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Ocoxin Oral Solution Exerts an Antitumoral Effect in Pancreatic Cancer and Reduces the Stromal-Mediated Chemoresistance

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Objectives: Pancreatic carcinoma is one of the most aggressive cancers overcoming chemoresistance. Thus, novel compounds to complement the current antitumor agents are in need. Ocoxin oral solution (OOS) has proven antioxidant, anti-inflammatory, and antistromagenic properties. The aim of this study was to analyze the effect of OOS in an experimental pancreatic cancer model and its implication in stroma-related chemoresistance to paclitaxel and gemcitabine.

Methods: Murine pancreatic carcinoma 266-6 cells were treated with OOS to analyze cell cycle and to perform a mRNA comparative microarray study. Then the viability was assessed in combination with paclitaxel and/or gemcitabine. Chemoresistance induced by the medium taken from fibroblast cultures was also investigated on 6 human pancreatic carcinoma cell lines. Furthermore, an experimental model of pancreatic cancer was carried out to study the effect of OOS in vivo.

Results: Ocoxin oral solution enhances the cytotoxic effect of paclitaxel and gemcitabine, while it ameliorates the chemoresistance induced by fibroblast-derived soluble factors in human pancreatic cancer cells. The OOS also promotes the regulation of the expression of genes that are altered in pancreatic carcinoma and slows down 266-6 cell pancreatic tumor development in vivo.

Conclusions: Ocoxin oral solution could be a potential complement to the chemotherapeutic drugs for pancreatic adenocarcinoma.

Key Words: CAFs, nutritional supplements, OOS, pancreatic cancer, stroma, PSCs

Pancreatic cancer is one of the most lethal diseases in the world because of its aggressiveness and the inexistence of markers for early detection and diagnosis.[1] That makes chemotherapy almost the only treatment available to cope with this disease.

However, the effectiveness of the compounds used currently is far away from being ideal because this malignancy becomes resistant to the majority of the utilized drugs.[2] Gemcitabine and paclitaxel are the most used first-line chemotherapeutic agents to treat pancreatic cancer, but the effect of these compounds is feeble. Thus, a combination of both gemcitabine and paclitaxel is currently used as a second-line treatment to improve patient's response to the tumor.[3]

The local microenvironment is an active participant in the process of cancer initiation, progression, and metastasis in many tumors.[4,5] Moreover, resistance to chemotherapeutics derives from both the tumor cell response and the tumor-associated stromal microenvironment.[6–9] In pancreatic carcinoma, the stroma is especially relevant because critical players in cancer development, such as cancer-associated fibroblasts (CAFs), endothelial cells, immune cells, and extracellular matrix, comprise 80% of tumor mass.[10,11]

Among all these cell types, CAFs have recently emerged as chemoresistance promoters by secreting cytokines and growth factors that alter tumor cell response to chemotherapeutic agents.[12,13]

Nutritional supplements are sometimes used in combination with the routine therapies to overcome the development of chemoresistance.[14,15] Several studies have shown that natural compounds not only increase the efficacy of chemotherapy but also relieve the adverse effects provoked by these agents when used as complementary therapy.[16,17] Glycyrrhizic acid is a biologically active substance, extracted from licorice root with anti-inflammatory and immunomodulatory properties. Furthermore, this substance inhibits the growth of leukemia, malignant glioma, colon cancer, and lung cancer.[18] Also, several vitamins and antioxidant compounds have been widely studied as anticancer agents with reasonably good results.[19–21] Recently, the nutritional complement Ocoxin oral solution (OOS) has demonstrated to exert antitumoral effects alone and as a coadjutant of irinotecan in the development of liver metastasis from colorectal cancer.[22,23] This compound comprises a mixture of several natural compounds such as green tea extract, glycyrrhizic acid, vitamin C, vitamin B_6, vitamin B_{12}, minerals, and amino acids and possesses immunomodulatory and antioxidant properties.[22,23] The results obtained show that the nutritional supplement OOS might be a potential complementary therapy to face the proliferative effects and reduce the stroma-mediated chemoresistance of pancreatic cancer.

MATERIALS AND METHODS

Animals

For the in vivo experimental model of pancreatic adenocarcinoma, 6- to 8-week-old male C57BL/6 mice were obtained from Charles River (Wilmington, Mass). Mice were fed with standard chow and water ad libitum. The Ethical Committee for Experimental Animal of the Basque Country University approved all the

proceedings with the reference number M20/2016/200, following institutional, national, and international guidelines regarding the protection and care of animal use for scientific purposes.

Cell Lines

The murine pancreatic adenocarcinoma 266-6 cell line (ATCC, LGC Standards S.L.U., Barcelona, Spain) was used for in vitro and in vivo experiments. This cell line contains the simian virus 40 (SV40) transgene that codes for small and large T-antigen.[24] Besides, 6 different human pancreatic carcinoma cell lines, BxPC-3, Capan-2, CFPAC-1, HPAF-II, Panc10.05, and SW1990, were used for further in vitro analyses. The human MRC-5 lung fibroblast cells (all purchased from ATCC, Barcelona, Spain) were used to obtain conditioned medium enriched with soluble factors. All cells were grown in a complete medium, RPMI-1640 supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 μg/mL), and amphotericin B (0.25 μg/mL) (Thermo Fisher Scientific, Waltham, Mass) under standard conditions. Panc10.05 cells were supplemented with recombinant human insulin (Life Technologies, Waltham, Mass) (10 U/mL).

OOS Solution

Ocoxin oral solution is a nutritional supplement that contains the following components (in 100 mL): 2 g of glycine, 2 g of glucosamine, 1.2 mg of malic acid, 640 mg of arginine, 204 mg of cysteine, 200 mg of monoammonium glycyrrhizinate, 120 mg of ascorbic acid, 80 mg of zinc sulfate, 25 mg of green tea extract, 12 mg of calcium pantothenate, 4 mg of pyridoxine, 4 mg of manganese sulfate, 3 mg of cinnamon extract, 400 μg of folic acid, and 2 μg of cyanocobalamin.

Fibroblast-Derived Conditioned Medium

Fibroblast-derived conditioned medium (fibroblast CM) was obtained from MRC-5, a cell line derived from human lung fibroblasts. Briefly, cells were cultured on 6-well plates at a concentration of 2×10^5 cells/mL in a complete RPMI medium. After 24 hours, the medium was replaced for a fresh one, and after another 24 hours, the medium enriched with soluble factors produced by fibroblasts was collected, centrifuged, and stored at −20°C.

Viability Assays

Tumor cells were cultured in 96-well plates at a concentration of 5×10^4 cells/mL in 10% FBS-supplemented RPMI medium with antibiotics-antimycotics to analyze the effect of OOS, paclitaxel, and gemcitabine on their viability. Once attached, 266-6 cells were treated separately with a concentration range of 0 to 1:50 (V/Vf) OOS (Catalysis S.L., Madrid, Spain), 1 to 10 μM paclitaxel (Sigma, St Louis, Mo), and 200 nM to 1 μM gemcitabine (Sigma). The human cells were treated with the concentration range of 0 to 1:50 (V/Vf) OOS, 1 to 15 μM for paclitaxel, and 1 to 5 μM for gemcitabine.

Next, to study the effect of OOS as a complementary therapy on the viability of cells, treatment combination assays were carried out. Cells were cultured as above and were treated with the most effective concentrations obtained in the previous tests, which was OOS 1:50 (V/Vf), combined with 1 μM paclitaxel and 1 μM gemcitabine. All the treatments were diluted in 10% FBS-supplemented RPMI medium with antibiotics-antimycotics. The controls were cultured with RPMI medium completed with 10% FBS, antibiotics, and antimycotics.

Finally, to analyze the effect of fibroblast-derived soluble factors on chemoresistance, human cancer cells were cultured at a concentration of 5×10^4 cells/mL in 96-well plates with fibroblast CM diluted 1:2 in RPMI medium supplemented with 10% FBS for 24 hours. Afterwards, cells were treated with OOS 1:50 (V/Vf), combined with 1 μM paclitaxel and 1 μM gemcitabine diluted in a complete fresh medium with fibroblast CM, whereas untreated cells were cultivated only with the whole medium. In all viabilities, after 48 hours with the treatments, PrestoBlue viability reagent (Thermo Fisher Scientific) was added to the cells for 3 hours, and the absorbance was measured with Fluoroscan Ascent (Thermo Labsystems, Waltham, Mass).

Cell Cycle Analyses

The 266-6 cells were cultured in 6-well plates for 18 hours to study the effect of OOS in the cell cycle in vitro. Then, cells were treated with 1:100, 1:200, and 1:500 (V/Vf) dilutions of OOS in complete RPMI for 48 hours. The control cells were cultured with the control medium. Then, cells were trypsinized, washed once with phosphate-buffered saline (PBS), and fixed with 70% ethanol for 15 minutes at 4°C. Afterwards, cells were washed with PBS 3 times and incubated with propidium iodide (PI) containing FxCycle PI/RNase Solution (Thermo Fisher Scientific) following the manufacturer's indications. Finally, cell cycle changes were analyzed by flow cytometry using the Gallios cytometer (Beckman Coulter, Brea, Calif). For cell division analysis, 266-6 cells were fluorescently labeled with carboxyfluorescein succinimidyl ester (CFSE) by incubating at 37°C for 30 minutes before seeding them into 6-well plates. Then, cells were treated as described above for cell cycle analysis. After 48 hours, cells were trypsinized, washed in PBS, and resuspended for flow cytometry studies by Gallios cytometer (Beckman Coulter).

Apoptosis Determination by Flow Cytometry

Human pancreatic cancer cells were cultured in a complete medium for 24 hours in 6-well plates at 3×10^5 cells/well concentration. Once the cells were attached, the old medium was replaced with fresh medium for control condition or with 1:2 diluted fibroblast CM for the chemoresistance studies and incubated for 24 hours. Afterward, cells were treated with OOS 1:50 (V/Vf), combined with 1 μM paclitaxel and 1 μM gemcitabine diluted in fresh medium or fibroblast CM for 48 hours. Then, cells were washed, trypsinized, pelleted by centrifugation, washed again with PBS, and double stained with the annexin V–fluorescein isothiocyanate apoptosis detection kit and PI following manufacturer's instructions (both purchased from Thermo Fisher Scientific). Finally, apoptosis was determined by the Gallios cytometer (Beckman Coulter). Note that the HPAF-II cell line was not included in this assay because of the high levels of cell death during the process.

mRNA Comparative Microarray Study of OOS-Treated Murine Pancreatic 266-6 Cells

A microarray study was carried out to analyze whether OOS altered gene expression in the 266-6 cells. To this end, cells were cultured in 8 independent T25 flasks; 4 of them were treated with 1:50 (V/Vf) of OOS, and 4 containers as controls were cultured in complete medium for 72 hours. In both cases, the media was replaced daily. Then, the total RNA was extracted according to Purelink RNA mini kit (Invitrogen, Carlsbad, Calif) manufacturer's instructions. Afterwards, RNA integrity was analyzed by using a Eukaryote Total RNA Nano Assay with the Lab-chip in the Agilent 2100 Bioanalyzer in combination with Agilent RNA

6000 Nano Chips. Subsequently, mRNA was labeled using the Agilent protocol One-Color Microarray-Based Gene Expression Analysis (Low Input Quick Amp Labeling). Messenger RNA was retrotranscribed with the AffinityScript Reverse Transcriptase (AffinityScript RT) in the presence of Cy3-CTP to obtain cDNA. These samples were hybridized using "SureHyb" hybridization chambers (Agilent Technologies, Inc, Santa Clara, Calif) according to the manufacturer's instructions and were washed with ozone-barrier slide covers according to the protocol of Agilent. Then, the slides were scanned using the DNA microarray scanner G2535CA of Agilent Technologies with the Agilent Scan control Software (version 8.5.1.) (default settings). Finally, feature extraction of the scanned images was made by using the Agilent Feature Extraction Software (version 10.7.3.1) (Agilent Technologies, Inc).

Experimental Development of Pancreatic Adenocarcinoma

Before the injection of 266-6 cells on mice to carry out an in vivo pancreatic cancer experimental model, a flow cytometry assay was performed to confirm the expression of the SV40 T-antigen on the tumor cells. To do so, cells were blocked in 5% FBS containing PBS for 30 minutes and labeled with the mouse anti-SV40 T-antigen antibody (1:500; Abcam, Cambridge, Mass) for 2 hours. Next, cells were washed with PBS 3 times and incubated with the corresponding secondary anti-antibody for 1 hour (1:2000, Alexa 595; Thermo Fisher Scientific). Finally, after extensive washing in PBS, the cells were processed by Gallios cytometer (Beckman Coulter).

Once the presence of the marker on cells was established, a murine orthotopic pancreatic adenocarcinoma model was developed for the in vivo analyses. Mice were anesthetized with 50 mg/kg of intraperitoneal injection of Nembutal, and a thin cut was made in the left flank to inject 100 μL of 266-6 cells directly into the pancreas at a concentration of 2×10^6 cells/mL diluted in PBS. Then, the pancreas was relocated, and the wound was sutured. The animals were divided into 3 experimental groups, consisting of 7 mice each one, according to the treatment administration pattern: group I comprised mice that received the vehicle treatment, used as control mice; group II involved mice treated daily with 100 or 200 μL of OOS, starting 10 days before tumor cell inoculation and until they were killed; group III involved mice that started treatment with 100 or 200 μL OOS 7 days after the tumor cell injection and continued the therapy until they were killed. All animals were killed 45 days after tumor cell injection (Fig. 1), and the pancreas was fixed in formaldehyde 3.7% to 4% vol/vol (Panreac AppliChem, Darmstadt, Germany) and embedded in paraffin. Four mice were inoculated with CFSE-labeled 266-6 cells, and they were killed after 48 hours to confirm the presence of the cells histologically in the organ by fluorescence.

T-Antigen Expression in Pancreatic Cancer-Bearing Mice by Quantitative Reverse Transcription–Polymerase Chain Reaction

The 266-6 cell marker SV40 T-antigen expression was quantified by quantitative reverse transcription–polymerase chain reaction (RT-qPCR) to analyze the tumor development in the pancreas. Total RNA was purified from the paraffin-embedded pancreas of all the treated and untreated mice using the Norgen FFPE RNA Purification Kit (Norgen Biotek, Thorold, Ontario, Canada) following the manufacturer's instructions, and we assessed the RNA concentration and quality by NANODROP spectrophotometer (ND-1000; Thermo Scientific, Rockford, Ill). Then, 2 μg RNA was retrotranscribed into cDNA using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, Calif) and amplified by using the Platinum-multiplex PCR mastermix (Applied Biosystems, Foster City, Calif).

The quantification of SV40 T-antigen gene expression was performed by RT-qPCR using the Itaq Universal SYBR Green Supermix (Bio-Rad) in CFX96 Real-Time System (Bio-Rad). The following SV40 T-antigen gene primers were purchased from Invitrogen (Thermo Fisher Scientific): forward: 5′-AAGCTCCAAC CCCTTTACCG-′3, reverse: 5′-ACATCAATGCTCACACGACG-′3. Relative expression of SV40 T-antigen was normalized to the internal control gene S15 by the ΔΔCt method.

Cytokine Levels in Mice Serum

The levels of interleukin (IL)-1β, IL-10, IL-12, and interferon γ (IFN-γ) cytokines were measured in untreated and OOS-treated tumor-bearing mice serum. A small sample of blood was extracted from the cava vein before the animals were killed, and the serum was obtained by centrifugation. Afterward, an enzyme-linked immunosorbent assay (ELISA) was carried out using Mouse IL1β and IL10 CytoSet kits (both purchased from Invitrogen) and Mouse IFN-γ and IL12 ELISA Set (both purchased from Abcam) according to the manufacturer's guidelines.

Statistical Analysis

The statistical analysis was carried out using the Student 2-tailed unpaired *t* test. All the in vitro experiments were performed in triplicate, and the in vivo assay was carried by duplicate with at least 7 animals in each group. Data are expressed as the mean value (standard deviation [SD]). The microarray assay was performed with 4 replicates for each treatment, and the statistics were analyzed with the multiExperiment Viewer version 4.9.0 (http://www.tm4.org/mev/). The comparison of expression profiles for differential
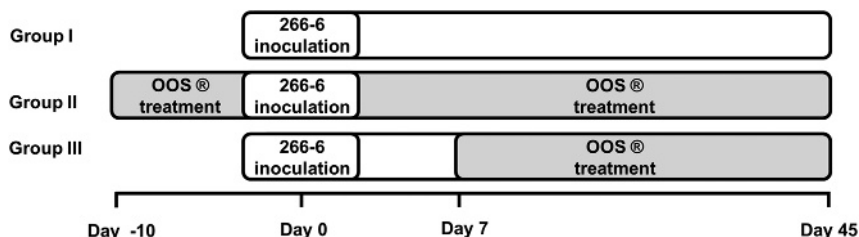

FIGURE 1. Scheme of experimental groups for in vivo pancreatic carcinoma development. Animals were divided into 3 groups as follows: group I: Untreated animals as control mice; group II: mice treated with 100 or 200 μL OOS starting 10 days before the tumor cell injection and continued the treatment until the animals were killed; group III: mice treated daily with 100 or 200 μL OOS from the 7 days after the tumor cell inoculation until the animals were killed.
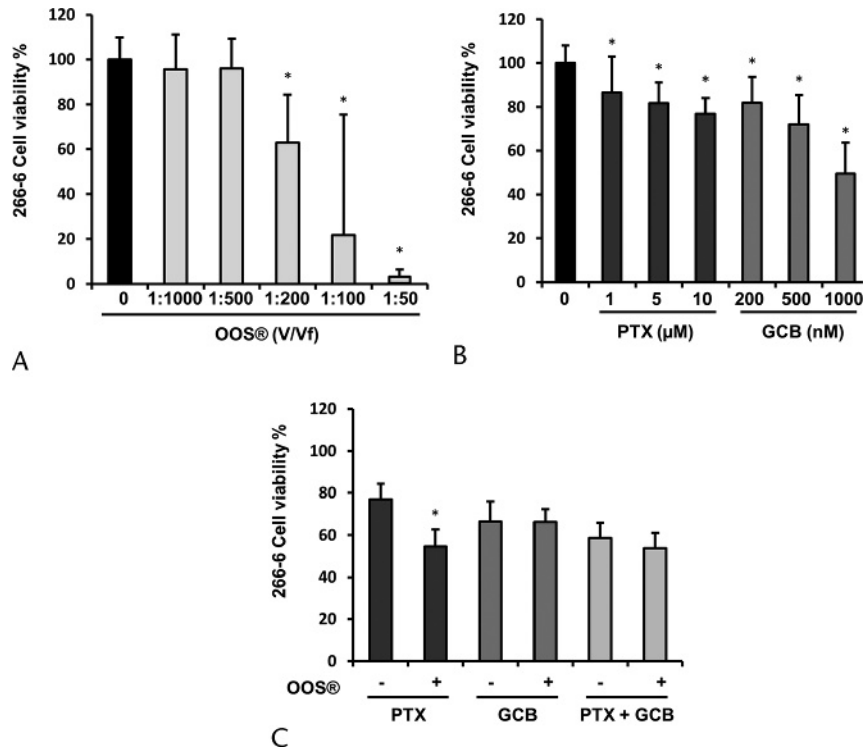
FIGURE 2. Ocoxin oral solution effect on the viability of the murine pancreatic adenocarcinoma 266-6 cell line. The viability of 266-6 cells was analyzed by means of the Presto Blue assay after 48 hours with different treatment combinations. A, Cell viability after OOS treatment according to 1:1000 to 1:50 (V/Vf) concentrations of (B) paclitaxel from 1 to 10 μM and gemcitabine from 200 to 1000 nM (C) combinations of all 3 of them: paclitaxel 1 μM + OOS 1:50 (V/Vf), gemcitabine 1 μM + OOS 1:50 (V/Vf), and paclitaxel 1 μM + gemcitabine 1 μM + OOS 1:50 (V/Vf). Data are expressed as the mean value (SD) of at least 3 independent experiments. Differences were considered significant for *$P < 0.05$.

expression analysis (Differential Expression) was carried out with LIMMA (Linear Models for Microarray Data) package. Results were considered statistically significant for $P < 0.05$.

RESULTS

Effect of OOS on the 266-6 Murine Pancreatic Adenocarcinoma Cells: Analysis of Tumor Cell Viability and Apoptosis Stage

First, the effect of OOS on the viability of the 266-6 murine pancreatic cancer cells was analyzed. The 266-6 cells were cultured with increasing concentrations of OOS. As shown in Figure 2A, OOS enhanced tumor cell death in a dose-dependent manner ranging from 4% using OOS 1:1000 (V/Vf) dilution to 95% using the OOS 1:50 (V/Vf) dose.

Then, 266-6 cells were treated as above with increasing concentrations of paclitaxel (1–10 μM) and gemcitabine (200–1000 nM) separately, to select the most effective drug dose to perform an OOS-chemotherapy combined assay. As shown in Figure 2B, paclitaxel 1, 5, and 10 μM provoked an overall 15% to 20% reduction in cell viability, and those cells treated with 200, 500, and 1000 nM of gemcitabine showed an 18%, 28%, and 50% viability decrease, respectively. Moreover, the addition of OOS as a complement to paclitaxel showed a 35% reduction in cell viability (Fig. 2C). No differences were detected when OOS was added in combination with gemcitabine or with paclitaxel and gemcitabine concomitantly.

Flow cytometry analyses were carried out to analyze the effect of OOS on the 266-6 cell cycle. As shown in Figure 3A,

PI incorporation was unchanged in cells treated with 1:500, 1:200, and 1:100 (V/Vf) of OOS compared with untreated cells. However, CFSE cell labeling showed that OOS 1:200 and 1:500 (V/Vf) dilutions slowed down 266-6 tumor cell division by 10% and 30% when the cells were treated with 1:100 (V/Vf) of OOS (Fig. 3B).

Comparative Microarray Study to Determine the Effect of OOS in Tumor Gene Expression

Bearing in mind that OOS treatment exerted antitumoral effects on 266-6 cells, a comparative microarray study was performed to analyze the molecular changes in gene expression promoted by OOS in 266-6 cells. The assay revealed a significantly altered gene profile compared with that of untreated cells. Fourteen of the identified genes had been previously associated with pancreatic adenocarcinoma in the Pancreatic Cancer Database[25] and Pancreatic Expression Database.[26] Interestingly, the expression levels of those genes went back to nonpathological values after OOS treatment (Table 1).

Effect of OOS Treatment in Pancreatic Adenocarcinoma Tumor Development

The expression of T-antigen on 266-6 cells was confirmed by flow cytometry (Supplementary Figure 1, http://links.lww.com/MPA/A713). Carboxyfluorescein succinimidyl ester detection by fluorescence microscopy demonstrated that tumoral cells were
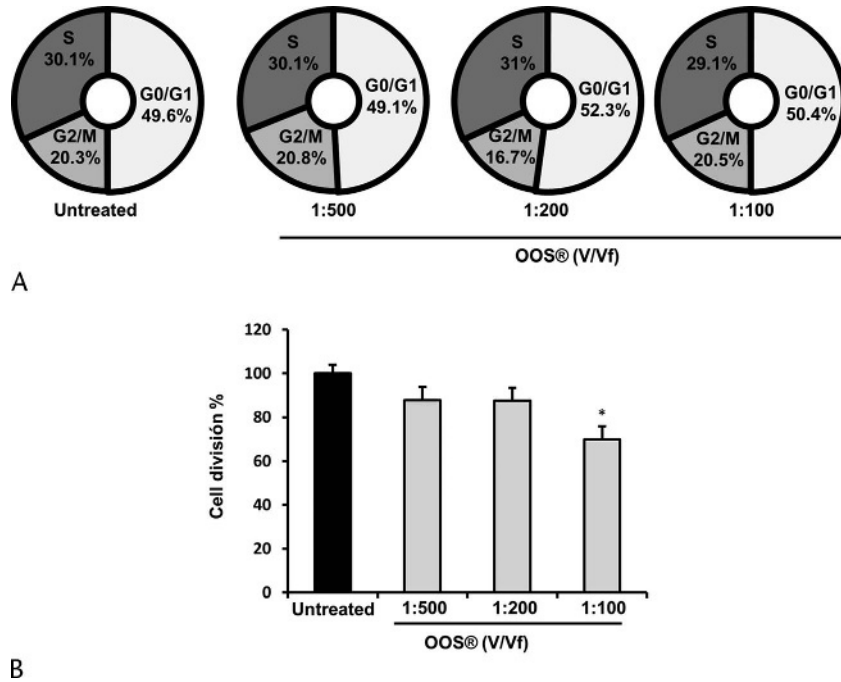
FIGURE 3. Cell cycle analysis of the 266-6 OOS-treated cells. 266-6 Cells were treated with 1:500, 1:200, and 1:100 OOS (V/Vf) for 48 hours the cell cycle was studied. A, Flow cytometry assay was carried out using PI (B) flow cytometry assay by labeling 266-6 cells with CFSE fluorescence probe. Data represent mean value (SD) of at least 3 independent experiments. Differences were considered significant for *$P < 0.05$.

TABLE 1. Comparative Microarray Study of Untreated and OOS-Treated 266-6 Cells

Gene	Pancreatic Carcinoma	OOS Treatment	Reference
Angptl4	Down	Up	Gadaleta et al,[27] 2011
Asns	Down	Up	Crnogorac-Jurcevic et al,[28] 2013
			Gadaleta et al,[27] 2011
Crabp1	Up	Down	Nakamura et al,[29] 2004
Creb1	Up	Down	Friess et al,[30] 2003
Eif4ebp1	Down	Up	Crnogorac-Jurcevic et al,[31] 2001
Fignl1	Up	Down	Gadaleta et al,[27] 2011
Foxf1	Up	Down	Gadaleta et al,[27] 2011
Grap2	Up	Down	Hustinx et al,[32] 2004
Hey1	Up	Down	Mann et al,[33] 2012
			Nakamura et al,[29] 2004
Nell2	Up	Down	Gadaleta et al,[27] 2011
			Nakamura et al,[29] 2004
Pak7	Up	Down	Capurso et al,[34] 2006
Rapgef3	Up	Down	Weeks et al,[35] 2008
Serpinf1	Down	Up	Yu et al,[36] 2005
Slc7a1	Down	Up	Gadaleta et al,[27] 2011
Thy1	Up	Down	Lowe et al,[37] 2007
			Badea et al,[38] 2008
			Friess et al,[30] 2003
			Gadaleta et al,[27] 2011
Trpc4	Up	Down	Capurso et al,[34] 2006
Wnt4	Up	Down	Lowe et al,[37] 2007

　　Total mRNA was extracted from 266-6 cells either untreated or treated with OOS 1:50 (V/Vf) for 72 hours to carry out an RNA microarray assay to detect differences in gene expression. The genes altered by the OOS treatment were compared with the data from the Pancreatic Expression Database v3.0 (available at http://www.pancreasexpression.org:9002) and the Pancreatic Cancer Database (available at http://pancreaticcancerdatabase.org/index.php).
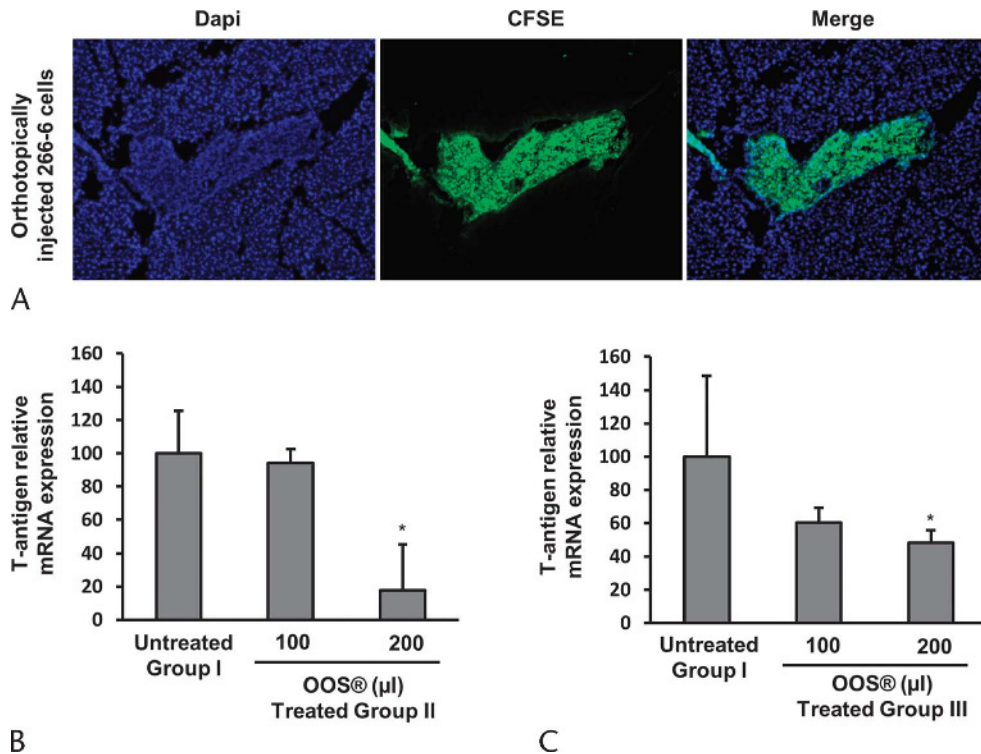
FIGURE 4. Ocoxin oral solution effect on T-antigen expression levels in pancreatic tissue. A, Carboxyfluorescein succinimidyl ester–labeled 266-6 cells (green) were inoculated in the pancreas of mice, and they were killed after 48 hours. The pancreas was frozen, processed, and contrasted with DAPI (4′,6-diamino-2-fenilindo) (blue) to analyze by fluorescence microscopy. B, T-antigen expression level by RT-qPCR in the pancreas of mice from groups I and II. C, T-antigen expression level by RT-qPCR in the pancreas of mice from groups I and III. Data are expressed as the mean value (SD) of at least 7 mRNA samples per each treatment and 2 independent assays. Differences were considered significant for *$P < 0.05$.

present in the pancreas as soon as 48 hours after tumor cell injection (Fig. 4A). The effect of OOS on the development of pancreatic adenocarcinoma in vivo was studied by the detection of SV40 T-antigen by RT-qPCR on the pancreas of untreated and OOS-treated tumor-bearing mice. As shown in Figure 4B, mice from group II showed a significant reduction of 82% in the expression of the tumor marker T-antigen in the pancreas when they were treated with 200 μL of OOS from the 10th day before tumor cell inoculation and until they were killed. Mice from group III, whose treatment started 7 days after tumor cell inoculation, showed 40% reduction of T-antigen expression when they received the treatment of OOS 100 μL and a significant decrease of 52% when the 200-μL dose was used (Fig. 4C).

Cytokine Levels in the Serum of Mice Bearing Pancreatic Adenocarcinoma and Treated With OOS

To analyze the inflammatory cytokine profile in the serum of OOS-treated mice, ELISAs were carried out. As shown in Figure 5, the same pattern of cytokine levels was observed in the serum of animals under group II or group III treatment schedules (Figs. 5A, B).

In both groups, IL-1β, IL-12, and IFN-γ were reduced in the serum, compared with serum levels in the untreated mice. In the case of group II, reductions in IL-1β, IL-12, and IFN-γ were significant in mice treated with OOS 100 μL or in the 200-μL dose (Fig. 5A). Regarding group III, the IL-1β reduction was substantial when OOS was administered at 200-μL doses and IL-12 at 100-μL doses. Either doses of OOS 100 and 200 μL significantly

reduced IFN-γ in serum (Fig. 5B). On the contrary, serum concentration of anti-inflammatory IL-10 cytokine significantly rose 5-fold in the serum of mice from groups II and III, treated with 200 μL OOS.

Effect of OOS on the Viability of Human Pancreatic Adenocarcinoma Cell Lines

As we showed in Figures 2 and 4, a cytotoxic role of OOS in mouse 266-6 pancreatic adenocarcinoma cells was probed in vivo and in vitro. Thus, we studied the potential of this compound in 6 human pancreatic adenocarcinoma cell lines. As shown in Figure 6, in vitro analyses revealed a pronounced cytotoxic effect of OOS in the BxPC-3, Capan-2, CFPAC-1, HPAF-II, Panc10.05, and SW1990 pancreatic cancer cell lines. All the cells cultured with the OOS 1:50 (V/Vf) dilution showed a strong decrease in cell viability ranging from 90% in BxPC-3 (Fig. 6A) to 70% in Capan-2 (Fig. 6B), 60% in CFPAC-1 (Fig. 6C), 37% in HPAF-II (Fig. 6D), 75% in Panc10.05 (Fig. 6E), and 60% in SW1990 (Fig. 6F).

Cytotoxic Effect of Paclitaxel and Gemcitabine in Human Pancreatic Adenocarcinoma Cell Lines

The cytotoxic effect of increasing concentrations of paclitaxel and gemcitabine was studied in 6 pancreatic adenocarcinoma cell lines. One-micrometer paclitaxel treatment reduced cell viability up to 57% in BxPC-3 (Fig. 7A), 62% in Capan-2 (Fig. 7B), 72% in CFPAC-1 (Fig. 7C), 31% in HPAF-II (Fig. 7D), 35% in Panc10.05 cells (Fig. 7E), and 50% in SW1990 (Fig. 7F). The
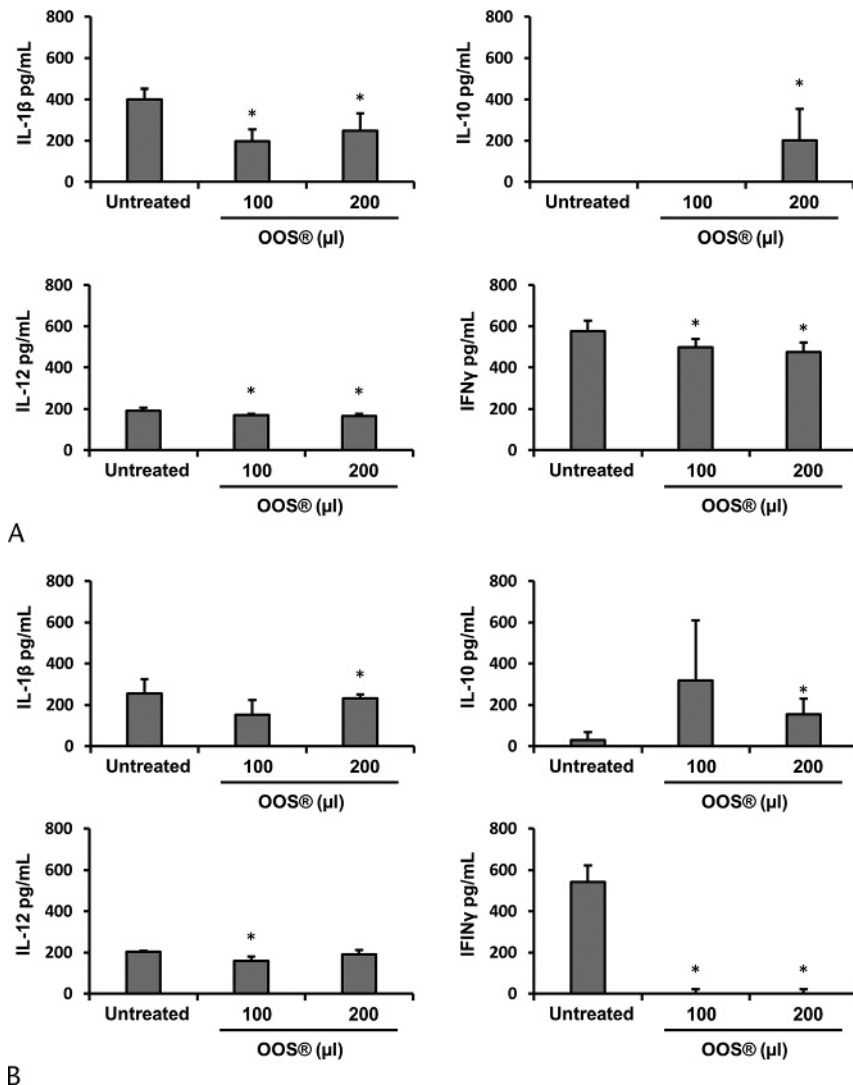
FIGURE 5. Cytokine levels in the serum of tumor-bearing mice. A, Cytokine levels in serum of tumor-bearing mice treated with 100 or 200 μL OOS according to group II administration pattern. B, Cytokine levels in serum of tumor-bearing mice treated with 100 or 200 μL OOS according to group III administration pattern. Data represent the mean value (SD) of at least 7 serum samples per each treatment and 2 independent assays. Differences were considered significant for *$P < 0.05$.

effect of gemcitabine is similar to that of paclitaxel, with a reduction on cell viability of 40% in BxPC-3 (Fig. 7A), 35% in Capan-2 (Fig. 7B), 70% in CFPAC-1 (Fig. 7C), 10% in HPAF-II (Fig. 7D), 33% in Panc10.05 (Fig. 7E), and 36% in SW1990 (Fig. 7F) cell lines. No differences in cell viability were observed with concentrations higher than 1 μM.

Effect of OOS as a Complement to Paclitaxel and Gemcitabine Adjuvant Therapy on the Viability of Human Pancreatic Adenocarcinoma Cells

Paclitaxel and gemcitabine are the most used chemotherapeutic agents in pancreatic adenocarcinoma patients. Therefore, we evaluate the potential of OOS as a complement for anticancer therapies when administering these compounds. In all the studied cell lines, the cytotoxicity of both drugs increased when combined with OOS 1:50 (V/Vf) dilution. Ocoxin oral solution improved the antitumor activity of paclitaxel by 25% in BxPC-3 (Fig. 8A), 71% in Capan-2 (Fig. 8B), 32% in CFPAC-1 (Fig. 8C), 15% in

HPAF-II (Fig. 8D), and 35% in Panc10.05 (Fig. 8E) and SW1990 (Fig. 8F) cells.

A similar effect was observed after treating cells with gemcitabine, since its anticancer activity was increased by 37% in BxPC-3 (Fig. 8A), 40% in Capan-2 (Fig. 8B), 17% in CFPAC-1 (Fig. 8C), 10% in HPAF-II (Fig. 8D), 39% in Panc10.05 (Fig. 8E), and 46% in SW1990 cells (Fig. 8F).

Effect of OOS as a Complement to Overcome Stromal-Mediated Chemoresistance of Human Pancreatic Adenocarcinoma Cell Lines

The potential of OOS as a complement to counteract fibroblast CM–induced chemoresistance to paclitaxel and gemcitabine in pancreatic cancer cells was studied. As shown in Figure 9, fibroblast CM increased the viability of all the tumor cell lines examined. Moreover, fibroblast CM abrogated the effect of paclitaxel, with a 90% to 100% viability in BxPC-3, CFPAC-1, HPAF-II, and SW1990 cells (Figs. 9A, C, D, and F), and low but
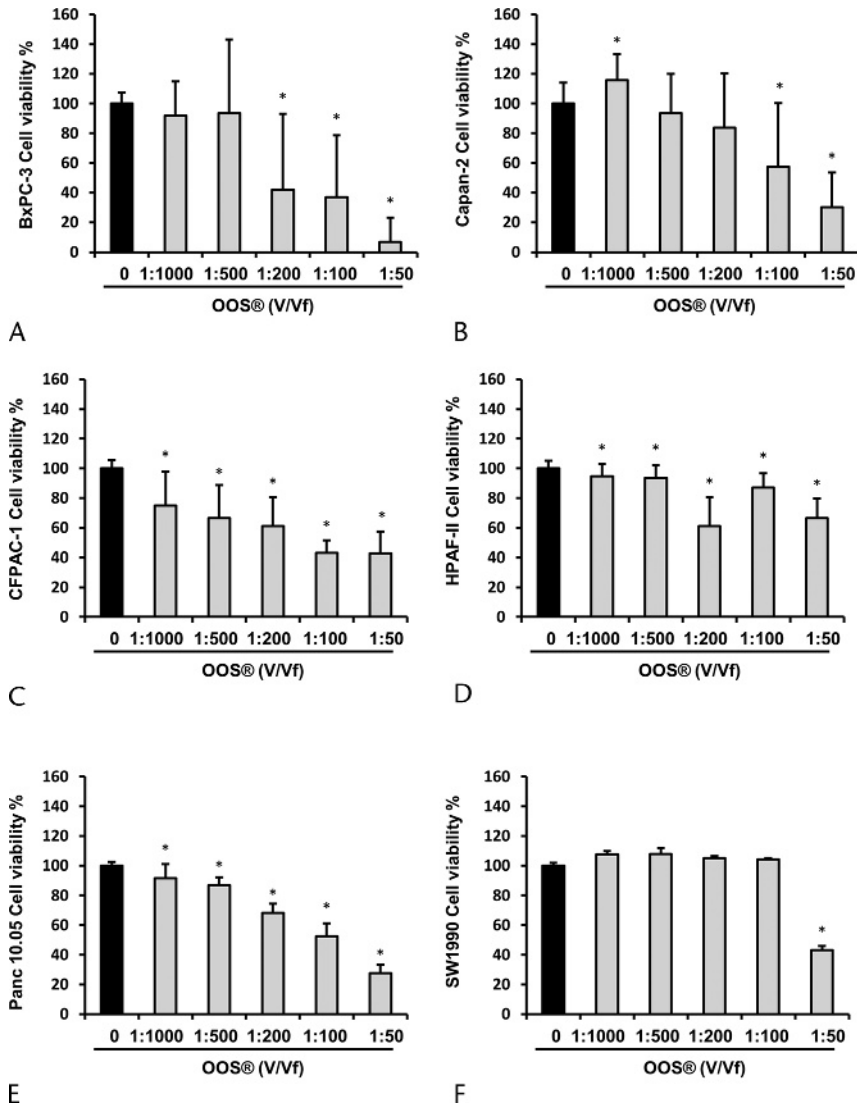
FIGURE 6. Cell viability of human pancreatic cancer cell lines in the presence of different concentrations of OOS. The viability of BxPC-3 (A), Capan-2 (B), CFPAC-1 (C), HPAF-II (D), Panc10.05 (E), and SW1990 (F) cells was tested by Presto Blue assay after treatment with increasing concentrations (1:1000 to 1:50 (V/Vf)) of OOS for 48 hours. Data represent the mean value (SD) of at least 3 independent experiments. Differences were considered significant for *$P < 0.05$.

sustained differences for Capan-2 and Panc10.05 cells (Figs. 9B and E). Regarding gemcitabine, fibroblast CM reverted the toxicity exerted by this drug in BxPC-3, Capan-2, CFPAC-1, HPAF-II, and SW1990 cell lines (Figs. 9A, B, C, D, and F). However, fibroblast CM did not modify gemcitabine's cytotoxicity in Panc10.05 cells (Fig. 9E).

In all the studied cell lines, the addition of the combination of gemcitabine or paclitaxel with OOS 1:50 (V/Vf) to fibroblast CM–stimulated cells reverted the chemoresistance produced by fibroblast CM and helped to recover the cytotoxic effect observed with paclitaxel and gemcitabine alone.

Mechanism of OOS to Reduce Cell Viability

To further confirm that the reduction in cell viability was accompanied by an increase in cell apoptosis in OOS-treated cells,

apoptosis levels were measured using annexin V–PI cell staining kit. As observed in Figure 10, OOS treatment increased the number of apoptotic cells in all the studied cell lines. We observed a 2-fold increase in BxPC-3 cell death (Fig. 10A), a 0.5-fold in Capan-2 (Fig. 10B), a 2-fold in CFPAC-1 (Fig. 10C), a 1.5-fold in Panc10.05 (Fig. 10D), and a 4.5-fold in SW1990 cells (Fig. 10E). Moreover, OOS acts as a synergistic agent for paclitaxel, increasing their apoptotic effect by 10% in Panc10.05 and 50% in SW1990 pancreatic cancer cell lines (Figs. 10D and E). When combined with gemcitabine, OOS promoted a further 15% apoptosis in Capan-2, 10% in Panc10.05, and 30% in SW1990 cells. Moreover, OOS administered as complement of paclitaxel and gemcitabine increased the apoptosis rate up to 25% in BxPC-3 cells (Fig. 10A) and CFPAC-1 cells (Fig. 10C) and up to 50% in SW1990 cells (Fig. 10E) and Panc10.05 cells (Fig. 10D) when cells were cultured with fibroblast CM, therefore abrogating stromal-mediated
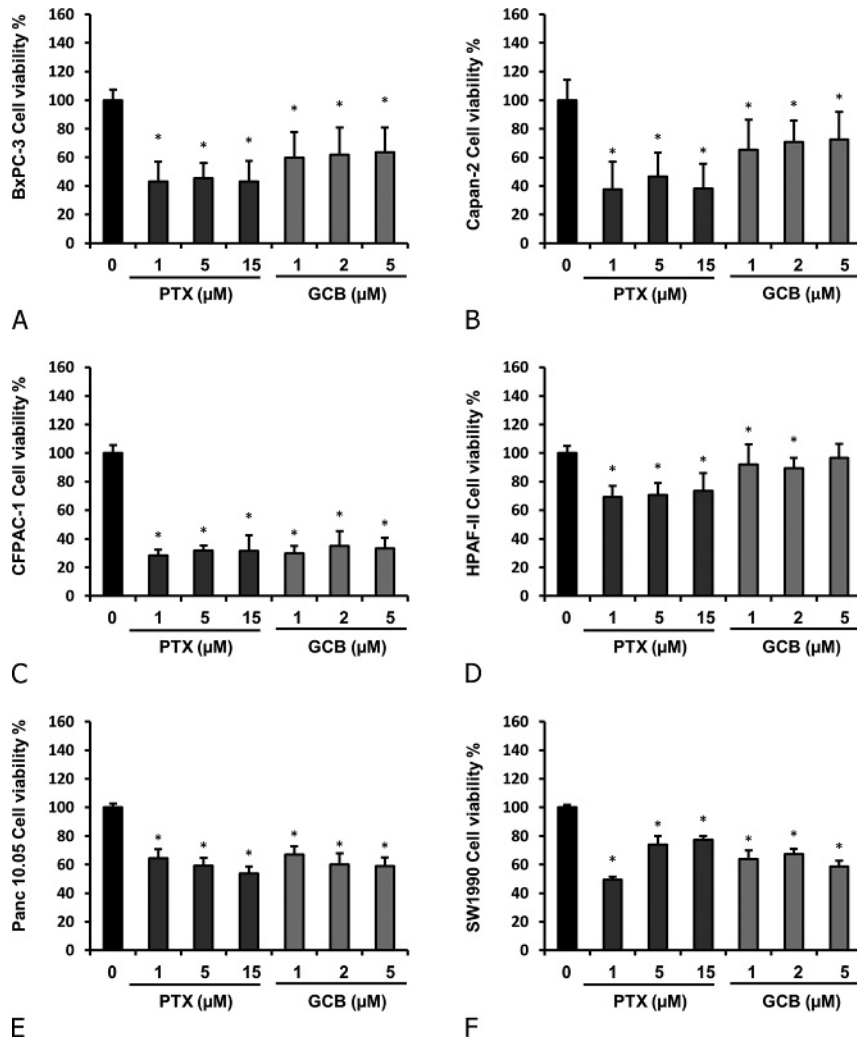
FIGURE 7. Cell viability of human pancreatic cancer cell lines in the presence of different concentrations of paclitaxel and gemcitabine. The viability of BxPC-3 (A), Capan-2 (B), CFPAC-1 (C), HPAF-II (D), Panc10.05 (E), and SW1990 (F) cells was tested by Presto Blue assay after treatment with paclitaxel (PTX) (1–15 μM) and/or gemcitabine (GCB) (1–5 μM) for 48 hours. Data represent mean values (SD) of at least 3 independent experiments. Differences were considered significant for *$P < 0.05$.

resistance (Fig. 10). The Capan-2 cell line only showed a 25% increased apoptosis when they were treated with gemcitabine (Fig. 10B). Thus, OOS exerted a synergistic effect with both chemotherapeutics almost diminishing fibroblast CM–mediated resistance in human pancreatic cancer cells.

DISCUSSION

Pancreatic cancer is an aggressive disease with intense stromal reaction,[39] which remains unresponsive to conventional therapies because of both the late detection and the acquired drug resistance. Accumulated evidence has suggested that the development of the desmoplastic stroma is a significant obstacle for the actual treatments for pancreatic cancer.[12,13,40,41] Thus, the combination of nutritional supplements and chemotherapy is a new therapeutic approach to support chemotherapy itself and to reduce the produced adverse effects targeting not only the tumor but also the components of its associated stroma.[42–44] In this way, natural compounds, such as curcumin, resveratrol, flavonoids, epigallocatechin-3-gallate, sulforaphane, or nimbolide, have shown promising antitumor effects in pancreatic cancer.[20,45,46] In this context, OOS consists of a mixture of several nutrients that have been proven to exert antitumor effects alone and in combination with chemotherapy, in vitro and in vivo, in the metastatic progression of colorectal cancer to the liver.[22,23] However, to our knowledge, there is no report on the efficacy of OOS in pancreatic cancer. In this article, we unveil the antitumoral effect of OOS in the development of pancreatic adenocarcinoma through the reduction of tumor cell viability, enhancement of the antitumor effect of chemotherapeutic agents, modulation of the expression of genes known to be associated with pancreatic adenocarcinoma, and by reverting chemoresistance conferred by CAFs. Moreover, OOS treatment reduced tumor development in vivo in the pancreas of mice inoculated with 266-6 cells and decreased the level of critical proinflammatory cytokines in their serum. Thus, these results support the need for further studies to clarify whether the use of OOS as a nutritional complement could be feasible during the treatment of pancreatic adenocarcinoma.
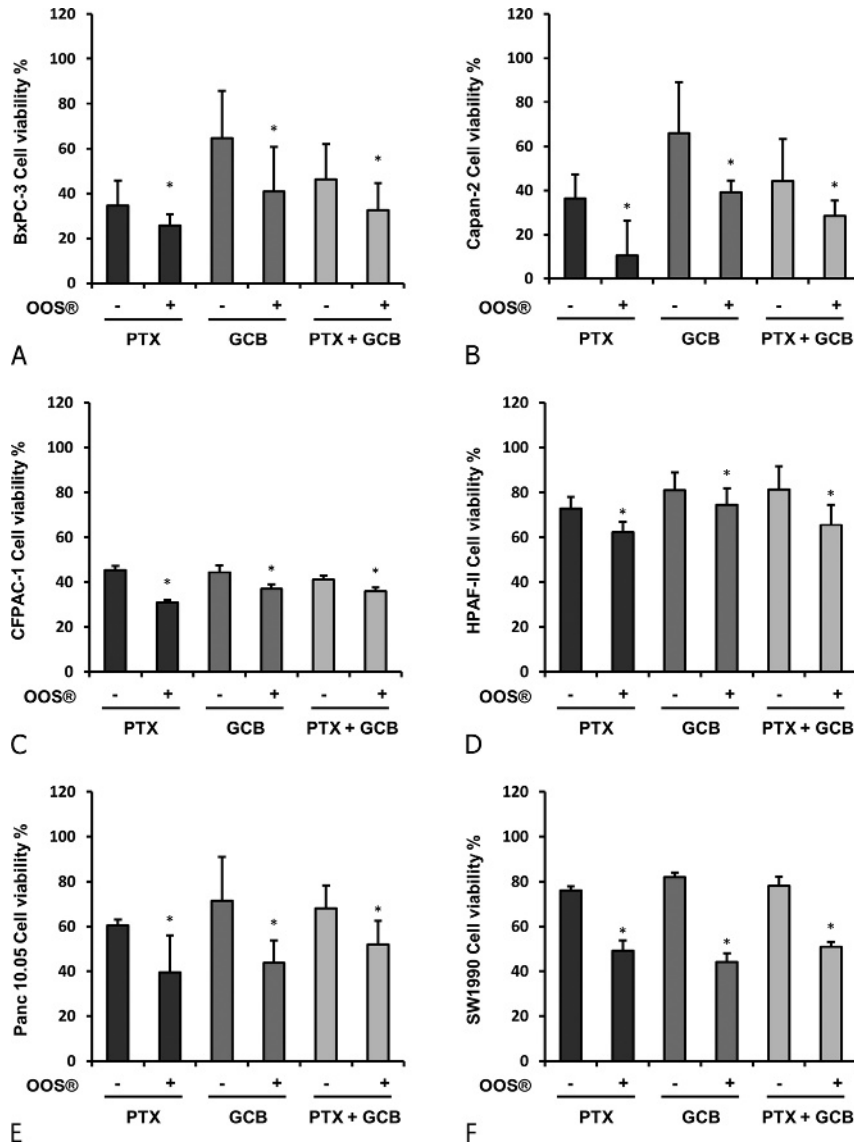
FIGURE 8. Cell viability of human pancreatic cancer cell lines treated with OOS as a complement to paclitaxel and gemcitabine. The most effective concentrations of paclitaxel (PTX), gemcitabine (GCB), and OOS were chosen, and viability of BxPC-3 (A), Capan-2 (B), CFPAC-1 (C), HPAF-II (D), Panc10.05 (E), and SW1990 (F) cells was tested for 48 hours in the presence of combinations of the 3 agents: paclitaxel 1 µM + OOS 1:50 (V/Vf), gemcitabine 1 µM + OOS 1:50 (V/Vf), and paclitaxel 1 µM + gemcitabine 1 µM + OOS 1:50 (V/Vf). Data are expressed as the mean value (SD) of at least 3 independent experiments. Differences were considered significant for $P < 0.05$ between the treatments alone and the treatments with OOS (*).

According to our results, OOS decreased the viability of the 266-6 murine pancreatic adenocarcinoma cells in vitro and reduced the tumor cell marker expression (SV40 T-antigen) in the pancreas in vivo, pointing out a reduction of tumor development in the pancreas. Besides, OOS also diminished significantly the in vitro viability of the human pancreatic cancer cell lines BxPC-3, Capan-2, CFPAC-1, Panc10.05, HPAF-II, and SW1990. Furthermore, the use of OOS as a complementary therapy to the agents, gemcitabine and paclitaxel, enhanced the cytotoxic effect in all the tested human cell lines.

Tumor growth and chemoresistance often correlate with the nature and the developmental degree of the microenvironment of the tumor itself.[47,48] In this context, CAFs are stromal cells associated with the developing tumor that play an essential role in tumor progression. Cancer-associated fibroblasts secrete multiple chemokines and inflammatory mediators that promote proliferation, invasion, and metastasis of cancer cells.[6] Moreover, accumulating evidence indicates the role of CAFs in the acquisition of drug resistance.[47] Following this, all the human cancer cell lines we tested showed an improvement in viability and a reduction in apoptosis levels in vitro when they were cultivated in the presence of supernatants obtained from fibroblast cultures, also becoming more resistant to paclitaxel and gemcitabine treatments.

Interestingly, when OOS was added to cancer cells cultured with fibroblast CM, the drug's effectiveness was reverted to basal conditions, whereas the number of apoptotic tumor cells increased. In our hands, OOS exerted an antioxidative and anti-inflammatory
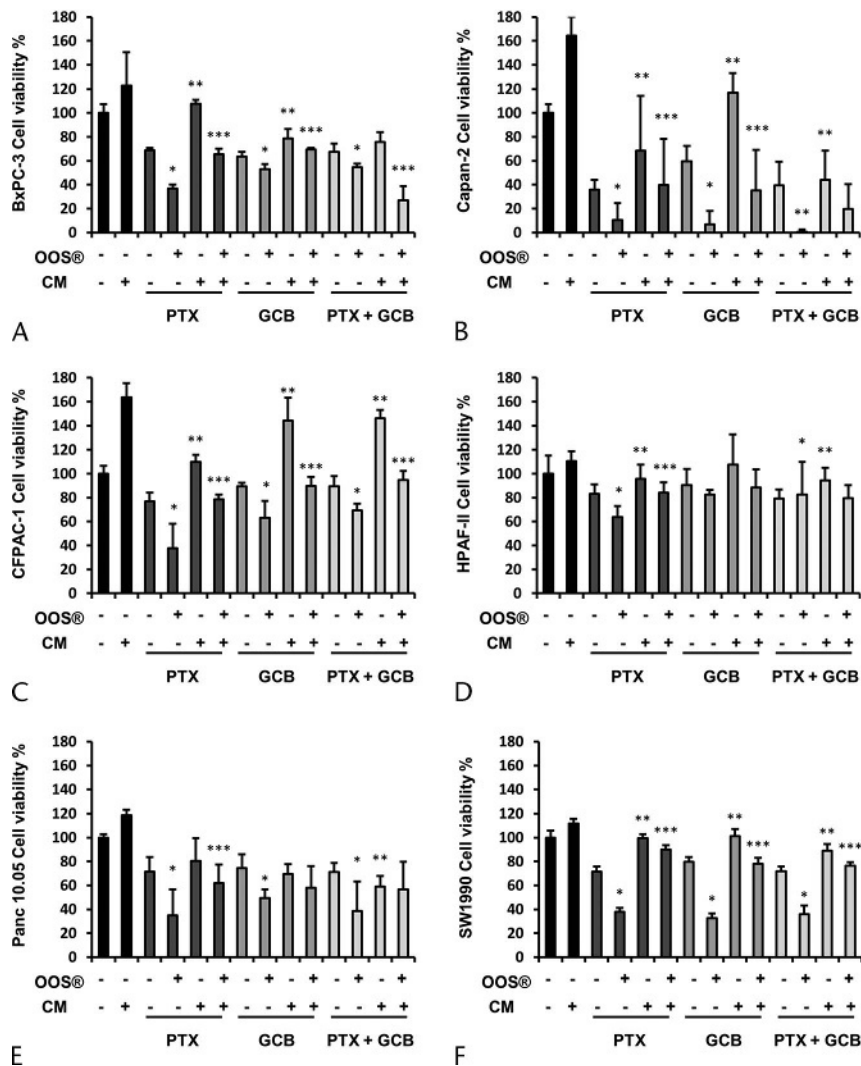
FIGURE 9. Cell viability of human pancreatic cancer cell lines in the presence of paclitaxel and gemcitabine alone and combined with OOS in cultures grown under normal and protumoral conditions. Human cancer cells were cultured with regular culture medium (–) and with fibroblast CM (CM). Then, the viability of BxPC-3 (A), Capan-2 (B), CFPAC-1 (C), HPAF-II (D), Panc10.05 (E), and SW1990 (F) cells was measured in the presence of paclitaxel (PTX) and gemcitabine (GCB) either alone or combined with OOS after 48 hours. Data are expressed as the mean value (SD) of at least 3 independent experiments. Differences were considered significant for $P < 0.05$ between the treatment alone and the treatment with OOS in normal conditions (*), the treatment alone and the treatment on protumoral conditions (**), the treatment on protumoral conditions and the treatment on protumoral conditions with OOS (***).

effect and reduced the in vitro viability and invasion of the murine 3T3 fibroblasts (data not shown), demonstrating that OOS exerts an impact not only on tumor cells but also on the stromal components.

Pancreatic stellate cells (PSCs) are the primary source of CAFs in the pancreas.[6,49] Pancreatic stellate cells stimulate cancer cell proliferation and inhibit cancer cell apoptosis, therefore acting as promoters of tumor growth.[50,51] Apte et al[51] utilized coculture of pancreatic cancer cell lines and PSCs or conditioned medium produced by PSCs to demonstrate that pancreatic cancer cells recruit PSCs promoting tumor growth and local invasion. Interestingly, our previous results showed that OOS reduced in vivo hepatic stellate cell infiltration into colorectal cancer liver metastasis.[22] Thus, a similar mechanism may be operating in our in vivo model of pancreatic carcinoma. Moreover, in vitro assays revealed that OOS slows down 266-6

cell cycle by 30%, therefore blocking in part, tumor cell proliferation. It is tempting to speculate that OOS may exert its antitumor effect by reducing both tumor cell proliferation and PSC infiltration into the pancreatic tumor. Further analysis is in need to clarify those results.

Inflammation and cancer progression are closely related.[52,53] In this work, OOS reduced the level of central proinflammatory cytokines, such as IL-1β, IL-12, and IFN-γ in the serum of pancreatic tumor-bearing mice, and increased anti-inflammatory IL-10. These results are in accordance with our previous results in a colorectal cancer liver metastasis model. On the other hand, the gene array performed in this study revealed that OOS reverted the expression of genes already reported as being altered in pancreatic cancer such as *Angptl4*, *Asns*, *Crabp1*, *Creb1*, *Foxf1*, *Thy1*, *Trpc4*, and *Wnt4*,[27–30,34,37,38] among others.
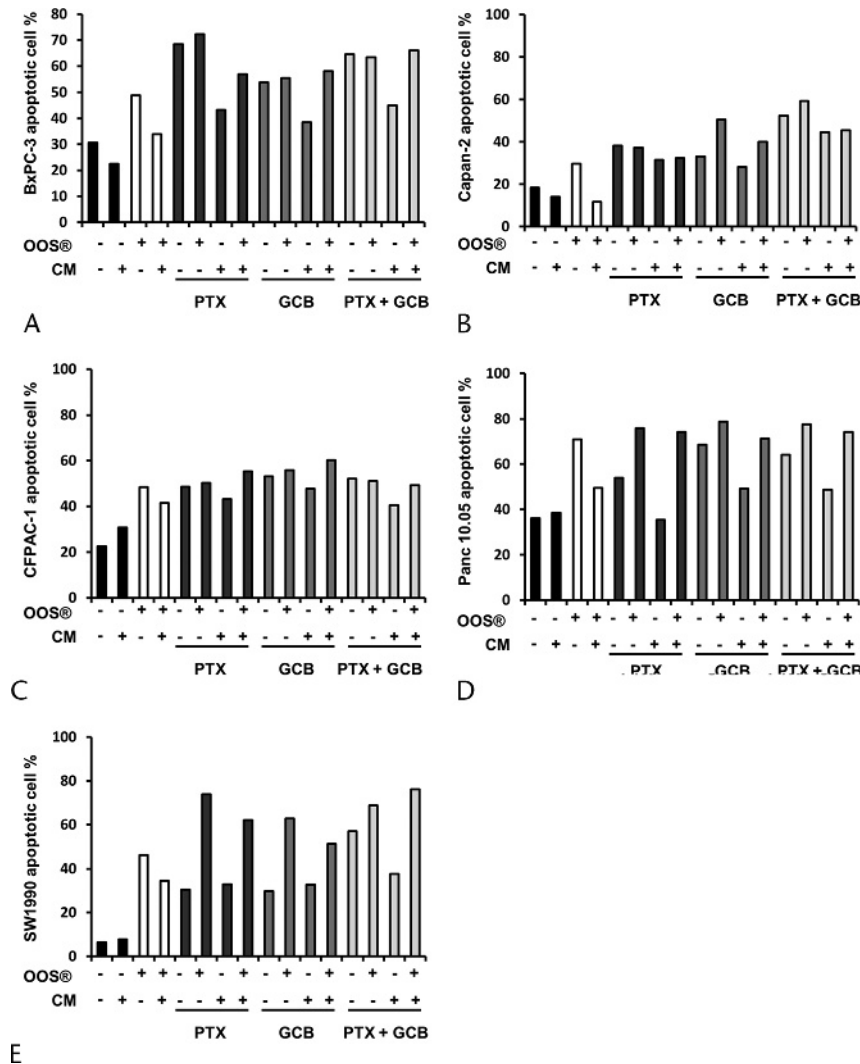
FIGURE 10. Effect of OOS alone or in combination with paclitaxel and gemcitabine in human pancreatic cancer cell apoptosis. Cell lines treated with paclitaxel and gemcitabine alone and in combination with OOS in both healthy and protumoral conditions. Apoptosis was analyzed by annexin V/PI to understand the mechanism of OOS that provoked the cell viability reduction. Cell death levels of BxPC-3 (A), Capan-2 (B), CFPAC-1 (C), Panc10.05 (D), and SW1990 (E) were measured in the presence of paclitaxel 1 μM and gemcitabine 1 μM either alone or combined with OOS 1:50 (V/Vf) after 48 hours. Cells were cultured with standard culture medium (–) and with fibroblast CM. Data show the most representative results.

In summary, OOS may slow down pancreatic cancer development, by decelerating cell division and increasing cell apoptosis. Besides, OOS may affect CAF infiltration into the tumor and reduce the level of inflammatory cytokines in the serum of mice. Moreover, OOS demonstrated the capacity to revert both the chemoresistance produced by the stromal cells and the expression of genes related to pancreatic cancer.

Therefore, OOS may constitute a nutritional supplement to combine with currently used chemotherapy to treat pancreatic cancer.

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