RESEARCH ARTICLE

# Epigallocatechin gallate affects survival and metabolism of human sperm

*Francesca De Amicis[1,2], Marta Santoro[1,3], Carmela Guido[1], Alessandra Russo[1] and Saveria Aquila[1,2]*

[1] Centro Sanitario, University of Calabria, Arcavacata di Rende (CS), Italy
[2] Department of Pharmaco-Biology, University of Calabria, Arcavacata di Rende (CS), Italy
[3] Post-graduate School in Clinical Pathology, University of Calabria, Arcavacata di Rende (CS), Italy

Received: April 3, 2012
Revised: August 1, 2012
Accepted: August 13, 2012

**Scope:** Green tea and its major constituent epigallocatechin gallate (EGCG) have been extensively studied as potential treatment for a variety of diseases. We assessed the influence of EGCG on male fertilizing potential by analyzing different features of human sperm involved in capacitation process.

**Methods and results:** Using human normozoospermic samples, we evaluated the effect of EGCG (2 μM, 20 μM, 60 μM) on sperm activities. Our results showed that lower doses of EGCG (from 2 to 20 μM) increased cholesterol efflux and tyrosine phosphorylation through the estrogen receptor (ER), since ICI 182,780, a specific ER antagonist, abrogated 20 μM EGCG effects. Besides, we evidenced that EGCG at similar concentrations, increased sperm motility, viability, and phosphorylation of proteins controlling cell survival such as Bcl2, Akt, and Src, via ER. Furthermore, we observed reduction of triglycerides content, induction of lipase, as well as the G6PDH activity. These results address to an increase in energy expenditure. On the contrary, treatment of 60 μM EGCG produced opposite effects that still appear after ICI cotreatment.

**Conclusion:** These results provide a novel mechanism involving ERs through which low doses of EGCG exerted benefits to sperm physiology, also detected data evidence the adverse action of high EGCG concentrations probably related to its prooxidant and antiestrogenic potential.

**Keywords:**
Epigallocatechin / Green tea / Reproduction / Sperm / Metabolism

## 1 Introduction

An expanding body of preclinical evidences suggests that epigallocatechin gallate (EGCG), the major catechin found in green tea, has the potential to impact a variety of human diseases. EGCG depending on concentrations, functions as a powerful antioxidant, preventing oxidative damage in healthy cells, but also as an antiangiogenic and antitumor agent with chemopreventative properties [1].

The green tea extracts (GTEs) show protection of cardiovascular system [2], reduction of blood glucose and cholesterol [3], and anti-inflammatory [4] effects. Besides in vitro research using human cancerous cell lines has shown EGCG to inhibit cell proliferation [5–7], to alter progression through the cell cycle [8, 9], to induce apoptosis [8–11], and to promote telomere shortening [12].

Moreover, catechins oral administration in rats has been reported many endocrinological actions. Among others, Divi and Doerge [13] demonstrated that catechin inhibits thyroid peroxidase (TPO), though in vivo long-term effect on thyroid functions has not been reported. Administration to rats of Polyphenone-60 (P-60), which are water extracts of green tea leaves, at high dose induced goiters and decreased weights of the testis and prostate gland in F344 rats [14]. However, the effect of catechins on endocrine system especially related to male fertility was seldom studied [15]. There was evidence that green tea polyphenols inhibited testosterone production in rat Leydig cells (LCs) in vitro by inhibitions of the PKA/PKC pathways, P450 side chain cleavage enzyme (P450scc), and 17b-hydroxysteroid dehydrogenase (17b-HSD) [16]. It is worth of notice that EGCG, via its gallate group, may act on the Estogen Receptor (ER-α and ER-β), thereby mimicking the

**Correspondence:** Professor Saveria Aquila, Centro Sanitario, University of Calabria, Arcavacata di Rende (CS) 87036, Italy
**E-mail:** aquisav@libero.it
**Fax:** +39 0984 496204

**Abbreviations:** **Bcl2**, B cell lymphoma gene-2; **EGCG**, epigallocatechin-gallate; **E2**, 17-β-estradiol; **ER-α**, estrogen receptor alpha; **ER-β**, estrogen receptor beta; **G6PDH**, glucose-6-phosphate dehydrogenase; **PI3K**, phosphoinositide 3-kinase; **PR**, progesterone receptor

© 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim 

7α-position of 17β-estradiol (E2) [17]. In target cells, it exerts opposite effects, eliciting antiestrogenic action and cytotoxicity [18, 19] at higher doses (100–150 μM), while coestrogenic for ER-α at lower doses (≤5 μM).

ER-α and ER-β are detected in germ cells from spermatogonia to spermatozoa [20, 21]. In human sperm, ERs were found differently located since both receptors are in the midpiece, while ER-β also in the flagellum. Sperm are able to synthesize estrogen [22] and in ejaculated sperm, E2/ERs activate the PI3K/Akt pathway, stimulates various sperm functions including motility, longevity, capacitation, and acrosome reaction [23, 24].

Immediately after ejaculation, human spermatozoa do not possess the ability to fertilize an oocyte, but they acquire this ability in the female reproductive tract. This process is known as capacitation [25–27]. Capacitation enables the sperm to bind to the zona pellucida (ZP) and undergo the acrosome reaction, to facilitate the penetration of the ZP and, subsequently, to aid the oocyte–sperm fusion process [25].

Despite the data supporting the beneficial effects of a diet rich in green tea polyphenols, few studies have shown their safety and potential toxicity when administered at high doses, as highly concentrated or nearly pure compounds [28].

Hence, by analyzing several biochemical changes of capacitation, we assessed the influence of different concentrations of EGCG on male fertilizing potential. Since the tyrosine kinase Src is a key player in the signal transduction cascade occurring during sperm capacitation [26] while Akt and Bcl2 in survival, we assessed their expression. In addition, as there is a close link between energy balance and reproduction [29], and we recently reported that sperm cells are able to modulate their own metabolism independently of systemic regulation [30], we evaluated the action of EGCG on lipid and glucose metabolism in human sperm.

## 2 Materials and methods

### 2.1 Chemicals

Percoll (colloidal PVP-coated silica for cell separation), sodium bicarbonate, sodium lactate, sodium pyruvate, DMSO, Earle's balanced salt solution, and (−)-EGCG from green tea were purchased from Sigma-Aldrich (# E4143) (Milan, Italy). ICI 182,780 (ICI, ER antagonist), and RU486 (PR antagonist) were purchased from Zeneca Pharmaceuticals (Cheshire, UK). Both are used to test the specific action of EGCG through ER but not through PR. Acrylamide bisacrylamide was from Labtek Eurobio (Milan, Italy). Triton X-100, Eosin Y was from Farmitalia Carlo Erba (Milan, Italy). ECL Plus Western blotting detection system, Hybond™ ECL™, Hepes Sodium Salt were from Amersham Pharmacia Biotech (Buckinghamshire, UK). Abs p-Src (Tyr139), p-Bcl2, p-Akt, total Src, total Bcl2, total Akt were from Santa Cruz Biotechnology (Heidelberg, Germany). Antiphosphotyrosine was from Calbiochem (Canada, US). EGCG was dissolved in water. ICI and RU486 were dissolved in ethanol (0.02% final concentration in culture). Ethanol was used as solvent control.

