Protective effect of the immunomodulator AS101 against cyclophosphamide-induced testicular damage in mice

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BACKGROUND: Cyclophosphamide (Cy), a widely used anticancer drug, is associated with significant testicular damage and sterility. Co-administration of the immunomodulating compound AS101 during chemotherapy treatments was previously shown to protect organs against cytotoxic damage, without attenuating the drug’s anticancer effect. In this animal study, we investigated the effect of AS101 on testicular damage, sperm DNA damage and infertility induced by Cy. Akt and glycogen synthase kinase-3β (GSK-3β) phosphorylation were investigated as a possible chemoprotective mechanism.

METHODS: Mature male mice, 10 in each group, were injected intraperitoneally with 200 mg/kg Cy once a week for 5 weeks, with or without concurrent treatment with 10 μg per mouse AS101 three times per week. Damage to testicular tubules and sperm production was determined, sperm chromatin damage was analyzed and fertility was gauged. Akt and GSK-3β phosphorylation were evaluated.

RESULTS: Co-treatment with AS101 during the course of Cy administration significantly reduced the percentage of damaged seminiferous tubules (76.0 ± 10.8% versus 40.3 ± 2.6%), and reduced sperm DNA fragmentation (%DFI) from 44.7 ± 1.0% to 25 ± 6.5%. Co-treatment with AS101 also partially protected against the decrease in numbers of impregnated females and litter size. AS101 increased Akt and GSK-3β phosphorylation.

CONCLUSIONS: Our results indicate that AS101 can significantly protect against Cy-induced testicular damage and sperm DNA damage, probably by acting through Akt/GSK-3β phosphorylation.

Key words: AS101 / testicular damage / sperm DNA damage / chemotherapy / fertility preservation

Introduction

For young male cancer patients, the success of treatment with regimens that are toxic to the gonads has made infertility an important consideration. Once the cancer is controlled, quality of life, which often includes the ability to have a normal child, then becomes a major concern (Meirow and Schenker, 1995; Schover, 2005). Alkylating agents are commonly used in the treatment of cancer. Cyclophosphamide (Cy) is extensively used as an anticancer drug as well as an immunosuppressive agent. However, Cy has significant detrimental effects on male reproduction. Cy exposure causes oligospermia and azoospermia in both animal models (Elangovan et al., 2006) and in humans (Garolla et al., 2006). Cy can cause a significant damage during a key point of sperm chromatin remodeling, thereby affecting the chromatin structure. In animals, this often results in embryonic loss, which can occur pre- or post-implantation (Arnon et al., 2001; Codrington et al., 2004; Elangovan et al., 2006).

The non-toxic immunomodulator AS101, developed and characterized by our group (Sredni et al., 1987), is a white crystalline, synthetic organic tellurium compound. The empirical formula is C₈H₇O₂NCl₃Te with a molecular weight of ~312. AS101 was shown to protect mice from hematopoietic damage caused by lethal and sublethal doses of chemotherapeutic drugs, including Cy, and to increase the survival of mice treated with various cytotoxic drugs or radiation (Kalechman et al., 1991; Kalechman et al., 1993a, b, 1995a; Sredni et al., 1996a, 2004b). Clinical trials in cancer patients treated with AS101 in...
combination with chemotherapy showed that treatment with AS101 resulted in a significant reduction in the severity of neutropenia, thrombocytopenia and alopecia that accompany chemotherapy (Kalechman et al., 1995b; Sredni et al., 1995, 1996b, 2004b).

However, treatment with AS101 did not negatively affect the efficacy of chemotherapeutic treatments. Moreover, AS101 co-treatment was found to have a synergistic effect on Cy in the treatment of tumor-bearing mice and increased their survival (Kalechman et al., 1991). In addition, AS101 itself exhibits a clear anti-tumoral effect in several tumor models (Kalechman et al., 2000; Sredni et al., 2004a; Hayun et al., 2006).

