Cardiovascular Pharmacology

Epigallocatechin-3-gallate relaxes the isolated bovine ophthalmic artery: Involvement of phosphoinositide 3-kinase-Akt-nitric oxide/cGMP signalling pathway

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ABSTRACT

The present study investigates the direct action and the underlying mechanism(s) of epigallocatechin-3-gallate (EGCG) vasomotor effects on the bovine isolated ophthalmic artery. Adjacent rings were cut from each artery and mounted in a wire miograph system for isometric recording. Concentration–response curves for EGCG were constructed by adding cumulative concentrations of the drug to arterial rings pre-contracted with 5-HT (1 μM). Effects of mechanical endothelial cell removal and of selective blockers of the nitric oxide (NO)/cGMP pathways were investigated on the EGCG relaxant responses. EGCG relaxed ophthalmic arteries and maximum relaxation was 78.4±2.64%. Mechanical removal of endothelium, blockade of soluble guanylyl cyclase by 1H-1,2,4-oxadiazolo [4,3-a]quinoxalin-1-one (ODQ, 1 and 5 μM) or inhibition of nitric oxide (NO) synthase by N\(^{-}\)-nitro-L-arginine (L-NAME, 50 and 100 μM) reduced significantly the relaxant response to catechin; moreover, the NO donor S-nitroso-N-acetylpenicillamine (SNAP, 100 μM) significantly increased the vasorelaxant responses to EGCG. Relaxation to EGCG was inhibited by iberiotoxin (200 nM), a blocker of big-conductance Ca\(^{2+}\)-activated K\(^{+}\) (BKCa) channel, whereas the blockade of K\(_{ATP}\) channel by glibenclamide (5 μM) and of small-conductance Ca\(^{2+}\)-activated K\(^{+}\) (SKCa) channel by apamin (100 nM) elicited no effect. Interestingly, also inhibition of phosphoinositide-3-kinase (PI3K) by wortmannin (100 nM) and of Akt by SH6 (1 μM) markedly decreased the EGCG-evoked vasorelaxation. These data suggest that EGCG induced vasorelaxation in ophthalmic arteries with endothelium-intact via the activation of the NO/cGMP signalling pathway and defined an intriguing role for PI3K and Akt as upstream mediators for activation of NO-mediated relaxant responses.

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1. Introduction

Green tea (Camellia sinensis) is the most popular beverage in the world and has only recently been studied extensively for its potential health beneficial effects (Mandel et al., 2005; Chen et al., 2008). Green tea is chemically characterized by the presence of high amounts of polyphenolic compounds known as catechins, whose most abundant is the epigallocatechin-3-gallate (EGCG), which appears to be the candidate responsible for biological effects.

Most of the research in the catechin field has been orientated on the ability of these molecules to reduce oxidative injury. However, biological reality is more complex and extends the role of these compounds to different biological processes. The results of numerous investigation indicate that EGCG has a potent antioxidant activity (Álvarez et al., 2002; Nagai et al., 2002; Xie et al., 2004; Morley et al., 2005), neuroprotective effects (Mandel et al., 2004) and anti-proliferative activity (Sanae et al., 2002; Shen et al., 2003; Álvarez-Castro et al., 2004). Additionally, it has been evidenced that EGCG has anti-proliferative (Löcher et al., 2002), anti-atherogenic (Ludwig et al., 2004), anti-thrombotic (Kang et al., 1999), platelet anti-aggregation (Zheng et al., 2004) and anti-inflammatory properties (Tedeschi et al., 2002).

