Preparation of SSCs recipients in sheep; effects of anticancer drugs

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Abstract

This in vitro investigation aimed to assess the impact of Taxol, Carboplatin, Vinblastine (VBL), and Vincristine (VCR) on ovine spermatogonial stem cells. Spermatogonial stem cells can be extracted from the testes of an animal and cultured in a laboratory to increase their quantity for transplantation into a recipient. Upon transplantation, these cells maintain their population and initiate sperm production in the recipient animal. Adequate preparation of the recipient is a crucial aspect of spermatogonial stem cell transplantation. Drawing upon the findings of in vitro investigations conducted on Spermatogonia Stem Cells, a tripartite regimen comprising Carboplatin, Taxol, and VCR was administered to four-month-old male Shal lambs at varying concentrations. The in vivo experiments included the culture of Spermatogonia Stem Cells, gfrα1 and c-myc specific gene expression analysis, and histological analysis. The results demonstrated that injection of the combination as mentioned above (at concentrations of 2.0632, 0.906, and 0.0072 mg, respectively) beneath the scrotums of each testicle of Shal lambs resulted in a significant reduction in the spermatogonial stem cells population. The findings suggest that a tripartite regimen consisting of Carboplatin, Taxol, and VCR may be suitable for preparing sheep recipients for spermatogonial stem cells, and based on the results of this study, the use of a combination of carboplatin, Taxol, and Vincristine is recommended for the preparation of SSC transplant receptors in sheep. Injection of a combination of carboplatin, Taxol, and Vincristine equivalent to twice the concentration recommended for cancer treatment by the drug manufacturer under the Scrotum of each of the testicles of Shal lambs, significantly reduces the cell population. The expression of gfrα1 and c-myc genes as specific markers of stem cells was reduced considerably in double and quadruple concentrations of Carboplatin + Taxol + Vincristine compared to other treatments.

Keywords: Expression, Ovine, SSCs, Transplantation, Vincristine.

1. Introduction

Spermatogonial stem cells (SSCs) exhibit a unique capacity for self-renewal within the seminiferous tubules, thereby ensuring uninterrupted sustenance of spermatogenesis throughout the lifespan of male organisms (1). In contemporary times, the transplantation of SSCs has proven to be an efficacious research tool for examining the interplay between Sertoli and germ cells in the process of spermatogenesis. Additionally, it has surfaced as a feasible approach for generating transgenic livestock (2). The ovine sector encounters difficulties in the transportation and dissemination of genetics, primarily attributed to impediments concerning cryopreservation of sperm, as well as insemination and embryo transfer procedures (3). Although homologous transplantation has demonstrated efficacy in certain animals, its effectiveness in larger animals remains uncertain in the absence of suitable recipients. Therefore, alternative methods for transporting genetics would be advantageous for the sheep industry (4). Preparation of the recipient for SSC transplantation necessitates the creation of space by eliminating the pre-existing germ cells (5). Various techniques, including irradiation, heat shock, and chemical treatment, have been employed to achieve this objective. Nevertheless, the intraperitoneal (i.p.) administration of Busulfan is the most frequently utilized approach owing to its ease of administration and accessibility (6). However, the use of Busulfan has been associated with severe systemic toxicity resulting from the destruction of hematopoietic cells and germ cells (7). As a result, alternative methods such as irradiation and heat shock have been explored. Nonetheless, these methods are limited by their requirement for specialized and expensive instruments as well as a narrow transplantation window (2).

In light of the resemblance between stem cells and cancer cells, this investigation employed herbal remedies, including Taxol, Vinblastine (VBL), and Vincristine (VCR), along with carboplatin, a platinum derivative utilized in cancer therapy, to establish recipients.

Taxol (Paclitaxel) is a chemotherapeutic agent that is extensively used to treat various types of cancers, including lung, neck, and breast cancers (8). Its mechanism of action involves binding specifically with tubulin β-subunits heterodimer, which promotes tubulin polymerization through its poly-oxygenated cyclic di-terpenoid with a characteristic taxane ring system (9). This effect is particularly pronounced during the G2/M phase of the cell cycle, where Taxol can halt cancer cell proliferation and trigger apoptosis (10).

