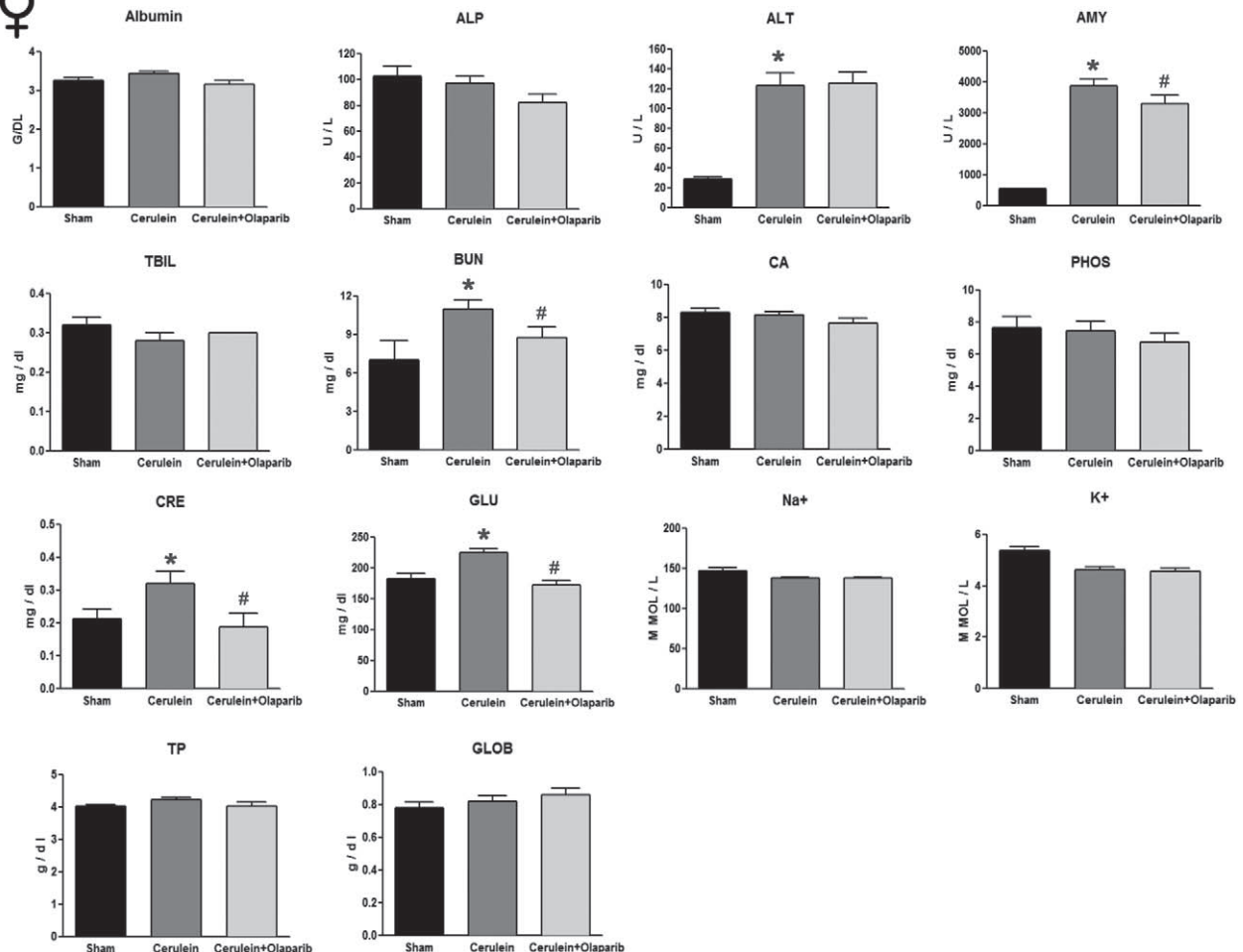

Fɪɢ. 6. **Effect of olaparib on selected parameters of organ injury in female Balb/c mice subjected to cerulein-induced pancreatitis.** Various physiological and organ injury marker levels: alkaline phosphatase (ALP), alanine aminotransferase (ALT), amylase (AMY), total bilirubin (TBIL), plasma blood urea nitrogen (BUN), plasma calcium (CA^{++}), plasma phosphate (PHOS), plasma creatinine (CRE), plasma glucose (GLU), plasma sodium (Na^+), plasma potassium (K^+), total protein (TP), and plasma globulin (GLOB) measured by Vetscan analysis, are shown in sham mice (not subjected to cerulein) ("Sham"), in vehicle-treated mice subjected to cerulein at 6 h ("Cerulein") and in mice subjected to cerulein in the presence of olaparib (2 mg/kg × 3 mg/kg) (Cerulein+Olaparib). Data are shown as mean ± SEM of 10 animals for each group; $^*P < 0.05$ shows significant increase in the respective parameter in response to cerulein, compared with the sham group; #$P < 0.05$ shows significant protective effect of olaparib in cerulein-pancreatitis mice compared with vehicle-treated cerulein-pancreatitis mice.
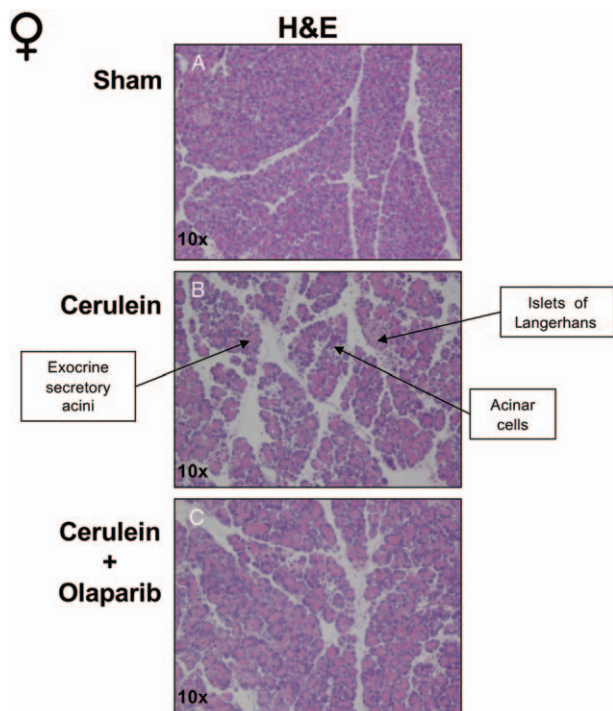
FIG. 7. **Effect of olaparib on histological alterations injury in the pancreas of female Balb/c mice subjected to cerulein-induced pancreatitis.** Representative histological pictures are shown in sham mice (not subjected to CLP) ("Sham"), in vehicle-treated mice subjected to cerulein at 6 h ("Cerulein") and in mice subjected to cerulein in the presence of olaparib (2 mg/kg × 3 mg/kg) (Cerulein+Olaparib). Pictures are selected from n = 5 animals for each group.

FIG. 8. **Effect of olaparib on PARP and PAR levels in HPDE cells subjected to hydrogen peroxide-induced oxidative stress.** Western blotting for PAR the enzymatic product of PARP activation and its actin loading control (top lanes) and PARP1 and its actin loading control (bottom lanes) in HPDE cells treated with olaparib (1μM, 3μM, 10μM, 30μM, or 100 μM) in control and in 300 μM H_2O_2 ("H") challenged cells at 1 h. Note the increase in PAR (indicative of PARP activation) in response to oxidative stress, and the full inhibition of this response at all olaparib concentrations tested. The expression of PARP1 was reduced by oxidative stress, and this was partially prevented by olaparib at 1μM to 10 μM. Western blots shown are representative of experiments performed on three different experimental days.

