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Expression of NA/1 symporter (NIS) in endometrial mucosa of fertile, sterile and post-menopausal women

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Summary. The expression of the Na/I Symporter (NIS) in the basolateral cell membrane of the thyroid follicular cells is responsible for the active accumulation of iodide within the thyroid gland and for the subsequent biosynthesis of thyroid hormones. However, several tissues, such as salivary glands, breast, stomach, colon, ovary and endometrium, express NIS even if they are unable to organify iodide.

In order to investigate a possible role of NIS in the endometrium, we analyzed, by immunochemistry, the expression of NIS in 44 endometrial samples of 20 patients with primary unexplained infertility, 14 fertile women and 10 in postmenopausal.

NIS immunostaining was detected in endometrial cells belonging to the majority of sterile, post-menopausal and fertile women. However, the sterile and post-menopausal patients showed a higher percentage of NIS reactive cells compared to the fertile women (60±21% and 57±18% vs 19±9%; p=0.0001). NIS immunostaining was localized on the membrane and cytoplasm of the endometrial cells. We could not find any correlation between endometrial thickness and NIS immunoexpression. Our results indicate that, in the absence of histological markers, a sterile endometrium can be recognized because of the high expressions of NIS. Moreover, NIS expressions, elevated in both sterile and menopause women, is not related to the estrogen levels, but it could be modulated by factors common to the two conditions. In conclusion, we speculate that NIS may play a role in the development of female sterility.

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Introduction

Na/I Symporter (NIS) drives the transcellular iodide uptake occurring from the basal to the apical membrane of the thyrocyte (Dai et al., 1996). This is an active process of transport because it operates against a NA^+ electrochemical gradient that is overcome by the NA^+/K^+ ATPase energy (Carrasco, 1993). NIS expression on the cellular membrane of thyrocytes is required to concentrate iodide (Carrasco, 1993). However, both NIS expression and iodide uptake are not limited to the thyroid follicular cells. Several epithelial cells belonging to different organs, such as the lactating mammary gland, the salivary glands, and the gastric mucosa share with the thyroid gland the ability to concentrate iodide (Carrasco, 1993; Ajjani et al., 1998; Jhiang et al., 1998, 1999; Spitzweg et al., 1998; Filetti et al., 1999; Spitzweg et al., 1999; Vayre et al., 1999; Kilbane et al., 2000; Spitzweg et al., 2000; Tazebay et al., 2000; Lacroix et al., 2001; Josefsson et al., 2002; Spitzweg and Morris, 2002). The peculiarity of thyroid follicular cells relies in the use of iodide once it has been accumulated inside them. Only thyrocytes, in fact, are able to organification of iodide in tyrosyl residues of thyroglobulin (Carrasco, 1993), and this process is catalyzed by the thyroid-specific enzyme thyroperoxidase (TPO), under the control of TSH (Robbins et al., 1980).

While the role of both iodide and NIS in the thyroid has been extensively evaluated, little is known about the function on NIS and of iodide in other NIS-expressing tissues.

The mammary gland, in the lactotrophic status, expresses high levels of NIS, mostly localized on the

epithelial alveolar cells. This expression seems to be necessary for supplying a sufficient amount of iodine to the nursing newborn (Ajjani et al., 1998; Tazebay et al., 2000). In addition, iodide uptake by mouse cultured mammary cells has been reported to be stimulated by prolactin (Rillema and Yu, 1996) and by estrogens. In mammary gland tissue, the stimulation of NIS expression and activity by prolactin and estrogens provides an important regulatory mechanism for iodide accumulation in milk during lactation (Carrasco, 1993; Rillema and Rowady, 1997; Tazebay et al., 2000; Spitzweg and Morris, 2002). Thus, in lactating breast, it has been postulated that once the iodide has been accumulated inside the mammary epithelial cells, it undergoes a process of organification, in a similar manner to that taking place in the thyroid, into the casein and other milk proteins by means of the lactoperoxidase; whereas, circulating estrogens have been implied in the regulation of NIS expression (Tazebay et al., 2000; Spitzweg and Morris, 2002).

