Article

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Inhibition Effects of Acetylsalicylic Acid with Nitric Oxide (NO-ASA) on Neoplastic Changes in the Exocrine Pancreas Acinar Cells of the Azaserine Injected Rats

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Background: Atypical acinar cell foci (AACF) seen in pancreatic cancer are fatal and have been studied with some causative agents. However, for the first time, the effect of acetylsalicylic acid with nitric oxide (NO-ASA) on AACF was examined in this study. Although NO-ASA has very successful inhibitory effects against some types of cancer, it has not been investigated whether they can exert their inhibition effects on AACFs.

Methods: For experimental purposes, 21 14-day-old male Wistar albino rats were used. Azaserine (30 mg/kg) was dissolved in 0.9% NaCl solution and injected intraperitoneally (i.p.) into 14 rats, except for the Control group (Cont) rats, for three weeks. Rats that were injected with azaserine once a week for three weeks and those that did not receive treatment were divided into experimental groups. 15 days after the end of the azaserine injection protocol, NO-ASA was applied to azaserine with NO-ASA (Az+NO-ASA) group rats three consecutive times with an interval of 15 days by gavage. At the end of the 5-month period, pancreatic tissue was dissected and weighed. Pancreas preparations prepared from histological sections were examined for AACF burden and analyzed via a video image analyzer. One-way analysis of variance (ANOVA) non-parametric statistical analyses were performed to test whether there was a difference between the averages of the experimental and Control groups.

Results: AACF burden in both groups injected with azaserine was found to be statistically significant in all categories compared to that of the Control group ($p < 0.05$). The average Calculated Estimated average AACF volume (mm^3) values, the Calculated estimated average AACF diameter (µm), the Estimated average number of AACF per unit volume, AACF rate as a % of Calculated Organ Volume were higher in the AzCont group rats than in the Az+NO-ASA group, when compared, and there was an important level statistical difference between the groups ($p < 0.05$). It was determined that for all parameters AACFs load in Az+NO-ASA group rats were significantly reduced compared to that of AzCont group rats ($p < 0.05$).

Conclusions: We observed that, as a result of the NO-ASA application, the experimental AACF focus ratio created by azaserine injection was significantly inhibited. The inhibitory effect of AACFs in Az+NO-ASA group rats may have resulted from the significant and independent chemopreventive and/or chemotherapeutic activity of NO-ASA against exocrine pancreatic AACF foci.

Keywords: NO-ASA; atypical acinar cell foci; exocrine pancreas; nitric oxide; rat

Introduction

There is an important relationship between radical changes in nutritional habits and neoplastic changes that occur in the exocrine part of the pancreas depending on the enzymes secreted by the digestive system. Thus, it is seen that pancreatic cancer develops depending on nutritional habits and environmental factors. Neoplastic changes due to mutagenic effects reach the metastatic stage after a development period of approximately five years, and the termination phase takes approximately two years [1]. Therefore, detecting the causes of pancreatic cancer in advance can make important contributions to the treatment. There are findings that the increase in the incidence of pancreatic cancer due to nutritional habits may be caused by high cyclooxygenase (COX) enzyme activity in the acinar cells in the exocrine pancreas [2].

Recently, the chemopreventive effects of acetylsalicylic acid observed in colorectal neoplasia have been demonstrated consistently for many years [3]. Acetylsalicylic acid with nitric oxide (NO-ASA) is a product of acetylsalicylic acid obtained by chemically adding nitric oxide to reduce side effects. There are very few studies on the mechanism of action of this type of acetylsalicylic acid on pancreatic enzymes and indirectly on the formation of pancreatic and bowel cancer. The Wnt signaling pathway is of critical importance in stem cell biology, tissue and organ regeneration, and maintaining homeostatic continuity.

However, mechanisms such as Wnt modulation of cancer through mitogen-activated protein kinase and nitric oxide synthetase signaling mechanisms, phase II enzyme induction, and oxidative stress enzyme induction that causes cell death are still the subject of research [4].

Previous studies have shown that NO-ASA may have a stronger inhibition effect than other acetylsalicylic acids. It has been shown that NO-ASA inhibits human pancreatic cancer cells to a greater extent than other acetylsalicylic acids *in vitro* culture cells. Although it is known that NO-ASA has antiproliferative and proapoptotic effects *in vitro*, its *in vivo* effect and possible neoplastic changes in exocrine pancreatic acinar cells are unknown [5]. Therefore, it is important to investigate any inhibitory effect of NO-ASA against neoplastic changes in exocrine pancreatic atypical acinar cell foci (AACF) with the help of a well-known experimental animal model (azaserine-rat).