integrity in control cells (i.e., cells not subjected to oxidative stress) (Fig. 11A) but reduced the degree of oxidative stress-induced mitochondrial DNA damage at 1 h postoxidant challenge (Fig. 11B). We attribute this finding to effects on the mitochondrial isoform of PARP, which has a qualitatively different role in the regulation of mitochondrial DNA repair than its nuclear counterpart (34, 35). While mitochondrial DNA
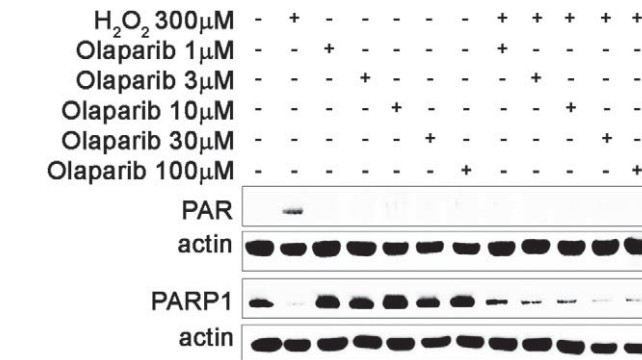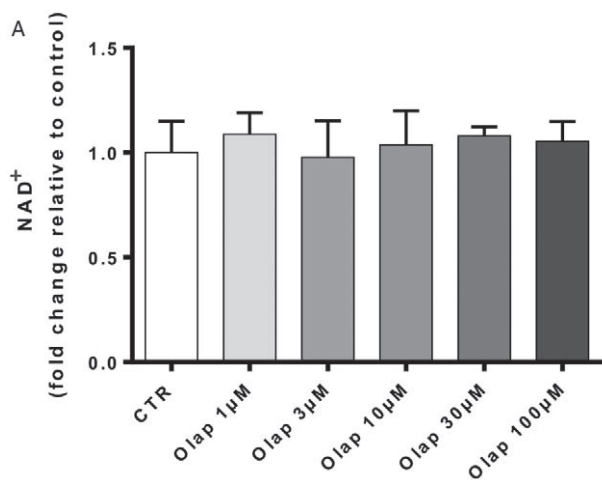
integrity partially recovered by 24 h post-H_2O_2 challenge, nuclear DNA damage became more extensive by 24 h. The nuclear DNA damage at 24 h was slightly exacerbated by the two highest concentrations of olaparib tested (30μM and 100 μM); however, the lower concentrations of olaparib did not have any adverse effects on DNA integrity at any of the time points and any of the experimental conditions tested. The latter findings are consistent with the role of PARP in the maintenance of nuclear DNA integrity, as well as with the idea that inhibition of DNA repair requires high concentrations of the PARP inhibitor (e.g., 30μM–100 μM in our study), while the cytoprotective and NAD^+-preserving effects of the same inhibitor manifest at lower concentrations (e.g., 1 μM in our study).

FIG. 9. **Effect of olaparib on PARP activation and cellular NAD^+ levels in HPDE cells subjected to H_2O_2-induced oxidative stress *in vitro*.** Cells were treated with olaparib (1μM, 3μM, 10μM, 30μM, or 100 μM) and then subjected to oxidative stress (H_2O_2) or its vehicle for 1 h. $^*P < 0.05$ shows significant decrease in NAD^+ levels in response to H_2O_2, compared with control cells; $^#P < 0.05$ shows significant protective effect of olaparib in H_2O_2 challenged cells compared with vehicle-treated H_2O_2 challenged cells. Data are shown as mean±SEM of n = 3 determinations.
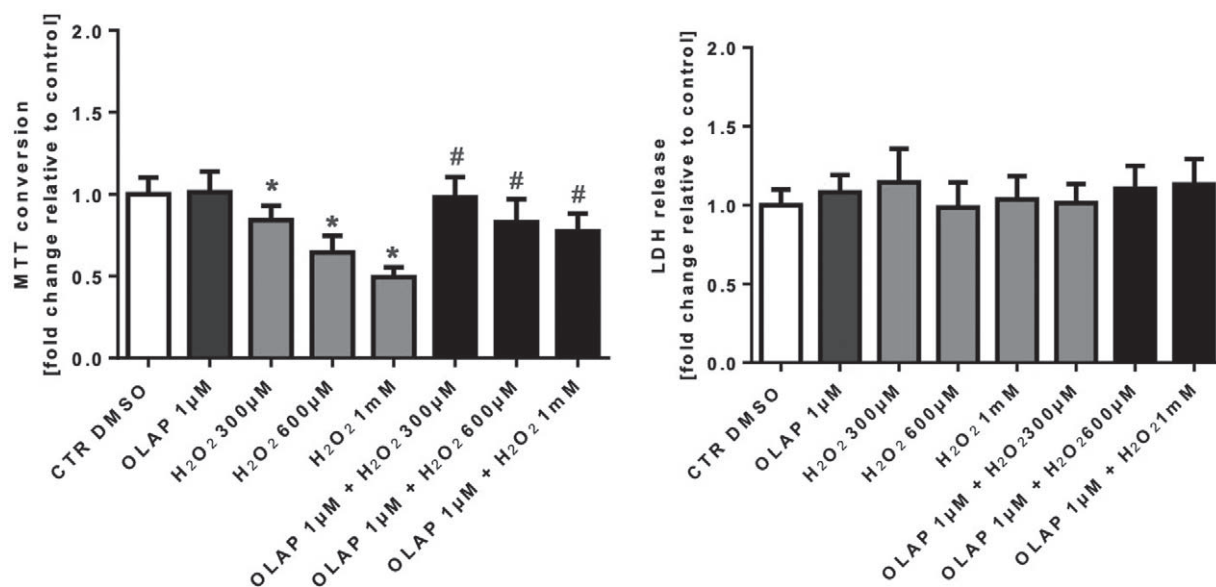
FIG. 10.   **Effect of olaparib on cellular viability in HPDE cells subjected to H₂O₂-induced oxidative stress *in vitro*.** A, Effect of olaparib on cellular MTT conversion in cells subjected to oxidative stress (300 μM, 600 μM, or 1 mM H₂O₂) in the presence of olaparib vehicle or 1 μM olaparib. Data are shown as mean±SEM of n = 3 determinations. *$P < 0.05$ shows significant decrease in MTT conversion in response to H₂O₂, compared with control cells; #$P < 0.05$ shows significant protective effect of olaparib in H₂O₂ challenged cells compared with vehicle-treated H₂O₂ challenged cells at the same H₂O₂ concentration. B, Effect of olaparib on LDH content (a marker of cell necrosis) in cells subjected to oxidative stress (300 μM, 600 μM, or 1 mM H₂O₂) in the presence of olaparib vehicle or 1 μM olaparib. Data are shown as mean±SEM of n = 3 determinations. Data are shown as mean±SEM of n = 3 determinations.