### 2.2 Semen samples and spermatozoa preparations

Human semen was collected, according to the World Health Organization [31] recommendations, by masturbation from healthy volunteer donors. Spermatozoa preparations were performed as previously described [22]. Each sperm sample was obtained by pooling the ejaculates of three different normozoospermic healthy donors. In our experience, this was necessary to obtain enough cells to perform all the tests [32, 33]. Final sperm concentration of resuspended samples during treatments was $20 \times 10^6$/mL.

After liquefaction, the normal ejaculates were pooled and subjected to centrifugation (800×g) for 5′ on a discontinuous Percoll density gradient (80:40% v:v) [31]. The 80% Percoll fraction was examined using an optical microscope at 100× magnification to ensure that a pure sample of sperm was obtained. Percoll-purified sperm was washed with uncapacitating medium (Earle's balanced salt solution medium) (t0), and incubated for 30 min at 37°C and 5% $CO_2$, without (−) or with the indicated concentrations of EGCG. Also, all the parameters were assessed at time 0 (t0). When combined treatments were performed, the cells were pretreated for 15 min with the ER antagonist ICI (1 μM) or RU486 (1 μM).

Each assay was performed using six different sperm samples. Histograms represent mean ± SD of six independent experiments each in duplicate. The study was approved by the local medical ethical committees, and all participants gave their informed consent.

### 2.3 Evaluation of sperm motility and viability

Sperm motility was assessed as previously described [33]. Viability was assessed by red eosin exclusion test using eosin Y [32].

### 2.4 Measurement of cholesterol in the sperm culture medium

Cholesterol was measured in duplicate in the incubation medium from human spermatozoa by a cholesterol oxidase–peroxidase (CHOD–POD) enzymatic colorimetric method accordingly to the manufacturer's instructions (Inter-Medical Biogemina, Naples, Italy). For each sperm, sample culture media were recovered by centrifugation, lyophilized, and subsequently dissolved in 1 mL of reaction buffer. The samples were incubated for 10 min at room temperature, then the cholesterol content was measured spectrophotometrically at 505 nm. The cholesterol standard used was 2 g $L^{-1}$. The limit of sensitivity for the assay was 0.005 mg $L^{-1}$. Inter- and intra-assay variations were 0.04 and 0.03%, respectively. Cholesterol results are shown as mg per $10^7$ spermatozoa.

© 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim 

### 2.5 Western blot analysis of sperm proteins

Each sperm sample treated as indicated was centrifuged for 5 min at 5000 × *g*. The pellet was resuspended in lysis buffer [22]. An equal amount of protein (80 μg) was boiled for 5 min and processed as previously described [33].

### 2.6 Triglyceride assay

Triglycerides were measured in duplicate as previously described [33] by a glycerol-3- phosphate oxidase–POD enzymatic colorimetric method according to the manufacturer's instructions (Inter-Medical). Data are presented as $\mu g/10^6$ spermatozoa.

### 2.7 Lipase activity assay

Lipase activity (Inter-Medical) was evaluated by the method of Panteghini et al. [34] based on the use of 1,2-*o*-dilauryl-rac-glycero-3-glutaric acid-(6′-methylresorufin) ester (DGGR) as substrate [33].

### 2.8 G6PDH activity

The G6PDH activity (Inter-Medical) was performed as previously described [32]. Data are expressed in nmol $min^{-1}/10^6$ spermatozoa. The enzymatic activity was determined with three control media: without glucose-6-phosphate as substrate, or without the coenzyme ($NADP^+$) and the third without either substrate or coenzyme (data not shown).

### 2.9 Measurement of glucose in the sperm culture medium

Glucose was measured in duplicate in the incubation medium from human spermatozoa by oxidase-POD reaction with a chromogen accordingly to the manufacturer's instructions (Inter-Medical). Ten microliters of the lysate were added to 1 mL of the buffer reaction and incubated for 10 min at room temperature. Then, the glucose content was measured at 500 nm using a spectrophotometer. Data are presented as $\mu g/10^6$ spermatozoa.

### 2.10 Statistical analysis

All data are presented as means ± SD for six experiments of six separate samples. Data were analyzed by two-way ANOVA test, using the GraphPAD Prism4 software (GraphPad Software, USA). Differences were considered statistically significant at $p < 0.05$.

## 3 Results

### 3.1 Effect of EGCG on human sperm motility and viability

We first determined flagellar sperm motility upon increasing concentrations of EGCG (2 μM, 20 μM, 60 μM) in human normozoospermic samples. Our data indicated that sperm motility was increased by EGCG at 2 and 20 μM (Fig. 1A), but not at higher concentration (60 μM). Similar results were obtained when we analyzed the effects of the same concentrations of EGCG on sperm viability (Fig. 1B).

It is reported that ECGC [35] is able to elicit antiestrogenic action [18, 19] at higher doses or coestrogenic for ER-α at lower doses. Since our previous studies [21] demonstrated that human sperm expresses the ERs mediating E2-induced capacitation and acrosome reaction in human sperm and aromatase, we hypothesized that both the "classical" ERs could be able to mediate EGCG action in sperm. Therefore, we pretreated the cells with ICI. This cotreatment abrogated the 20 μM EGCG effects either on sperm motility or viability (Fig. 1A and B) while no change was evidenced on 60 μM EGCG. Also, RU486 had no effect.

### 3.2 Double effects of EGCG on cholesterol efflux and protein tyrosine phosphorylation in human spermatozoa

We then investigated the influence of different concentrations of EGCG on sperm membrane cholesterol efflux and protein tyrosine phosphorylation [26, 27] in human normozoospermic samples. Our results showed that lower EGCG treatment (from 2 to 20 μM EGCG) increased the cholesterol efflux compared with untreated samples as shown in Fig. 2A, and ICI, but not RU486, abrogated 20 μM EGCG effect. No change was observed upon 60 μM EGCG also after ICI or RU 486.

It was reported that cholesterol efflux initiates signaling events leading to tyrosine phosphorylation of sperm proteins [26, 27]. In our study, it appears that an increase in the protein tyrosine phosphorylation was obtained upon EGCG, from 2 to 20 μM (Fig. 2B) and the combination of ICI markedly reduced the 20 μM EGCG action. On the contrary, treatment with higher EGCG dose produced a significant decrease of tyrosine phosphorylation compared with the untreated cells as indicated by densitometric evaluation.

### 3.3 EGCG modulates Src, Akt, and Bcl2 phoshorylation in human spermatozoa

We then evaluated the impact of EGCG on Src activity, evaluating the levels of tyrosine phosphorylation at position 139. The results of this study clearly showed that 2–20 μM EGCG significantly induced Src phosphorylation.

