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# Occurrence of sulfonated steroids and ovarian expression of steroid sulfatase and SULT1E1 in cyclic cows

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ABSTRACT

Historically sulfonated steroids were primarily considered as inactive metabolites destined for elimination. However, more recently they have been increasingly recognized as precursors for the production of bioactive steroids in target tissues and as functional molecules without preceding hydrolysis. In order to comprehensively characterize their occurrence in cyclic cows and their formation and hydrolysis in bovine ovarian steroidogenesis, ovaries from cyclic cows were screened for the expression of oestrogen sulfotransferase (SULTE1) and steroid sulfatase (STS) by Western blot and immunohistochemistry. Moreover, a broad spectrum of 13 sulfonated steroids was measured applying liquid chromatography-tandem mass spectrometry (LC–MS/MS) in blood samples collected from three cycling heifers during defined stages of the ovarian cycle and in fluid obtained from ovarian follicles of different size. SULT1E1 was undetectable in ovarian tissues. For STS only a weak immunostaining was found predominantly in granulosa cells of larger follicles. However, no specific band occurred in Western blot. In blood, concentrations of all sulfonated steroids investigated were below the limit of quantification (LOQ). In follicular fluid, only cholesterol sulfate was measured in considerable concentrations (328.3 ± 63.8 ng/ml). However, the role of cholesterol sulfate in bovine follicular steroidogenesis remains unclear as concentrations were obviously unrelated to follicular size. The remaining sulfonated steroids investigated were undetectable or only slightly exceeded LOQ in a minor proportion of samples. The results are clearly contrary to a role of sulfonated steroids as important precursors, intermediates or products of bovine ovarian steroidogenesis.

## 1. Introduction

The ovarian follicle can be considered as an extremely fragile microenvironment. In antral follicles, numerous factors may affect oocyte maturation and the acquisition of developmental competency. Steroid hormones produced by cooperation between theca and granulosa cells are among the essential regulators in the well-orchestrated process of follicular development. Theca cells provide the androgens which are converted to oestrogens by the granulosa cells [1]. Unconjugated steroids are able to diffuse across biological membranes in order to cooperate with their respective nuclear receptors and to initiate a biological response [2,3].

Sulfonation of steroids, which in humans is mainly performed in the liver and adrenal gland, was primarily considered to generate biologically inactive metabolites provided for elimination due to their increased water solubility and restricted diffusion volume [4]. The responsible enzymes belong to the superfamily of cytosolic sulfotransferases (SULTs). In mammals, the so far identified SULT genes

*Abbreviations:* A4, androstenedione; AnDiolS, 5-androstene-3β,17β-diol sulfate; An, androsterone; AnS, androsterone sulfate; APCI, atmospheric pressure chemical ionization; CholS, cholesterol sulfate; CL, corpus luteum; COCs, cumulus-oocyte-complexes; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate; 16OHDHEAS, 16α-hydroxydehydroepiandrosterone sulfate; DHTS, 5α-dihydrotestosterone sulfate; eAnS, epiandrosterone sulfate; E1S, oestrone sulfate; E2, oestradiol-17β; E2S, 17β-oestradiol-3-sulfate; ESI, electrospray ionization; eTS, epitestosterone sulfate; FF, follicular fluid; GC/MS, gas chromatography-mass spectrometry; IgG, immunoglobulin G; IRS, immunoreactive score; IVM, in vitro maturation; LOQ, limit of quantification; LC–MS/MS, liquid chromatography-tandem mass spectrometry; 17OHP4, 17α-hydroxyprogesterone; 17OHP5, 17α-hydroxypregnenolone; 17OHP5S, 17α-hydroxypregnenolone sulfate; P4, progesterone; P5, pregnenolone; P5S, pregnenolone sulfate; PBST, phosphate buffer saline with Tween-20; RIA, Radioimmunoassay; SDS, sodium dodecyl sulfate; STS, steroid sulfatase; SULT, cytosolic sulfotransferase; SULTE1, oestrogen sulfotransferase; T, testosterone; TS, testosterone sulfate