Expressions of NIS have been detected in the salivary gland as well as in gastric mucosa (Ajjani et al., 1998). In the salivary gland, NIS expression has been mainly found in epithelial ductal cells, while in the stomach, NIS is strongly expressed from mucin-secreting cells of the gastric antrum, even if chief cells may express NIS (Jhiang et al., 1998; Filetti et al., 1999; Spitzweg et al., 1999; Vayre et al., 1999; Josefsson et al., 2002). In both tissues, the active iodide transport has been correlated with a possible conservation of iodine by an entero-thyroid recirculation of iodide carrying out a protective role against iodine deficiency (Josefsson et al., 2002).

Few data are available regarding NIS expression in human or mouse uterus (Kotani et al., 1998; Spitzweg et al., 1999; Lacroix et al., 2001; Perron et al., 2001; Wapnir et al., 2003; Di Cosmo et al., 2006). Spitzweg et al. did not observe any expression of NIS in the uterus but they did not specify on whether the absence of expressivity concerned the muscular or mucosa portion of the uterus (Spitzweg et al., 1999). Locroix et al., detected a clear NIS expression in endometrium, but not in myometrium (Lacroix et al., 2001). Wapnir et al., and Di Cosmo et al., found expression of NIS localized inside the endometrial cells (Wapnir et al., 2003; Di Cosmo et al. 2006). So far, the function of iodide and the possible role of NIS in this tissue is not clear. The endometrial mucosa is characterized by variability of the mucosal thickness and histological findings as well as the capacity to receive the conception product because of the estrogens-progesterone hormonal axis. We have analyzed the role of NIS in endometrial estrogenic mucosa by evaluating women with menstrual cycles and in the post-menopausal period. Moreover, among the menstruating women we have analyzed NIS expression in both the fertile and sterile female. We found a similar increase in NIS expression in the endometrial cells of sterile and post-menopausal women compared to fertile ones, suggesting a role of NIS in the regulation of fertility.

Materials and methods

Subjects of study

Forty-four women were selected for the study and evaluated at the Department of Gynecologic and Obstetric Science and Reproduction Medicine of the University of Messina or at the Human Reproduction Center of Messina. They did not show hypothyroidism signs or any enlargement of the thyroid gland and were healthy with respect to chronic illnesses. Ten of these women with an age ranging between 56 and 74 years (mean age ± SD: 63.5±6.5) were in menopause and thirty-four were in fertile age, had regular menstrual cycles and were not taking any contraceptive drugs or using an IUD. Twenty of the menstruating woman, with an age ranging between 20 and 40 years (mean age ± SD: 31.5±5.7), suffered from idiopathic sterility of at least two years duration; and 14 women, with an age ranging between 31 and 42 (mean age ± SD: 37.2±3.9), have had at least one birth.

Formal consent was obtained, and the study was approved by the Ethics Committee of the Faculty of Medicine, University of Messina, Italy.

Idiopathic sterility was defined as the absence of an apparent cause for sterility after a series of medical examinations, which included estradiol levels, histopathological evaluation of the endometrium, ultrasonography, semen analyses, hysterosalpingography and laparoscopy.

Menstruating and post-menopausal women underwent a transvaginal sonographic examination of the thickness of the uterine cavity by a transvaginal probe with 7.5 MHz transducer, which in menstruating women was performed in the pre-ovulatory phase. Endometrial thickness was measured at the point of maximal thickness (junction of the endometrium and myometrium) and an endometrial biopsy was performed in all subjects.

Tissue collection

Endometrial biopsy samples were obtained from the anterior wall of the uterine cavity. Each sample was immediately fixed in 4% formalin and then routinely processed at the Department of Human Pathology, University of Messina, Italy. Paraffin blocks of the endometrial samples were cut into 5-μm serial sections to perform histochemical stains, such as Haematoxylin-eosin (H&E) and Giemsa, as well as immuno-histochemistry.