© 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim 

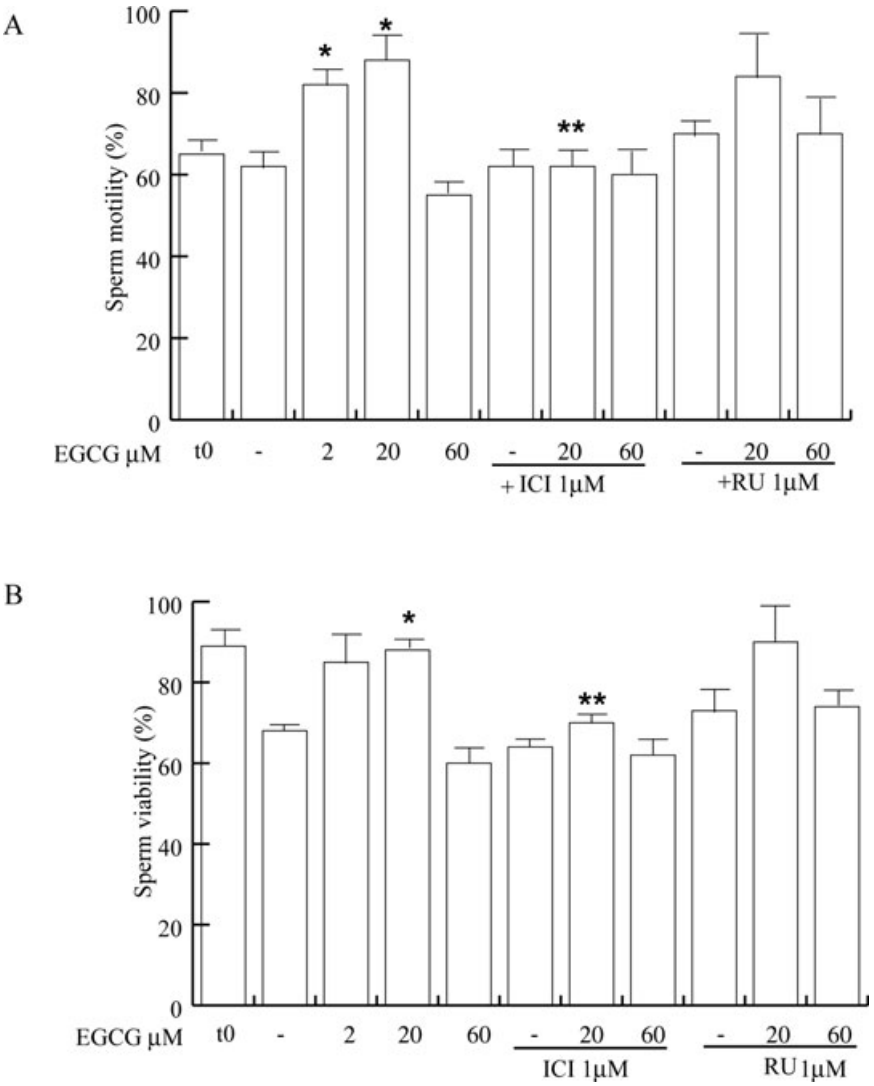


**Figure 1.** EGCG affects sperm motility and viability. Spermatozoa were incubated in unsupplemented Earle's medium (uncapacitating medium) for 30 min at 37°C and 5% $CO_2$, in the absence (−) or in the presence of increasing concentrations of EGCG, and/or ICI (1 μM) or RU486 (1 μM). Sperm motility (A) and viability (B) were assessed also at t0. *$p < 0.05$ versus untreated (−); **$p < 0.05$ versus 20 μM EGCG.

While RU486 cotreatment had no significant effect, the combination with ICI attenuated the 20 μM EGCG-induced effects (Fig. 3). At the opposite, high dose of EGCG reduced p-Src expression levels.

In order to provide further insights on the molecular action of EGCG, we also evaluated phosphorylation levels of key proteins controlling cell survival such as Akt and Bcl-2. Our findings indicate (Fig. 3) that EGCG at the concentration of 2–20 μM induced Akt protein phosphorylation and this disappear upon ICI cotreatment. At the concentration of 60 μM EGCG, Akt phoshorylation levels were reduced.

Furthermore, low concentrations of EGCG exposure enhanced Bcl-2 phosphorylation at Serine 70, which is a physiologically relevant phosphorylation site, necessary for a full and potent antiapoptotic function. This effect was greatly inhibited in the presence of ICI. All these results suggest that low concentrations of EGCG through ERs ameliorate survival in human sperm.

Although phosphorylation levels of the above-mentioned proteins were influenced by EGCG stimulus, the expression of total Akt and total Bcl-2 remains unchanged.

### 3.4 EGCG influences both lipid and glucose metabolism in human sperm

We investigated the effects of increasing stimuli of EGCG in the intracellular levels of triglycerides and lipase activity. A significant decrease in sperm triglycerides levels was observed (Fig. 4A) at the concentrations of 20 μM and ICI, but not RU486, reverted the effect. On the contrary, 60 μM EGCG did not influence tryglicerides levels either in the ICI or RU.

These results well correlate with data obtained on lipase activity that was significantly enhanced by 20 μM EGCG (Fig. 4B).

To gain more insight on sperm energy management, we evaluated the EGCG action on glucose metabolism. As shown