Immunohistochemical detection of PARylation in human clinical samples obtained from pancreatitis patients

In pancreas histological sections of patients who died of acute or chronic pancreatitis, accumulation of PAR (the product of PARP) was demonstrated, while in healthy control pancreatic tissues no PAR immunostaining was detectable (Fig. 12). The PAR staining was primarily nuclear, but also perinuclear and cytosolic; it was apparent both in acute and chronic pancreatitis sections.

DISCUSSION

We conclude that in mice subjected to cerulein-induced pancreatitis, the PARP clinically approved PARP inhibitor olaparib improves pancreatic function, beneficially modulates the systemic inflammatory response (reduction of the plasma level of various pro-inflammatory mediators, but no inhibition of the anti-inflammatory cytokine IL-10), and reduces the biochemical markers of renal injury. The protective effects of olaparib were evident at 3 mg/kg, which is a relatively low dosage of the inhibitor, compared with the higher doses (typically 50 mg/kg–100 mg/kg) used in the oncological context (34–41). The olaparib dose in the present study was selected based on prior studies using this PARP inhibitor in rodent models in various nononcological contexts including sepsis, stroke, and acute lung injury (22–27). There are a limited number of published pharmacokinetic studies with olaparib in rodents. A 100 mg/kg oral dosing of olaparib resulted in plasma levels of 2,000 ng/mL to 200 ng/mL over the first 12 h (42), which corresponds to approximately 5 μM to 0.5 μM olaparib plasma concentration. Based on these data, we estimate that in the current study (with 3 mg/kg olaparib dosing,

intraperitoneally, as opposed to orally), the achieved plasma concentrations of olaparib were likely in the high nanomolar range, perhaps up to 0.5 μM. (However, it should be noted that for PARP inhibitors it is the intracellular concentrations that are relevant and not the plasma levels; tissue levels can be in fact higher than plasma levels, especially at later time points—12 to 24 h—after administration (42), but no published data exist specifically regarding pancreatic tissue olaparib levels).

The *in vitro* experiments, complementing the *in vivo* studies, established that olaparib also exerts protective effects against oxidant-induced cell injury—once again, at a low concentration of 1 μM—which, once again, is markedly lower than the concentrations of the PARP inhibitor used in cancer cells to exert cytotoxic effects and/or to enhance the cytotoxic action of various chemotherapeutic agents (30μM–100 μM or higher) (40–48). As discussed previously in our position paper (20), this difference between the protective/nononcological dose and concentration of olaparib versus the oncological dose and concentration of the same molecule is very important, because one can predict that the lower doses of olaparib that would be used in the context of therapeutic repurposing would be associated with a more advantageous safety and adverse effect profile than the adverse effect profile of the drug that is documented in cancer patients.