A role for EGCG in the modulation of vasomotor responses is emerging from a series of interesting reports. EGCG seems to produce opposite vascular effects, relaxant and contractile, in various vascular beds. The catechin can induce vasorelaxant responses via coupling to the inhibition of Ca\(^{2+}\) influx in smooth muscle cells (Huang et al., 1998), the stimulation of endothelial nitric oxide (NO) synthase (NOS) (Huang et al., 1999; Lorenz et al., 2004) or by involving an increased production of prostacyclin (Mizugaki et al., 2000). The EGCG-evoked vasorelaxant effects appear to be both endothelium-dependent and -independent. In bovine aortic endothelial cells, EGCG-induced relaxation in an endothelium-dependent manner and occurred in response to activation of NOS (Lorenz et al., 2004); by contrast, in rat aortic rings EGCG-induced relaxation was endothelium-independent and was mediated by an inhibition of phosphodiesterase (PDE) 1–5 isofrom activity (Álvarez et al., 2006). Moreover, it is reported that EGCG is able to induce contractile responses in isolated rat aorta (Sanae et al., 2002; Shen et al., 2003; Álvarez-Castro et al., 2004).
Although the effects of EGCG in various vascular beds have been well documented (Huang et al., 1999; Álvarez-Castro et al., 2004; Lorenz et al., 2004; Álvarez et al., 2006), studies on the vascular actions of EGCG in ocular tissues are still missing. Emerging clinical evidence support the hypothesis that vascular component exerts a significant role in the etiology of glaucoma and negatively influences the evolution of disease by suggesting that treatments aimed to improve ocular blood flow may benefit glaucoma patients. The present in vitro study was designed to examine the direct effect of EGCG on the ophthalmic artery, to evaluate the signalling mechanism(s) involved in the vasomotor activity and to provide new insights into the role of catechins in ocular tissue.

2. Materials and methods

2.1. Preparation of tissue

Experiments were performed in accordance with the European Community guidelines for the use of experimental animals and were approved by the institutional ethics committee. The techniques for isolation and preparation of ophthalmic arterial rings have been performed as previously described (Romano and Lograno, 2006). In brief, bovine eyes, including the immediate retro-bulbar structures, were obtained from local slaughterhouse, enucleated within 5 min after death and immediately immersed in ice-cold modified Krebs solution (mM composition: NaCl 136.8, KCl 5.4, MgSO4 0.8, NaH2PO4 1.4, NaHCO3 12, CaCl2 2.7, d-glucose 5, Na-ascorbate 0.2) that had been pre-gassed with a mixture of 95% O2 and 5% CO2 and kept at 4 °C during transportation. They were brought to the laboratory within 30 min.

The main ophthalmic artery was carefully dissected free of adherent connective and adipose tissue. Two adjacent rings were cut from each ophthalmic artery and were mounted on fine tungsten wires on a myograph system (Fort 10, WPI, Sarasota, FL, USA). Tension was measured and recorded on a dual channel electronic display wires on a myograph system (Fort 10, WPI, Sarasota, FL, USA). Tension was assessed by precontracting the vessel with 1 μM 5-HT and maximally relaxed with 10 μM carbachol; relaxations greater than 60% were designated as endothelium-intact. In some arterial rings, endothelium was removed by gentle rubbing the intimal surface of the vessel with a human hair; carbachol-induced relaxation of <10% indicated successful removal. All experiments were carried out in the presence of indomethacin (10 μM).

2.2. Experimental procedure

The effects of EGCG on 5-HT-induced tone were examined in paired rings derived from the same arteries. Ophthalmic arteries were contracted by an addition of 5-HT (1 μM) to the organ bath, and once a stable contraction was achieved and maintained, the vasorelaxant responses of EGCG were assessed as cumulative concentration–response curves by the addition of stock solutions to the buffer. In some experiments, the EGCG vasorelaxation was repeated after 40 min, to confirm the reproducibility of the response. All protocols were carried out in both the presence and the absence of endothelium. The steady-state response to EGCG was taken at each concentration and expressed as a percentage relaxation of the imposed 5-HT contraction. To investigate the role of endothelium in the EGCG-induced relaxation response, endothelium was mechanically removed as above described.

The mechanisms involved in EGCG-elicted vasorelaxation actions were studied in bovine ophthalmic arterial rings by adding suitable drugs before EGCG was administered. The vasorelaxation responses to EGCG were investigated in the presence of the NOS inhibitor Nω-nitro-L-arginine methyl ester (L-NAME, Pfieffer et al., 1996) (50 and 100 μM), the selective neuronal NOS inhibitor N-[(4S)-4-amino-5-[aminooethyl] aminopentyl]-N'-nitroguanidine (AAAN, Hah et al., 2001) (100 μM), the potent inhibitor of endothelial NOS Nω-[(1-iminoethyl)-l-ornithine (L-NIO, Rees et al., 1990) (100 μM), the NO donor S-nitroso-N-acetylpenicillamine (SNAP, Ferrero et al., 1999) (100 μM), the guanylyl cyclase inhibitor 1H-[1,2,4]xodiazolo-[4,3-a] quinoxalin-1-one (ODQ, Gérthwaite et al., 1995) (1 and 5 μM), the phosphoinositol-3-kinase (PI3K) inhibitor wortmannin (Arcaro and Wyman, 1993) (100 nM) and the Akt inhibitor SH6 (Kozikowski et al., 2003) (1 μM). All these drugs were added 30 min before and remained present throughout the construction of the concentration–effect curves.