© 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim 

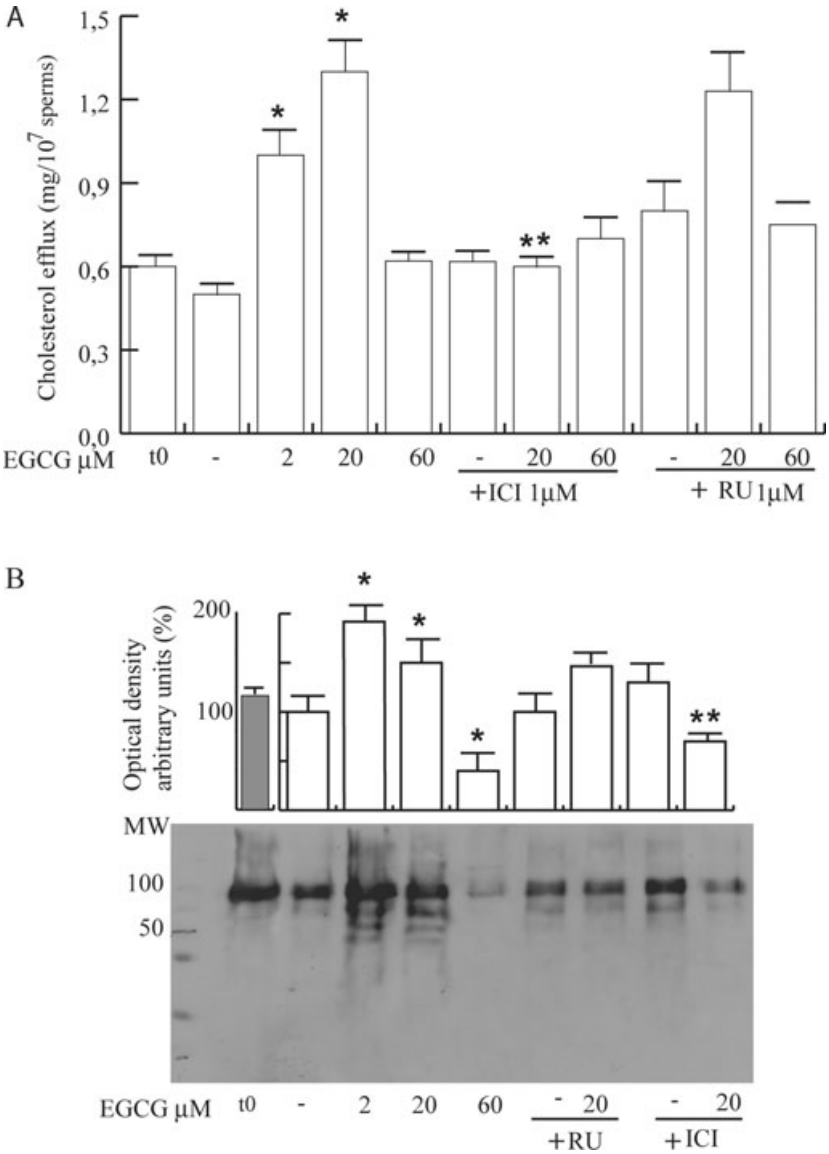


**Figure 2.** EGCG affects cholesterol efflux and protein tyrosine phoshorylation. Sperm cells were incubated in unsupplemented Earle's medium for 30 min at 37°C and 5% $CO_2$, in the absence (−) or in the presence of increasing concentrations of EGCG, and/or ICI (1 μM) or RU 486 (1 μM). Cholesterol efflux (A) was measured also at t0. *$p < 0.05$ versus untreated (−); **$p < 0.05$ versus 20 μM EGCG. (B) Sperm lysates were used for Western blot analysis performed to determine protein tyrosine phosphorylation. Representative Western blots of tyrosine phosphorylation are shown. Sperm cells were incubated in unsupplemented Earle's medium for 30 min at 37°C and 5% $CO_2$, in the absence (−) or in the presence of increasing concentrations of EGCG, and/or ICI (1 μM) or RU 486 (1 μM). Time 0 (t0). Densitometric analysis (mean ± SD) of the 95 kDa band/actin of six independent experiments performed. *$p < 0.05$ versus untreated (−),**$p < 0.05$ versus 20 μM EGCG. Autoradiograph presented is a representative example. MW, molecular weight marker.

in Fig. 5A, EGCG at low concentrations was effective to increase the enzymatic activity while at the high concentration (60 μM) did not produced significant change compared with the untreated samples. ICI cotreatment partially counteracted the above-described results obtained by 20 μM EGCG suggesting that other signaling pathways could be also involved in the regulation of sperm metabolism by EGCG.

These results were confirmed by the data obtained by evaluating the glucose levels (Fig. 5B).

## 4 Discussion

Although the bioactive component of green tea, EGCG, has received considerable attention [1–4] particularly as antiangiogenic and antitumor agent with chemopreventative properties, the effects on male fertilizing capacity are seldom studied. EGCG could offer a protective role against cell oxidative damage involved in the pathogenesis of male infertility [36] but besides to studies promoting the health benefits of green tea consumption [37], it is also known that EGCG has prooxidant potential [28, 38].

Hastak et al. [39] and Weinreb et al. [40] noted that low concentrations of EGCG induced an antiapoptotic pattern of gene expression thereby modulating cell survival. Whereas high concentrations of EGCG induced a proapoptotic pattern thereby modulating cell death in different cell models.

Numerous molecules are proposed to be the target of EGCG action and several studies demonstrated that green tea catechins can bind and downregulate ER-α and ER-β [35, 41], which are expressed from spermatogonia to spermatozoa [20, 21]. ERs mediate mammalian sperm capacitation,

