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EMBRYO BIOLOGY

The investigation of the role of sirtuin-1 on embryo implantation in oxidative stress–induced mice

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Abstract

Purpose Implantation is essential for a successful pregnancy. Despite the increasing number of studies, implantation is still an unknown process. This study aimed to determine whether sirtuin-1 has a role in embryo implantation in oxidative stress–induced mice.

Methods Pregnant mice were separated into 5 groups: control, vehicle, paraquat, SRT1720, and SRT1720+Paraquat. Paraquat is a herbicide and is used to induce oxidative stress. SRT1720 is a specific sirtuin-1 activator. Implantation and inter-implantation sites were removed in the morning of the 5th day of pregnancy after Chicago blue injection was performed. Sirtuin-1 and Forkhead box O1 (FoxO1) were detected by immunohistochemistry and Western blot while acetylated lysine was evaluated by Western blot analysis. Reactive oxygen and nitrogen species (ROS/RNS) and superoxide dismutase (SOD) activity were determined by fluorometric and spectrometric methods, respectively.

Results Although there was no embryo implantation in paraquat-treated mice, 5 out of 9 SRT1720+Paraquat-treated mice had implantation sites which were significantly higher compared to the paraquat-treated group. Sirtuin-1 and FoxO1 expressions were increased at implantation sites of SRT1720-treated mice. ROS/RNS levels were decreased, while deacetylated FoxO1 levels and SOD activity were increased in SRT1720-treated mice.

Conclusion Our findings suggest that sirtuin-1 may play a role in embryo implantation against oxidative stress through FoxO1-SOD signaling.

Keywords Implantation · Oxidative stress · Sirtuin-1 · FoxO1 · SOD

Introduction

Human fertility is an inefficient process whereby 30% of pregnancies cease before the second trimester [1]. Although assisted reproductive technology (ART) provides solutions to some fertility problems, the implantation rate has not been greatly improved; and challenges continue regarding the poor accuracy of the methods for assessing embryonic viability and endometrial receptivity. Thus, more investigations are needed to provide practical solutions to these problems [2]. The implantation of the blastocyst into the maternal uterus is a crucial step in establishing pregnancy and thus ensuring further embryonic development. Similar to many developmental processes, implantation involves an intricate succession of molecular and cellular interactions which must be executed within an optimal time frame [3]. Embryonic development to the blastocyst stage and uterine differentiation to the receptive environment are essential to the establishment of the embryo-uterine "cross-talk," which ultimately leads to the initiation and progression of successful implantation [4]. Uterine sensitivity to implantation-competent blastocysts is classically divided into three stages: pre-receptive, receptive, and refractory phases [5]. Uterine receptivity for implantation that supports blastocyst growth, attachment, and the subsequent events of implantation is time-limited [6].

In a healthy body, ROS and antioxidants remain in balance. When the balance is disrupted towards an overabundance of ROS, oxidative stress occurs [7, 8]. Some of the causes of oxidative stress in the uterus are decreased estrogen and progesterone levels, and it triggers a decrease in SOD expression, leading to endometrial shedding and implantation deficiency [9]. ROS is generated by several endogenous and exogenous

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processes [10]. One of the oxidative stress–generating exogenous agents is paraquat [11]. Paraquat produces oxidative damage in organisms by producing superoxide anions and subsequently causes an imbalance in the redox state of the cell [12]. Alterations in the oxidative stress mediated by ROS accumulation cause a significant increase in the transcriptional activity of FoxO1, and this has a major impact on SOD and catalase expression [13].

Forkhead box O transcription factor is a key player in an evolutionarily conserved pathway [14]. The members of the mammalian FoxO family consist of FoxO1, 3, 4, and 6, and they are all highly similar in their structure, function, and regulation [14]. These four genes are involved in multiple cellular pathways, which regulate proliferation (FoxO1, FoxO3, and FoxO4), oxidative stress resistance (FoxO1 and FoxO3), metabolism (FoxO1 and FOXO3), cellular differentiation (FoxO3), inflammation (FoxO1, FoxO3, and FoxO4), aging (FoxO1, FoxO3, and FoxO4), and apoptosis (FoxO1, FoxO3, and FoxO4) in mammals [15]. Therefore, the function of these important molecules is tightly controlled by a wide range of protein-protein interactions and post-translational modifications including phosphorylation, acetylation, and ubiquitination [16]. Sirtuin-1 activates several members of the FoxO family of transcription factors which promote the expression of stress response genes including SOD [17].

Sirtuins also play a pivotal role in FoxO function via NAD-dependent deacetylation in response to oxidative stress and thereby may contribute to cellular stress resistance [18]. Sirtuins comprise a highly conserved family of proteins present in virtually all species from bacteria to mammals. Sirtuins are class III histone/protein deacetylases and seven sirtuin genes (sirtuins 1–7) have been identified and characterized in mammals [19, 20]. Sirtuin-1 plays an important role in inflammation, stress resistance, and cellular senescence/aging through the deacetylation of histones, transcription factors, and signaling molecules [20]. Sirtuin activity is linked to metabolic control, apoptosis, cell survival, development, inflammation, and healthy aging [19]. Sirtuin-1 can be induced with several activators. There is a specific sirtuin-1 activator, SRT1720, of which health and lifespan benefits were shown in adult mice fed a high-fat diet [21]. Also, SRT1720 improves the follicle reserve and prolongs the ovarian lifespan of diet-induced obesity in female mice via activating sirtuin-1 [22]. There is only one study showing the role of sirtuin-1 in the regulation of uterine receptivity conducted in vitro [23]. However, it is still unknown whether SRT1720 can promote embryo implantation through activating sirtuin-1 signaling. In this study, we induced oxidative stress in mice via paraquat to characterize the effect of SRT1720 on embryo implantation in order to investigate the roles of sirtuin-1 and FoxO1 in this mechanism.

Material and methods

Animals

Six- to 8-week-old female Balb/C mice (n=49 for all groups) and 12-week-old male Balb/C mice (n=20) were obtained from Akdeniz University Animal Research Unit, Antalya, Turkey. Mice were left at 22–24 °C room temperature with a 12-h light/dark cycle and with free access to mice chow and tap water ad libitum.

Two or three female mice were kept with a male mouse overnight for mating, and the female mice were checked for a vaginal plug the next morning. Female mice observed to have vaginal plugs were admitted on the 1st day of pregnancy [24]. Pregnant mice were randomly separated into 5 groups: control group (n=10), vehicle group (n=10), paraquat group (n=10), SRT1720 group (n=10), and SRT1720+Paraquat group (n=9). The control group received no injection; the vehicle group was injected with the solvent of sirtuin-1 activator SRT1720 on the 1st, 2nd, 3rd, and 4th days of pregnancy, and the solvent of paraquat on the 4th day of pregnancy. The paraquat group was injected with 30 mg/kg paraquat (#36541, Sigma Aldrich, St. Louis, MO, USA) (prepared in 0.9% NaCl #106404, Merck, Darmstadt, Germany) on the 4th day of pregnancy; SRT1720 group was injected with 50 mg/kg SRT1720 (#A10862, Generon, Whittle Parkway, Slough, UK, prepared in 5% DMSO, 30% PEG400 -Polyethylene Glycol 400- #8074850050, Merck, Darmstadt, Germany, and 2% Tween-80 #P4780, Sigma Aldrich, St. Louis, MO, USA) on the 1st, 2nd, 3rd, and 4th days of pregnancy; and SRT1720+Paraquat group was injected with 50 mg/kg SRT1720 on the 1st, 2nd, 3rd, and 4th days of pregnancy along with 30 mg/kg paraquat in the morning of the 4th day of pregnancy, 30 min after SRT1720 injection. All injections were done early in the morning (Table 1).