Finally, the possible contribution of potassium channel to EGCG relaxant action was studied. A pre-treatment for 30 min with potassium channel inhibitors, such as iberiotoxin (Galvez et al., 1990) (200 nM), glibenclamide (5 μM) and apamin (100 nM) was assessed on arterial rings to offer further knowledge for the identification of the potassium channel(s) involved. Thereafter, arterial rings were pre-contracted with 5-HT and maximally relaxed to EGCG as cited above.

2.3. Data and statistical analysis

Values are given as the mean ± S.E.M. and n represents the number of vessels studied. All vasorelaxation responses were expressed as percentage relaxation of the tone induced by 1 μM 5-HT. The concentration of vasorelaxation, giving a half-maximal response (EC50), was obtained by fitting four-parameter sigmoidal concentration–response curves using Prism GraphPad (version 3.0, San Diego, CA, U.S.A.) and expressed as its negative logarithm, pEC50. Rmax refers to the maximum relaxation achieved.

Statistical analysis was performed by analysis of variance (ANOVA), followed by Bonferroni’s post hoc test (Prism 3.0). Student’s t-test for paired observations was used when appropriate. P-values of <0.05 were considered as statistically significant.

2.4. Chemicals

Drugs used in the present study were obtained from Tocris Bioscience (Bristol BS11 9JX, U.K.) except where indicated: 5-HT (5-hydroxytryptamine) creatinine sulphate, carbachol hydrochloride, iberiotoxin, N-[(4S)-4-amino-5-[aminooethyl] aminopentyl]-N'-nitroguanidine (AAAN) (Sigma Aldrich, St. Louis, MO, USA); SH6 (Merk Chemicals Ltd, U.K.); EGCG (granted by SIFI S.p.A., Catania, Italy) was daily dissolved in distilled water to a concentration of 10 mM. All other reagents were of analytical grade. Stock solutions (10 mM) of each drug were prepared in dimethyl sulfoxide or ethanol as appropriate and subsequent concentrations were diluted in the Krebs solution. Serial dilutions were prepared daily in Krebs solution. The final bath concentration of dimethyl sulfoxide or ethanol was 0.1% which we have found elsewhere to have no effect on the tonus or mechanical function of preparation.

3. Results

3.1. Vasorelaxant effects of EGCG on arterial rings

EGCG (1 nM to 100 μM) evoked a significant concentration-dependent relaxation (P<0.001; n=18) of bovine ophthalmic artery
pre-contracted with 1 μM 5-HT (Fig. 1A). The threshold concentration for vasorelaxation was 100 nM and the highest concentration utilized (100 μM) elicited a maximum relaxation of about 78% (Fig. 1B). The potency (\( \rho_{0.001} \)) and reactivity (\( \rho_{0.001} \)) of EGCG significantly were greater in endothelium-intact arterial rings compared with endothelium-denuded rings (in the presence of endothelium: \( pEC_{50}=6.21\pm0.06, R_{\text{max}}=78.4\pm2.64 \); in the absence of endothelium: \( pEC_{50}=6.03\pm0.50, R_{\text{max}}=16.1\pm3.59 \); Fig. 1B). Relaxant effect to EGCG was not affected by the presence or the absence of indomethacin.

3.2. Involvement of nitric oxide in the relaxant effects of EGCG

The contribution of NO to the EGCG response of isolated ophthalmic arteries was assessed by its inhibitor. In endothelium-intact arterial rings, the NOS inhibitor L-NAME (50 μM) significantly inhibited the relaxation to EGCG compared to control (\( P<0.05, n=8 \), Fig. 2). The EGCG-induced vasorelaxation was completely abolished by 100 μM L-NAME (\( n=8 \), Fig. 2). In addition, specific inhibitor of different NOS isoforms has been used. The selective neuronal NOS inhibitor \( \text{N}-(4\text{S})-(4\text{-amino-5-[aminoethyl] aminopentyl})-\text{N}^\prime\)-nitroguanidine (AAAN) (100 μM) had no effect on vasorelaxation induced by EGCG (Fig. 3, \( n=6 \)) whereas the selective endothelial NOS inhibitor L-NIO (100 μM) completely inhibited this response (Fig. 3, \( n=6 \)).