It has been determined that pancreatic intraepithelial neoplasia (PanIN), which is one of the precursor neoplastic changes that is frequently seen in pancreatic cancer patients, and AACFs are found in the pancreas in 80% of these cases. [6]. NO-ASA is a product of acetylsalicylic acid, which is widely used for medical purposes, by chemically adding nitric oxide to reduce possible side effects, and there are very few experimental studies on its mechanism of action. It is suggested that NO-ASA, which was found to have an inhibitory effect on neoplastic cancer cells in an *in vitro* study, has a stronger antiproliferative effect than traditionally used acetylsalicylic acid applications. Due to the combination of acetylsalicylic acid with nitric oxide, the antiproliferative and proapoptotic effects of the component occur [5]. In the current study, it is investigated whether NO-ASA has any inhibitory effect on exocrine pancreatic acinar cells with the help of the well-known azaresine-rat model [6,7]. This study aims to investigate the inhibitory effects of NO-ASA on neoplastic changes in exocrine pancreatic acinar cells, utilizing the azaserine-rat model.

Materials and Methods

The study was carried out following the approval of the Experimental Animals Ethics Committee of the Experimental Medical Research and Implementation Center (project No: 161210004, date: 12/2016). For experimental purposes, 21 14-day-old male Wistar albino rats weighing 22–30 g were used, and the rats used in the study were obtained from Necmettin Erbakan University KONÜDAM Experimental Medicine Application and Research Center, Konya, Turkey. The care, feeding and preservation of the rats were carried out in the same center. The azaserine-rat model has recently been used by many researchers to investigate the origin of pancreatic AACF [8,9].

Experimental Materials

As experimental materials, azaserine (CAS No.: 115-02-6), hematoxylin (CAS No.: 517-28-2), eosin (CAS No.:17372-87-1), NO-ASA (CAS No.: 175033-36-0), acetone (CAS No.: 67-64-1), formalin (Product No.: 1.04002), HCl (CAS No.: 7647-01-0), methanol (CAS No.: 67-56-1), and chloroform (CAS No.: 67-66-3) were obtained from Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada.

Experimental Protocol

Except untreated Control group (Cont), to initiate neoplastic differentiation in the exocrine pancreas of rats, totally a total of 14 rats were intra-peritoneally (IP) injected with azaserine (30 mg/kg) dissolved in 0.9% NaCl solution. After three consecutive weeks of injection, all of the rats were divided into three experimental groups:

(1) Control group not treated with azaserine (n = 7);

(2) Group injected with (i.p.) azaserine only (30 mg/kg) (n = 7);

(3) Group injected intraperitoneally (i.p.) azaserine (Az) and gavaged with Az+NO-ASA (40 mg/kg b.w.) (n = 7).

15 days after the end of the azaserine injection protocol (45 days after the beginning of azaserine injection) NO-ASA dissolved in 1% carboxymethyl cellulose solution (40 mg/kg b.w.) was administered to Az+NO-ASA group rats for three consecutive times with an interval of 15 days by gavage [10]. During the study, all rats were fed in the same room and with the same standard feed and a special diet. The rats used for the experiment were housed in a standard animal shelter, with room humidity and temperature (temperature: 21 ± 2 °C and 55% humidity) under control, with *ad libitum* 12-hour light and 12-hour dark cycle, with 4 rats in each cage. In this study, using azaserine which is a chemical well known to have a mutagenic feature in the induction of cancer in the rat pancreas. Pancreatic cancer model (azaserine-rat) developed by Longnecker and Curphey [11] was employed to induce hyperplastic differentiation in the acinar cells of the subjects [12]. At the end of the 5-month period, the rats were anesthetized with 50 mg/kg (sc) Ketamine HCl (NDC No.: 00409-2053-10, Pfizer inc., Lake Forest, IL, USA) and 15 mg/kg xylazine HCL (sc) (NDC No.: 64189-9002-1, Bioveta Co., Ivanovice na Hané, Czech Rep) and the rats were sacrificed by cervical dislocation. At autopsy, the rat pancreas was removed as a whole. The weights of whole resected pancreases were recorded before fixing in 10% buffered neutral formalin. Before being immersed in the fixative solution, each pancreas was spread out on a piece of porous paper to provide maximum cross-sectional area for subsequent sections.