Implantation sites on the 5th day of pregnancy were visualized by an intravenous injection of Chicago Blue dye solution (1% Chicago blue dye #C8679, Sigma Aldrich, St. Louis, MO, USA, was prepared in 0.9% NaCl) before sacrification and the number of implantation sites, demarcated by distinct blue bands was recorded [25]. Uterine horns that did not exhibit any implantation sites were flushed with phosphate-buffered saline (PBS) to recover blastocysts. Uteri and implantation and inter-implantation sites were collected and fixed in formalin for immunohistochemistry, stored in liquid nitrogen for Western blotting, and stored at −80 °C for biochemical analysis.

Tissue processing

Tissues were fixed in 10% formalin (#15512, Merck, Darmstadt, Germany) (100 mL 37% formaldehyde, 900 mL distilled water, pH~7) at room temperature for 24 h. Formalin

Table 1 SRT1720 or paraquat injection to female mice on the 1**st**, 2**nd**, 3**rd**, and 4**th** days of pregnancy

	GD1	GD2	GD3	GD4	GD5
Control group	-	-	-	-	Sacrification
Vehicle group	SRT1720 solvent	SRT1720 solvent	SRT1720 solvent	SRT1720 solvent + paraquat solvent	Sacrification
Paraquat group	-	-	-	30-mg/kg paraquat	Sacrification
SRT1720 group	50-mg/kg SRT1720	50-mg/kg SRT1720	50-mg/kg SRT1720	50-mg/kg SRT1720	Sacrification
SRT1720+Paraquat group	50-mg/kg SRT1720	50-mg/kg SRT1720	50-mg/kg SRT1720	50-mg/kg SRT1720 30-mg/kg Paraquat	Sacrification

was cleaned with tap water; then, tissues were dehydrated with immersion in 70%, 80%, and 90% ethanol (#100986, Merck, Darmstadt, Germany) for 24 h each and 100% ethanol for 4 h. After dehydration, tissues were cleared in xylene (#16446, Sigma Aldrich, Italy) and embedded in paraffin wax (#107337, Merck, Darmstadt, Germany). Cleared tissues were kept in paraffin in an oven at 56 °C and then embedded in paraffin.

Immunohistochemistry

Formalin-fixed and paraffin-embedded tissues were cut into 5-μm sections and placed on slides coated with poly-l-lysine (#J1800AMNZ, Thermo Scientific, Braunschweig, Germany). They were kept in an oven at 56 °C overnight the day before immunostaining. Sections were deparaffinized in xylene and rehydrated through a graded series of ethanol, rinsed in distilled water, and washed three times in PBS (pH: 7.2–7.4). To unmask antigens, an antigen retrieval procedure was performed by treating the samples in 10-mM citrate buffer (#100242, Merck, Darmstadt, Germany), pH 6.0, in a microwave oven at 750 W for 5 min, three times. After cooling for 20 min at room temperature, the sections were washed in PBS for 5 min. Endogenous peroxidase activity was blocked by incubation in methanol containing 3% H_2O_2 (#108597, Merck, Darmstadt, Germany) for 20 min and washed with PBS three times. Sections were incubated with a blocking solution (Ultra UV Block, #TA-125UB, LabVision Corporation, Fremont, CA, USA) for 7 min at room temperature to block non-specific binding. Excess serum was drained, and sections were incubated with rabbit polyclonal anti-sirtuin-1 (#15404; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:50 dilution and rabbit monoclonal anti-FoxO1 (#2880, Cell Signaling Technology, Danvers, MA, USA) at 1:100 dilution at +4 °C overnight. The primary antibodies were substituted with normal rabbit IgG (#sc-2027, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in the same dilutions as the specific antibodies. The next day, sections were washed with PBS three times. Then, they were incubated with biotinylated anti-rabbit secondary antibody (#BA-1000, Vector Laboratories, Burlingame, USA) at 1:500 dilution in a humid environment at room temperature for 45 min, followed by incubation with HRP-conjugated streptavidin complex (#TS125HR, Thermo Scientific, Fremont, CA, USA) for 20 min at room temperature. Sections were washed 3 times with PBS after each treatment. Sections were treated with diaminobenzidine (DAB) chromogen (#D4168, Sigma Aldrich, St. Louis, MO, USA) for 2 min to improve signaling and then washed in tap water. Mayer's hematoxylin (#109249 Merck, Darmstadt, Germany) was utilized for counterstaining of the sections, and they were dehydrated and mounted with Entellan (#107961; Merck, Darmstadt, German) mounting medium. Sections were examined and photographed by Nikon Eclipse E200 microscope.

H-SCORE evaluations

In sections of experimental groups, evaluation of the immunohistochemical labeling of FoxO1 in the luminal epithelium was performed using H-SCORE [26]. Immunopositive cells were counted in 5 different areas under ×200 original magnification under the Nikon Eclipse E200 microscope. Three sections were randomly selected from every group. The evaluations were recorded as percentages of positively stained cells of all types in each of four intensity categories which were denoted as 0 (no staining), 1+ (weak but detectable above control), 2+ (distinct), 3+ (intense). The evaluation was conducted by two blinded observers, and an H-SCORE value was derived by summing the percentages of cells that stained at each intensity multiplied by the weighted intensity of the staining (H-SCORE = $\sum \cdot Pi\ (i + 1)$ for each tissue, where i is the intensity score and Pi is the corresponding percentage of the cells).

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and Western blotting

Uteri and implantation and inter-implantation sites were frozen in liquid nitrogen, then thawed and subjected to protein isolation. Protein extraction and immunoblot analysis were