Most of the activities of AS101 have been attributed primarily to the direct inhibition of the anti-inflammatory cytokine interleukin-10 (IL-10). The AS101-induced down-regulation of IL-10 has been shown to induce the up-regulation of glial cell-derived neurotrophic factor (GDNF), which induced the activation of Akt (Kalechman et al., 2003; Sredni et al., 2007). The activation of the Akt pathway has been shown to play a significant role in AS101-induced protection in these models. In the tests, Akt activation can induce radio- and chemo-protection by enhancing spermatogonial stem cell survival and increasing stem cell self-renewal (Rasoulpour et al., 2006). Glycogen synthase kinase-3β (GSK-3β) is a ser-thr kinase that is phosphorylated and inhibited by Akt. GSK-3β regulates cell metabolism, cell cycle and cell fate through the phosphorylation of a diverse array of substrates (Guo et al., 2003) and is potentially very important for conferring chemo-resistance.

The objective of this animal experiment was to investigate whether AS101 has a protective effect on male fertility against the damage induced by acute administration of Cy. As Akt activation has previously been shown to play a critical role in the mechanism of protection conferred by AS101, an additional objective was to investigate whether the Akt/GSK-3β signaling pathway is involved in the AS101-induced chemo-protection.

Materials and Methods

Reagents, animals and experimental design

Cy (CAS no. 6055-19-2; Sigma, St Louis, MO, USA) was dissolved in phosphate buffered saline (PBS) to a concentration of 25 mg/ml. AS101 was dissolved in PBS and stored at 4°C until use. Mice were housed in specific pathogen free conditions. Ethical approval for animal experimentation was received from the Institutional Ethics Committee. To assess male fertility, each mouse was placed with two young healthy females. Only males that fertilized at least one female and had proven fertility were chosen for the experiment. A group of 40 inbred Balb/c male mice, aged 5–6 weeks, were used for this study.

Twenty mice were injected intraperitoneally (i.p.) with 200 mg/kg of Cy once a week for a period of 5 weeks (Cy group). This dose has previously been shown to have a devastating effect on male fertility and corresponds to the therapeutic dose of humans (Elvangano et al., 2006).

Ten of these mice were co-treated with i.p. injections of AS101 at a dosage of 10 µg per mouse every other day, starting 1 week before the first Cy injection and continuing during the 5 weeks of Cy treatment (Cy + AS101 group). An additional group of 20 mice were injected with PBS and did not receive Cy: 10 male mice received PBS only and 10 were treated with AS101, as previously described. The animals from all groups were sacrificed by cervical dislocation 5 weeks after the last injection, and body weights were determined, whereupon the epididymides and testes were quickly dissected out.

For determination of Akt and GSK-3β phosphorylation, five mice were injected i.p. with 200 mg/kg of Cy once (Cy group). Five mice were injected i.p. with 200 mg/kg of Cy and pre-treated with i.p. injections of AS101 every other day 1 week before the Cy injection, for a total of four injections, as previously described (Cy + AS101 group). An additional group of five mice were injected with PBS vehicle and another group of five male mice were treated with AS101 as described above. The animals from all groups were sacrificed by cervical dislocation 24 h after the last Cy injection; the testes were quickly dissected out, snap-frozen in liquid nitrogen and stored at −80°C until use.

Histological evaluation of testicular damage

The removed testes were pierced with a needle and fixed in Bouin’s fixative. After 24 h, testes were washed three times and maintained in 70% ethanol. Samples were then embedded in paraffin and sectioned. Random tissue sections (5 µm) at different areas of the testes were then stained using hematoxylin/eosin and examined under a light microscope (AX70, Olympus, Tokyo, Japan) under ×100 magnification. A total of 200 cross-sections of seminiferous tubules from each sample were examined for evidence of damage, as indicated by empty and atrophic tubules.

Sperm extraction and concentration

In order to recover the motile epididymal spermatozoa, the two cauda epididymides were placed in M2 medium (Sigma Chemical Company, St Louis, MO, USA), minced with fine scissors and incubated at 37°C (95% air and 5% CO2) for 15 min prior to careful removal of all the medium. Samples were then diluted to a final volume of 1 ml in M2. The concentration of sperm cells in the medium was then evaluated using a hemocytometer, and the samples frozen at −80°C.