The NO donor SNAP (100 μM) caused a small but significant increase in relaxant responses to EGCG in a concentration-dependent manner most notably at high concentrations of EGCG (control: \( pEC_{50}=6.23\pm0.19, R_{\text{max}}=78.5\pm2.38 \); EGCG: \( pEC_{50}=6.38\pm0.07, R_{\text{max}}=93.6\pm2.11, P<0.01, n=8 \), Fig. 4).

3.3. Role of guanylyl cyclase

To evaluate the possibility of EGCG to activate the soluble guanylyl cyclase in order to induce vasorelaxation, the endothelium-intact ophthalmic arterial rings were pretreated with the selective guanylyl cyclase inhibitor ODQ for 30 min. As shown in Fig. 5, ODQ (1 μM) produced a significant inhibition of the vasorelaxant action evoked by EGCG (\( P<0.001 \); control: \( pEC_{50}=6.19\pm0.18, R_{\text{max}}=82.3\pm3.9, n=6 \); EGCG: \( pEC_{50}=6.21\pm0.06, R_{\text{max}}=78.4\pm2.64 \); Fig. 1B).

Fig. 1. Effects of EGCG on the bovine ophthalmic artery. (panel A) Typical original tracing showing one experiment in which EGCG induced a concentration-dependent relaxant response in arterial rings pre-contracted to 5-HT (1 μM) in the absence (a) and in the presence (b) of endothelium. (panel B) The EGCG vasorelaxant responses in arterial rings with (●) endothelium-intact and (□) endothelium-denuded, pre-contracted to 5-HT (1 μM). Data are given as means with error bars representing S.E.M.

Fig. 2. Effects of L-NAME pre-treatment on EGCG-induced vasorelaxation in bovine ophthalmic artery with intact endothelium. Increasing concentrations of EGCG in the absence of endothelium, shown in panel A, were compared with the effect of L-NAME (50 μM). Data are given as means with error bars representing S.E.M. *\( P<0.05 \), **\( P<0.001 \) significance when compared with the control relaxation using one-way ANOVA followed by Bonferroni’s post hoc test.

Fig. 3. Effects of AAAN (\( N-(4\text{S})-(4\text{-amino-5-[aminoethyl] aminopentyl})-\text{N}^\prime\)-nitroguanidine) (100 μM), a neuronal NOS inhibitor, and L-NIO (100 μM), an endothelial NOS, on vasorelaxation to EGCG in bovine ophthalmic artery with endothelium-intact pre-contracted with 5-HT (1 μM). Increasing concentrations of EGCG in the absence and in the presence of inhibitors. Data are given as means with error bars representing S.E.M. *\( P<0.01 \) significance when compared with the control relaxation using one-way ANOVA followed by Bonferroni’s post hoc test. **\( P<0.001 \).

Fig. 4. Effects of nitric oxide donor SNAP on vasorelaxation evoked by EGCG in bovine ophthalmic artery with intact endothelium. Concentration–response curves to (■) EGCG (control) and (▲) in the presence of SNAP (100 μM). Data are given as means with error bars representing S.E.M. *\( P<0.01 \) significance when compared with the control relaxation using Student’s \( t \)-test for paired observations.
in the presence of inhibitor; pEC50 was not evaluated, Rmax = 20.9 ± 0.14, n = 6) but did not modify the 5-HT response. Moreover, a pre-treatment with 5 μM ODQ for 30 min completely abolished the vasorelaxation to EGCG (Fig. 5; n = 6).

3.4. Involvement of potassium channel in EGCG vasorelaxation

In order to identify the role of specific types of K+ channel in the EGCG-induced relaxation, ophthalmic arterial rings were incubated with different inhibitors of K+ channels for 30 min prior to the application of EGCG. In the presence of the selective blocker of the Ca2+-activated K+ (BKCa) channels iberiotoxin (200 nM), the EGCG-induced vasorelaxation was significantly inhibited for endothelium-intact arterial rings (P < 0.01, Fig. 6, n = 6). The selective blocker of small-conductance Ca2+-activated K+ (SKCa) channels apamin (100 nM, n = 4) and the selective blocker of ATP-sensitive (KATP) K+ channels glibenclamide (5 μM, n = 4) failed to modify the vasorelaxation induced by catechin suggesting that SKCa channels and KATP channel might not be responsible for the EGCG-evoked vasorelaxation (Fig. 6).