performed as described previously [27]. All samples were weighed and put into a homogenization buffer supplemented with a Complete R protease inhibitor cocktail (#11697498001; cOmplete Merck, Mannheim, Germany). After homogenization, samples were centrifuged at 10.000*g* for 10 min. Supernatants were collected and stored at −80 °C. The protein concentration was determined by BCA kit (bicinchoninic acid solution (#B9643) and copper (II) sulfate solution (#C2284); Sigma Aldrich, St. Louis, MO, USA), and 50 μg of protein was applied per lane. The samples prepared were mixed with distilled water and Laemmli (#S3401, Sigma Aldrich, St. Louis, MO, USA) in line with the protein content of the protein lysates prepared. The samples were then boiled in water at 100 °C. Samples were subjected to SDS polyacrylamide gel electrophoresis (30% acrylamide in 10% gel) at 80 V for approximately 2 h. At the end of electrophoresis, PVDF (polyvinylidene difluoride) membrane (#1620177, Bio-Rad, Portland, ME, USA) was kept in methanol (#106009, Merck, Merck, Darmstadt, Germany) for 30 s. The proteins were transferred onto the membrane in a buffer containing 0.2 mol/L glycine (#1610718, Bio-Rad, Portland, ME, USA), 25 mmol/L Tris (#108387, Merck, Darmstadt, Germany), and 20% methanol overnight at +4 °C and 32 volts and 65 A. The next day, the membranes were blocked for 1 h with 5% BSA (#A9647, Sigma Aldrich, St. Louis, MO, USA) and 0.1% Tween 20 (#TA-125-TW, Lab Vision, Thermo Fisher Scientific, Rockford, USA) in 0.14 mol/L Tris-buffered saline (TBS; pH 7.2–7.4). Membranes were incubated with 1:500 dilution of rabbit polyclonal anti-sirtuin-1 (#15404, Santa Cruz Biotechnology), 1:2000 dilution of rabbit monoclonal anti-FoxO1 (#2880, Cell Signaling Technology, Danvers, MA, USA), and 1:500 dilution of rabbit polyclonal acetylated lysine (#9441, Cell Signaling Technology, Danvers, MA, USA) overnight at +4 °C. Primary antibodies were diluted in 5% BSA. After incubation, they were washed with TBS-T for 30 min. The membranes were incubated with horseradish peroxidase (HRP)–conjugated secondary antibody (#PI-1000, Vector Laboratories, Burlingame, USA) diluted in 5% BSA on a rocker for 1 h at room temperature. They were then washed with TBS-T for 30 min. The membranes were incubated for 5 min within the Super Signal Chemiluminesans (CL)-HRP substrate system (#34080, Thermo Fisher Scientific, Rockford, USA), and the signals on membranes were then transferred on to hyper film (#28-9068-37 Amersham Hyperfilm ECL, GE Healthcare, Buckinghamshire, UK) in a dark room. The film was processed through a developer (#1757855, Ilford, England) and fixative (#1984565, Ilford, England) followed by washing with distilled water and drying. Thus, the quantities of sirtuin-1, FoxO1, and acetylated lysine protein expressions were determined. Membranes were also labeled by an identical protocol for binding of a 1:5000 dilution of a rabbit monoclonal anti-beta actin antibody (#4970, Cell Signaling Technology, Danvers, MA, USA) as an internal control to confirm the equal loading of the samples. The bands were quantified using NIH image analysis software (Image J Version 1.36b; National Institutes of Health, MD, USA).

Determination of ROS/RNS levels

OxiSelect™ In Vitro ROS/RNS Assay Kit (#STA-347, Cell Biolabs, Inc. San Diego, CA) was used to measure the levels of ROS and RNS in uteri and implantation sites of experimental groups according to the protocol provided by the company. Briefly, samples in PBS buffer were lysed using a homogenizer. The lysates were collected and centrifuged at 10,000*g* for 15 min at +4 °C.

Protein concentrations were measured at 595 nm by a modified Bradford assay using Coomassie Plus reagent with bovine serum albumin as a standard (Pierce, Thermo Fisher Scientific, Roskilde, Denmark).

The lysates were adjusted to equal protein concentrations (1:100 dilution) using PBS. We added 50 μL of each sample (triplicate) and 50 μL of catalyst solution to a black 96-well plate and then incubated for 5 min at room temperature. We added 100 μL of dichlorodihydrofluorescein (DCFH) solution to the samples and incubated the mixture at room temperature for 20 min protecting it from the light. Finally, fluorescence was read with a fluorometric plate reader at 480 nm/530 nm [28].

Determination of SOD levels

Total tissue SOD activity was measured using a commercially available kit (#706002, Cayman Chemical, Ann Arbor, USA) that measures all three types of SOD (Cu/Zn-, Mn-, and EC-SOD). One unit of SOD is defined as the amount of enzyme that dismutates 50% of the superoxide radical. Briefly, xanthine oxidase and hypoxanthine generate superoxide radicals that are dismutated by SOD, and in the process, tetrazolium salt is converted to a formazan dye that is read at 450 nm. SOD activity of the samples was calculated from the linear regression of a standard curve that was determined using SOD activity of bovine erythrocytes at various concentrations run under the same conditions. The SOD activity was expressed as units per milliliter of tissue extract [29].

Statistical analysis

Statistical analyses were performed using one-way ANOVA. Probability values of less than 0.05 were considered significant; values are presented as mean ± SEM (standard error of the mean).

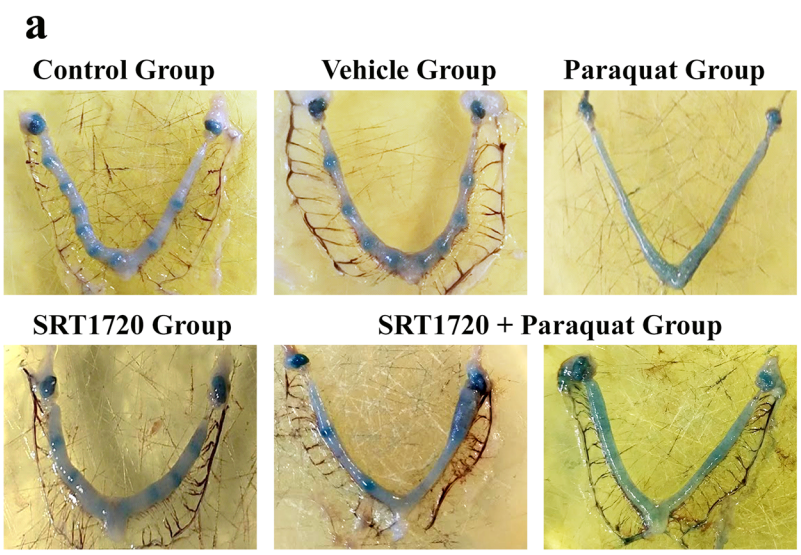
Results

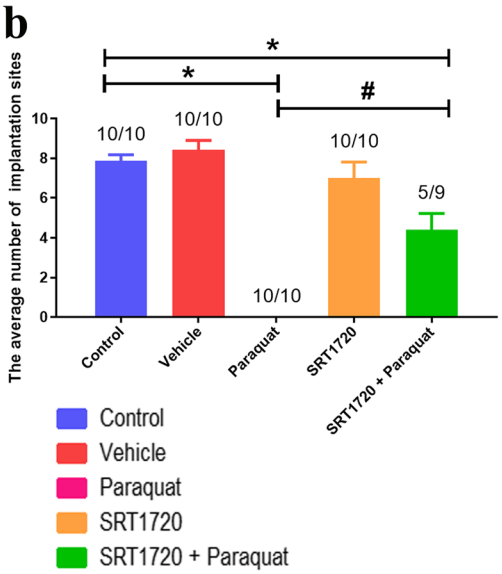
Paraquat exposure inhibits embryo implantation

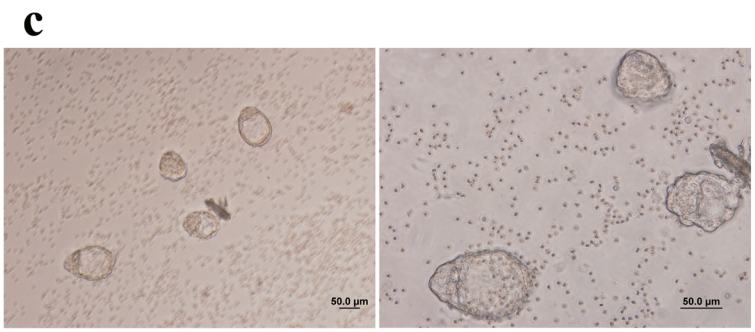
Blastocysts position in the implantation chamber (crypt) in the afternoon of day 4 of pregnancy (day 1 = vaginal plug) [30]. The receptive state of the uterus is defined as the limited time when the uterine milieu is favorable to blastocyst acceptance and implantation [24]. It is known that paraquat can adversely impact the development and implantation of embryos [31, 32]. To minimize the damaging effects of paraquat on embryo growth and activation, paraquat was injected at a 30-mg/kg dose that prevents implantation but does not damage the embryo [32]. Control and vehicle group mice showed implantation sites as demarcated by blue bands. Paraquat group mice did not have any implantation sites (Fig. 1a and b). To eliminate the possibility of lack of implantation due to failure of fertilization, one of the uterine horns was washed with PBS, and the presence of blastocysts was observed. Normal morphology of blastocysts demonstrated that the administered paraquat dose did not adversely affect embryo development (Fig. 1c).