Immunohistochemistry

Immunohistochemistry was performed using a mouse monoclonal antibody (MAb) raised against the peptide enclosed among residues 625-643 of human Sodium Iodide Symporter (hNIS, clone SPM186, Spring Bioscience, Fremont, CA, USA). Antigen retrieval technique was carried out as described by Gown et al.

(Gown et al., 1993). The sections were deparaffinized in xylene, re-hydrated through serial passage in graded alcohol. Endogenous biotin was inactivated by addition of 0.05% (v/v) solution of streptavidin in phosphate-buffered saline (PBS) and endogenous peroxidase activity was quenched by 30 min incubation with 0.3% (v/v) H_2O_2 in absolute methanol. Subsequently, the slides were placed in 10 mM citrate buffer (pH 6.0) and heated for 15 min in a microwave oven (Whirlpool AVM 300, power set at 500 watts). Microwave exposure was broken into three equal time periods and, at the end of the first cycle, 50 μl of distilled water were added to the slide holder to prevent loss of fluid from boiling. To each slide was added hNIS primary antibody, diluted 1:50 in 10nM PBS. After, the slide was incubated overnight in a moist chamber at +4°C. The staining was visualized using the LSAB system (LSAB kit from Dako Corporation, Carpinteria, CA) and the colour reaction was developed by 3,3'-diaminobenzidine (DAB, Sigma) activated with 0.05% hydrogen peroxide. Sections were counterstained with Mayer's haematoxylin, dehydrated and mounted. Specificity of the binding was assessed either by omitting the primary antiserum or by replacing the primary antiserum with normal mouse serum. In neither of these conditions was staining evident. An immunoabsorption test was performed to confirm the specific immunoreactivity of hNIS. Thyroid specimens showing Graves' disease were used as positive controls of the hNIS immunoreaction (Saito et al. 1997; Filetti et al., 1999). For the evaluation of the results, the following criteria were used: (i) number of positive cases; (ii) number of reactive cells per case, the number of cells was estimated counting 600 cells/case and using 50 X magnification; (iii) sub-cellular location of the staining: membrane and cytoplasm; (iv) semiquantitative staining grade using a score system from 0 to 4+ (0 = absent; 1 = weak; 2 = moderate; 3 = intense; 4 = very intense).

Histological and immunohistochemical evaluations were performed twice and blindly by three different pathologists (MT, MG and GB), with an inter-observer concordance of nearly 100%. Where minimal inter-observer discrepancies were present, the mean value was considered as the result.

Table 1. NIS expression in sterile, fertile and menopause women.

Endometrial samples	Reactive endometrial samples	% Reactive Cells[a,b]	Intensity[c]
Sterile women	17/20	60±21	3±1
Fertile women	12/14	19±9	3±1
Post-menopausal women	8/10	57±18	3±1
p value		p=0.0001	

[a]: Data are expressed in mean ± DS and analyzed by two-tailed Student T test with Yates correction for continuity; [b]: The proportion of positive cells was calculated based on evaluation of 600 cells using x 50 magnification as specified under Materials and Methods; [c]: Staining was scored on a five-point scale from 0 (absent) through 4+ (very intense) as specified in Materials and Methods.

Statistical analysis

Data were expressed in mean ± SD. The normal distribution, variance and two-tailed Student T test with Yates correction for continuity were tested by the Primer statistical program. Correlation between two variables has been analyzed with the linear tendency of Microsoft Excel program. The level of statistical significance was set at $p<0.05$.

Results

Histological evaluation

In both sterile and fertile women, the histological features of endometrial samples were compatible with the mid-late proliferative phase and showed quite similar characteristics. In particular, all specimens, showed a large prevalence of glands covered by one or more lines of epithelial cells with eccentric, large and stratified nuclei, nucleolar prominence and moderate mitotic activity.

In post-menopausal women, the endometrial mucosa showed few glands of regular contours and small caliber, lined by single layers of small endometrial cells. Cystic dilatations were focally encountered in all post-menopausal endometrial samples.

No signs of inflammation or benign or malignant proliferations were observed in any endometrial sample examined.

NIS immunohistochemistry

The expression of NIS was seen in the majority of endometrial samples of the sterile, fertile and post-menopausal women (17/20 or 73% vs 12/14 or 86% vs 8/10 or 80%); (Table 1).

In all groups, the NIS immunostaining was observed in endometrial cells. Comparison of the number of NIS reactive cells detected among the three groups revealed that the expression of NIS was quite similar in sterile and menopause women; both groups showed a significantly higher NIS immunoreaction compared to fertile women ($p = 0.0001$); (Table 1).

In all positive samples, NIS immunostaining showed a membranous and cytoplasmatic sub-cellular localization (Fig. 1A-C); whereas, thyroid control tissue expressed NIS only on the membrane of follicular cells (data not shown).

In reactive samples, the grade of NIS immuno-staining was moderate, intense or very intense (Table 1).

Correlation of NIS expression with endometrial thickness

Average endometrial thickness, measured in both sterile and fertile menstruating women, was 7±2 mm. A thinner endometrium (4±1 mm) was seen in post-menopausal women. We tried to correlate the

endometrial thickness with the expression of NIS, but no correlation was found in any sterile (r = 0.48), fertile (r = 0.36), or post-menopausal (r = 0.19) group (Fig. 2A-C).

Discussion

Unexplained sterility is a growing phenomena that affects approximately 10–15% of couples (ASRM, 2004). Therefore, it represents a relevant medical, social and economic health problem. Many efforts are currently being made to find new biomarkers and to identify the causes of sterility (Acosta et al., 2000; Lindhard et al., 2002). Female causes account for 40%–50% of the cases (Duckitt, 2003). The endometrial mucosa is particularly involved in the pathogenesis of female sterility because it receives the conception product. The cyclic maturation of endometrium depends mainly on the interplay between the gonadal steroids estrogen and progesterone, but numerous other endocrine, paracrine, and autocrine factors have been implied too (Krussel et al., 2003). Hormonal factors during the late proliferation and the luteal phases are crucial for the preparation of endometrium to the blastocyst implantation (Krussel et al., 2003).