The Vince alkaloids, procured from the Madagascar periwinkle plant, have demonstrated efficacy against a diverse range of cancer cell types. VCR and VBL were the pioneering agents in this class of chemotherapeutics. Their mechanism of action entails binding to a distinct site on the tubulin protein and impeding cellular division. This impediment is attributed to the hindrance of the protein's ability to form requisite structures within the cell (11).

Carboplatin is a platinum-based chemotherapeutic agent that shares a mechanistic similarity with Cisplatin, albeit with distinct structural and toxicological features. It represents the second generation of platinum-based drugs following Cisplatin. Both agents exert their antineoplastic effects by inducing DNA damage, which leads to the inhibition of DNA replication, transcription, and cellular division (12).

This study initially examined the effects of Taxol, carboplatin, VBL, and VCR on ovine SSCs. Subsequently, a combination of carboplatin, Taxol, and VCR was administered to four-month-old male Shal lambs at three concentrations based on the results of in vitro experiments.

2. Material and Methods

2.1. Chemicals

The procurement of chemicals was executed through Sigma (St. Louis, MO, USA), while the acquisition of plastics was conducted at Corning (USA).

2.2. Experiment

The procurement of the 4-month Shal male lambs was carried out from Rasouli Farm, located in Tehran, Iran. Furthermore, it is important to note that the protocol for animal usage in this study had received approval.

The present study is centered on an in vitro experiment that was conducted to assess the impact of various drug treatments. The drugs employed in the experiment included Taxol, Carboplatin, VBL, and VCR, which were tested at different concentrations ranging from 7.5 to 240 µg/mL using the MTT test. Furthermore, the experiment investigated the combined effect of Taxol and Carboplatin, both individually and in combination with either VBL or VCR. Based on the results of the in vitro study, a combination of Carboplatin, Taxol, and VCR was administered to four-month-old male Shal lambs. Three different concentrations of the drug combination were used to treat 12 lambs, with each treatment being replicated three times. The effective concentrations (T1: 1.0316, 0.453, and 0.0036 mg of Carboplatin, Taxol, and VCR, respectively) were determined in accordance with the manufacturer's guidelines. In addition to administering the effective concentrations, double (T2) and quadruple (T3) concentrations were also administered to the left testis of the lambs included in the study. Accordingly, as pointed out by Lin et al. on both sides of the left testis, the studied treatments were performed by injection under the scrotum once a week being repeated for four weeks (4). The control group included 0 mg/kg (T0), and there was no difference regarding the injection volume between the experimental and control groups. The collection of the lambs' testes was performed four weeks after the treatment. Subsequently, the testes were transported to the laboratory for histological examination, isolation, and SSC culture.

2.3. Histology

Small sections of testicular tissue were preserved in modified Davidson's fluid overnight at a temperature of 4 ^{oC}. The specimens were first cleaned with 70% ethanol and then coated with paraffin wax. Afterward, they were sliced into thin 5 mm segments using typical procedures. These sections were then treated with xylene and ethanol. In the final phase, hematoxylin and eosin were used for staining the sections in order to do histological analysis.

2.4. Isolating and culturing SSCs

Four weeks post-Busulfan treatment, the researchers obtained the left testis from the male sheep. To isolate SSCs, a two-step enzymatic digestion process was employed, following a modified version of the method described by Rasouli et al (13). In the initial enzymatic digestion step, the tunica albuginea was removed. Subsequently, approximately 50g of tissue was finely cut into small fragments using fine scissors. These minced seminiferous tissue fragments were then suspended in Dulbecco's Modified Eagle Medium (DMEM) obtained from Inoclon, Iran. The tissue was incubated in a shaker incubator at 37$^{°C}$ for 45 min, resulting in separation. The supernatant above the tissue was collected, and the remaining solid material was washed with DMEM. To undergo a second round of enzymatic digestion, the solid material was combined with DMEM containing hyaluronidase type II, collagenase, and DNase. This mixture was also incubated in a shaker incubator at 200 cycles/min for 30 min. The suspension was then centrifuged at 1000 rpm for 2 min.