SRT1720 improves embryo implantation in paraquat-treated mice

Activation and increased expression of sirtuin-1 play an important role in uterine receptivity [23]. To stimulate sirtuin-1 activity, we used SRT1720, a synthetic activator specific to sirtuin-1 [21]. To evaluate whether SRT1720 has harmed embryo implantation, the number of implantation sites was compared to that of the control group and no significant differences were observed (Fig. 1a and b). Then, we evaluated whether SRT1720 improves embryo implantation in paraquat-injected mice. While some of the SRT1720-treated mice did not have implantation sites (4/9), the rest of the mice (5/9) had implantation sites. Although the number of implantation sites in the SRT1720+Paraquat group was significantly lower than that in the control group (p <0.05), it was significantly higher than that in the paraquat group (p <0.05) (Fig. 1a and b).







	No of mice	No. of mice with IS	No of IS
Control Group	10	10 (%100)	7,875±0,301
Vehicle Group	10	10 (%100)	8,416667±0,488
Paraquat Group	10	0 (%0)	0±0
SRT1720 Group	10	10 (%100)	7±0,816
SRT1720 + Paraquat Group	9	5 (%55.5)	4,4±0,818

Fig. 1 Implantation sites of experimental groups on the 5**th** day of pregnancy. **a**–**b** The average number of implantation sites of experimental groups on the 5**th** day of pregnancy. **c** Blastocysts obtained by washing one uterine horn on the 5**th** day of pregnancy in the paraquat group. Data are plotted as mean ± SEM. Different symbols indicate a significant difference compared to that group (*: Control, #: Paraquat). All the scale bars represent 50 µm

Sirtuin-1 expression in uteri and implantation sites of experimental groups

The sirtuin-1 activator SRT1720 improved embryo implantation in paraquat-injected mice; thus, we determined sirtuin-1 spatial distribution and expression in uteri and implantation and inter-implantation sites of experimental groups via immunohistochemistry and Western blotting, respectively. Sirtuin-1 exhibited mainly nuclear expression. Sirtuin-1 expression was in the luminal and glandular epithelium and stroma. Embryos in control, vehicle, SRT1720, and SRT1720+Paraquat groups also showed sirtuin-1 expression (Fig. 2a). Sirtuin-1 expression was significantly increased in SRT1720-treated groups compared to that in the control group (Fig. 2a and b).

FoxO1 expression in uteri and implantation sites of experimental groups

Recent studies indicate the roles of FoxO1 in the female reproductive system, especially in the maternal endometrium [33]; and a very recent study shows that *FoxO1*-ablated mice were infertile due to defective implantation and invasion [1]. Sirtuin-1 is known to modulate upstream signaling of FoxO1 [34] and deacetylates FoxO1, promoting its transcriptional activity [35]. Therefore, we determined FoxO1 spatial distribution and expression in uteri and implantation sites of experimental groups via immunohistochemistry and Western blotting, respectively. FoxO1 exhibited both nuclear and cytoplasmic expression. FoxO1 expression was mainly observed in the luminal and glandular epithelium; there was no expression in stroma except endothelial cells (Fig. 3a). FoxO1 expression was higher at implantation sites compared to that at inter-implantation sites for control, vehicle, SRT1720, and SRT1720+Paraquat groups, and was statistically significant. Also, FoxO1 expression at implantation sites of SRT1720-treated groups was significantly increased compared to that of the control group (Fig. 3b).

Cellular distribution of FoxO1 in the luminal epithelium of uteri and implantation sites of experimental groups

We observed unique cellular distributions of FoxO1 in the luminal epithelium of uteri and implantation sites of experimental groups. FoxO1 was mainly nuclear at implantation sites and cytoplasmic at inter-implantation sites of control,

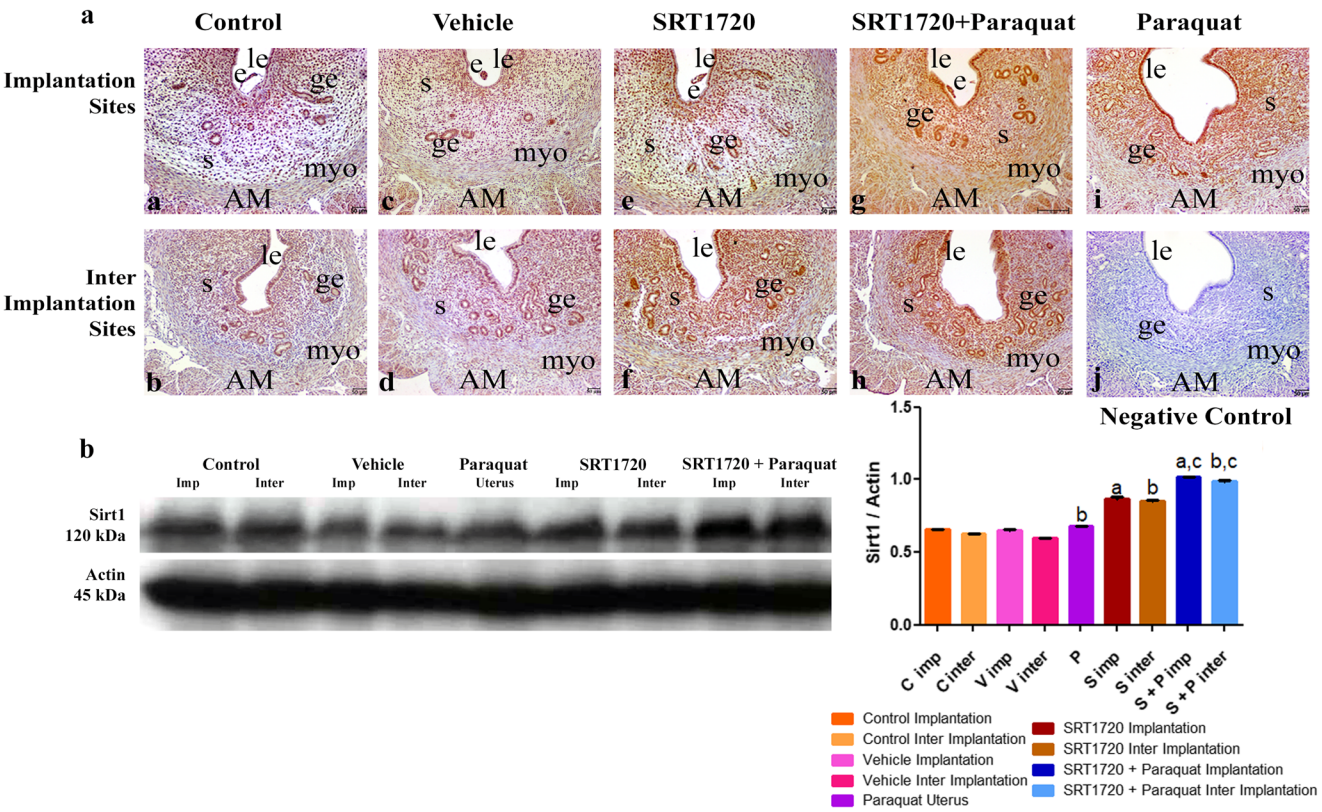


Fig. 2 Sirtuin-1 expression in the endometrium of experimental groups on the 5**th** day of pregnancy. **a** Immunohistochemistry of sirtuin-1. **b** Western blot and relative densitometric analysis of sirtuin-1. e, embryo; le, luminal epithelium; ge, glandular epithelium, s, stroma; myo, myometrium; AM, antimesometrium; imp, implantation sites; inter, inter implantation sites. Data are plotted as mean ± SEM. Different letters indicate a significant difference compared to that group (a, Control Implantation; b, Control Inter Implantation; c, Paraquat Uterus). All the scale bars represent 50 μm