Flow cytometry sperm chromatin structure assay

To measure the Cy-induced DNA damage to sperm, we assayed the susceptibility of sperm nuclear DNA to low pH-induced denaturation in situ (sperm chromatin structure assay (SCSA)) as previously described (Evenson et al., 2002). Briefly, the frozen samples (n = 5) were thawed and diluted to a concentration of 1–2 × 108 sperm/ml with TNE buffer solution (0.15 M NaCl, 0.01 M Tris–HCl and 1 mM disodium EDTA, pH 7.4). From the diluted samples, 200 µl was then placed in a Falcon tube (BD Immunocytometry Systems, San Jose, CA, USA) and mixed with 400 µl low-pH detergent solution (0.1% Triton X-100, 0.15 M NaCl and 0.08 N HCl, pH 1.2). After 30 s, the cells were stained with 1.2 ml of acridine orange (AO) staining solution containing 6 µg/ml of AO in staining buffer (0.1 M citric acid, 0.2 M Na2HPO4, 1 mM EDTA and 0.15 M NaCl, pH 6.0). Samples were analyzed using a FACScan flow cytometer (BD Immunocytometry Systems, San Jose, CA, USA). A total of 1 × 106 spermatozoa from each sample were collected and analyzed at a flow rate of 100–200 cells/s. Green fluorescence emission of AO was measured at 515–530 nm with a band pass filter, and red fluorescence of AO was detected through a 630–650 nm long-pass filter. The resulting fluorescence was quantified by the flowjo software (TreeStar, Oregon, USA). The extent of DNA denaturation of each cell was quantified by the calculated parameter DNA fragmentation index (DFI), using the following formula: red fluorescence/(red + green fluorescence). The percentage of cells with abnormal chromatin structure was defined as the percent of cells with high DFI values (%DFI) (Evenson et al., 2002).

Study of male fertility

To investigate the effects of AS101 on the deterioration of reproductive performance after Cy administration, preliminary fertility assays were
peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG incubated for 1 h at room temperature with a secondary antibody washed three times for 10 min each time in TBS-T, the membranes were with the appropriate diluted primary antibody overnight at 4°C. After being washed three times for 10 min each time in TBS-T. The primary rabbit monoclonal antibodies used were raised against Ponceau S (0.005% in 1% acetic acid) to confirm equal amounts of protein, and blocked with 5% non-fat dry milk in TBS-0.1% Tween (TBS-T) for 1 h at room temperature, washed three times for 10 min each time in TBS-T. The primary rabbit monoclonal antibodies used were raised against the following antigens: anti-human phospho-Akt1/2/3 (Ser473, sc-7985-R; Santa Cruz, Santa Cruz, CA, USA) (diluted 1:400), anti-human GSK-3β and phospho-GSK-3β (Ser9, #9315 and #9336, respectively; Cell Signaling, Danvers, MA, USA) (diluted 1:300). To confirm that the protein load was similar in all lanes, we used an antibody raised in mouse against the housekeeping protein α-tubulin (Sinuani et al., 2006) from chicken (T6199, Sigma, diluted 1:500). Antibodies against phospho-proteins were diluted in TBS-T containing 5% bovine serum albumin, whereas all other antibodies were diluted in TBS-T containing 1% skim milk. Membranes were incubated with the appropriate diluted primary antibody overnight at 4°C. After being washed three times for 10 min each time in TBS-T, the membranes were incubated for 1 h at room temperature with a secondary antibody peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG (#111-033-003 or #115-035-003, respectively, diluted 1:5000; Jackson Laboratories, Bar Harbor, ME, USA), depending on the type of primary antibody used. After washing, the membranes were analyzed by the enhanced chemiluminescence system according to the manufacturer’s protocol (Pierce, Waltham, MA, USA). Densitometry was conducted to quantify differences in band intensity using the Image J (NIH, Bethesda, MD, USA) image-processing program. Results were normalized by comparison to the values in the PBS control group in each membrane.