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were divided into five families, SULT 1–5 [5]. These enzymes catalyse the transfer of a sulfonate group from 3`-phosphoadenosine-5´phosphosulfate (PAPS) to an acceptor hydroxyl group of the substrate. In the pre-molecular biological era the characterization of SULTs was difficult as they are structurally closely related, generally accept several substrates and may exhibit overlapping substrate specificities. Commonly, SULT1E1 is considered as highly specific for oestrogens. In addition to its expression in classical excretion organs (liver, kidney) it may also occur in oestrogen target tissues to fine-tune the local oestrogen activity [6]. The occurrence and role of SULT1E1 in growing and maturing follicles is still widely unclear. SULT1E1 expression was detected in mouse ovaries and it has been concluded that oestrogen inactivation may enable the expression of prostaglandin-endoperoxide synthase-2 (PTGS-2; synonym cyclooxygenase-2, COX-2) and subsequent cumulus expansion to allow normal ovulation [7]. Ovarian SULT1E1 expression has also been detected in equine follicles [8] during human chorionic gonadotropin (hCG)-induced ovulation/luteinisation. After hCG injection, SULT1E1 transcripts significantly increased after 30–39 h in granulosa cells indicating that in the equine ovary luteinisation is not only accompanied by a decrease in E2 biosynthesis but also by an inactivation of previously formed oestrogens [8].

In addition to their role as inactive metabolites, during the past years sulfonated steroids have been increasingly considered as a pool of precursors for the local production of bioactive steroids. The removal of the sulfate moiety from the sterane backbone, which is catalysed by the enzyme steroid sulfatase (STS), may directly provide active forms or unconjugated substrates for further subsequent conversions [4]. In humans direct conversions of some sulfonated steroids into other subspecies without cleavage of the sulfo group are possible as demonstrated [9]. STS is a member of the arylsulfatase enzyme family [10] and mainly occurs as a membrane-bound protein in the endoplasmatic reticulum. In the human ovary, high STS expression was found in cumulus cells from patients with endometriosis suggesting local steroidal regulation mechanisms for the environment of oocytes [11]. Moreover, the ovarian expression of STS has been detected in rat ovary [12] and in the equine follicle during hCG-induced ovulation/luteinisation. STS transcripts isolated from granulosa cells of equine pre-ovulatory follicles showed a significant decrease 24–39 h post-hCG application [13]. It was suggested that this down-regulation in equine luteinizing preovulatory follicles may explain a further possibility of the decrease in E2 biosynthesis after the LH surge by an insufficient hydrolysis of sulfonated precursors and not only by a decrease of steroid synthesis from unconjugated substrates. Taken together, these observations point to a possible utilization of sulfonated substrates as an alternative or complementary pathway of ovarian steroidogenesis depending on STS activity. Moreover, the expression of oestrogen receptors indicates that the ovary is sensitive to estrogenic activity [14], which may be locally modulated by an interplay of STS and SULT1E1. To our knowledge, no information is currently available on the expression of these two enzymes for the bovine ovary. As information about the formation of sulfonated oestrogens and their hydrolysis within follicular development and ovarian steroidogenesis may contribute to a better understanding of bovine ovarian function and could be useful for the optimization of in vitro systems applied for bovine embryo production, major aims of this study were to characterize the expression patterns of STS and SULT1E1 in the bovine ovary as a function of the ovarian cycle and to monitor concentrations of various sulfonated steroids in the systemic circulation throughout the ovarian cycle applying liquid chromatography-tandem mass spectrometry (LC–MS/MS). To date no information is virtually available on the occurrence of sulfonated steroids in non-pregnant female bovines. Moreover, to assess steroid sulfonation in bovine ovarian follicles in a more direct manner, concentrations of sulfonated steroids were also determined in ovarian follicular fluid. In addition to oestradiol-17β (E2) and progesterone (P4), information on concentrations of unconjugated steroids is only very limited in cycling cattle, dehydroepiandrosterone (DHEA) and 17α-hydroxyprogesterone (17OHP4) were also included in the measurements.