The selection of the cell line used in the present paper requires some qualification. The pancreas consists of many different cell types, and each of these cell types can be affected by the inflammatory processes. When attempting to model pancreatitis *in vitro*, some investigators use acinar cell lines, while others use ductal epithelial cells. The utilization of the single-cell line in our study is a clear limitation of the current study. Even though PARP is expressed in most
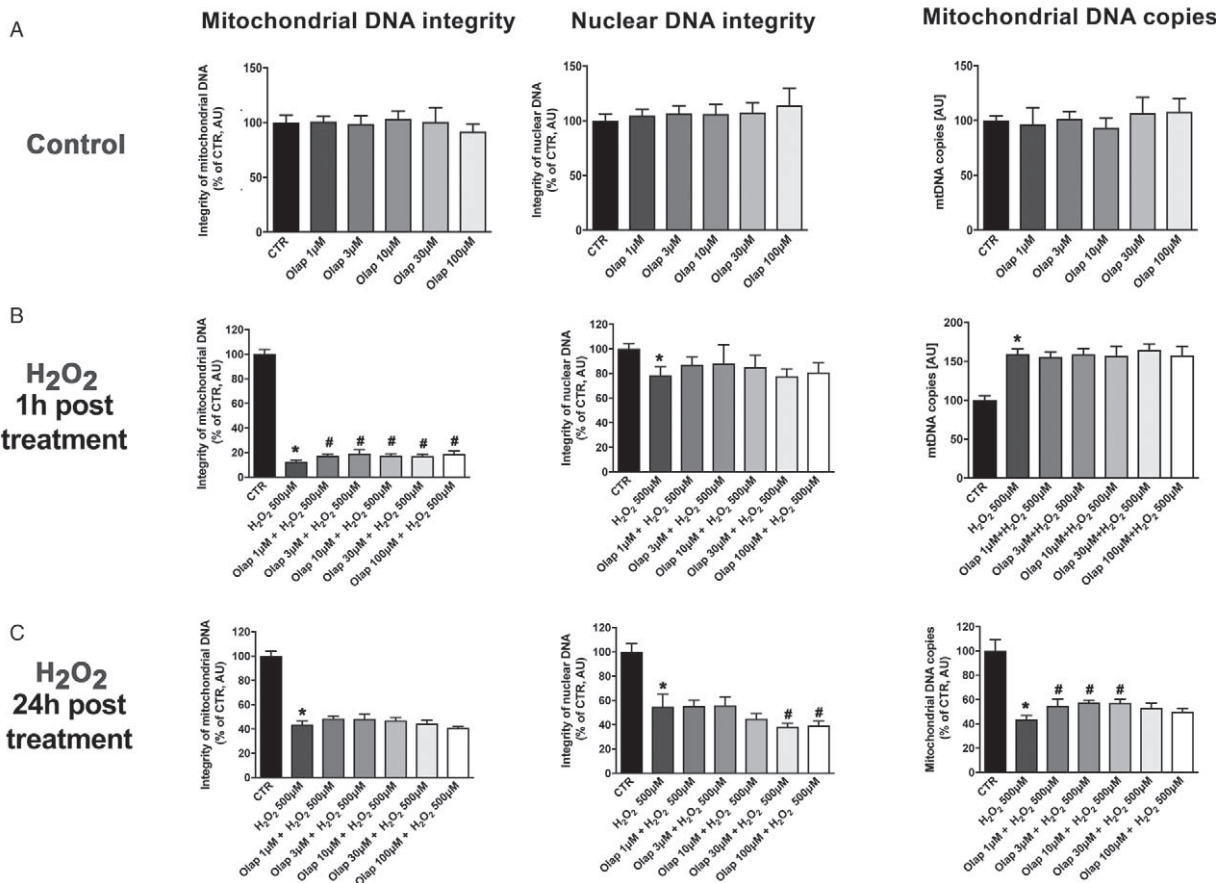
FIG. 11. **Effect of olaparib on mitochondrial DNA integrity, mitochondrial DNA copy number, and nuclear DNA integrity in HPDE cells subjected to H_2O_2-induced oxidative stress *in vitro*.** A, Mitochondrial DNA integrity, mitochondrial DNA copy number, and nuclear DNA integrity in control HPDE cells (without oxidative stress) subjected to vehicle or various concentrations of ($1\mu M$, $3\mu M$, $10\mu M$, $30\mu M$, or $100\mu M$) olaparib for 24 h (data expressed as % of control). B, Mitochondrial DNA integrity, mitochondrial DNA copy number, and nuclear DNA integrity in control HPDE cells (without oxidative stress) subjected to vehicle or various concentrations of olaparib ($1\mu M$, $3\mu M$, $10\mu M$, $30\mu M$, or $100\mu M$), followed by treatment with H_2O_2 ($500\mu M$) for 1 h (data expressed as % of control). C, Mitochondrial DNA integrity, mitochondrial DNA copy number and nuclear DNA integrity in control HPDE cells (without oxidative stress) subjected to vehicle or various concentrations of olaparib ($1\mu M$, $3\mu M$, $10\mu M$, $30\mu M$, or $100\mu M$), followed by treatment with H_2O_2 ($500\mu M$) for 24 h (data expressed as % of control). Results are shown as mean $\pm$ SEM of n = 3 per condition. $^*P < 0.05$ shows a significant change in response to H_2O_2, compared with control cells; #$P < 0.05$ shows significant effect of olaparib in H_2O_2 challenged cells compared with vehicle-treated H_2O_2 challenged cells.