2.5. Enriching SSCs

To increase the number of SSCs, the liquid above the original sample was passed through two filters: first an 80 µm filter and then a 60 µm filter made of nylon net. The cells that passed through the filters were then placed in 60 mm Petri dishes coated with a mixture of lectin and bovine serum albumin (BSA), using a technique previously explained by Jafarnejad et al (14). To make the lectin-BSA coated dishes, the Datura stramonium agglutinin lectin was mixed with DPBS at a concentration of 5 µg/mL. The mixture was left at room temperature for 2 h, and then the dishes were washed with DPBS containing 0.6% BSA. After that, the dishes were coated with BSA and left at room temperature for another 2 h.

The cells were put onto dishes that had been covered with lectin and left to sit for roughly 5-6 h at 37°C in a CO2 incubator with 5% CO2 in the air. This procedure helped to ensure that any extraneous cells would attach to the lectin-BSA. Following the incubation period, the liquid containing the stem cells was transferred to a 15 mL tube and spun at 1000 rpm for 5 min. The liquid above the cells was then disposed of, and the solid material was mixed with DMEM.

2.6. Feeder layers preparation and culturing SSC

Fresh DMEM mixed with 10% FBS (Gibco, USA) was used for the preparation of feeder layers and SSC culture to revive the remaining cells in lectin-coated dishes. The cells were then placed in a CO_2 incubator with 5% CO_2 in air at 37°C for 2-3 days. The purpose of incubation was to encourage the growth of these cells, which were mainly Sertoli cells until they formed a confluent monolayer. To grow the cells, they were moved to a 50 mL cell culture flask after being treated with 0.25% trypsin-EDTA. A layer of feeder cells was created by deactivating Sertoli cells with 10 µg/mL of mitomycin-C for three h. The cells were then rinsed five times with DPBS and finally washed with DMEM containing 10% FBS. The isolated SSCs were grown in a culture flask containing DMEM medium with 10% FBS, on top of a Sertoli cell feeder layer. The flask was then placed in a CO2 incubator with a 5% CO2 and 37°C temperature. After 10 days, the primary culture hosted SSC colonies.

2.7. Characterizing and analyzing SSCs

The researchers employed alkaline phosphatase staining and measured the presence of *gfra1* and *c-myc* genes to characterize SSCs. To carry out the alkaline phosphatase staining, two-time washing of the SSC colonies with DPBS was carried out, and their staining was done with a kit from Sigma (Catalogue No.86C) following the provided guidelines. The colonies were then counted using an inverted microscope.

2.8. The reduction assay of MTT (Methylthiazolyldiphenyl-tetrazolium bromide)

To create the feeder layer, 96-well dishes were coated with Sertoli cells treated with mytomicin. Then, SSCs were added to the layer after one day. The cells were then cultured for 48 h in 96-well dishes with

different concentrations of Taxol, Carboplatin, VBL and VCR (7.5 to 240 µg/mL of each drug) with 5000 cells per well. To assess the cells' viability, a Thermo Fisher Scientific kit was utilized following the manufacturer's guidelines.

In the beginning, the combination of 5 mg of MTT and 1 mL of sterile PBS was done to produce a stock solution that contains 12 mM MTT. Then, each well received the addition of 10 µL of the stock solution. Furthermore, 100 µL of medium received a negative control. The incubation of wells, which was conducted with the presence of 5% CO2 at 37°C, took place for 4 h. In the next phase, each well was provided with 100 µL of SDS-HCl solution. The small plate was kept in a damp chamber at 37°C overnight. The concentration of formazan was measured using optical density at 570 nm. To eliminate the influence of Sertoli cells, 96-well dishes without SSCs were coated for each treatment. The cell viability's toxic effects were expressed as a percentage of the control's cell viability. The viability of untreated cells is considered 100% in all studies related to cell toxicity. The program ratio (www.aatbio.com) was used to estimate IC50 values via a nonlinear regression method.

2.9. Isolation of RNA, reverse transcription and real time PCR

The process of isolating total RNA from a sample has been a significant procedure. Trizol reagent was used for the isolation, and DNase was then used to prevent DNA contamination. The RNA concentration was measured by detecting its absorbance at 260 nm. To create the first-strand complementary DNA, 0.5 mg of the total RNA was utilized, along with MMLV enzyme and oligo dT primers for reverse transcription. The researchers employed real-time PCR to investigate the expression of certain markers. The PCR was formed with a final volume of 10µL. To begin the real-time PCR process, the polymerase was activated by exposing it to a temperature of 94°C for 15 min. This was followed by 40 amplification cycles, each consisting of denaturation at 95°C for 10 s, annealing of specific primers at 60°C for 15 s, and extension at 72°C for 20 s. The process was concluded with a final extension at 72°C for 5 min.

Sequences of custom primers were employed for analyzing gene expression through real-time PCR as follows:

β *actin* [5' ACCCAGCACGATGAAGATCA 3' (forward) and 5' GTAACGCAGCTAACAGTCCG 3' (reverse)]; *gfra1* [5' CCACCAGCATGTCCAATGAC 3' (forward) and 5' GAGCATCCCATAGCTGTGCTT 3' (reverse) *c-myc* [5' AGAATGACAAGAGGCGGACA 3' (forward) and 5' CAACTGTTCTCGCCTCTTC 3' (reverse)].

The comparative threshold cycle (ΔΔCT) method was the analytical approach for analyzing the data, and for an endogenous control, β actin was adopted.

2.10. Statistical Analysis

The data collected for the study were analyzed using SPSS 16, a statistical software program developed by IBM in the USA. Duncan's multiple-range test and one-way ANOVA were the inferential measures to compare multiple numeric datasets.

3. Results

In this study, the effect of different concentrations of Taxol, Carboplatin, VBL and VCR separately and in combination on the survival of SSCs in vitro was studied. Based on these results the most effective combination of the studied drugs was evaluated in an in vivo study on the survival of testicular spermatogonia in lambs.

3.1. The effects of each of the compounds of Taxol, Carboplatin, VBL and VCR on the viability of SSCs

In this experiment, the effects of toxicity and inhibitory concentration of 50% of each of the drugs Taxol, Carboplatin, VBL and VCR on SSCs were investigated. For this purpose, concentrations of 7.5 to 240 µg/mL of each drug were applied to the studied cells for 72 h (Figure 1). The results showed that the survival

of SSCs at equal concentrations and more than 30 µg/mL was significantly reduced compared to the control (P <0.05). Carboplatin at a concentration of 7.5 µg/mL increased survival and at equal concentrations and more than 30 µg/mL significantly reduced SSC viability (P <0.05). Whereas cell viability was significantly reduced by VBL and VCR at equal concentrations and more than 15 and 7.5 µg/mL, respectively.

3.2. Combination effect of Taxol with VBL or VCR on the viability of SSCs

Figure 2. illustrates the impact of the combination of Taxol with VBL and VCR on the survival of SSC. The combination of Taxol and VBL at equal concentrations, exceeding 60 µg/ mL, resulted in a significant reduction of SSC viability. Similarly, the combination of Taxol and VCR at equal concentrations, exceeding 15 µg/ mL, significantly decreased the viability of these cells (P <0.05).

3.3. Combination effects of Carboplatin with Taxol, VBL, or VCR on the viability of SSCs

Figure 3. displays the findings of treating SSCs with a mixture of Carboplatin and Taxol, VBL, or VCR. When used in equal concentrations of more than 30, 7.5, and 7.5 µg/ mL, respectively, these combinations considerably reduced the viability of stem cells (P <0.05). Carboplatin + Taxol + VBL and Carboplatin + Taxol + VCR treatments at equal concentrations and more than 30 µg / mL significantly reduced the viability of these cells (P <0.05).

3.4. The effects of Taxol, Carboplatin, VBL and VCR, both individually and in combination, on the viability of SSCs by assessing their cytotoxicity

The results of the experiment show that the combination of Carboplatin, Taxol, and VCR at a concentration of 26 µg/ mL had the highest inhibitory effect on the SSCs. The details of the results can be found in Table 1.

3.5. Effect of Carboplatin + Taxol + VCR on SSCs in vivo

Based on the results of the effects of Taxol, Carboplatin, VBL and VCR treatments on the survival of SSCs under in vitro conditions, the combination of Carboplatin + Taxol + VCR for in vivo study was selected and the effective concentration (T1: 1.0316, 0.453 and 0.0036 mg of Carboplatin, Taxol, and VCR,

respectively) was calculated based on the manufacturer's recommendation. In addition to injecting effective concentrations, double (T2) and quadruple (T3) concentrations were also studied on the left testis of the lambs under study.

3.6. Testicular weights after Carboplatin + Taxol + VCR treatment

The results of injecting three levels of Carboplatin + Taxol + VBL into the left testis of the lambs under study showed that injecting concentrations of twice (T2) and four times (T3) significantly decreased the mean testicular weight of the testes compared to other treatments and control so that the largest decrease was related to T3 treatment ($P < 0.05$) (Figure 4).

3.7. Histological analysis

The testicular histological results of the studied lambs four weeks after the application of three levels of Carboplatin + Taxol + VBL are shown in Figure 5. As the concentration of the treatments increased, the number of spermatocytes decreased. The lowest cell count was observed at the highest concentration, which was four times the baseline level (T3).

3.8. Formation of SSCs colony in vitro and their expression of specific gene

Four weeks after injection of Carboplatin + Taxol + VCR at three levels into the testes of the studied lambs, stem cells were extracted from the testes and cultured for 10 days. The results of staining the colonies with alkaline phosphatase as a specific marker of stem cells as well as the formation of bridges between the colonies are shown in Figure 6. It was found that double and quadruple concentrations of Carboplatin + Taxol + VCR led to a significant reduction of colony formation and the bridges between them ($P < 0.05$) (Figure 7). Also, the expression of *gfrα1* and *c-myc* genes as specific markers of stem cells was significantly reduced in double and quadruple concentrations of Carboplatin + Taxol + VCR compared to other treatments ($P < 0.05$) (Figure 8).

4. Discussion

Previous studies have proposed that SSCT is possible in livestock such as pigs, cattle, sheep, and goats. However, none of them have simplified the approach to make it useful in an agricultural production context. A difficulty in creating this technology lies in the preparation of appropriate recipients (15). Previously, in rodents, Busulfan, a DNA alkylating agent, was utilized to eradicate germ cells in transplant recipients before transplantation. Nonetheless, the effective dose of Busulfan varies depending on the species and strain, and the treatment may cause severe bone marrow depression, which can be fatal (16). Due to the similarity of stem and cancer cells, in this current study, herbal medicines such as Taxol, VBL, and VCR, as well as Carboplatin, which is a platinum derivative for cancer treatment, were used to prepare recipients. Results reveal that the use of a combination of Carboplatin, Taxol, and VCR is recommended for the preparation of SSCs Recipients in Sheep.

The combination of anticancer drugs with complementary mechanisms demonstrates strong synergistic effects in cancer treatment. For instance, the combination of carboplatin, taxol, and gemcitabine (17), taxol and verapamil (18), eribulin and taxol (19), and coralyn and taxol (20) inhibits the growth of cancer cells and induces apoptosis. Additionally, combinations such as etoposide with cisplatin and mitomycin C with vinblastine exhibit potent synergistic effects in breast cancer treatment (21). The combination of vincristine and SAHA (a histone deacetylase inhibitor) strongly synergizes by arresting the cell cycle and inducing apoptosis (22). Furthermore, combining taxol with flavonoids reduces drug resistance and enhances therapeutic efficacy (23). The combination of taxol and vincristine also shows strong synergistic effects in inhibiting cancer cell growth by disrupting cell division (24). However, while sequential administration of taxol and vinblastine results in strong synergy, co-administration may lead to antagonistic effects (25). These findings highlight that combining drugs with complementary mechanisms can enhance treatment efficacy and reduce drug resistance.

The results of Borovskaya et al (26), showed a significant reduction in the maturity of the seminiferous tissue and the population of SSCs. Taxol inhibits DNA, RNA, and protein synthesis and arrests cells in the G2-M phases of the cell cycle, which results in the formation of genetically abnormal aneuploid cells. The

antitumor effect of Taxol is based on its capacity to bind formed microtubules and induce their polymerization.

VCR and VBL are types of plant alkaloids that prevent the formation of microtubules (27). This leads to a halt in the mitosis process and ultimately results in the death of the cell. These alkaloids also have a minor impact on pachytene spermatocytes, while higher doses mainly affect Sertoli cells by destroying their microtubules and mitochondria (28).