© 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim 

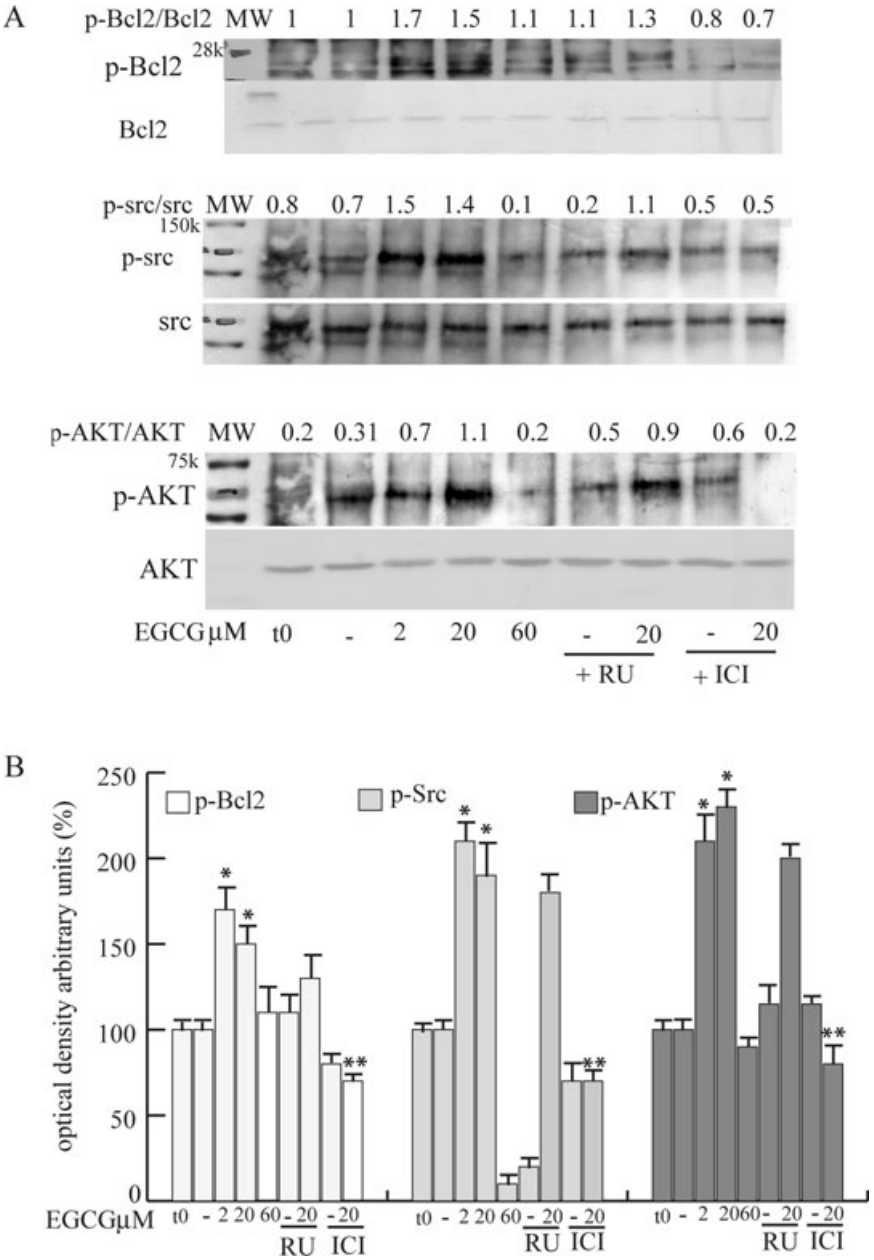


**Figure 3.** EGCG influences Src, Akt, and Bcl2 phosphorylation in sperm cells. Washed spermatozoa were incubated in uncapacitating medium for 30 min at 37°C and 5% $CO_2$, in the absence (−) or in the presence of increasing concentrations of EGCG, and/or ICI (1 μM) or RU 486 (1 μM). (A) Representative Western blots of p-Akt (p-Akt) and p-Bcl2 (pBcl2) and p-Src are shown. (B) Densitometric analysis (mean ± SD) of six independent experiments are reported as pSrc/Src, pAkt/Akt, and pBcl2/Bcl2 relative intensity. *$p < 0.05$ versus untreated (−); **$p < 0.05$ versus EGCG 20 μM.

acrosome reactions, and fertilizing ability stimulated by E2 and environmental estrogens, with the environmental estrogens being much more potent than E2 [23], suggesting our hypothesis that EGCG could influence sperm capacitation through ERs.

It is reported that EGCG, depending on concentrations, can exert estrogenic or antiestrogenic activities.

Particularly in HeLa cells expressing ectopic ER-α or ER-β, EGCG resulted in significant decreases in the E2-/ER-α-mediated signal while, at concentrations lower than 5 μM, resulted in an increase. On the other hand, catechins enhanced the E2-/ER-β-mediated luc activity at either of the concentrations tested [18, 19].

Further studies reported that relatively low concentration of EGCG could induce a pronounced ER-α re-expression in ER-α-negative breast cancer cells, suggesting that EGCG can reactivate the estrogen signal pathways via ER-α [42].

All these findings sustaining estrogenic and antiestrogenic dual properties of EGCG, support the results observed in the present study, in which we evidence the different effects of the catechin on the most relevant human spermatozoa functions for a successful fertilization. Specifically, we demonstrate for the first time that low concentrations of EGCG (2 and 20 μM) through ER increased motility, viability, and known hallmarks of sperm capacitation while high doses exert opposite actions. Our data are in agreement with

© 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim 

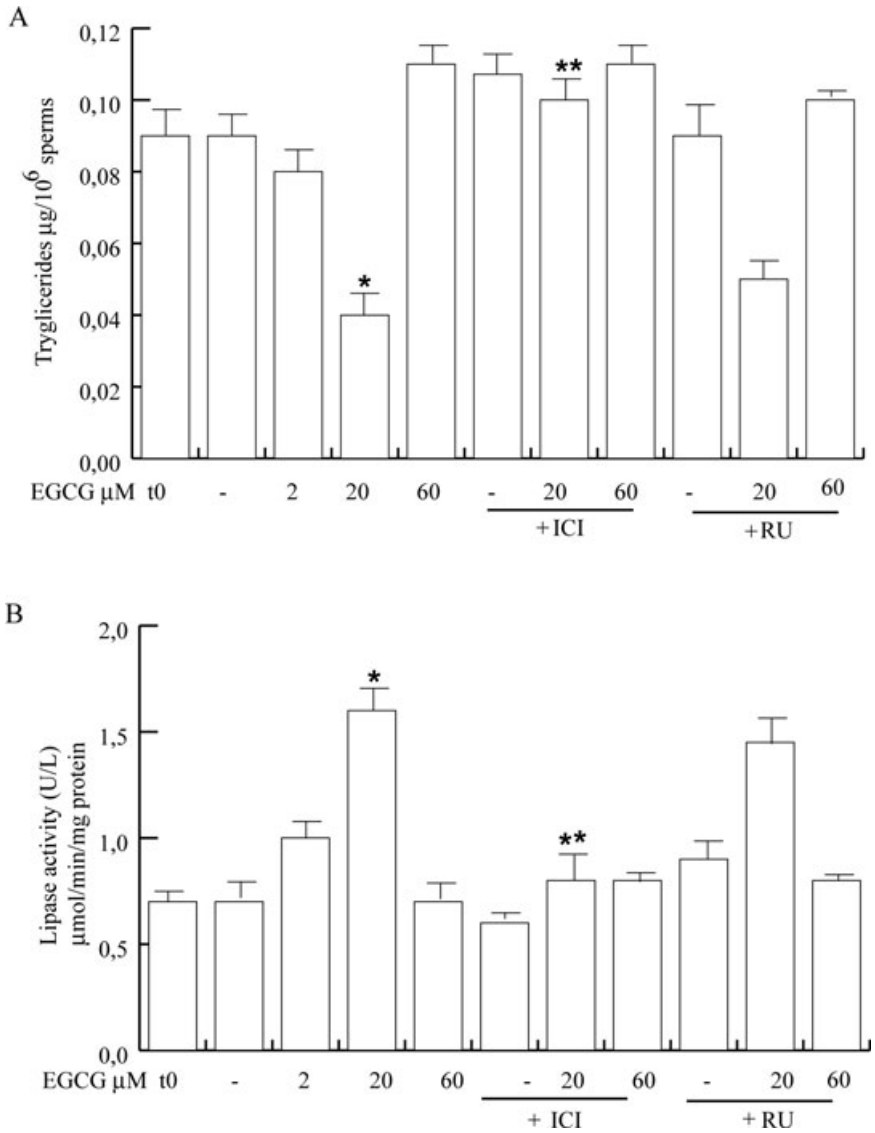


**Figure 4.** EGCG effects on lipid metabolism in human sperm. Washed spermatozoa were incubated in uncapacitating medium for 30 min at 37°C and 5% $CO_2$, in the absence (−) or in the presence of increasing concentrations of EGCG, and/or ICI (1 µM) or RU 486 (1 µM). Triglyceride content (A) and lipase activity (B) were determined also at t0. *$p < 0.05$ versus untreated (−); **$p < 0.05$ versus EGCG 20 µM.

previous report [38], although we also defined the molecular mechanisms.

During its life, mammalian spermatozoa goes through two different physiological conditions: uncapacitated condition, during which spermatozoa maturation occurs in a resting state accumulating and/or economizing energy substrates and the capacitated condition, which allows spermatozoa to achieve the final competence to fertilize the oocyte.