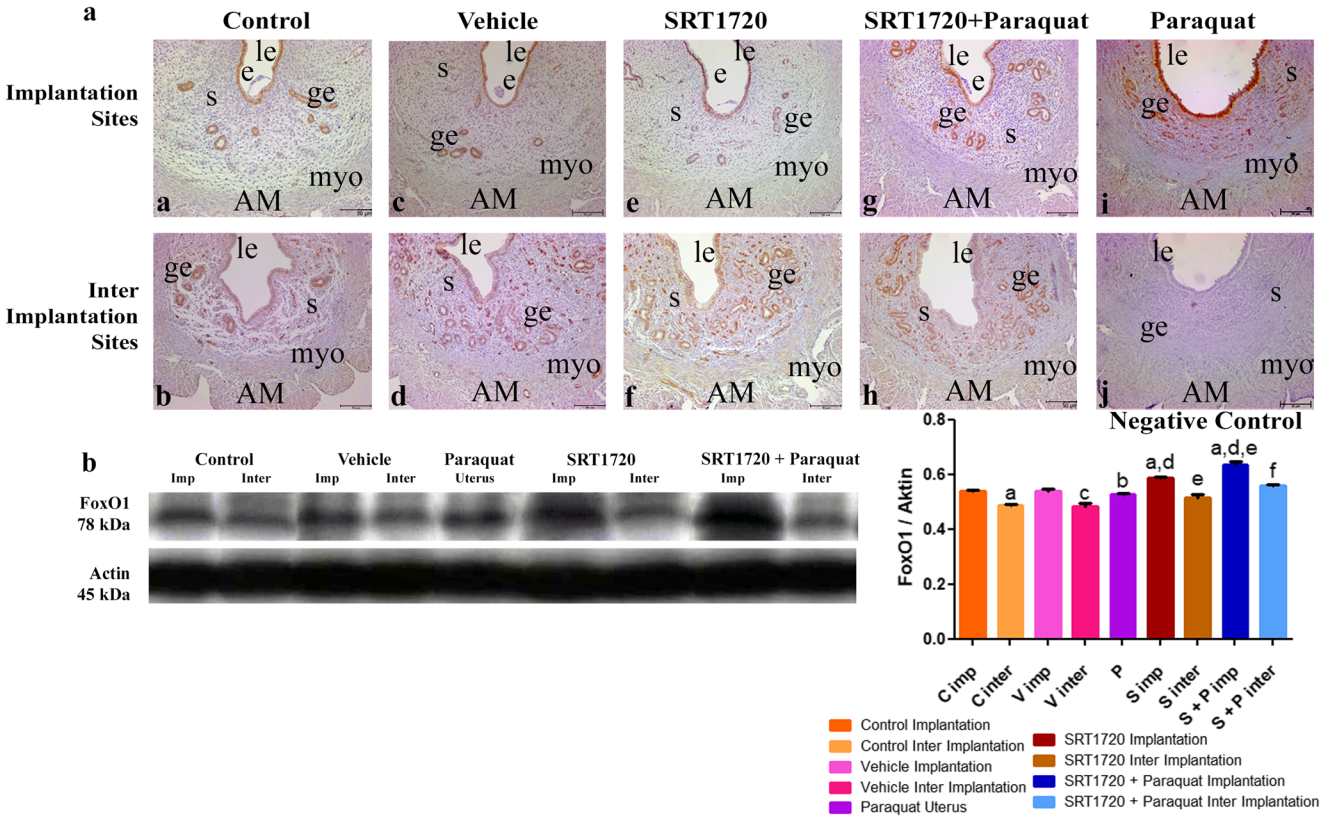


Fig. 3 FoxO1 expression in the endometrium of experimental groups on the 5**th** day of pregnancy. **a** Immunohistochemistry of FoxO1. **b** Western blot and relative densitometric analysis of FoxO1. e, embryo; le, luminal epithelium; ge, glandular epithelium; s, stroma; myo, myometrium; AM, antimesometrium; imp, implantation sites; inter, inter implantation sites. Data are plotted as mean ± SEM. Different letters indicate a significant difference compared to that group (a, Control Implantation; b, Control Inter Implantation; c, Vehicle Implantation; d, Paraquat Uterus; e, SRT1720 Implantation; f, SRT1720+Paraquat Implantation). All the scale bars represent 50 μm

vehicle, SRT1720, and SRT1720+Paraquat groups. When examined in detail, we observed that FoxO1 was mainly cytoplasmic only in the blastocyst attachment region of control, vehicle, SRT1720, and SRT1720+Paraquat groups. Furthermore, FoxO1 was mainly nuclear in the antimesometrium and cytoplasmic in the mesometrium side of the luminal epithelium of the paraquat group. We evaluated FoxO1 expression in the luminal epithelium of all groups by H-SCORE analysis. FoxO1 expression in the luminal epithelium of implantation sites was higher than that of inter-implantation sites of all experimental groups with a statistically significant difference. SRT1720- and paraquat-treated groups had higher FoxO1 expression than the control group. On the other hand, paraquat and SRT1720 co-treated groups had much higher FoxO1 expression compared to the control group (Fig. 4).

Deacetylated FoxO1 levels in uteri and implantation sites of experimental groups

FoxO activity is tightly regulated at the post-translational level affecting its localization, stability, DNA binding, or protein-protein interactions [36]. In the presence of oxidative stress, sirtuin-1, located in the nucleus, associates with and deacetylates nuclear FoxOs [37]. The deacetylated form is necessary for the post-translational modification of this transcription factor which is needed for its active modification and cellular defense against oxidative stress [38]. Sirtuin-1 stimulates the deacetylation of FoxO1, which, in turn, plays an essential role in mediating the sirtuin-1-induced upregulation of SOD and the suppression of oxidative stress [39]. Thus, we determined deacetylated FoxO1 levels by subtracting acetylated lysine values from FoxO1 values obtained via Western blot. As indicated in Fig. 5a and b, deacetylated FoxO1 levels at implantation sites were higher than those at inter-implantation sites of control, vehicle, SRT1720, and SRT1720+Paraquat groups and it was statistically significant. Deacetylated FoxO1 levels in the paraquat group were lower than those in the control group. Deacetylated FoxO1 levels in the SRT1720 treated group were higher than those in the control group. Moreover, paraquat and SRT1720 co-treated groups had much higher deacetylated FoxO1 levels than the control group.

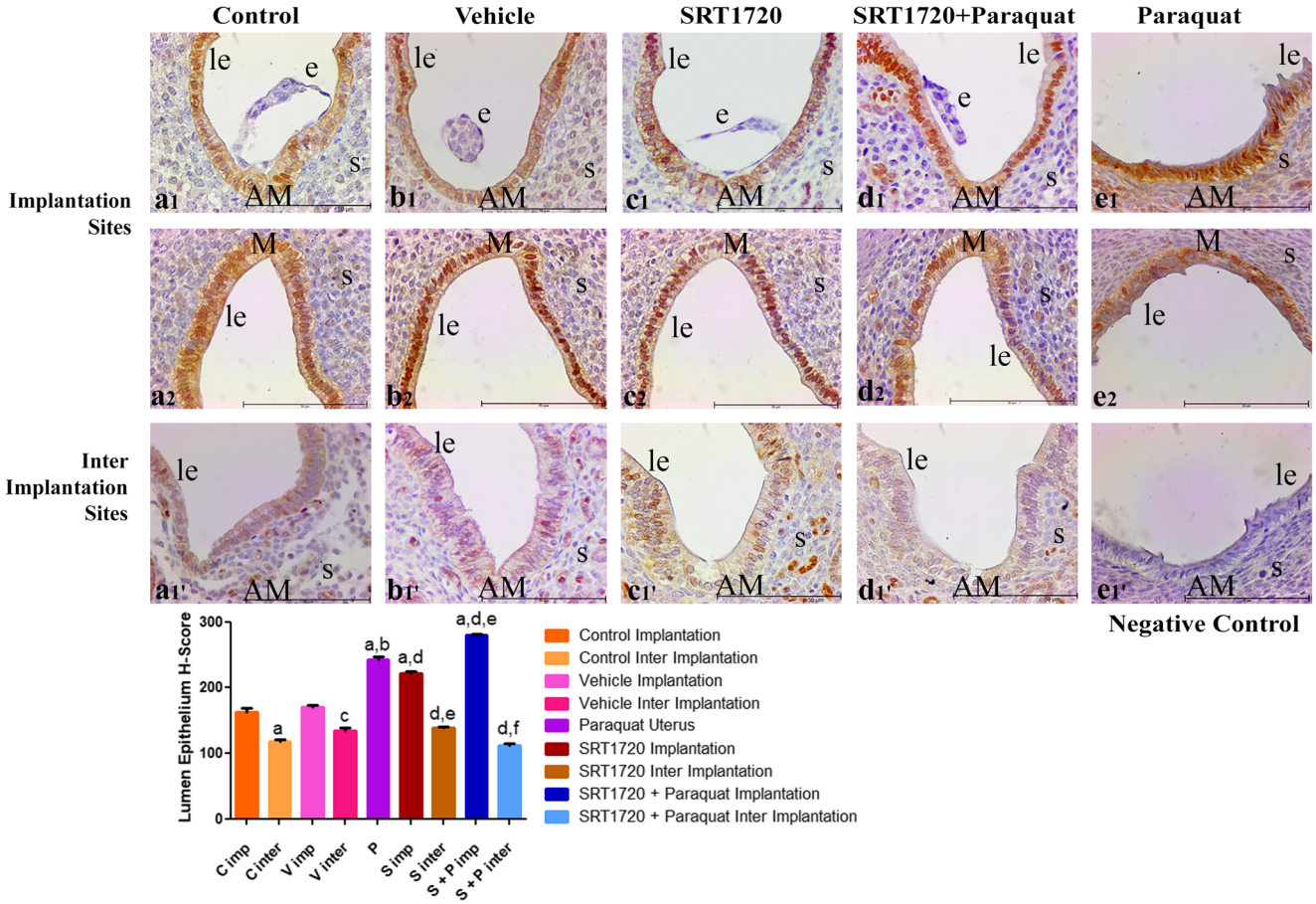


Fig. 4 Cellular distribution of FoxO1 at embryo homing sites in experimental groups on the 5**th** day of pregnancy. e, embryo; le, luminal epithelium; ge, glandular epithelium; s, stroma; myo, myometrium; AM, antimesometrium; imp, implantation sites; inter, inter implantation sites. Data are plotted as mean ± SEM. Different letters indicate a significant difference compared to that group (a, Control Implantation; b, Control Inter Implantation; c, Vehicle Implantation; d, Paraquat Uterus; e, SRT1720 Implantation; f, SRT1720+Paraquat Implantation). All the scale bars represent 50 μm

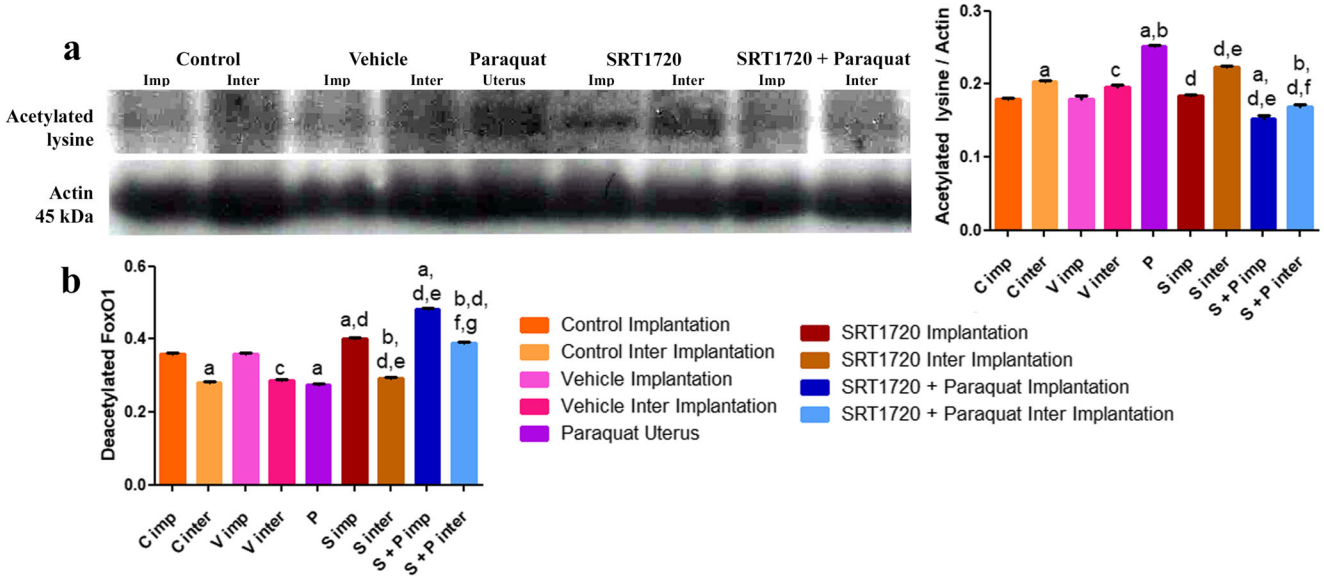


Fig. 5 Deacetylated FoxO1 levels in uteri or implantation sites of experimental groups. **a** Western blot and relative densitometric analysis of FoxO1. **b** Deacetylated FoxO1 levels. Data are plotted as mean ± SEM. Different letters indicate a significant difference compared to that group (a, Control Implantation; b, Control Inter Implantation; c, Vehicle Implantation; d, Paraquat Uterus; e, SRT1720 Implantation; f, SRT1720 Inter Implantation; g, SRT1720+Paraquat Implantation)

ROS/RNS levels in uteri and implantation sites of experimental groups

SOD is one of the antioxidant enzymes directly involved in the neutralization of ROS and RNS [40]. We determined the levels of ROS/RNS which cause oxidative stress, in uteri and implantation and inter-implantation sites of experimental groups. As indicated in Fig. 6, ROS/RNS levels were similar at implantation and inter-implantation sites for control, vehicle, SRT1720, and SRT1720+Paraquat groups. ROS/RNS levels significantly increased in paraquat and SRT1720+Paraquat groups compared to those in the control group. In the SRT1720+Paraquat group, the levels of ROS/RNS were higher than those in the control group, but they were significantly lower than those in the paraquat group. In the SRT1720 group, the levels of ROS/RNS were significantly lower than those in the control group. These results indicated that the levels of ROS/RNS were decreased in SRT1720-treated groups.

SOD levels in uteri and implantation sites of experimental groups

Sirtuin-1-mediated deacetylation of FoxO1 directly controls the expression of SOD [41]. In our study, we determined SOD activity in uteri and implantation and inter-implantation sites of experimental groups. As indicated in Fig. 7, SOD activity was similar at implantation and inter-implantation sites for control, vehicle, SRT1720, and SRT1720+Paraquat groups. SOD activity was significantly increased in paraquat, SRT1720, and SRT1720+Paraquat groups compared to that in the control group. The increase in SOD activity was higher in the SRT1720+Paraquat group compared to that in the paraquat and SRT1720 groups.

Discussion

The mechanisms involved in embryo implantation have not been clarified and are still an intriguing subject in this regard. In one study performed with Ishikawa and RL95-2 endometrial carcinoma cell lines, sirtuin-1 was shown to play an important role in uterine receptivity [23]. However, there is no in vivo study in the literature regarding whether sirtuin-1 is involved in embryo implantation in mice. In this study, we show that embryo implantation can be improved by SRT1720, a specific activator of sirtuin-1, in paraquat-treated mice. To our knowledge, this should be the first *in vivo* study demonstrating the involvement of sirtuin-1 in embryo implantation.

It has been suggested that oxidative stress affects the entire reproductive life and pregnancy-related disorders [7, 42]. Dietary antioxidants and oxidative stress may influence the timing and maintenance of a viable pregnancy [43]. Additionally, restraining stress leads to a decrease in implantation success in mice [44]. Previous studies showed that paraquat adversely affected embryo development and implantation via inducing oxidative stress [31, 32, 45, 46]. In one of those studies, paraquat prevented embryo implantation without harming embryo development [32]. Likewise, in our study, implantation did not occur in 30-mg/kg paraquat–injected mice. Meanwhile, the normal morphology of blastocysts obtained by washing one of the uterine horns indicated that embryos are healthy and the administered paraquat dose did not affect embryo development.

The sirtuin-1 activator, SRT1720, has been reported to show positive roles in female reproduction, such as improving follicle reserve and extending ovarian life [22]. In our study, although embryo implantation was inhibited by paraquat, the occurrence of embryo implantation with the use of SRT1720 suggests that sirtuin-1 activity may have an important role in this process. There are studies in the literature using different concentrations and applications of SRT1720 [22, 47–49]. In our study, 5 out of 9 SRT1720+Paraquat mice had implanta-

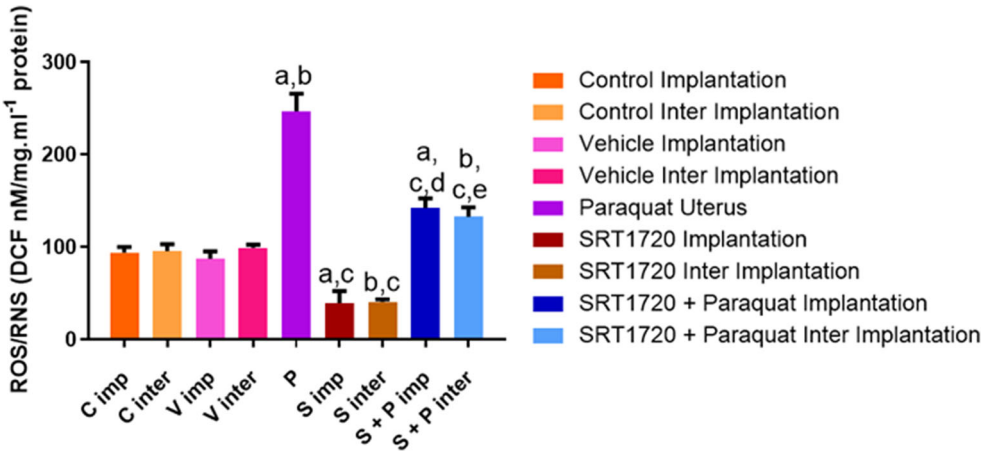


Fig. 6 ROS/RNS levels in uteri or implantation sites of experimental groups on the 5**th** day of pregnancy. Data are plotted as mean ± SEM. Different letters indicate a significant difference compared to that group (a, Control Implantation; b, Control Inter Implantation; c, Paraquat Uterus; d, SRT1720 Implantation; e, SRT1720 Inter Implantation)

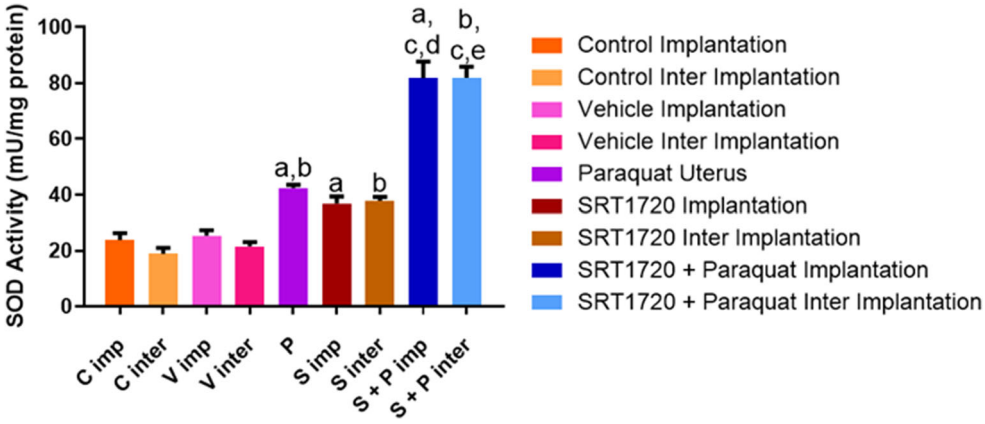


Fig. 7 SOD activity in uteri or implantation sites of experimental groups on the 5**th** day of pregnancy. Data are plotted as mean ± SEM. Different letters indicate a significant difference compared to that group (a, Control Implantation; b, Control Inter Implantation; c, Paraquat Uterus; d, SRT1720 Implantation; e, SRT1720 Inter Implantation)

tion sites while implantation did not occur in 4 out of 9 mice. This might be a result of the applied dose of SRT1720. Administration of higher doses of SRT1720 could result in higher implantation site numbers.

The fact that paraquat inhibited implantation and was partially improved by SRT1720 made us think that sirtuin-1 might play a role in embryo implantation. In general, sirtuin-1 is localized in nuclei but is also known to shuttle between cytoplasm and nuclei in some cell types [50, 51]. The nucleocytoplasmic shuttling of sirtuin-1 has been described in both physiological and pathological conditions where oxidative stress occurs [52]. Sirtuin-1, a protein deacetylase, regulates cellular oxidative stress and its toxicity. Furthermore, sirtuin-1 is also regulated by oxidative stress [52–54]. H_2O_2-mediated oxidative stress may result in cytoplasmic localization of sirtuin-1 in cervix and lung cells [51, 55]. On the other hand, while sirtuin-1 was expressed mainly in the nucleus of rat nucleus pulposus cells, no significant cytoplasmic translocation of sirtuin-1 was observed with H_2O_2 treatment [54]. In our study, sirtuin-1 was nuclear in SRT1720 and SRT1720+ Paraquat mice. This confirms that the nucleocytoplasmic shuttle of sirtuin-1 is specific to cells and tissues. In one study, paraquat exposure upregulated the expression of sirtuin-1; long-term exposure to paraquat significantly decreased sirtuin-1 expression in the lungs of mice. Another sirtuin-1 activator, resveratrol, strongly enhanced sirtuin-1 expression and attenuated lung injury induced by paraquat exposure [56]. In our study, increased expression of sirtuin-1 in SRT1720-treated groups may indicate that SRT1720 enhances sirtuin-1 activity. Mouse type II alveolar epithelial cells (AECs-II) were exposed to low-dose paraquat, and sirtuin-1 expression was upregulated. On the other hand, sirtuin-1 expression gradually decreased with increasing paraquat concentrations [53].

Sirtuins catalyze the deacetylation and adenosine diphosphate (ADP) ribosylation of target proteins using the oxidized form of nicotinamide adenine dinucleotide (NAD^+) as a coenzyme [57]. Sirtuin-1 targets histones, transcription factors, and transcriptional regulators in the nucleus, which control gene expression by deacetylation [58]. FoxO1 is a direct and functional target for sirtuin-1 in mammalian systems [59]. Activating sirtuin-1 can promote FoxO1 expression and transition from cytoplasm to nucleus [60]. We observed that the endometrial distribution of FoxO1 was in the luminal and glandular epithelium; although expression was weak in stromal cells, it was distinct in endothelial cells in the stroma. Interestingly, the expression of FoxO1 differed in the luminal epithelium through the mesometrial-antimesometrial (M-AM) axis. Trophoblast cells directly communicating with the luminal epithelium during implantation swallow the luminal epithelial cells without caspase-3 activation (entosis); then, apoptosis occurs in the luminal epithelial cells so the luminal epithelium surrounding the blastocyst is breached on day 5 evening of pregnancy [30]. FoxO1 expression was cytoplasmic in the luminal epithelial cells of the antimesometrium, notably in the blastocyst attachment region, and it was nuclear in the luminal epithelium of the mesometrium [33]. In other words, FoxO1 distribution was only cytoplasmic at embryo homing sites in the luminal epithelium and nuclear in the luminal epithelial cells excluding those surrounding the blastocyst. FoxO1 expression was higher at implantation sites than that at inter-implantation sites of all groups. Similarly, Adiguzel et al. also showed that FoxO1 expression was weak at inter-implantation sites on day 5 of pregnancy [33]. This suggests that FoxO1 is an important molecule for embryo implantation. FoxO1 can be retained in the nucleus by decreasing its acetylation; conversely, FoxO1 can be translocated to the cytoplasm by increasing its acetylation [61]. Cytoplasmic localization of FoxO1 at the mesometrial side of the luminal epithelium might be related to increased acetylation of FoxO1, and nuclear localization of FoxO1 at the antimesometrial side of the luminal epithelium might be an adaptive response to oxidative stress against possible embryo implantation in paraquat-treated mice.

FoxO1 deacetylation by sirtuin-1 regulates transcription activity and consequently provides resistance to oxidative stress [59, 62, 63]. We indicate that FoxO1 acetylation increases in uteri of paraquat-treated mice, and therefore deacetylation decreases. However, there was increased

FoxO1 deacetylation both in SRT1720 and SRT1720+Paraquat mice. Furthermore, FoxO1 deacetylation increased more in SRT1720+Paraquat mice compared to that in only SRT1720-treated mice. This might indicate that FoxO1 deacetylation can further be increased by SRT1720 treatment in addition to the induced oxidative stress.

FoxO1 mediates protective effects against oxidative stress through the regulation of antioxidant genes, such as catalase and SOD, [64] and FoxO1 activity is regulated by sirtuin-1 [13, 17]. SOD is responsible for the detoxification of superoxide anions and is required for normal health and reproduction [65]. Oxygen radicals and SOD regulate cellular function by controlling the production or activation of biologically active substances [66]. Our findings show that SOD activity was increased in SRT1720 and SRT1720+Paraquat mice. This increase might be due to increased FoxO1 deacetylation. Furthermore, an increase in SOD activity despite low deacetylation of FoxO1 in the paraquat group may suggest that another mechanism is involved in this increase. ROS and SOD play important roles in the process of reproductive physiology [67]. ROS are like a double-edged sword; they serve as key signaling molecules in physiological processes but also have roles in pathological processes involving the female reproductive tract [7].

We determined ROS/RNS levels to evaluate the relationship between SRT1720 and oxidative stress. As expected, paraquat treatment significantly increased ROS/RNS levels since it causes oxidative stress. In paraquat and SRT1720 co-treated groups, ROS/RNS levels were higher than those in the control group but less than those in only the paraquat-treated group. Besides, SRT1720-treated mice had less oxidative stress. It is possible to state that SRT1720 performs an important role in reducing oxidative stress in the female reproductive system. Reducing the damage caused by paraquat in the female reproductive system by SRT1720 seems promising for embryo implantation. These findings show that SRT1720 might improve reproductive failures caused by increased oxidative stress in the female reproductive system.

Nevertheless, this study has some limitations. The main limitation of our study is the lack of different SRT1720 dose treatments. We used SRT1720 at 50 mg/kg dose. It might be possible that SRT1720 treatment with doses greater than 50 mg/kg may lead to a higher percentage of embryo implantation. The second limitation of our study was that we were unable to investigate whether sirtuin-1 plays a role in decidualization and whether paraquat and SRT1720 treatments had any effects on decidualization. However, a very recent study revealed the essential role of sirtuin-1 in regulating differentiation and maintaining ROS homeostasis in human endometrial stromal cells during decidualization in vitro [68]. Thirdly, we did not follow the mice until the end of pregnancy to compare birth rates. It will be interesting to explore whether SRT1720 has protective roles in paraquat-treated mice regarding pregnancy progression after implantation. Lastly, although animal studies provide a valuable source of knowledge, the need to perform respective human studies is still apparent. The fundamental limitation of research on humans is the availability of human tissue during implantation. We believe our results are fundamental for the potential roles of sirtuin-1 in mouse embryo implantation and will lead to further studies in this field.

Conclusion

Infertility is a social and economic problem. Approximately 30% of human fertilization results in miscarriage. In some individuals, infertility problems can be solved by assisted reproductive techniques, but if the uterus is not receptive, embryo transfer results in implantation failure. Based on these findings, sirtuin-1 may protect embryo implantation against oxidative stress through FoxO1-SOD signaling. It will be worthy to investigate sirtuin-1-FoxO1-SOD signaling in implantation failure and recurrent pregnancy loss cases.

Author contribution KA: investigation, formal analysis, writing – original draft, visualization; EG: formal analysis; MAA: investigation, formal analysis; IU: review and editing; NA: conceptualization, writing – review and editing, funding acquisition

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Data availability Available upon request.

Declarations

Ethics approval The experimental protocol was approved by the Animal Care and Use Committee of Akdeniz University Faculty of Medicine (Number of the Ethical Approval 2017.02.15).

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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