Statistical analysis
Student’s t-test with Bonferroni correction was performed to assess the differences between groups. In order to adjust for the dependent study design of the mating experiments, a generalized logistic model, which takes the analysis of the cluster structure of the experiment into consideration, was performed (Rao and Scott, 1992). Differences in litter size were assessed with the Kruskal–Wallis ANOVA test. Statistical significance was determined at \( P < 0.05 \).

Results
Effect of treatments on reproductive organ weight and sperm count
The mean body weight of animals treated with Cy was significantly lower than that of the control animals and that of the Cy + AS101 groups (Table I). The mice, however, remained vital; no deaths occurred during the course of the experiment. The absolute and relative average testicular and epididymal weights in the Cy + AS101 group were significantly higher (\( P < 0.05 \)) than those of the Cy only group, but lower than the control group (\( P < 0.05 \)). The concentration of sperm recovered from mice epididymis was significantly lower in the Cy-treated group than in the controls. The Cy + AS101 group had a higher concentration of sperm cells than did the Cy group, but it was lower than in the control group (Table I).

Effect of treatments on histological evaluation
The cross-sections of Cy-treated mice were characterized by empty and atrophic seminiferous tubules, compared with the normal cellular content of the controls (Fig. 1), which was also reflected in the decrease in their testicular weights (Table I). In the Cy + AS101 group, the damage to the seminiferous tubules was considerably less severe, with many tubules showing undamaged spermatogenesis.

| Table I | Effect of 5 weekly injections of 200 mg/kg cyclophosphamide, with or without concurrent AS101 treatment, on body, testis and epididymal weight, and sperm concentration of fertile mice |
|-----------------|-----------------|-----------------|-----------------|
| **PBS (control)** | **AS101** | **Cy** | **Cy + AS101** |
| Body weights (g) | 26.0 ± 0.6 | 26.2 ± 1.0 | 22.8 ± 2.1* | 25.6 ± 1.1** |
| Testes weight (mg) | 107.2 ± 12.7 | 105.7 ± 14.7 | 46.3 ± 8.7* | 31.5 ± 2.0** |
| Testes weight (mg/g body wt) | 4.15 ± 0.5 | 4.3 ± 0.3 | 1.98 ± 0.16* | 2.9 ± 0.3** |
| Epididymides weight (mg) | 39.1 ± 4.8 | 34.2 ± 4.2 | 25.1 ± 2.9* | 1.0 ± 0.1** |
| Epididymides weight (mg/g body wt) | 1.5 ± 0.2 | 1.3 ± 0.1 | 1.1 ± 0.1* | 1.2 ± 0.1** |
| Sperm concentration (10^6 cells/ml/two epi.) | 22.1 ± 5.4 | 22.5 ± 3.8 | 8.7 ± 1.6* | 15.6 ± 2.9** |

PBS, phosphate buffered saline; Cy, cyclophosphamide.

*\( P < 0.05 \) compared with the PBS and AS101 control groups.

**\( P < 0.05 \) compared with the Cy group.
The average percent of damaged seminiferous tubules in the control group was 2.5 \pm 1.7\%\). The average percent of damaged seminiferous tubules in the Cy-treated group was significantly higher, 76.0 \pm 10.8\%\). In contrast, histological sections of seminiferous tubules in the testis from Cy + AS101-treated animals showed significantly fewer damaged tubules, 40.3 \pm 2.6\%\) (Fig. 2). Administration of only AS101 had no effect on testicular histology.

**Effect of treatments on DNA fragmentation**

The administration of Cy caused a significant increase in the percent of sperm cells with abnormal chromatin structure (%DFI), from 6.5 \pm 2.3\% in the control group to 44.7 \pm 1.0\% in the Cy-treated group (Fig. 2). In the Cy + AS101 group, the %DFI value was 25.4 \pm 6.5\% (Fig. 3). This percentage is significantly higher than in the PBS-treated group, but significantly lower than that observed in the Cy only group.