The process of sperm maturation, capacitation, and fertilization occurs in different molecular milieu provided by epididymis and female reproductive tract including oviduct. Specifically, sperm are exposed to estrogens within the male and female tract particularly in tubal fluid following follicle rupture and when in close vicinity to ovulated oocytes. A body of evidences reports that E2 and environmental estrogens induce both capacitation and acrosin activity in normal sperm [21, 23]. Our results demonstrate that 20 µM EGCG, mimicking a coestrogenic action, induces cholesterol efflux and this effect was abrogated by ICI cotreatment. Sixty micromolar EGCG exerted opposite action probably inducing oxidative cellular damage due to antiestrogenic performance. It has been suggested that similar compounds at high concentrations may be responsible for a variety of reproductive disturbances in men, including possible declines in sperm concentration [43, 44].

Proteins phosphorylation provides cells with a "switch" that can turn on or turn off the function of various proteins. Several studies have correlated the degree of tyrosine phosphorylation with the capacitative state of spermatozoa. Visconti et al. [26] observed a time-dependent increase in protein tyrosine phosphorylation of a set of specific proteins in the molecular range of 40–120 kDa, which was correlated with the capacitation state of spermatozoa [21]. Later studies reported that the protein tyrosine phosphorylation increases in spermatozoa during capacitation in various species [45].

© 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim 

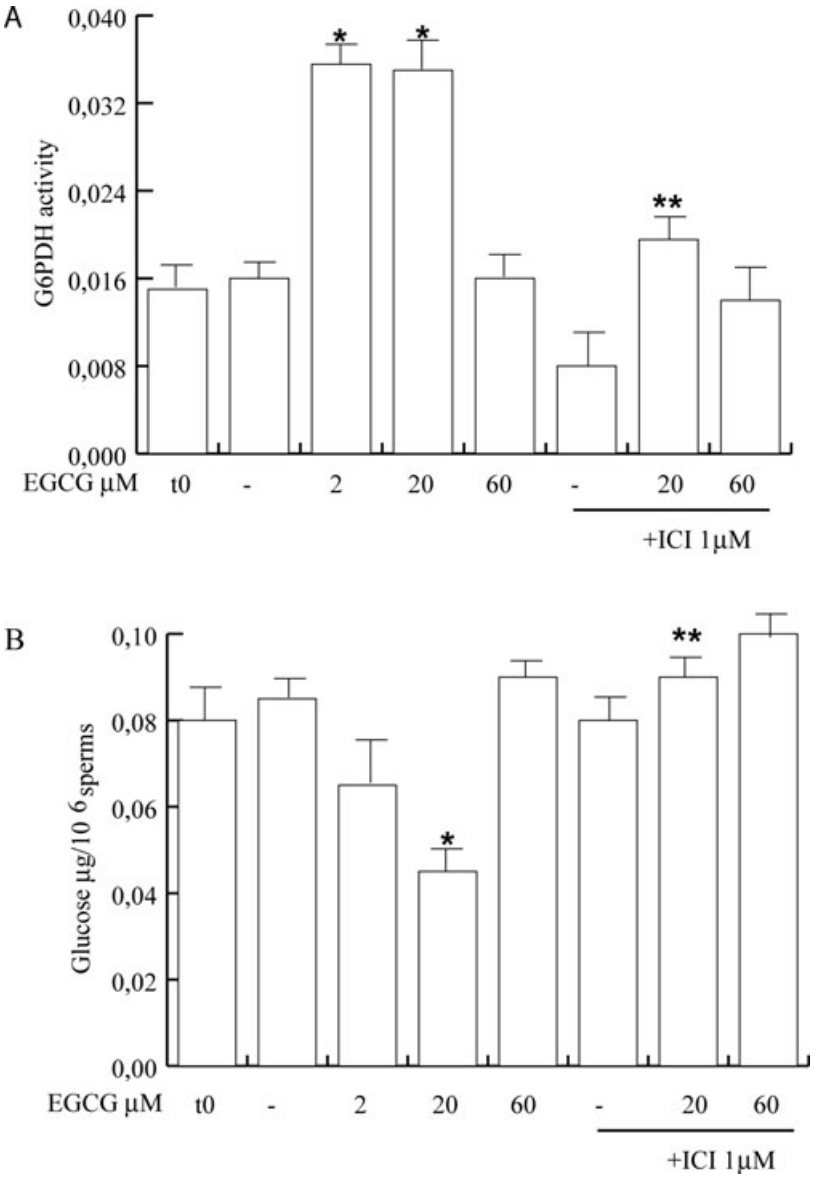


**Figure 5.** EGCG action on glucose metabolism in human sperm. Sperm samples, washed twice with an uncapacitating medium, were incubated in the same medium for 30 min at 37°C and 5% $CO_2$, and treated in the absence (−) or in the presence of increasing concentrations of EGCG, and/or ICI (1 μM). (A) The conversion of $NADP^+$ to NADPH catalyzed by G6PDH was measured by the increase of absorbance at 340 nm every 20 s for 1.5 min. The assay was performed also at t0. Data are expressed in nmol·min−$1/10^6$ spermatozoa, and represent mean ± SD. *$p < 0.05$ versus untreated (−); **$p < 0.05$ versus EGCG 20 μM. (B) Glucose content was determined also at t0, and data shown represent mean ± SD of six independent experiments each in duplicate. *$p < 0.05$ versus untreated (−); **$p < 0.05$ versus EGCG 20 μM.

We show that low concentrations of EGCG induced protein tyrosine phosphorylation indicating a stimulation of the adenylate cyclase/cAMP/PKA signaling, which plays a crucial role in capacitation [22]. Furthermore, treatment with both 20 μM EGCG and ICI, but not RU486, reversed the effect demonstrating that EGCG action is specifically mediated by ERs. This is not surprising since we previously demonstrated that ER activation induces tyrosine phosphorylation in human sperm [21]. At the opposite, after 60 μM EGCG exposure, we evidenced inhibition of tyrosine phoshorylation pattern, probably also related with cytotoxic effects due to the higher concentrations. The biphasic effects observed in our phoshorylation studies may be partly explained by the dual properties of EGCG evidenced in several studies demonstrating that EGCG, starting from concentrations of 50 μM [46] binds strongly to many molecules and affects a variety of enzyme activities via inhibition of protein phosphoylation thus inducing cell death.

In somatic cells, EGCG influences Src and AKT activity and these two kinases play an important role in capacitation, whereas Akt in survival [21, 47]. From our data, given the activation of Src and Akt by low concentrations of EGCG we may support, in part, beneficial effects of green tea consumption. Indeed, drinking 8–10 cups of green tea is sufficient to increase serum levels of EGCG into the low micromolar range investigated in our study, thus exerting increase of sperm motility and viability [48] necessary in female genital tract. The high concentrations of EGCG produced the drastic reduction of p-Src and p-Akt probably due to the generation of $H_2O_2$ in culture medium causing inhibition of phosphorylation of these proteins [46].