3.5. Role of phosphoinositide-3-kinase

An important role for NO in vessels is to stimulate soluble guanylyl cyclase that synthesizes cGMP. To elucidate whether Akt was an important downstream target of PI3K involved in the activation of endothelial NOS-dependent NO production (Dimmel et al., 1999), the EGCG effect with known Akt modulator was investigated. A pre-treatment of ophthalmic arterial rings with the Akt inhibitor SH6 (1 μM) for 30 min, significantly inhibited the EGCG-evoked vasorelaxant responses (P < 0.001, n = 5, Fig. 7). In addition, the same result was found by pre-treating the ophthalmic arterial rings with wortmannin (100 nM), a PI3K inhibitor (P < 0.001, n = 5, Fig. 7), by suggesting that Akt/PI3K pathway might be affected in the EGCG-induced vasorelaxation.

4. Discussion

Our results show that EGCG produces a relaxant responses on the bovine ophthalmic arterial rings pre-contracted with 5-HT. Although previous studies have reported the vasorelaxant effects of EGCG in several vascular beds (Huang et al., 1998, 1999; Lorenz et al., 2004; Kim et al., 2007), we have extended these findings to the vascular actions of this catechin in ocular vascular tissues. It is intuitive how these observations have interesting physiologic and pathophysiologic implications since blood flow to the optic nerve head is functionally regulated and supplied by ophthalmic artery.

**Fig. 5.** Effects of the guanylyl cyclase inhibitor ODQ (1 μM) on vasorelaxation evoked by EGCG in bovine ophthalmic artery with intact endothelium, pre-contracted with 5-HT (1 μM). Data are given as means with error bars representing S.E.M. *P < 0.05, **P < 0.001 significance when compared with the control relaxation using Student’s t-test for paired observations.

**Fig. 6.** Effects of potassium channel blockers on vasorelaxation to EGCG in bovine ophthalmic artery with intact endothelium, pre-contracted with 5-HT (1 μM). Increasing concentrations of EGCG in the absence and in the presence of inhibitors. Data are given as means with error bars representing S.E.M. *P < 0.001 significance when compared with the control relaxation using one-way ANOVA followed by Bonferroni’s post hoc test.

**Fig. 7.** Effects of PI3K inhibitor wortmannin (100 nM) and Akt inhibitor SH6 (1 μM) on vasorelaxation to EGCG in bovine ophthalmic artery with intact endothelium pre-contracted with 5-HT (1 μM). Increasing concentrations of EGCG in the absence and in the presence of inhibitors. Data are given as means with error bars representing S.E.M. *P < 0.05, **P < 0.001 significance when compared with the control relaxation using one-way ANOVA followed by Bonferroni’s post hoc test.