**Figure 1** The effect of AS101 co-treatment on the testicular damage induced by 5 weekly injections of 200 mg/kg cyclophosphamide (Cy) (n = 5). Representative histological sections of testis from mice injected with (A) phosphate buffered saline, (B) AS101, (C) Cy or (D) Cy + AS101. The cross-sections of Cy-treated mice were characterized by empty and atrophic seminiferous tubules, compared with the normal cellular content of the controls. In the Cy + AS101 group, the damage to the seminiferous tubules was considerably less severe, with many tubules showing undamaged spermatogenesis.

**Figure 2** The average percent of damaged tubules in the testes of mice injected with phosphate buffered saline (PBS), cyclophosphamide (Cy), or Cy + AS101. Cy induced a significant increase in the percent of damaged seminiferous tubules. Significant protection was conferred by AS101 against the Cy-induced testicular damage to the tubules. *P < 0.05 compared with the PBS control group. **P < 0.05 compared with the Cy-treated group.

**Figure 3** The effect of AS101 co-treatment on the percentage of sperm with abnormal chromatin structure (%DFI) (n = 5). The extent of DNA denaturation of each cell was quantified by the calculated parameter DNA fragmentation index (DFI). The percentage of cells with abnormal chromatin structure was defined as the percentage of cells with high DFI values (%DFI) (Evenson et al., 2002). Significant protection was conferred by AS101 against the cyclophosphamide-induced increase in the percentage of sperm with abnormal chromatin (%DFI). *P < 0.05 compared with the phosphate buffered saline control group. **P < 0.05 compared with the Cy-treated group.
25.6% of the PBS group (level of phosphorylated Akt (pAkt) to 175.3 ± 28.4% relative to the PBS group (P < 0.05). Cy decreased the level of pAkt to 55.3 ± 25.6% of the PBS group (P < 0.05). In the Cy + AS101 group, the level of pAkt was 164.3 ± 5.7% of the PBS group, which was significantly higher than in the Cy group but smaller than in the control group (P < 0.05). No significant change was found in the level of α-tubulin, which served as control (Fig. 5).

AS101 also caused a significant increase in the level of phosphorylated GSK-3β (pGSK-3β) to 130.3 ± 16.9% of the PBS group (P < 0.05). Cy decreased the level of pGSK-3β to 36.4 ± 12.5% of the PBS group (P < 0.05). In the Cy + AS101 group, the level of pGSK-3β was 114.5 ± 9.2% of the PBS group, which was significantly higher than in the PBS and Cy groups (P < 0.05). No significant changes were found in levels of unphosphorylated GSK-3β and α-tubulin, which served as controls (Fig. 6).

**Effect of treatments on male fertility**

The administration of Cy resulted in a significant reduction in the percent of impregnated females (20.0 ± 4.2%), compared with 93.3 ± 6.1% of impregnated females in the control group. In the Cy + AS101 group, the percent of impregnated females was 80.0 ± 8.2%. The litter size of the females who did achieve pregnancy was reduced in the Cy when compared with the control group, from 9.9 ± 1.0 offspring/female to 4.3 ± 1.4, respectively. In the group injected with Cy + AS101, the litter size was 8.3 ± 0.6, which was significantly higher than in the Cy group but smaller than in the control group (P < 0.05, Fig. 4).

**The effect of treatments on Akt and GSK-3β phosphorylation**

Administration of AS101 induced a significant increase in the average level of phosphorylated Akt (pAkt) to 175.3 ± 28.4% relative to the PBS group (P < 0.05). Cy decreased the level of pAkt to 55.3 ± 25.6% of the PBS group (P < 0.05). In the Cy + AS101 group, the level of pAkt was 164.3 ± 5.7% of the PBS group, which was significantly higher than in the PBS and Cy groups (P < 0.05). No significant change was found in the level of α-tubulin, which served as control (Fig. 5).