parenchymal cells (including acinar cells and epithelial cells, and vascular cells), and PARP-associated pathological processes have been previously reported in a wide variety of cell types during acute pancreatitis—including acinar cells (49), there may well be cell-type-specific differences in the relative contribution of PARP to the cell dysfunction or mediator production; this remains to be determined in follow-up experiments.

The PARP inhibitor's beneficial effects in various nononcological models of cell and organ injury and local and systemic inflammation are likely to have multiple mechanistic components. One aspect is likely related to direct cytoprotection (i.e., preservation of cellular NAD^+ and ATP levels); another aspect likely relates to the selective modulation of the expression and production of multiple pro-inflammatory mediators and circulating factors; these effects are likely to lead to secondary actions *in vivo*, e.g., the prevention of the infiltration of organs with activated mononuclear and polymorphonuclear cells, and maintenance of vascular integrity (20). We hypothesize that some combination of the above actions is responsible for the beneficial effects of olaparib in the current study.

Our findings are largely consistent with the results of prior studies focusing on PARP and pancreatitis that utilized PARP inhibitors of various structural classes. The first-generation PARP inhibitor 3-aminobenzamide was found to improve pancreatic histology and reduce bacterial translocation in a model of taurocholate-induced pancreatitis in rats; it also reduced serum amylase levels and attenuated renal dysfunction; moreover, serum concentrations of proinflammatory cytokines were also reduced by the inhibitor (12, 15, 50). In another study, utilizing a structurally related compound, benzamide, once again, improved issue oxidative stress indices, better histological injury scores, reduced bacterial translocation, and improved survival were reported (14). One of the obvious problems with studies using 3-aminobenzamide or benzamide is the low potency of these compounds (for instance, benzamide was used at a dose of 100 mg/kg); another is their low selectivity; as discussed elsewhere (51) they exert many non-PARP-related pharmacological actions. Moreover, they are not clinically approved and therefore they are not translationally relevant. Second-generation PARP inhibitors, for instance the phenanthridinone compound