**Fig. 8.** The scheme represents a plausible signalling pathway involved in the ophthalmic artery vasorelaxant effects by EGCG. The principal mechanism involves the activation by EGCG of PI3K, through a yet not identified receptor site localized on the cell membrane or in the cytoplasm, which stimulates the NO/cGMP signalling cascade and in turn elicits vasorelaxation.
The vasorelaxation of EGCG in bovine ophthalmic artery appears to be pharmacologically relevant, since our findings reported an EC50 value approximately 620 nM evidencing the potent action of EGCG in this tissue. Moreover, mechanical endothelial disruption significantly reduces the vasorelaxant effect induced by catechin suggesting a central function of the endothelium in EGCG vasorelaxation, prompting us to undertake a series of pharmacological studies to investigate the roles of endothelial relaxing factors in the EGCG response. Given the NO as the primary relaxing factor of the endothelium (Moncada et al., 1991), we first targeted the NO/cGMP pathway. A pre-treatment of arterial rings, with the nonelective inhibitor of NO, L-NAMe yielded a blockade of the vasorelaxant effects induced by EGCG indicating a clear involvement of NO in the vasorelaxation induced by the catechin. Although L-NAMe caused the blockade of relaxant response to EGCG, it is a nonelective NO inhibitor and acts on all isoforms of the enzyme. To identify the NO isoenzymes involved in EGCG vasorelaxation we compared the effects of L-NAMe on bovine ophthalmic artery with those of some more recently introduced NO-specific inhibitors. The two agents we examined were L-NIO, a selective inhibitor of endothelial NO (Rees et al., 1990) and AAAN which has a 2500-fold greater selectivity for neuronal NO over endothelial NO (Hah et al., 2001). These findings demonstrate that a pre-treatment with L-NIO completely inhibited the EGCG-induced vasorelaxation response suggesting that endothelial NO plays an interesting role in mediating the relaxant effect to catechin. Moreover, we found that in the bovine ophthalmic artery, AAAN at concentrations up to 100 μM failed to affect vasorelaxation evoked by the catechin. The lack of effect on endothelium-dependent relaxation might have been given by its poor Ki (314 μM) (Hah et al., 2001) for bovine endothelial NO. In addition, the treatment of endothelium-intact ophthalmic artery with the nitric oxide donor SNAP amplified relaxation of catechin supporting the central role for the nitric oxide in EGCG-vasorelaxation. These findings are consistent with previous reports that EGCG induces relaxation in a variety of vascular tissues by involving NO production (Huang et al., 1999; Lorenz et al., 2004; Kim et al., 2007). Among the signalling pathways that support a NO-mediated vasorelaxation effect, activation of the soluble guanylyl cyclase/cGMP pathway is considered to be a major vasodilatatory mechanism for nitric oxide. We demonstrated that the selective guanylyl cyclase inhibitor ODQ completely abolished the relaxation to EGCG at the highest concentration utilized in the bovine ophthalmic artery. All evidence taken together point to a pivotal role of endothelium component and soluble guanylyl cyclase/cGMP pathway in mediating the NO-dependent relaxant response to EGCG in bovine ophthalmic artery.

The possibility that K+ channels, which hyperpolarize and synergistically act with NO to relax smooth muscle, contributed to EGCG vasorelaxation was evaluated in the current study. This premise is supported by previous studies which show the involvement of K+ channels in the endothelium-dependent vasorelaxation catechin-induced (Huang et al., 1999). Selective blocker of BKCa channels, but not SKCa or KATP channels, effectively reduced EGCG vasorelaxation suggesting that endothelial NO and K+ channels are important mediators of catechin relaxation.

The present study also shows that inhibition of PI3K signalling by wortmannin (100 nM) or inhibition of Akt (1 μM) by SH6 significantly abolished the vasorelaxation induced by EGCG. Previous studies have provided experimental evidence that EGCG activates the key enzyme of vascular homeostasis endothelial NO and parallelly PI3K/Akt pathway and induce endothelium-dependent vasorelaxation (Lorenz et al., 2004; Kim et al., 2007). Here, we have demonstrated that the PI3K/Akt pathway is an important upstream mediator of the NO/cGMP signals involved in vasorelaxant action of EGCG in ophthalmic artery.

The use of multiple inhibitors for the same pathway intermediates has enabled us to elucidate the mechanisms of this signalling cascade, emphasizing and providing further support on impact mechanism by which EGCG activates vasorelaxation in the bovine ophthalmic artery. More importantly, as depicted in Fig. 8, one possible mechanism may involve the interaction of EGCG with a receptor site not yet identified triggering a signalling pathway that sequentially activates PI3K, Akt and NO; this in turn stimulates guanylyl cyclase with the final enhancement of the cGMP-dependent protein kinase pathway. In conclusion, the micrographs, lot, immunohistochemical analysis, yield a potent relaxation of bovine ophthalmic artery, which consists of an endothelium-dependent component; the PI3K/Akt pathway is the major upstream activator for the NO and cGMP for vasorelaxant response. The emerging function of EGCG in ocular vascular tissue rises the questions of how this catechin can have a future in the treatment of ocular neurodegeneration disease and paves the way to a more integrated therapeutic approach in glaucoma disease.

Acknowledgements

We thank Dr. Antonio Gattulli, veterinarian of the slaughterhouse of the AUSL/Ba (Bari, Italy) and SIFI S.p.A. (Catania, Italy) for having provided EGCG. Support was granted by “Ministero dell’Università e della Ricerca Scientifica e Tecnologica” (Cofinanziamento 2006).

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