Delessard et al (29) discovered that the testicular tissues of mice treated with VCR contained Sertoli cell-only tubules. It was suggested that there may be a depletion of SSC in certain tubules as a result of prepubertal exposure to VCR. Exposure to cyclophosphamide, VCR, and doxorubicin at concentrations used in humans can lead to a significant reduction in SSCs. Intraperitoneal injection of VCR was found to be more effective in eliminating the SSCs compared to intravenous injection (30).

Delessard et al. reported that pre-pubertal exposure to VCR or CYP caused sperm morphological abnormalities and DNA damage in adult mice. VCR also had a negative impact on RNA synthesis (29). Exposure to VCR may cause pachytene arrest in some seminiferous tubules by activating the pachytene checkpoint. Furthermore, they observed DNA damage in spermatocytes and spermatids, which hampers the progression of germ cells and leads to the demise of non-proliferating spermatid cells in mice (31). Al-Ahmed, found that some degenerated meiotic cells might cause damage to young spermatids' acrosomic system and cytoplasmic bridges. In mouse spermatogenesis, VCR has been observed to inhibit thymidine uridine and l-leucine (32).

The human fetal testis is negatively impacted by chemotherapeutic drugs, specifically cisplatin and Carboplatin, leading to a reduction in the quantity of germ cells, including gonocytes and pre-Spermatogonia, as well as a decrease in germ cell proliferation. The inhibition of cell division during spermatogenesis, particularly in the phases following treatment with Carboplatin, is likely attributed to the interaction between platinum-based compounds present in Carboplatin and DNA molecules. This disruption in cell division phases may have detrimental effects on the maturation of sperm cells (33).

In summary, both GFRα1 and Myc play pivotal roles in regulating stem cell behavior; GFRα1 acts as a receptor for GDNF signaling, essential for the survival and self-renewal of SSCs, while Myc controls the balance between self-renewal and differentiation, thereby modulating the dynamics and functionality of stem cells within various microenvironments (34; 35; 36). Both c-myc and gfra1 play key roles in spermatogonial stem cells (SSCs). C-myc, as a transcription factor, regulates self-renewal and cell proliferation, and its expression is upregulated by GDNF through the PI3K/Akt pathway. gfra1, as the GDNF receptor, provides essential signaling for the survival and function of these cells. In summary, cmyc is involved in proliferation, while gfra1 supports signaling and survival in SSCs (37).

In conclusion, it is imperative to explore strategies for restoring sperm production in cases of impaired SSC. One potential approach under extensive investigation involves the combination of pharmaceutical intervention and transplantation of testicular cells, aiming to augment the regenerative potential of the testes (26). The findings from in vivo experiments, including SSC culture, gene expression analysis, and histology, demonstrated that the injection of a combination of Carboplatin, Taxol, and VCR at concentrations of 2/0632 mg, 0/906 mg, and 0/0072 mg respectively, equivalent to twice the recommended concentration for cancer treatment by the drug manufacturer, resulted in a significant reduction in the cell population when administered under the scrotum of four-month-old Shal lambs. The results of the present study recommended utilizing a combination of Carboplatin, Taxol, and VCR for the preparation of SSC transplant receptors in sheep.

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Author's Contributions

Study concept and design: M. Z.

Analysis and interpretation of data: M. Z.

Drafting of the manuscript: M. Z. and A. Gh.

Critical manuscript revision for important intellectual content: M. Z. and A. Gh.

Statistical analysis: M. Z. Writing, review, and editing: A. Gh.

The Ethics

This article is based on a project approved by the Iran Research Institute of Science and Technology with the code 1011097005. No live human samples were used in this study.

Conflict of Interest

The authors declare that they have no conflict of interest.

Data Availability

The data that support the findings of this study are available on request from the corresponding author.

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٤٥٨ **Table 1.** Cytotoxicity of Carboplatin, Taxol, VCR, or VBL or against the SSCs.

Treatments	IC50
Taxol	59.92
Carboplatin	99.17
VBL	52.81
VCR	98.75
Taxol+ VCR	56.41
Taxol+ VCR	57.36
Carboplatin+Taxol	47.24
Carboplatin+ VBL	30.96
Carboplatin+ VCR	48.32
Carboplatin+Taxol+ VBL	36.68
Carboplatin+Taxol+ VCR	25.99

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