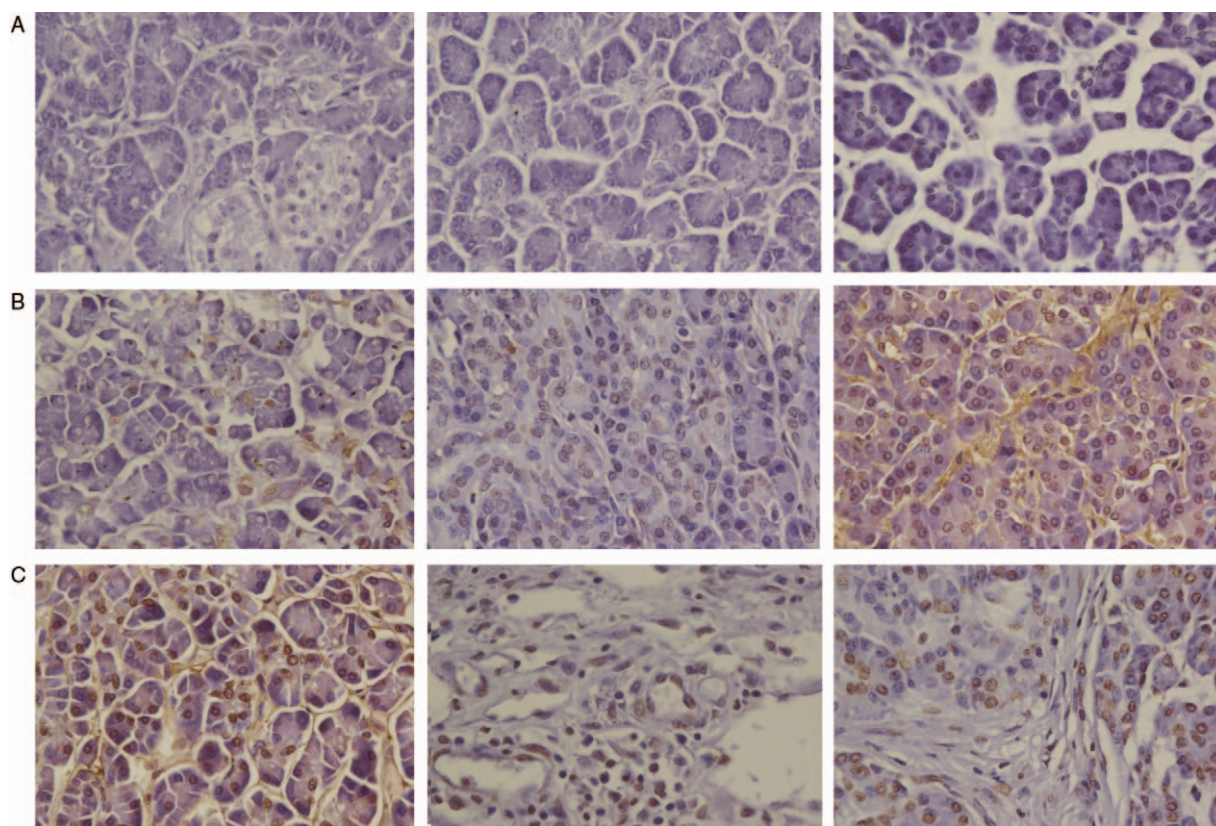
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Fɪɢ. 12. **Human pancreatitis is associated with PARP activation in the pancreatic tissue.** A, PAR staining in normal control pancreatic tissue samples. B, PAR staining in pancreatic tissue samples obtained from patients with acute pancreatitis. C, PAR staining in pancreatic tissue samples obtained from patients with chronic pancreatitis. PAR accumulation in (B) and (C) is visible as a brown intracellular (nuclear, perinuclear, and cytoplasmic) staining. ×40 magnification.

PJ34, are more potent, more selective to PARP (as opposed to other ribosylating enzymes) (51); PJ34, too, was reported to improve the outcome of acute pancreatitis in mice in a model of pancreatitis induced by choline/methionine-deficient/ethionine-supplemented (CMDE) diet: CMDE-induced increases in serum amylase, lipase, IL-1β, IL-6, ALT, and AST levels, as well as the histological changes in pancreas, liver and lung were all improved by PJ34 therapy (15 mg/kg/day) (11). Similar findings with PJ34 were also reported in a cerulein-induced pancreatitis model (13). The current report adds to this body of information and demonstrates that the clinically approved PARP inhibitor, olaparib, also exerts significant beneficial effects in an acute pancreatitis model. Interestingly, one of the prior studies, published in 2007 by Mota et al. (11), has also used a second-generation PARP inhibitor that was designated as KU0058684. This molecule was tested side-by-side with PJ34 (at the same dose of 15 mg/kg/d) in the CMDE model and exhibited comparable efficacy. This molecule is now also a clinically approved anticancer drug similar to olaparib (its current designation is niraparib/Zejula) (52). Thus, the body of evidence suggests that at least two of the currently clinically approved PARP inhibitors (niraparib and olaparib) may be potentially suitable for repurposing for the experimental therapy of acute pancreatitis.