© 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim 

The interaction between energy balance and reproduction is subject of intensive investigations [29]. Capacitated sperm displays an increased metabolic rate and overall energy expenditure, presumably to affect the changes in sperm signaling and function during capacitation. For example a successful gamete fusion requires glucose to produce NADPH through the pentose phosphate pathway (PPP) [49]. From this point of view, we investigated whether the double action produced by the different concentration of EGCG on capacitated status, survival, and motility could involve specific changes in glucose and lipid metabolism.

EGCG has been shown to increase the expression of genes related to fat oxidation in the skeletal muscle [50] but data evidencing an influence on sperm metabolism are still lacking.

We found that low doses of the compound induced human sperm energy expenditure, by stimulating lipase activities, with a concomitant reduction of triglycerides levels. As for glucose metabolism, we observed an enhanced G6PDH activity at 2 and 20 μM EGCG, also addressing to an induction of energy consumption via ERs. These results well correlate with our previous data demonstrating that E2/ERs induce insulin secretion closely related with GDPDH activity [51].

Taken together all our data acquire more emphasis corroborating previous findings indicating that, at medium dosages, EGCG increases the number of porcine sperms that bind to ZP [38, 51]. We defined the molecular mechanisms of EGCG action on male fertilizing potential thus suggesting that EGCG may be an effective supplement for improvement in human ART.

In conclusion, in the present study, we demonstrate for the first time that tightly depending on the used concentration, EGCG/ERs are able to improve fertilization potential of the human male gamete, evidencing the specific effects on motility, viability, and energy expenditure in human sperm. This could be explained by the coestrogenic action upon low concentration of EGCG required for capacitation and the acrosome reaction. Paradoxically, the use of elevated concentrations of the antioxidant EGCG might instead has a negative effect on fertility due to antiestrogenic potential at high concentrations. Therefore, it cannot be totally excluded that excessive EGCG concentrations seem to be harmful for parameters related to reproduction.

*This work was supported by MIUR Ex 60% – 2011.*

*The authors have declared no conflict of interest.*

## 5 References

[1] Singh, B. N., Shankar, S., Srivastava, R. K., Green tea catechin, epigallocatechin-3-gallate (EGCG): mechanisms, perspectives and clinical applications. *Biochem. Pharmacol.* 2011, *82*, 1807–1821.

[2] Basu, A., Lucas, E. A., Mechanisms and effects of green tea on cardiovascular health. *Nutr. Rev.* 2007, *65*, 361–375.

[3] Hara, Y., Physiological functions of tea polyphenols: part 2. *Am. Biotechnol. Lab.* 1994, *12*, 18.

[4] Shapiro, H., Lev, S., Cohen, J., Singer, P., Polyphenols in the prevention and treatment of sepsis syndromes: rationale and pre-clinical evidence. *Nutrition* 2009, *25*, 981–997.

[5] Mukhtar, H., Ahmad, N., Green tea in chemoprevention of cancer. *Toxicol. Sci.* 1999, *52*, 111–117.

[6] Uesato, S., Kitagawa, Y., Kamishimoto, M., Kumagai, A. et al., Inhibition of green tea catechins against the growth of cancerous human colon and hepatic epithelial cells. *Cancer Lett.* 2001, *170*, 41–44.

[7] Takada, M., Nakamura, Y., Koizumi, T., Toyama, H. et al., Suppression of human pancreatic carcinoma cell growth and invasion by epigallocatechin-3-gallate. *Pancreas* 2002, *25*, 45–48.

[8] Ahmad, N., Feyes, D. K., Nieminen, A. L., Agarwal, R. et al., Green tea constituent epigallocatechin-3-gallate and induction of apoptosis and cell cycle arrest in human carcinoma cells. *J. Natl. Cancer Inst.* 1997, *89*, 1881–1886.

[9] Salucci, M., Stivala, L. A., Maiani, G., Bugianesi, R. et al., Flavonoids uptake and their effect on cell cycle of human colon adenocarcinoma cells (Caco2). *Br. J. Cancer* 2002, *86*, 1645–1651.

[10] Chen, C., Shen, G., Hebbar, V., Hu, R. et al., Epigallocatechin-3-gallate-induced stress signals in HT-29 human colon adenocarcinoma cells. *Carcinogenesis* 2003, *24*, 1369–1378.

[11] Hsu, S., Yu, F. X., Huang, Q., Lewis, J. et al., A mechanism-based in vitro anticancer drug screening approach for phenolic phytochemicals. *Assay Drug Dev. Technol.* 2003, *1*, 611–618.

[12] Naasani, I., Oh-Hashi, F., Oh-Hara, T., Feng, W. Y. et al., Blocking telomerase by dietary polyphenols is a major mechanism for limiting the growth of human cancer cells in vitro and in vivo. *Cancer Res.* 2003, *63*, 824–830.

[13] Divi, R. L., Doerge, D. R., Inhibition of thyroid peroxidase by dietary flavonoids. *Chem. Res. Toxicol.* 1996, *9*, 16–23.

[14] Sakamoto, Y., Mikuriya, H., Tayama, K., Takahashi, H. et al., Goitrogenic effects of green tea extract catechins by dietary administration in rats. *Arch. Toxicol.* 2001, *75*, 591–596.

[15] Kao, Y. H., Hiipakka, R. A., Liao, S., Modulation of endocrine systems and food intake by green tea epigallocatechin gallate. *Endocrinology* 2000, *141*, 980–987.

[16] Figueiroa, M. S., César Vieira, J. S., Leite, D. S., Filho, R. C. et al., Green tea polyphenols inhibit testosterone production in rat Leydig cells. *Asian J. Androl.* 2009, *11*, 362–370.

[17] Fang, H., Tong, W., Shi, L. M., Blair, R. et al., Structure-activity relationships for a large diverse set of natural, synthetic, and environmental estrogens. *Chem. Res. Toxicol.* 2001, *14*, 280–294.

[18] Belguise, K., Guo, S., Sonenshein, G. E., Activation of FOXO3a by the green tea polyphenol epigallocatechin-3-gallate induces estrogen receptor alpha expression reversing invasive phenotype of breast cancer cells. *Cancer Res.* 2007, *67*, 5763–5770.

[19] Farabegoli, F., Barbi, C., Lambertini, E., Piva, R., (−)-Epigallocatechin-3-gallate downregulates estrogen receptor alpha function in MCF-7 breast carcinoma cells. *Cancer Detect. Prev.* 2007, *31*, 499–504.

© 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim 

[20] Hess, R. A., Bunick, D., Bahr, J. M., Sperm, a source of estrogen. *Environ. Health Perspect.* 1995, *103*, 59–62.

[21] Aquila, S., Sisci, D., Gentile, M., Middea, E. et al., Estrogen receptor (ER) alpha and ER beta are both expressed in human ejaculated spermatozoa: evidence of their direct interaction with phosphatidylinositol-3-OH kinase/Akt pathway. *J. Clin. Endocrinol. Metab.* 2004, *89*, 1443–1451.

[22] Aquila, S., Sisci, D., Gentile, M., Middea, E. et al., Human ejaculated spermatozoa contain active P450 aromatase. *J. Clin. Endocrinol. Metab.* 2002, *87*, 3385–3390.