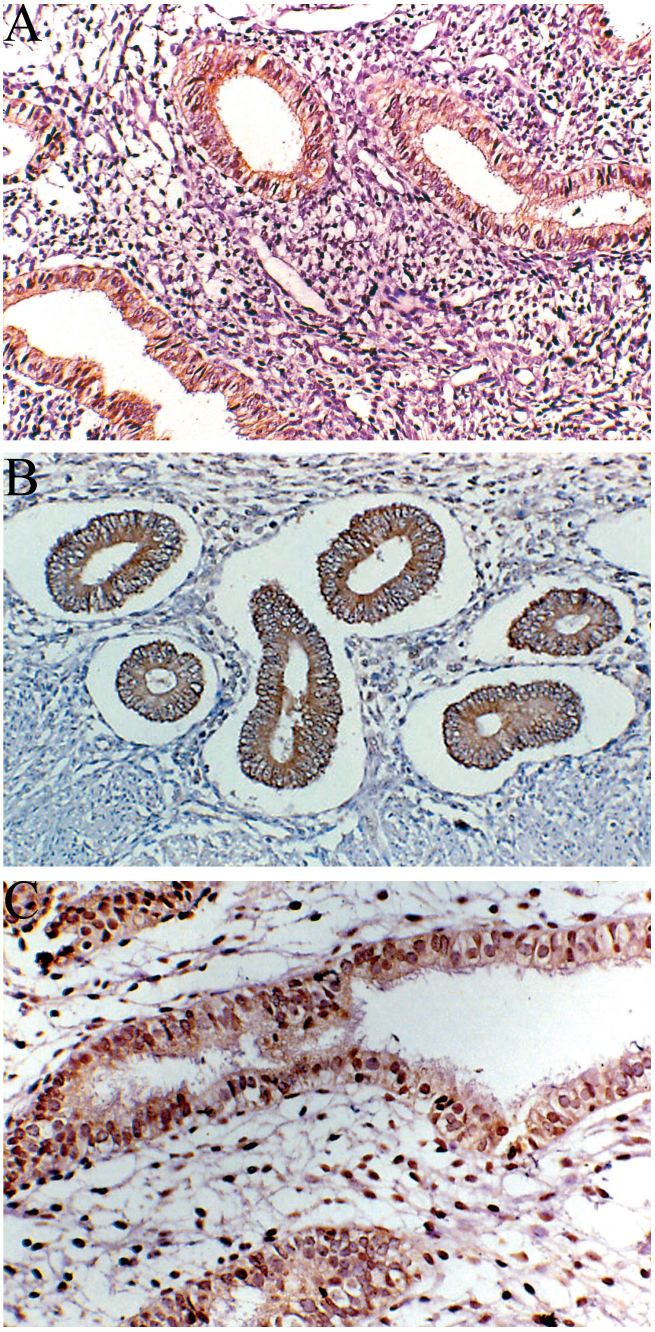

Fig. 1. NIS immunostaining in endometrial cells of fertile, post-menopausal and sterile women. Membranous and cytoplasmatic NIS immunostaining in endometrial cells belonging to a fertile (**A**), post-menopausal (**B**) and sterile woman (**C**). A, B, x 130; C, x 250

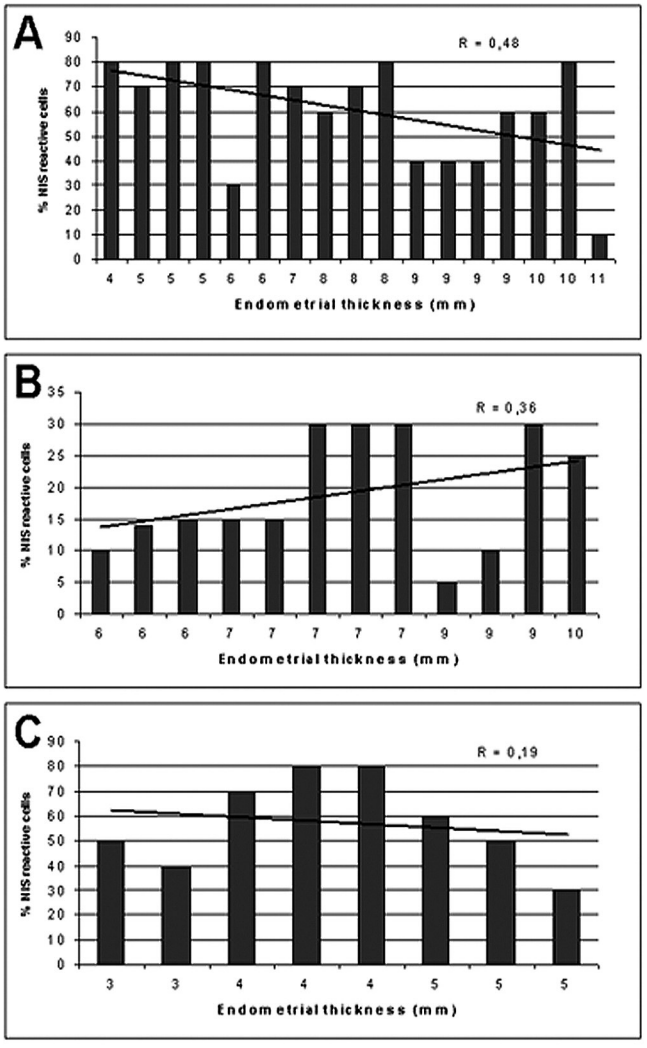

Fig. 2. Linear tendency between the increase of endometrial thickness and NIS expression in sterile (**A**), fertile (**B**) and post-menopausal (**C**) women group. Linear tendency analysis was performed by Microsoft Excel program.

Endometrial biopsies analysis after H&E and Giemsa staining represents the best way to evaluate the maturation stage. Immunohistochemical approaches can give additional quantitative and qualitative information about the distribution of those factors regulating the endometrial mucosa maturation.

In this study, we report our experience on the immunohistochemical expression of NIS on the endometrial mucosa of sterile and fertile women in mid-late proliferative phase. Our results demonstrate that NIS expression is significantly higher in sterile women compared to fertile women. Immunohistochemical expression of NIS in endometrial mucosa may therefore represent a new putative marker, capable of distinguishing the sterile from the fertile endometrial mucosa. On the basis of this evidence, and since sterile and fertile endometrium are indistinguishable from the histological point of view, we suggest that NIS expression could be introduced among the recommended examinations suggested in the evaluation of an idiopathic sterility.

Our results also indicate that the expression of NIS is higher in the post-menopausal period compared to fertile women (60% vs 20% of endometrial cells). It appears likely that estrogens could be responsible for this effect and that they may play a role in the regulation of NIS expression in endometrial mucosa. Indeed, these conclusions are in agreement with previously reported results, obtained on thyroid cells, and indicating that estradiol downregulates NIS expression and upregulates thyroid cell growth (Furlanetto et al., 1999, 2001; Dohan et al., 2003). However, we observed quite similar expressions of NIS in both post-menopausal and sterile patients, and, more importantly, the sterile women did not show any estradiol deficiency. These data seem to exclude a direct action of this hormone on NIS endometrial expression. The lack of correlation between estrogen levels and NIS expression is also confirmed by the absence of any correlation between the increase of endometrial thickness, a well known biological target of estrogen stimulation, and the expression of NIS in all our women, analyzed in sterile, fertile or post-menopausal conditions. These data indicate that the correlation between estradiol levels and NIS expression reported in thyroid tissue, does not occur in human endometrial mucosa. Therefore, overexpression of NIS in endometrial mucosa of sterile and post-menopausal women is modulated by other yet-to-be-discovered factors.

According to previous reports, we found that NIS expression was mainly localized in both membrane and cytoplasm of endometrial cells (Lacroix et al., 2001; Wapnir et al., 2003). In thyroid tissue the expression of NIS was recognized only on the basolateral part of the cell membrane. The expression of NIS in both membrane and cytoplasm has been largely debated because only the membranous NIS appears to be functionally active in the transport of iodide. In this regard, an active transport of iodide through NIS was demonstrated only when a NIS expression was localized on the plasma membrane, while the intracellular NIS appears to be functionally inactive (Wapnir et al., 2003). Recently, a possible role of cytoplasmic NIS was postulated in cancer cells. It has been hypothesized that NIS could also function as a reverse carrier of iodide, transporting iodide from inside toward the outside of the cells (Dingli et al., 2004). Specifically, several studies have examined the uptake of a high-dose of radioiodine and they have demonstrated that cancerous cells without the ability for iodide organification show the expression of NIS related with the duration of iodide retention (Nakamoto et al., 2000; Dingli et al., 2004). Thus, NIS expression has been implied in the carrier mechanism responsible for the iodide leak out. Considering these data, we may suppose that NIS expressions in the endometrium may be related with a dynamic trapping of iodide. Therefore, in post-menopausal and sterile women the over-expression of NIS could be associated with a major retention of iodide, altering the fertility of the endometrial mucosa. However, further studies on the mechanism of action of iodide are needed to demonstrate the exact role of NIS in endometrium, either in normal fertile or in sterile conditions.

In conclusion, we found that the expression of NIS is increased in sterile women, compared to fertile ones. This finding suggests a possible role of NIS immunoexpression in the diagnostic evaluation of female with unexplained fertility problems, and open a new line of investigation regarding the possible role of NIS in female fertility in either high- or sufficient iodide intake areas or in iodide-deficient regions.

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