Histopathological Examinations

Standard detection and staining methods used in the routine hematoxylin-eosin staining method were applied for the detection and staining of tissue samples. They were

blocked in hard paraffin and routine hematoxylin-eosin staining was applied to 4 µm thick sections taken from the prepared blocks with a Rotary microtome (Thermo Scientific, Waltham, MA, USA). The prepared preparations were examined with a Zeiss brand research microscope (Axio Imager model Zeiss light microscope, Rostock, Germany), measurements were made and photographs of AACFs detected in the pancreas were taken. Hematoxylin-eosin tissue preparations prepared from the pancreas of rats were previously reported [13,14]. Neoplastic lesions (AACF) focal changes occurring in the exocrine pancreas of rats were classified according to the criteria determined by Reddy and Rao [13] according to their size, histological features, and morphology. Accordingly, each analytical focus was measured and evaluated analytically. Longnecker [15] stated that AACFs are carcinogen-induced pancreatic lesions, and therefore AACF was considered as a possible precursor of cancer cell growth in the rat exocrine pancreas.

Stereological Analysis

Pancreas preparations prepared from histological sections were examined for AACF, acinar cell adenoma and acinar cell carcinoma, with the help of Zeiss Brand Video Image Analysis device (Axio Imager model Zeiss light microscope, Rostock, Germany). By counting all the foci detected in the preparations, the pancreas in each preparation was compared with the perimeter of the total area. There were no any acinar cell adenoma and acinar cell carcinoma in pancreas. The perimeter of each focus was measured digitally. This system includes a video system that records images of the preparations examined and a digital mechanism that can measure the areas of foci and tumors. It seems possible to calculate the approximate total tumor burden in a preparation based on the three-dimensional structures of AACF, tumors and adenomas measured with the VOLUGEN package program, which was developed based on this model modified by [16,17] and used in many similar studies. Accordingly, according to the number of rats used in the experiment, it can be calculate the Estimated mean AACFs diameter (µm), Estirmated Mean Values AACF volume (mm^3) and Estimated number of AACF per unit volume.

Statistical Analysis

One-way analysis of variance (ANOVA) nonparametric statistical analyses were performed to test whether there was a difference between the averages of the experimental and Control groups. The numbers of atypical acinar cell foci that were likely to form in pancreatic tissues removed from animals in the experimental and Control groups, the mean values and standard deviations of these numbers were calculated in the (ProStat version 5.04 for Windows, Poly Software Int., Pearl River, NY, USA) program. In the same package program, the Student-Newman-Keuls Multiple Comparison statistical analysis (ANOVA) method was used to test whether there was a difference between the averages of the experimental and Control groups [18]. The results of the data are given in the table as arithmetic mean ± standard deviation. For descriptive statistics, mean values are expressed as median, and for statistical significance; values of $p < 0.05$ were considered significant.

Table 1. The main body and pancreatic weights of the experimental groups (mean ± standard deviation).

Groups	Cont	AzCont	Az+NO-ASA
Body weights (g)	410 ± 40.3	339 ± 60.8	376.8 ± 44.9
Pancreatic weights (g)	1.380 ± 0.13	1.400 ± 0.14	1.400 ± 0.15

Cont, Control group; Az, azaserine; NO-ASA, acetylsalicylic acid with nitric oxide.

Results

According to the findings, no significant difference was found between the average body and pancreas weights of the Cont, AzCont and Az+NO-ASA experimental groups (Table 1). However, the average body weight of Cont group rats was measured to be higher than those of the other groups. When the average pancreas weights were investigated there was a difference in the average pancreas weight between the rats belonging to the AzCont and Az+NO-ASA groups. It seems that the differences between the groups were at limited levels, not reaching statistically significant levels.

In the histopathological examinations, AACFs were observed in all AzCont and Az+NO-ASA group rats injected with azaserine, but no AACFs were found in Cont group rats. AACF burden in both groups injected with azaserine (AzCont and Az+NO-ASA) was found to be statistically significant in all categories compared to that in the Control group ($p < 0.05$). The estimated average AACF diameter (µm) values calculated by the stereological calculation method for the AzCont and Az+NO-ASA groups measured from the exocrine pancreas of rats were different, and the average AACF diameter was smaller in the Az+NO-ASA group rats. (311 ± 81) compared to the AzCont (382 ± 74) group rats in the two groups (Fig. 1). It was determined that the difference between them was not statistically significant, but the AACF amounts were statistically significant when both groups were compared with the Control group ($p < 0.05$). No AACF was found in the untreated Control group.