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**Discussion**

The results of this study indicate that co-treatment with AS101 can significantly protect against Cy-induced reproductive damage. In the group co-treated with AS101, we found less reduction in absolute and relative testicular and epididymal weight and significantly fewer damaged seminiferous tubules. Although the body weights of Cy + AS101-treated animals were significantly higher than those of the Cy only group, the protection against the decrease in relative testicular weights indicated that AS101 protected specifically against the damaging effects of Cy in the testis, and did not only affect general well-being. AS101 also protected against the decrease in the amount of sperm cells recovered from the epididymides. Furthermore, the results of the mating experiment indicated that AS101 prevented the decrease in the percent of impregnated females, and the females who did become pregnant produced larger litters than did those exposed to Cy alone.

Our results indicated that AS101 significantly protects against the increase in sperm cells with abnormal chromatin structure, induced by Cy. The protective effect of AS101 against the adverse effect of Cy on the number of impregnated animals and on the litter size could be attributed to the protection against the significant increase in sperm chromatin damage, which has been shown to reduce fertilization rates and cause post-implantation embryo loss in animals (Codrington et al., 2004, 2007; Elangovan et al., 2006). The SCSA method has defined 27–30% DFI as the point at which men are infertile (Evenson and Wixon, 2006). In the Cy + AS101-treated group, the average %DFI values are below this threshold.

The mechanism of the prevention of sperm DNA damage, however, is unclear. It is possible that by preventing the damage to the process of spermatogenesis AS101 also prevented the observed damage in sperm chromatin compaction. It is also possible that the observed activation of the Akt/GSK-3β pathway may be responsible for the improved chromatin structure, since it is known to play a crucial role in DNA repair (Kao et al., 2007).

The protective capability of AS101 on sperm chromatin structure is very important for patients undergoing anti-cancer treatments. Until now, no increase in malformations among children naturally conceived to parents who have previously undergone chemotherapy has been reported (Tempest et al., 2008). A selection against the abnormal sperm cells during the natural fertilization process may explain the contradiction (Arnon et al., 2001). However, the use of assisted reproductive technologies such as intracytoplasmic sperm injection (ICSI) might increase the risk of fertilization by abnormal sperm cells, since no natural sperm cell selection occurs (Arnon et al., 2001).
**Figure 5** The effect of treatments on phosphorylated Akt (pAkt) levels (n = 5). (A) AS101 significantly increased pAkt levels, whereas cyclophosphamide (Cy) significantly decreased pAkt levels, compared with the phosphate buffered saline (PBS) controls. Co-treatment with AS101 prevented the Cy-induced decrease in pAkt levels, which remained higher than in the controls. No significant change was found in α-tubulin levels, which served as controls. (B) A sample of a western blot analysis showing the results obtained from testicular lysates of two mice from each group. *P < 0.05 compared with the PBS control group. **P < 0.05 compared with the Cy-treated group.

**Figure 6** The effect of treatments on phosphorylated glycogen synthase kinase-3β (pGSK-3β) levels (n = 5). (A) AS101 significantly increased pGSK-3β levels, whereas cyclophosphamide (Cy) significantly decreased pGSK-3β levels, compared with the phosphate buffered saline (PBS) controls. Co-treatment with AS101 prevented the Cy-induced decrease in pGSK-3β levels, which were similar to the PBS control. No significant change was found in levels of unphosphorylated GSK-3β and α-tubulin, which served as controls. (B) A sample of a western blot analysis showing the results obtained from testicular lysates of two mice from each group. *P < 0.05 compared with the PBS control group. **P < 0.05 compared with the Cy-treated group.
2001; Revel et al., 2005). The ability of AS101 to protect against DNA damage in these cases, therefore, is particularly important.

In spite of numerous studies reporting infertility in cancer patients, we still do not fully understand the exact mechanisms of chemotherapy-induced fertility impairment (Agarwal and Allamaneni, 2005). Previous studies have shown that Akt activation can induce radio- and chemo-protection by enhancing spermatogenic stem cell survival and increasing stem cell self-renewal (Rasoulpour et al., 2006). GSK-3β regulates cell metabolism, cell cycle and cell fate through the phosphorylation of a diverse array of substrates. Akt inhibits GSK-3β activity by phosphorylation at Ser9. Guo et al. (2003) have shown evidence suggesting that GSK-3β has a critical role in mammalian meiosis and spermatogenesis, and its inhibition results in down-regulation of meiotic DNA synthesis.

In this study, we found that AS101 can increase Akt and phosphorylation of GSK-3β in the testis. In previous studies, we have shown that this effect is partly responsible for AS101-induced protection of neuronal cells (Kalechman et al., 2003; Okun et al., 2007; Sredni et al., 2007). It is most likely, therefore, this effect also partly responsible for the chemo-protective effect of AS101 on the testis.

The mechanism of AS101-induced Akt activation, however, is unclear.

In the testis, Akt phosphorylation can be induced by GDNF, which activates the PI3K/Akt pathway (Lee et al., 2007). AS101 was shown to increase the level of GDNF in various models (Kalechman et al., 2003; Okun et al., 2007; Sredni et al., 2007). We suggest, therefore, that the activation of the PI3K/Akt pathway may be the result of an AS101-induced increase in GDNF levels. In addition, we have previously demonstrated a direct mechanism of Akt phosphorylation by AS101 in vitro (Makarovsky et al., 2003). The direct route of Akt activation, therefore, cannot be ruled out.

Since phosphorylated Akt and GSK-3β levels in the testes were evaluated from frozen—thawed homogenized tissue, it was not possible to determine whether the differences observed were only due to drug effects on cell signaling or also due to changes in cell populations and different proportions of cell types in post-chemotherapy treatments. However, injection of AS101 alone, which according with histological examination did not change cell composition, induced an increase in phosphorylated Akt and GSK-3β levels. These results suggest that the changes observed in phosphorylated Akt and GSK-3β levels are due to effects of AS101 on the cells and not due to changes in cell populations. Localization of these alterations by immunohistochemical studies should be further investigated.

An additional possible mechanism of AS101 chemoprotection is the ability to directly inhibit the enzymatic activity of caspases, such as interleukin-1β converting enzyme and caspase-3. This inhibition results in a significant reduction in the levels of the active form of IL-1β and IL-18 in lipopolysaccharides-treated mice, and therefore may result in an anti-inflammatory and anti-apoptotic effect (Brodsky et al., 2007). Additional research is needed in order to evaluate the role of these effects in AS101-induced chemoprotection of the testis.

To prevent infertility in adult patients, semen cryopreservation is offered before the initiation of treatment, but in many cases, the quality of the sperm cells obtained is poor (Howell and Shalet, 2001; Revel et al., 2005). In order to use cryopreserved thawed semen in these cases, assisted reproduction and ICSI technique are often required to achieve pregnancies. In cured males with severely impaired semen parameters, assisted reproduction and ICSI provide a means of overcoming infertility. However, these methods are expensive, have substantial drawbacks and limitations, and raise concerns about genetic safety issues using sperm that was exposed to chemotherapy (Arnon et al., 2001; Howell and Shalet, 2001; Revel et al., 2005). Therefore, other approaches that prevent damage and protect male fertility are expected to be of great benefit to many patients.

The results presented in this study suggest that AS101 could be used to provide significant protection against chemotherapy-induced infertility and sperm DNA damage. AS101 could offer an alternative method of fertility preservation, with no significant side effects, and without negatively affecting the efficacy of anti-cancer treatment. This will significantly improve the patient’s quality of life. Further research needs to be conducted in order to study the protective effect of AS101 co-administration on pre- and post-implantation loss, as well as on impaired embryonic development and birth defects.

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References


Elangovan N, Chiou Tj, Tseng WF, Chu ST. Cyclophosphamide treatment causes impairment of sperm and its fertilizing ability in mice. Toxicology 2006;222:60–70.