There are several lines of preclinical studies that suggest that not all populations of animals subjected to various models of

critical illness may benefit equally from PARP inhibition. For instance, in several models (ranging from stroke and endotoxemia and sepsis), in female mice the protective effect of PARP inhibition or PARP deficiency is diminished or non-significant; this difference may be related to a physiological inhibitory effect of estrogen on PARP activation (20). Surprisingly, in our current study, we found that olaparib was efficacious in our pancreatitis model both male and female animals; this is an important observation when considering future clinical trials focusing on therapeutic repurposing.

It has been known for several decades that PARP activity plays a role in the maintenance of DNA integrity, chiefly through the recruitment of various DNA repair enzymes and the assembly of nuclear and mitochondrial DNA repair complexes (4, 53, 54). Inhibition of DNA repair is, in fact, the principal mode of action of olaparib's approved indication in the field of oncology, an effect that is more prominent when the cancer cells have certain mutations (e.g., mutations in the BRCA gene), or when cancer therapy is performed in combination with treatment with cytotoxic and/or genotoxic chemotherapeutics. Because of this important function of PARP, it is essential to separate the doses/concentrations of PARP inhibitors that are cytoprotective (and therefore relevant for potential therapeutic repurposing) and the doses/concentrations that are genotoxic or cytotoxic (or enhance anticancer therapy). Our experiments in the HPDE cell line have begun defining these concentration differences. Importantly, we have demonstrated

that in these cells, $1 \, \mu M$ of olaparib already suppresses all detectable PARylation (at least, as it is detectable by Western blotting; it is theoretically possible that a small residual PARylation still remains and the method used does not have sufficient sensitivity to detect it, and to inhibit this residual PARP activity, one requires higher concentrations of olaparib). The same concentration of olaparib ($1 \, \mu M$) was also sufficient to fully restore cellular NAD^+ levels to near-normal levels in oxidatively challenged cells. At the $1 \, \mu M$ olaparib concentration, cells were also protected from the decline in MTT conversion (an index of mitochondrial function and cell viability). At the lowest tested olaparib concentration ($1 \, \mu M$), as well as 3 and 10 times higher ($3 \, \mu M$ and $10 \, \mu M$), olaparib exerted no adverse effects on mitochondrial or nuclear DNA integrity in normal cells or in oxidatively stressed cells: neither acutely (at 1 h) or later (at 24 h). At the 24 h time point—when the cells exhibited a significant degree of nuclear DNA damage, the highest concentrations of olaparib tested ($30 \, \mu M$ and $100 \, \mu M$)—but not the lower concentrations of olaparib—induced a further impairment of nuclear DNA integrity, perhaps indicating that these concentrations of the drug (while commonly used in the oncological context) are to be avoided in the therapeutic repurposing/pancreatitis context. (Nevertheless, please note that in this experiment, the majority of the nuclear DNA damage was induced by H_2O_2 *per se,* and not by the addition of olaparib.) When considering PARP inhibitors' effects on DNA integrity, it should also be mentioned that the method used here to estimate DNA integrity or DNA damage does not assess all forms of DNA damage (for instance, it does not measure DNA base modifications, or larger scale aberrations like micronucleus instability or chromosomal alterations) (55). To assess the effect of olaparib on these parameters, further studies are required.

The current experimental design utilized a relatively short-term model, where longer-term events (e.g., later-time mortality) was not evaluated. This is a clear limitation of the current report Nevertheless, it should be noted that in other models of critical illness (e.g., cecal ligation and puncture induced polymicrobial septic shock), olaparib treatment, in fact, had significant beneficial effects on a variety of parameters including organ injury as well as mortality (27).

Taken together, on the results of the current studies, we conclude that the clinically approved PARP inhibitor olaparib can improve organ function and can exert beneficial effects in pancreatitis and its efficacy can be demonstrated both in male and female animals. Based on the results of the *in vitro* experiments, we can also predict that there are concentrations of olaparib (e.g., $1 \, \mu M$) at which the PARP inhibitor exerts beneficial and cytoprotective effects without adversely affecting cell viability or nuclear or mitochondrial DNA integrity. Taken together, the current results support the concept of repurposing and clinical introduction of this PARP inhibitor for the experimental therapy of pancreatitis. This conclusion is further strengthened by our data demonstrating that in pancreatitis (both acute and chronic forms) — similar to a variety of other forms of critical illness including sepsis (56, 57)—increased accumulation of PAR (the product of PARP) occurs.

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