The Estimated main values AACF volume (mm^3) were higher in the AzCont group rats (0.1151 ± 0.135) than in the Az+NO-ASA group (0.01338 ± 0.09) when compared, and there was an important level of statistical difference in the values between the groups ($p < 0.05$). The Estimated average AACF volume was markedly higher in the AzCont group than the Az+NO-ASA group (Fig. 1).

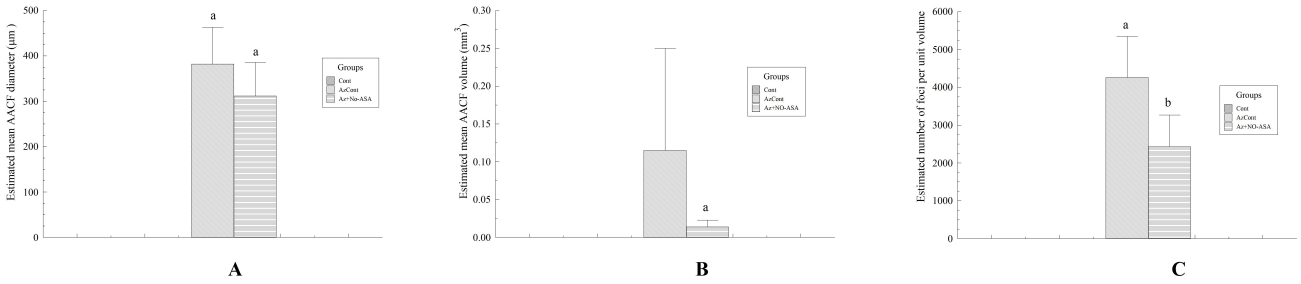

Fig. 1. Calculated mean quantitative values of atypical acinar cell foci (AACF) among the groups (mean ± standard deviation). (A) Estimated mean AACFs diameter (μm) (error bar: standard deviation). [a] A group that is statistically different from the Cont group. (B) Estimated Mean Values AACF volume (mm^3) ($p < 0.05$) (error bar: standard deviation). [a] Az+NO-ASA group (n = 7) values are statistically different from the AzCont group (n = 7). (C) Estimated number of AACF per unit volume ($p < 0.05$) (error bar: standard deviation). [a]A group that is statistically different from the Cont group, [b] A group that is statistically different from the AzCont group.

When comparing estimated number of AACF per unit volume for each group, the values of the AzCont group rats (4258 ± 1088) were found to be higher than the values calculated for the Az+NO-ASA group rats (2431 ± 836). It was determined that the average values of the AzCont group and the Az+NO-ASA group were significantly different ($p < 0.05$), and the AACF loads of rats given NO-ASA decreased. AACF rate as a % of Calculated Organ Volume values decreased in Az+NO-ASA group rats (2.405 ± 2.05) when compared with the AzCont group rats (8.815 ± 4.51), and there was an important level of statistical difference in the average AACF volume values between the groups (Fig. 2). Differences between two groups were statistically meaningful ($p < 0.05$).

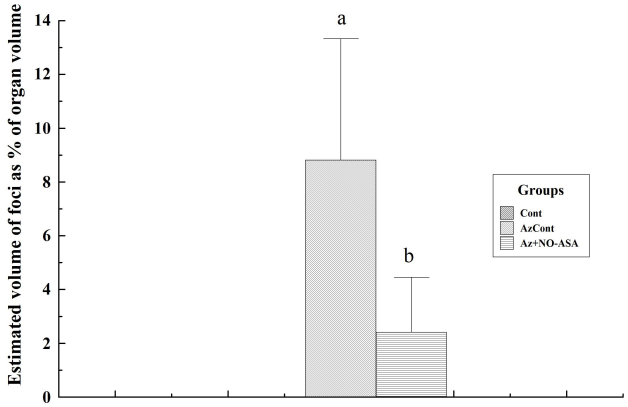

Fig. 2. Estimated mean volume of AACF as % of organ volume ($p < 0.05$) (error bar: standard deviation). [a] A group that is statistically different from the Cont group; [b] A group that is statistically different from the AzCont group (n = 7).

Acidophilic AACF was identified in the pancreas as early as one month after azaserine treatment. In this study, the majority of AACF foci are characterized by zymogen-rich cytoplasm and basal nucleus, round to oval shape, which is slightly larger than normal (Fig. 3A). AACFs were slightly compressed of surrounding parenchyma. The foci in Az+NO-ASA rats, mostly non-encapsulated and close to a normal acinar cell in appearance. It is observed that the foci characterized by larger volumes and acidophilic properties in the AzCont Group rats turn into relatively smaller and basophilic foci in the Az+NO-ASA group (Fig. 3B).

Discussion

According to the World Health Organization data, cancer-related deaths among non-communicable diseases have risen to the 2nd place after cardiovascular system diseases [19]. Pancreatic cancer ranks seventh among the causes of cancer-related death worldwide. Global trends show that cancer cases are increasing and are expected to soon become the leading cause of deaths in Western countries [20]. Therefore, detecting the changes that lead to pancreatic cancer in advance can make important contributions to the treatment. Smoking and family-related genetic factors increase the risk factor by 5–10% [21]. Obesity, diabetes mellitus, chronic pancreatitis, race, periodontal diseases, occupational diseases, nutritional habits, Helicobacter pylori and gallbladder stones may increase the risk factor [22]. It is thought that epigenetic changes may also play an important role in the development of pancreatic cancer. Depending on eating habits, excessive intake of some nutrients may lead to DNA methylation and modification of histone proteins in pancreatic cells [23].

NO-ASA is safer than other known types of non-steroidal anti-inflammatory drugs (NSAIDs) and inhibits the growth of colon cancer cells at a higher rate than traditional NSAIDs. It has been demonstrated that NO-ASA inhibits the growth of human pancreatic, colon, prostate, lung and tongue cancer cell lines treated. NO-NSAIDs inhibit cell proliferation, induce apoptosis and change the cell cycle phase distribution (from G2/M to G0/G1 block), and NO-aspirin induces nuclear lysis of atypical cells [5]. NO-aspirin showed similar effects on two pancreatic cancer cell lines, BxPC-3 (expresses COX) and MIA PaCa-2

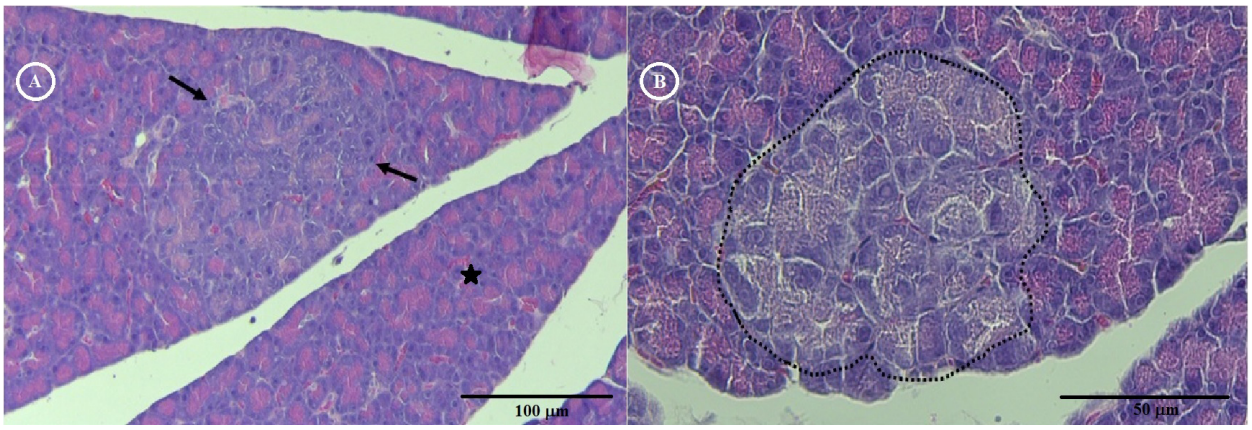

Fig. 3. Characteristics of AACFs formed in the exocrine pancreas of rats as a result of azaserine and Az+NO-ASA applications (H&E). (A) The exocrine pancreas from AzCont group rats shows an AACF (between arrows) and a normal acinar cell population (asterisk). AACF is characterized by large volume and acidophilic properties. Scale bar: 100 µm. (B) AACF from Az+NO-ASA rats. AACF is characterized by having basophilic cytoplasm due to decreased zymogen and paler stains by hematoxylin and eosin (H&E). Scale bar: 50 µm.

(no COX expression), suggesting a COX-independent effect. These results raise the possibility that NO-NSAIDs possess chemopreventive and/or chemotherapeutic activity against a wide variety of human cancers.

Although human pancreatic cancer is a disease of major importance, no similar studies appear to have been carried out hitherto to determine whether NO-ASA can modify exocrine pancreatic carcinogenesis *in vivo*. The present study was designated to test the hypothesis that NO-ASA administration azaserine-initiated rats (Az+NO-ASA Group) may inhibit neoplastic developments sourced acinar cells.

It is generally accepted that a small number of precursor AACF that arise from pancreatic acinar cells due to mutagenic and other carcinogenic effects develop and lead to exocrine pancreatic cancer through a multistep development process [24]. Recognizing the precursor lesions that lead to pancreatic cancer can play an important role in early diagnosis and prevention. AACF, which are the subject of this study, have been shown in previous studies to cause cancer in the exocrine pancreas through experiments conducted on rats [15]. Revealing the possible neoplastic development inhibitory potential of no-acetylsalicylic acid may enable the development of new applications to prevent the development of exocrine pancreatic cancer in the early stages. It has long been shown that acetylsalicylic acid may have a reducing or inhibiting effect on tumor development, at least in bowel cancer studies.

The results obtained confirm our hypothesis that NO-ASA can reduce tumor burden in the exocrine pancreas. In the present study, quantitative stereological assessment of the azaserine-initiated NO-ASA fed rats were found to statistically reduce AACF burden when compared to AzCont rats ($p < 0.05$). Azaserine (o-diazoacetyl-L-serine) is an antimetabolite isolated from cultures of *Streptomycetes sp.* and is a mutagen in Ames *Salmonella typhimerium* assay [7] demonstrated that the pancreas of suckling rats is sensitive to carcinogenic effects of azaserine. It appears to be activated in mammalian cells by a pyridoxal-dependent enzyme system. Previously, N-7-carboxymethyl guanine has been identified in DNAs of exposed azaserine [25]. Oncogenic studies suggest that the KRAS oncogene has a potential role in pancreatic cancer formation. It is thought that any change in this gene triggers neoplastic developments. It is accepted that the KRAS oncogene causes the inactivation of the tumor suppressor genes *CDKN2A*, *TP53*, *DPC4* and *BRCA2,* and as a result, these changes in neoplastic cells cause chromosome loss, gene amplification and telomere shortening in pancreatic cells, resulting in cancer formation [22].

This effect was due to the inhibition of proliferation as well as the induction of programmed cell death. NO-NSAIDs also act on checkpoints (G1-S) in the cell cycle. Effective NSAIDs used to date, these properties allow them to be used as chemopreventive agents against colon cancer. Present findings of our study may indicate that AACFs initiated by azaserine injection may reduce via NO-ASA uptake Their strong effectiveness compared with conventional NSAIDs, combined with their reported safety, makes them promising candidates for chemopreventive agents against acinar cell sourced neoplastic developments. This study has some limitations. Although our histopathological results show the inhibitory effect of NO-ASA on AACFs, they are not sufficient to explain the pathways that reveal this effect. Undoubtedly, the fact that it is not supported by biochemical data is another limiting effect. It is important to support it with immunohistological and biochemical studies that will reveal these pathways in order to guide the next studies.

Conclusions

As a result, with the use of NO-ASA, a statistically significant decrease was observed in the AACfs of rats in the Az+NO-ASA group compared to the AzCont group. It may be an indicator of the chemopreventive and/or chemotherapeutic activity of NO-ASA against exocrine pancreatic cancer. The results obtained from this research may contribute to studies on the treatment or inhibition of exocrine pancreatic cancer formation.

Availability of Data and Materials

All data are available from the corresponding author upon reasonable request.

Author Contributions

SD: conception and design, supervising, technical and material support, statistical analysis, drafting of the manuscript, literature review, writing the article; HY: statistical analysis, writing the article and critical revision of the manuscript. Both authors contributed significantly to editorial changes of important content, read and approved the final manuscript. Both authors participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The study was carried out following the approval of the Experimental Animals Ethics Committee of the Experimental Medical Research and Implementation Center (project No: 161210004, date: 12/2016). For experimental purposes, 21, 14-day-old male Wistar albino rats were used, and the rats used in the study were obtained from Necmettin Erbakan University KONÜDAM Experimental Medicine Application and Research Center.

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Conflict of Interest

The authors declare no conflict of interest.

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