## 2. Materials and methods

### 2.1. Sample collection

Bovine genital tracts were collected from a local abattoir (Fleischmarkt Olpe, Olpe, Germany). They were categorized according to their oestrus cycle stage based on morphological criteria (ovaries: presence of dominant follicles and/or corpus luteum (CL), size and number of miscellaneous follicles, uterus: closure of the cervix, amount of mucus) and following the nomenclature published by Senger [15]: pro-oestrus: phase between luteolysis and the onset of oestrus; oestrus: the period during which the female tolerates copulation; postoestrus: the stage between ovulation and formation of a functional CL; interoestrus: period characterized by the dominance of luteal progesterone. For each cycle stage three pairs of ovaries were preserved and transported to the laboratory on ice. Moreover, ovaries corresponding to the interoestrus were used to collect follicular fluid (FF) samples. They were classified according to their diameter into the following categories I: 3–5 mm, II: 6-8 mm, III: 9-14 mm and IV: ≥ 15 mm. The fluid was withdrawn from the follicles by aspiration and fluids from the same follicle category and individual animal were pooled. Granulosa cells and the cumulus-oocyte-complexes (COCs) were removed via centrifugation for 3 min at 300 × *g*. The collected FF was stored at −20 °C until the analyses. Additionally, for the preparation of COCs, suitable ovaries without obvious pathological alterations were transported to the laboratory in 30 °C warm PBS within 3 h after slaughter.

### 2.2. Sample processing for immunohistochemistry, staining procedure and evaluation of immunostained sections

For use in immunohistochemistry, ovaries were gently cut and tissue samples (ovarian stroma with or without parts of larger follicles; corpora lutea) with a maximum size of 1 $cm^3$ were prepared from different regions of the ovaries. Samples were fixed for 24 h in 10% neutral phosphate buffered formalin, followed by dehydration through a graded ethanol series and finally embedded in paraffin wax (Histo-Comp-Vogel, Giessen, Germany).

Standard indirect immunoperoxidase staining methods were applied using the avidin-biotin technique for signal enhancement. Tissue sections (3 µm) were mounted on coated slides (Superfrost Plus®, Thermo Scientific, Germany) and boiled for 20 min at 95–99 °C in 10 mM citrate buffer pH 6.0 for antigen retrieval. As primary antibodies, a rabbit antiserum against human STS [16] and a polyclonal rabbit antibody against recombinant bovine SULT1E1 (#MM-0115-P, MediMabs, Montreal, Canada; [17]) were used at a dilution of 1:2000, respectively. Blocking serum (from goat), biotinylated secondary antibody (goat anti-rabbit immunoglobulin G; IgG) and avidin-biotin-complex-horseradish peroxidase were taken from the Vectastain Elite ABC Kit (PK-6105; Vector Laboratories, Burlingame, USA) and applied as specified by the supplier. NovaRED was used as a substrate (Substrate Kit for Peroxidase, Vector Laboratories, Burlingame, USA). As a positive control tissue, bovine placenta from late pregnancies was applied (STS: [18]; SULT1E1: [19]). In negative controls, the primary antibody was replaced by non-specific purified rabbit IgG (Invitrogen, Camarillo, CA, USA) at the same dilution as the primary antibodies. For a better differentiation between weak signals and nonspecific background, counterstaining was omitted.

For each animal, one slide was evaluated per ovary, except for ovaries with a large follicle or CL. In these cases two slides per ovary were evaluated. Semi-quantitative evaluation of immunostaining in ovarian stroma, granulosa cells, theca cells, oocytes, CL and endothelium of blood vessels was performed using a modification of the immunoreactive score (IRS) described by Ellenberger et al. [20]. For