[23] Adeoya-Osiguwa, S. A., Markoulaki, S., Pocock, V., Milligan, S. R. et al., 17beta-Estradiol and environmental estrogens significantly affect mammalian sperm function. *Hum. Reprod.* 2003, *18*, 100–107.

[24] Aquila, S., Sisci, D., Gentile, M., Carpino, A. et al., Towards a physiological role for cytochrome P450 aromatase in ejaculated human sperm. *Hum. Reprod.* 2003, *8*, 1650–1659.

[25] Suarez, S. S., Control of hyperactivation in sperm. *Hum. Reprod.* 2008, *14*, 647–657.

[26] Visconti, P. E., Westbrook, V. A., Chertihin, O., Demarco, I. et al., Novel signaling pathways involved in sperm acquisition of fertilizing capacity. *J. Reprod. Immunol.* 2002, *53*, 133–150.

[27] Travis, A. J., Kopf, G. S., The role of cholesterol efflux in regulating the fertilization potential of mammalian spermatozoa. *J. Clin. Invest.* 2002, *110*, 731–736.

[28] Isbrucker, R. A., Edwards, J. A., Wolz, E., Davidovich, A. et al., Safety studies on epigallocatechin gallate (EGCG) preparations. Part 3: teratogenicity and reproductive toxicity studies in rats. *Food Chem. Toxicol.* 2006, *44*, 651–661.

[29] Chehab, F. F., Leptin as a regulator of adipose mass and reproduction. *Trends Pharmacol. Sci.* 2000, *21*, 309–314.

[30] Andò, S., Aquila, S., Arguments raised by the recent discovery that insulin and leptin are expressed in and secreted by human ejaculated spermatozoa. *Mol. Cell. Endocrinol.* 2005, *256*, 49.

[31] World Health Organization, *WHO Laboratory Manual for the Examination and Processing of Human Semen*, 5th edn. Cambridge University Press, Cambridge, UK 2010.

[32] Aquila, S., Gentile, M., Middea, E., Catalano, S. et al., Autocrine regulation of insulin secretion in human ejaculated spermatozoa. *Endocrinology* 2005, *146*, 552–557.

[33] Aquila, S., Guido, C., Laezza, C., Santoro, A. et al., A new role of anandamide in human sperm: focus on metabolism. *J. Cell. Physiol.* 2009, *221*, 147–153.

[34] Panteghini M., Bonora R., Pagani F, Measurement of pancreatic lipase activity in serum by a kinetic colorimetric assay using a new chromogenic substrate. *Ann. Clin. Biochem.* 2001, *38*, 365–370.

[35] Goodin, M. G., Fertuck, K. C., Zacharewski, T. R., Rosengren, R. J., Estrogen receptor-mediated actions of polyphenolic catechins in vivo and in vitro. *Toxicol. Sci.* 2002, *69*, 354–361.

[36] Lombardo F., Sansone A., Romanelli F., Paoli D. et al., The role of antioxidant therapy in the treatment of male infertility: an overview. *Asian J. Androl.* 2011, *13*, 690–697.

[37] Webb, T., Green tea experiments in lab, clinic yield mixed results. *J. Natl. Cancer Inst.* 2000, *92*, 1038–1039.

[38] Spinaci M., Volpe S., De Ambrogi M., Tamanini C., Galeati G., Effects of epigallocatechin-3-gallate (EGCG) on in vitro maturation and fertilization of porcine oocytes. *Theriogenology* 2008, *69*, 877–885.

[39] Hastak, K., Gupta, S., Ahmad, N., Agarwal, M. K. et al., Role of p53 and NF-kappaB in epigallocatechin-3-gallate-induced apoptosis of LNCaP cells. *Oncogene* 2003, *22*, 4851–4859.

[40] Weinreb, O., Mandel, S., Youdim, M. B., Gene and protein expression profiles of anti- and pro-apoptotic actions of dopamine, R-apomorphine, green tea polyphenol (−)-epigallocatechine-3-gallate, and melatonin. *Ann. NY Acad. Sci.* 2003, *993*, 351–361.

[41] Kuruto-Niwa, R., Inoue, S., Ogawa, S., Maramatsu, M. et al., Effect of tea catechins on the ERE-regulated estrogenic activity. *J. Agric. Food Chem.* 2000, *48*, 6355–6361.

[42] Li, Y., Yuan, Y. Y., Meeran, S. M., Tollefsbol, T. O. Synergistic epigenetic reactivation of estrogen receptor-α (ER-α) by combined green tea polyphenol and histone deacetylase inhibitor in ER-α-negative breast cancer cells. *Mol. Cancer* 2010 *14*, 274.

[43] Sharpe, R. M., Hormones and testis development and the possible adverse effects of environmental chemicals. *Toxicol. Lett.* 2001, *120*, 221–232.

[44] Skakkebaek, N. E., Rajpert-De Meyts, E., Main, K. M., Testicular dysgenesis syndrome: an increasingly common developmental disorder with environmental aspects. *Hum. Reprod.* 2001, *16*, 972–978.

[45] Bragado, M. J., Gil, M. C., Martin-Hidalgo, D., Hurtado de Llera, A. et al., Src family tyrosine kinase regulates acrosome reaction but not motility in porcine spermatozoa. *Reproduction.* 2012, *144*, 67–75.

[46] Fujiki, H., Suganuma, M., Okabe, S., Sueoka, N. et al., Cancer inhibition by green tea. *Mutat. Res.* 1998, *402*, 307–310.

[47] Aquila, S., Middea, E., Catalano, S., Marsico, S. et al., Human sperm express a functional androgen receptor: effects on PI3K/AKT pathway. *Hum. Reprod.* 2007, *22*, 2594–2605.

[48] Lee, M. J., Maliakal, P., Chen, L., Meng, X. et al., Pharmacokinetics of tea catechins after ingestion of green tea and (−)-epigallocatechin-3-gallate by humans: formation of different metabolites and individual variability. *Cancer Epidemiol. Biomarkers Prev.* 2002, *11*, 1025–1032.

[49] Urner, F., Sakkas, D., Characterization of glycolysis and pentose phosphate pathway activity during sperm entry into the mouse oocyte. *Biol. Reprod.* 1999, *60*, 973–978.

[50] Sae-tan, S., Grove, K. A., Kennett, M. J., Lambert, J. D., (−)-Epigallocatechin-3-gallate increases the expression of genes related to fat oxidation in the skeletal muscle of high fat-fed mice. *Food Funct.* 2011, *2*, 111–116.

[51] Guido, C., Perrotta, I., Panza, S., Middea, E. et al., Human sperm physiology: estrogen receptor alpha (ER-α) and estrogen receptor beta (ER-β) influence sperm metabolism and may be involved in the pathophysiology of varicocele-associated male infertility. *J. Cell Physiol.* 2011, *226*, 3403–3412.

© 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim 